

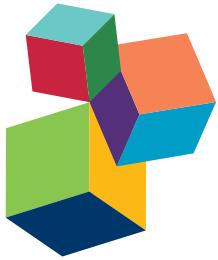
# **SURVEYING ANTIMICROBIAL RESISTANCE: APPROACHES, ISSUES, AND CHALLENGES TO OVERCOME**

**EDITED BY:** Gilberto Igrejas, José Luis Capelo, Alexandre Gonçalves

and Patrícia Poeta

**PUBLISHED IN:** Frontiers in Microbiology





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**ISSN 1664-8714**

**ISBN 978-2-88945-141-8**

**DOI 10.3389/978-2-88945-141-8**

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# SURVEYING ANTIMICROBIAL RESISTANCE: APPROACHES, ISSUES, AND CHALLENGES TO OVERCOME

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Cover Picture: Windmill: Pico Island, Azores. The red biotechnology. Antimicrobial molecules are not restricted to treated patients, either animals or humans, but rather circulate throughout the whole ecosystem. The “One Health” concept recognizes that the health of humans is connected to the health of animals and the environment. The use of antibiotics in human and veterinary medicine may have consequences beyond their intended applications.

Cover image by Gilberto Igrejas and Patrícia Poeta

## Why Antibiotic Resistance?

The use of antibiotics in human and veterinary medicine may have consequences beyond their intended applications. The “One Health” concept recognizes that the health of humans is connected to the health of animals and the environment. Progress in molecular genetics is facilitating the rapid evaluation of the essentiality of these targets on a genomic scale. In 2015, a group of researchers established the International Conference on Antibiotic Resistance (IC2AR). The primary objective of this meeting is to bring together scientists involved in antibiotic resistance prevention and control. The IC2AR conducted its inaugural world congress in January 2015 at Caparica (Portugal).

Antimicrobial resistance presents a significant challenge to scientists in the field of infectious diseases. The full knowledge of how antibiotics resistance is evolving and being transmitted between hosts in different ecosystems is taking on great importance. Necessary action includes research to define the scope of the problem including its various sources.

This eBook comprises a series of original research and review articles dealing with the epidemiology of resistance in animal and zoonotic pathogens, mobile elements containing resistance genes, the omics of antimicrobial resistance, emerging antimicrobial resistance mechanisms, control of resistant infections, establishing antimicrobial use and resistance surveillance systems, and alternatives strategies to overcome the problem of antimicrobial resistance worldwide.

Gilberto Igrejas, José Luis Capelo and Patrícia Poeta  
Scientific Committee of IC2AR, February 20th, 2017

**Citation:** Igrejas, G., Capelo, J. L., Gonçalves, A., Poeta, P., eds. (2017). Surveying Antimicrobial Resistance: Approaches, Issues, and Challenges to Overcome. Lausanne: Frontiers Media.  
doi: 10.3389/978-2-88945-141-8

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# Editorial: Surveying Antimicrobial Resistance, Approaches, Issues, and Challenges to Overcome

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**Keywords:** genomics, antimicrobial resistance, surveillance, molecular microbiology, epidemiology

## OPEN ACCESS

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**Specialty section:**

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 02 December 2016

**Accepted:** 13 January 2017

**Published:** 01 February 2017

**Citation:**

Igrejas G, Capelo JL, Gonçalves A  
and Poeta P (2017) Editorial:  
Surveying Antimicrobial Resistance,  
Approaches, Issues, and Challenges  
to Overcome. *Front. Microbiol.* 8:90.  
doi: 10.3389/fmicb.2017.00090

## Editorial on the Research Topic

### Surveying Antimicrobial Resistance, Approaches, Issues, and Challenges to Overcome

The 1st International Caparica Conference in Antibiotic Resistance, was held in Caparica, Portugal in January 26–28, 2015. This very successful meeting attracted nearly 110 attendees, from 32 countries and involved a total of over 70 oral presentations, 16 shotguns presentations and 27 posters. The results and insights from this meeting are being made accessible to the general scientific community by this special dedicated issue of the *Frontiers in Microbiology Research Topic*.

Antimicrobial resistance within populations of different infectious agents is a worldwide public health threat. Already the available treatment options for common infections in some settings are becoming ineffective. There are now reports of bacterial resistance to all antibiotic classes used in either human or veterinary medicine, and in several cases, of an association between antibiotic use and the development of clinical resistance. To counter this emergent problem, the World Health Organization has appealed for urgent and concerted action by governments, health professionals, industry, civil society and patients to slow down the spread of drug resistance, limit its impact today and preserve medical advances for future generations.

The present Research Topic brings together a group of leading researchers from all over the world who have described different aspects of antimicrobial resistance patterns found in diverse ecosystems. The articles in this topic seek to address this question, including the epidemiology of resistance in animal and zoonotic pathogens, mobile elements containing resistance genes, the omics of antimicrobial resistance, emerging antimicrobial resistance mechanisms, control of resistant infections, establishing antimicrobial use and resistance surveillance systems, and alternatives strategies to overcome the problem of antimicrobial resistance worldwide.

We want to thank the reviewers for their many thoughtful and insightful comments, and the authors for their high-quality contributions. In closing, we would like to encourage all those who have not yet had a chance to participate in a “Caparica Meeting” and remind all to participate in the 2nd edition of the International Caparica Conference in Antibiotic Resistance that will be held in Caparica on 12–15th June 2017 (<http://www.ic2ar2017.com>).

## AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# A Decade-Long Commitment to Antimicrobial Resistance Surveillance in Portugal

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## OPEN ACCESS

### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 18 June 2016

**Accepted:** 04 October 2016

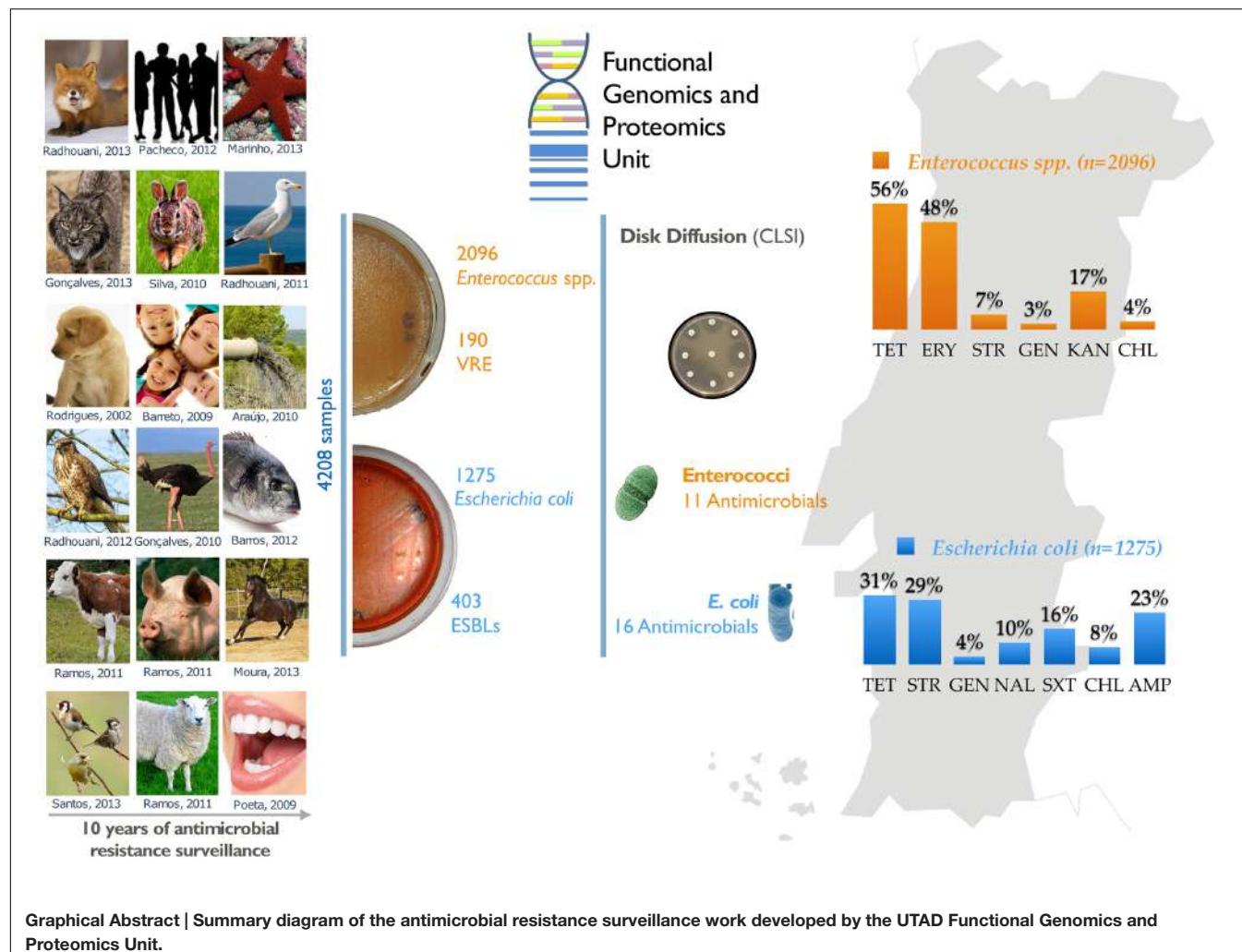
**Published:** 31 October 2016

### Citation:

Marinho CM, Santos T, Gonçalves A, Poeta P and Igrejas G (2016) A Decade-Long Commitment to Antimicrobial Resistance Surveillance in Portugal. *Front. Microbiol.* 7:1650.  
doi: 10.3389/fmicb.2016.01650

Antimicrobial resistance (AMR) is a worldwide problem with serious health and economic repercussions. Since the 1940s, underuse, overuse, and misuse of antibiotics have had a significant environmental downside. Large amounts of antibiotics not fully metabolized after use in human and veterinary medicine, and other applications, are annually released into the environment. The result has been the development and dissemination of antibiotic-resistant bacteria due to many years of selective pressure. Surveillance of AMR provides important information that helps in monitoring and understanding how resistance mechanisms develop and disseminate within different environments. Surveillance data is needed to inform clinical therapy decisions, to guide policy proposals, and to assess the impact of action plans to fight AMR. The Functional Genomics and Proteomics Unit, based at the University of Trás-os-Montes and Alto Douro in Vila Real, Portugal, has recently completed 10 years of research surveying AMR in bacteria, mainly commensal indicator bacteria such as enterococci and *Escherichia coli* from the microbiota of different animals. Samples from more than 75 different sources have been accessed, from humans to food-producing animals, pets, and wild animals. The typical microbiological workflow involved phenotypic studies followed by molecular approaches. Throughout the decade, 4,017 samples were collected and over 5,000 bacterial isolates obtained. High levels of AMR to several antimicrobial classes have been reported, including to  $\beta$ -lactams, glycopeptides, tetracyclines, aminoglycosides, sulphonamides, and quinolones. Multi-resistant strains, some relevant to human and veterinary medicine like extended-spectrum  $\beta$ -lactamase-producing *E. coli* and vancomycin-resistant enterococci, have been repeatedly isolated even in non-synanthropic animal species. Of particular relevance are reports of AMR bacteria in wildlife from natural reserves and endangered species. Future work awaits as this threatening yet unsolved problem persists.

**Keywords:** antimicrobial resistance, surveillance, molecular microbiology, enterococci, *Escherichia coli*, wildlife



Graphical Abstract | Summary diagram of the antimicrobial resistance surveillance work developed by the UTAD Functional Genomics and Proteomics Unit.

## INTRODUCTION

The early XX century witnessed the beginning of the modern antibiotic era with the first true antibiotic treatment, an asfernamina (Salvarsan) discovered by Paul Ehrlich that was successfully used to treat Syphilis (Elsner, 1910; Aminov, 2010). The reign of antibiotics in clinical practice is commonly associated with the discovery of penicillin produced by the fungus *Penicillium notatum* by Fleming (2001). However, the first wide use of antibiotics was with a sulfanamide antibiotic powder carried by Second World War soldiers with effectiveness against a wide range of infections (Davenport, 2012). The use of antibiotics to treat bacterial infections became one of the main scientific accomplishments; leading many scientists to believe that the threat of infectious diseases had ended (Jones, 1999). However, this golden age has come to an end in recent decades as we have become aware of the evolution of antibiotic resistance in bacterial strains, particularly in pathogens developing resistance to an extensive range of antibiotics (Davies and Davies, 2010).

Antibiotics are small secondary metabolites, either naturally produced by microorganisms or chemically synthetized,

to mediate competition among bacterial populations and communities (Allen et al., 2010; Cordero et al., 2012). Antibiotics are commonly found in the environment in sub-inhibitory concentrations diminishing the growth rate of competing populations rather than killing them. Natural or synthetic antibiotics can also act as signal molecules regulating expression of a large number of transcripts in different bacteria. Moreover, antimicrobial compounds might target self-regulation of growth, virulence, sporulation, motility, mutagenesis, stress response, phage induction, transformation, lateral gene transfer, intrachromosomal recombination, or biofilm formation (Goh et al., 2002; Yim et al., 2007; Baquero et al., 2013).

Currently, antibiotics available for consumption are produced either by microbial fermentation, semi- or full synthetically manufactured. Antibiotics cause bacterial death or inhibit growth by different mechanisms of action: (a) disrupting cell walls, e.g.,  $\beta$ -lactam and glycopeptides; (b) targeting the protein synthetic machinery, e.g., macrolides, chloramphenicol, tetracycline, linezolid, and aminoglycosides; (c) affecting the synthesis of nucleic acids, e.g., fluoroquinolones and rifampin; (d) inhibiting metabolic pathways, e.g., sulphonamides and folic

acid analogs; or (e) disrupting the membrane structure, e.g., polymyxins and daptomycin (Sengupta et al., 2013).

Antimicrobial resistance (AMR) contributes to the homeostasis of microbial populations and communities by modulating the effect of naturally produced antibiotics (Cordero et al., 2012; Baquero et al., 2013). Human misuse of antibiotics has unbalanced this natural genetic system of resistance (Osterblad et al., 2001; Xi et al., 2009). In some developed countries, livestock alone represent about 50–80% of antibiotics consumption; crops, pets and aquaculture collectively account for an estimated 5%, and human therapy the remainder (Cully, 2014). The use of antibiotics in human and veterinary medicine exerts a major selective pressure leading to the emergence and spread of resistance because about 30–90% of antibiotics used are not metabolized and are discarded basically unchanged (Sarmah et al., 2006). The period of time that an antibiotic takes to be degraded after release into the environment might influence how it spreads and accumulates. For instance, penicillins are easily degraded, while fluoroquinolones and tetracyclines persist for longer. Direct discharges from antibiotics industries into water effluents can be shockingly high, a reality in both developed and developing countries (Li et al., 2009; Joakim Larsson, 2014). Antibiotics are also released into the environment via agricultural application of manure and sewage water as fertilizers, and leakage from waste storage facilities (Sarmah et al., 2006; Le-Minh et al., 2010; Barrett, 2012).

The high concentration of antibiotics used prophylactically by humans might have led to contamination of human waste streams and exerted selection pressure on commensal and pathogenic bacteria. It is not surprising that non-metabolized antibiotics, resistance genes and mobile genetic elements are frequently detected in wastewaters and sewage treatment plants (Pellegrini et al., 2011). Often these compounds are not removed during sewage treatment to the extent that incidences of resistance genes are used as indicators of human impact on aquatic ecosystems (Zuccato et al., 2000; Araujo et al., 2010). Although antibiotics might be considered simply as chemical pollutants, genetic mobile elements are capable of self-replication and can be transmitted horizontally even between phylogenetically distant bacteria (Gillings and Stokes, 2012). Consequently, increased concentration of antibiotics in the environment raises the diversity and the abundance of resistance genes.

The consequences of antibiotic pollution are being actively examined, and there is an imperative need for novel strategies to curtail the release of antibiotics and bacteria from human sources (Baquero et al., 2013). Furthermore, the long-term consequences of spreading antibiotics and resistance genes cannot be predicted without quantitative analysis over suitable timescales (Chee-Sanford et al., 2009). The spread of AMR into the environment occurs not only through the contamination of natural resources but also through the food chain and direct contact between human and animals.

Currently, AMR has been reported for all antibiotic classes used both in human or veterinary medicine. An association between antibiotic use and clinical AMR proliferation has also been described (Skurnik et al., 2006; Pallecchi et al., 2008). AMR

is now considered a major challenge requiring urgent action in the present to prevent a worldwide catastrophe in the future.

Portugal boasts some of the most diverse fauna and flora in Europe, and in relation to its size is considered one of the 25 biodiversity hotspots of the world (Pereira et al., 2004). Its farming systems are also numerous and varied. However, Portugal is at high risk of losing this diversity. With the mission of long-term surveillance of AMR in bacteria from different sources, the Functional Genomics and Proteomics Unit, based at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal), has recently completed a decade of unceasing research. Over the years, we have investigated the resistomes of several bacteria, mainly commensal indicator bacteria such as enterococci and *Escherichia coli* sampled from the microbiota of different animals or from other sources. Here, we present an overview of the resistance profiles of these bacteria in relation to where they were found.

## AMR AS A PUBLIC HEALTH CONCERN

Antimicrobial resistance has become recognized as a major clinical and public health problem (Gillings, 2013). Every year about 25,000 patients perish in the European Union (EU) because of hospital-acquired resistant bacterial infection (ECDC, 2011). Likewise, in the United States of America (USA), infections caused by antibiotic resistant bacteria are more common among the population than cancer, with at least 2 million people infected every year, at least 23,000 of whom die (CDC, 2013). Besides the clinical consequences of AMR, there are also large societal costs. The number of new antibiotics coming to the market over the past three decades has dramatically declined as several pharmaceutical companies stopped searching for antibiotics in favor of other antimicrobial drugs (Spellberg et al., 2004; Walsh, 2013). When antimicrobial drugs are discovered and developed, it is imperative to understand and predict how resistance mechanisms might evolve in order to find a method to control dissemination (Walsh, 2013). Genome-scale research may offer insight into unknown mechanisms of AMR (Tenover, 2006; Rossolini and Thaller, 2010). This will then inform decisions on appropriate policies, surveillance, and control strategies. The ideal AMR surveillance system should be able to track long-term antibiotic resistance trends, regularly alert healthcare professionals to novel resistance profiles, identify emerging resistance patterns, and create an easy-access database for physicians and scientists (Jones, 1999). To avoid a crisis, governments must recognize the importance of this valuable resource and implement a wiser and more careful use of antibiotics, raising everyone's awareness of this issue.

Investigating the zoonotic AMR problem in its full complexity requires the collection of many types of data. Identifying the most efficient points at which intervention can control AMR mainly depends on quality collaboration between all the stakeholders involved (Wegener, 2012). The One Health concept is a universal strategy for improving connections between stakeholders in all aspects of health for humans, animals and the environment. The aim is that the synergism achieved between

interdisciplinary collaborations will push forward healthcare, accelerate biomedical research breakthroughs, increase the effectiveness of public health, extend scientific knowledge and improve clinical care. When properly implemented, millions of lives can be protected and saved in the near future (Pappaioanou and Spencer, 2008). The One Health initiative is therefore an ideal framework for addressing zoonotic transmission of AMR bacteria by monitoring and managing agricultural activities, food safety professionals, human and animal clinicians, environment and wildlife experts, while maintaining a global vision of the problem.

## RESISTOME

The antibiotic resistome defines the pool of genes that contribute to an antibiotic resistance phenotype (D'Costa et al., 2006). Bacterial populations have exquisite mechanisms to transfer antibiotic resistance genes by horizontal transfer, mainly in densely populated microbial ecosystems. The human gut, for instance, offers ample opportunities for bacteria of different provenance to share genetic material, including the many antibiotic resistance genes harbored by gut microflora (van Schaik, 2015).

Currently, there is urgent focus on the natural resistome. A resistome database was set up in Liu and Pop (2009) and contains information on about 20,000 genes reported so far. Conclusions from several metagenomics approaches are that those 20,000 genes are just a small portion of all resistomes, since other genes with different purposes not directly related to antibiotic resistance may be implicated (Gillings, 2013). Recently, a new bioinformatics platform has been developed, the comprehensive antibiotic research database (CARD), which integrates molecular and sequence data allowing a fast identification of putative antibiotic resistance genes in newly annotated genome sequences (McArthur et al., 2013). Environmental and human associated microbial communities have been shown to harbor distinct resistomes, suggesting that antibiotic resistance functions are mainly constrained by ecology (Gibson et al., 2015). However, to date, studies investigating the roles of antibiotics and AMR outside of the clinical environment are scarce. To know more about resistome composition and the dynamics among AMR genes, new studies should be performed on AMR communities in the environment.

## AMR SURVEILLANCE IN INDICATOR BACTERIA

Living organisms are defined by the genes they possess, while the control of expression of this gene set, both temporally and in response to the environment, determines whether an organism can survive changing conditions and compete for the resources it needs to reproduce. Changes to a bacterial genome are likely to threaten the microbe's ability to survive, but acquisition of new genes may enhance its chances of thriving by allowing growth in a formerly hostile environment. If a pathogenic bacteria gains resistance genes it is more likely to survive in

the presence of antimicrobials that would otherwise eradicate it, thus compromising clinical treatments. Bacterial genomes are dynamic entities evolving through several processes, including intrachromosome genetic rearrangements, gene duplication, gene loss and gene gain by lateral gene transfer, and several other chemical and genetic factors can trigger changes by locally altering nucleotide sequences (Lawrence, 2005). AMR strains emerge and spread as a combined result of intensive antibiotics use and bacterial genetic transfer, and the incidences of resistant bacteria from diverse habitats have been increasing (Coque et al., 2008).

Recently, several pathogens have been reported as being multidrug-resistant (MDR) and believed to be 'unbeatable' (Grundmann et al., 2006). Incidences of MDR strains of *Staphylococcus aureus*, *E. coli*, *Salmonella*, and *Enterococcus* are of international concern and have been reported thoroughly by global health organizations (ECDC, 2013; WHO, 2014). Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the main causes of AMR associated clinical infections. Resistant isolates are still being reported at a high rate, as in Portugal where MRSA is recovered from more than 50% of isolates from humans (ECDC, 2013). In 2011, the percentage of *E. coli* isolates resistant to third-generation cephalosporins, through production of extended-spectrum beta-lactamases (ESBLs), ranged from 3 to 36% in Europe. Over the last 4 years, MDR in *E. coli* has been reported to be on the increase (ECDC, 2013). Also on European territory, enterococci resistant to high-level aminoglycosides (25–50% of recovered isolates) and vancomycin (VRE) (less than 5% of isolates) have been reported with some countries noting an increase (ECDC, 2013). *Salmonella* are considered potential sources of zoonosis. MDR *Salmonella* strains are often recovered from human and animal isolates in Europe, with high incidence in children (ECDC, 2013).

Human and animal commensal gastrointestinal bacteria are continuously subject to diverse antimicrobial pressures (Levy and Marshall, 2004). Resistant strains may act as reservoirs of resistance genes that can easily be spread to pathogenic strains (Allen et al., 2013). The potential pathogens *E. coli* and enterococci are commonly used as indicators of the selection pressure and AMR evolution through the environment (Radhouani et al., 2014).

*Escherichia coli* are facultative anaerobic Gram-negative bacteria belonging to the *Enterobacteriaceae* family. As a commensal bacterium *E. coli* colonizes the gastrointestinal tract of humans and animals, but it is found ubiquitously in soil, plants, vegetables and water (van den Bogaard and Stobberingh, 2000). *E. coli* is a potential pathogen and the leading cause of human urinary tract infection, bacteraemia and gastroenteritis, among many other infections that require therapeutic intervention (Fluit et al., 2001; Kaper et al., 2004). *E. coli* has an outstanding capacity to acquire and transfer antibiotic resistance genes from or to other bacteria, which can later be disseminated from humans to animals and to the natural environment, and vice versa (Teuber, 1999). AMR *E. coli* strains, particularly those resistant to important antibiotics, have been increasing (Collignon, 2009).

Likewise, *Enterococcus* are commensal bacteria from the intestinal flora of humans and animals, and are frequently

monitored as indicators of fecal contamination of food products (Teuber and Perreten, 2000; Ramos et al., 2012). Enterococci are facultative anaerobes capable of persisting in extreme conditions caused by temperature and pH variations, dehydration, and oxidative stress. In the last two decades they have become dangerous nosocomial pathogens, persisting in hospital environments where selective pressure from the presence of antibiotics improves their resistome (Franz et al., 2011; Arias and Murray, 2012). Enterococci can cause urinary tract, wound and intra-abdominal infections, endocarditis, and bacteraemia. *Enterococcus faecalis* and *Enterococcus faecium* are the major species responsible for enterococci infections. Enterococci are intrinsically resistant to some antibiotics ( $\beta$ -lactams and aminoglycosides) and some species have specific AMR, such as *E. faecalis* to lincosamides and streptogramins A, and *Enterococcus gallinarum* and *E. casseliflavus* to vancomycin. They can also easily exchange resistance genes with other bacteria. Use of avoparcin, a glycopeptide antibiotic chemically similar to vancomycin, as an animal feed additive in Europe, and of high amounts of vancomycin in USA hospitals has led to the spread of VRE in animals, humans, food, and the environment (Kirst et al., 1998; Wegener, 1998). However, banning avoparcin in 2006 has not been sufficient to eradicate the AMR mechanisms present in bacteria and some VRE still persist in animals and the environment (Poeta et al., 2007b; Araujo et al., 2010; Ramos et al., 2012).

## COMPILED OF UTAD AMR SURVEILLANCE DATA

Antibiotic prescribing practices in the EU differ widely, and the introduction of standardization would be one way to monitor use more closely and possibly reduce the emergence of resistant bacteria. The antimicrobials market has been increasing with an average annual growth of 6.6% between 2005 and 2011. Worldwide demand for antibiotics is conjectured to reach about €34.1 billion in 2016. Currently, the market is dominated by aminoglycosides, which account for 79% of demand, while penicillins account for 8%, erythromycin 7%, tetracyclines 4%, and chloramphenicol 1%. Market expansion is expected to slow down to 4.6% in coming years (GR & DS, 2012). Between 2000 and 2010, consumption of antibiotic drugs increased by 36% (from 54 billion standard units to 73 billion standard units). The greatest increase was in low- and middle-income countries, but in general, high-income countries still use more antibiotics per capita (Van Boeckel et al., 2014). Portugal is in the top 10 European countries with the greatest consumption of antibiotics, at around 21–25 doses per 1,000 habitants each year (Hede, 2014). Nonetheless, exposure to antibiotics it is not only of antibiotic consumption through prescribed human treatments but also by their use in animal production. The antibiotic environmental contamination can contribute further to the increased emergence of resistance in pathogenic and environmental bacteria. The antibiotics used in animal production may be excreted directly into the environment, or accumulate in manure which could later be spread on land as fertilizer (Almeida et al., 2014). The

global total consumption of antibiotics in livestock was estimated in 2010 to be around 63,151 tons and it is projected to rise 67% by 2030. Increase driven by the growth in consumer demand for livestock products in middle-income countries and a shift to large-scale farms where antimicrobials are used routinely (Van Boeckel et al., 2014).

The Portuguese annual amount of antibiotics used in human and animal consumption was discussed in a report with data from 2010 to 2011. A first comparison of the antibiotic usage in human and veterinary medicine in Portugal indicates that two-thirds of the consumed antibiotics are used in veterinary medicine whereas the rest one-third in human medicine. For human medicine antibiotic prescription increased 4.5 tons (81.4–85.9 tons). In these two registered years, the annual amount of the different antibiotic groups was markedly larger for penicillins, which alone accounted for more than 65.0%, followed by quinolones (13.0%), macrolides (7.0%), cephalosporins (6.0%), and sulfonamides (5.0%). In veterinary medicine the annual amount of antibiotics sold was 179 tons in 2010 and 163 tons in 2011. Tetracyclines and penicillins were the most sold therapeutic solutions for the both years (Almeida et al., 2014).

Although it has been registered a decrease in the use of antibiotics in Portugal, their consumption and use is still high. Surveying AMR trends among bacteria is necessary to inform risk analyses and guide public policy. Over one decade at University of Trás-os-Montes and Alto Douro (UTAD), the antimicrobial profiles of indicator bacteria *E. coli* (Table 1) and enterococci (Table 2) have been studied in samples isolated from more than 75 different sources in Portugal. Figure 1 summarizes the data according to the four groups of sources from which samples were collected: humans, pets, food-producing animals, and wild animals.

The fecal samples used in all studies, either from Human or animal origin, were obtained following similar guidelines. The fecal samples (one per animal) were recovered from patients (in the case of human studies) and directly from the intestinal tract in the animal studies. Samples were transported in Cary-Blair medium from the place of recovery to the Centre of Studies of Animal and Veterinary Sciences (CECAV) facilities for processing. For *E. coli* selection, fecal samples were seeded onto Levine agar plates and incubated for 24 h at 37°C. One colony per sample with typical *E. coli* morphology was selected and identified by classical biochemical methods (Gram-staining, catalase, oxidase, indol, Methyl-Red, Voges-Proskauer, citrate, urease, and triple sugar iron), and by the API 20E system (BioMerieux, La Balme Les Grottes, France). As for the enterococcal isolation, fecal samples were sampled onto Slanetz-Bartley agar plates and incubated at 37°C for 24 h. One colony with typical enterococcal morphology were identified to the genus level by cultural characteristics, Gram staining, catalase test, and bile-aesculin reaction. Antibiotic susceptibility tests were made by the disk diffusion method following CLSI recommendations. 16 antibiotics were tested against *E. coli* isolates: ampicillin (10 mg), amoxicillin + clavulanic acid (20 mg + 10 mg), cefoxitin (30 mg), cefotaxime (30 mg), ceftazidime (30 mg), aztreonam (30 mg), imipenem (10 mg), gentamicin (10 mg), amikacin (30 mg), tobramycin (10 mg),

**TABLE 1 | Description of all sources of *Escherichia coli* isolates analyzed in a decade of antimicrobial resistance (AMR) surveillance in Portugal by the UTAD Functional Genomics and Proteomics Unit.**

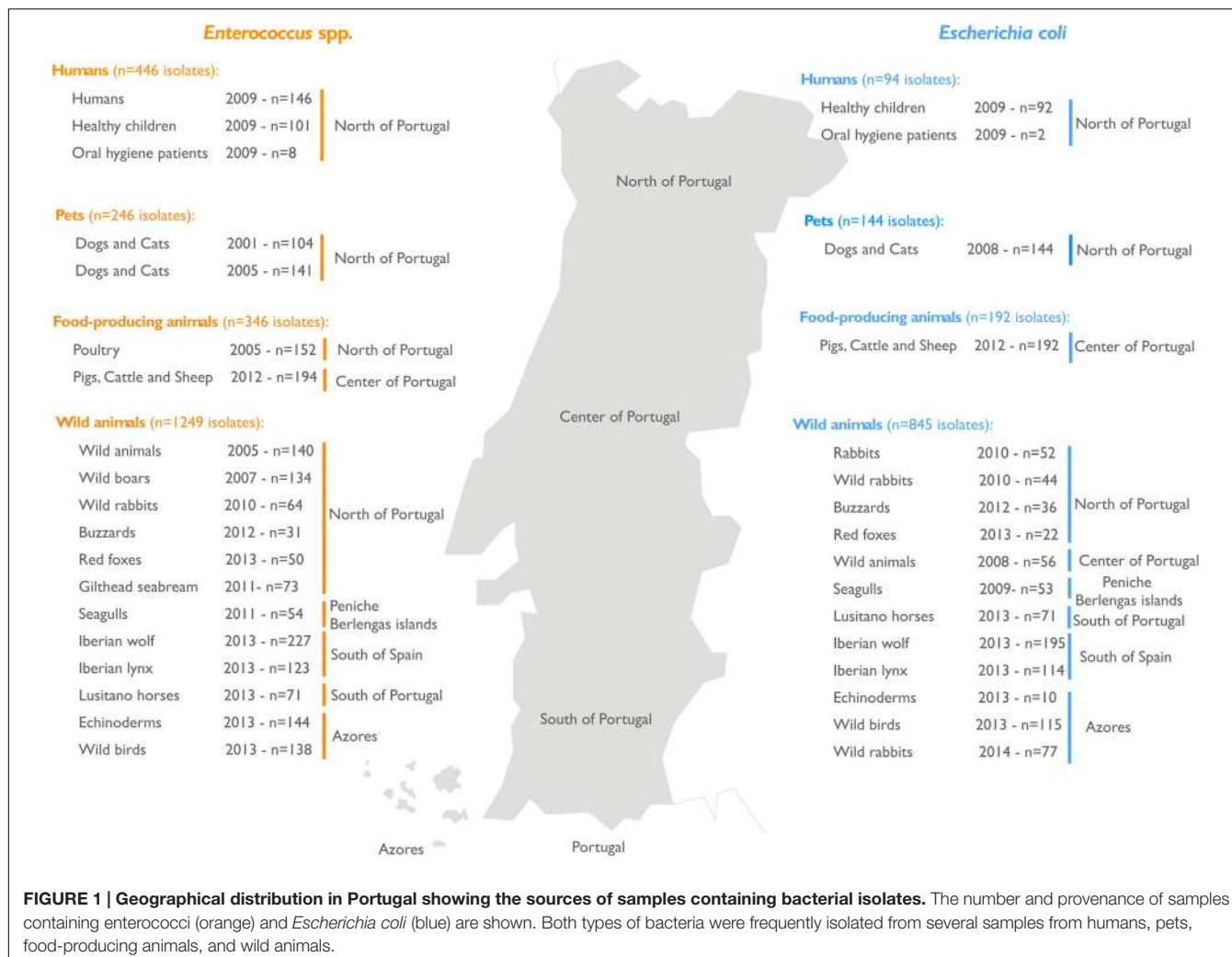
Sampling group	Sample source	Recovered samples (n = 1,841)	Recovered isolates (n = 1,275)	Reference
Pets	Dogs and cats	75	144	Costa et al., 2008
Humans	Children	118	92	Barreto et al., 2009
	Oral hygiene patients	46	2	Poeta et al., 2009
Food-producing animals	Pigs, cattle, and sheep	198	192	Ramos et al., 2012
Wild animals	Diarrheic rabbits	52	52	Poeta et al., 2010
	Wild rabbits	77	44	Silva et al., 2010
	Buzzards	42	36	Radhouani et al., 2012
	Red foxes	52	22	Radhouani et al., 2013
	Several wild animals	72	56	Costa et al., 2006
	Seagulls	53	53	Radhouani et al., 2009
	Lusitano horses	90	71	Moura et al., 2010
	Iberian wolf	237	195	Goncalves et al., 2013a
	Iberian lynx	27	18	Goncalves et al., 2012
	Iberian lynx	98	96	Goncalves et al., 2013b
	Echinoderms	250	10	Marinho et al., 2013
	Wild birds	218	115	Santos et al., 2013
	Wild rabbits	136	77	Silva et al., 2010

**TABLE 2 | Description of all sources of *Enterococcus* spp. isolates analyzed in a decade of AMR surveillance in Portugal by the UTAD Functional Genomics and Proteomics Unit.**

Sampling group	Sample source	Recovered samples (n = 2,730)	Recovered isolates (n = 2,287)	Reference
Pets	Dogs and cats	104	104	Rodrigues et al., 2001
	Dogs and cats	220	142	Poeta et al., 2005a
Humans	Humans	220	146	Poeta et al., 2005a
	Children	118	101	Barreto et al., 2009
	Oral hygiene patients	46	8	Poeta et al., 2009
Food-producing animals	Poultry	220	152	Poeta et al., 2005a
	Pigs, cattle, and sheep	198	194	Ramos et al., 2012
Wild animals	Wild animals	77	140	Poeta et al., 2005b
	Wild boars	67	134	Poeta et al., 2007b; Silva et al., 2010
	Wild rabbit	77	64	Silva et al., 2010
	Gilthead seabream	118	73	Barros et al., 2011
	Seagulls	57	54	Radhouani et al., 2011
	Buzzards	42	31	Radhouani et al., 2012
	Iberian wolf	237	227	Goncalves et al., 2013a
	Iberian lynx	30	27	Goncalves et al., 2011
	Iberian lynx	98	96	Goncalves et al., 2013b
	Echinoderms	250	144	Marinho et al., 2013
	Lusitano horses	90	71	Moura et al., 2010
	Red foxes	52	50	Radhouani et al., 2013; Santos et al., 2013
	Wild birds	218	138	Santos et al., 2013

streptomycin (10 mg), nalidixic acid (30 mg), ciprofloxacin (5 mg), sulfamethoxazole-trimethoprim (1.25 mg + 23.75 mg), tetracycline (30 mg), and chloramphenicol (30 mg). The identification of phylogenetic groups was performed as described by Clermont *E. coli* phlo-typing method (Clermont et al., 2000). In the case of enterococci, the isolates were tested for 11 antibiotics: [vancomycin (30 µg), teicoplanin (30 µg), Ampicillin (10 µg), streptomycin (300 µg), gentamicin (120 µg), kanamycin

(120 µg), chloramphenicol (30 µg), tetracycline (30 µg), erythromycin (15 µg), quinupristin-dalfopristin (15 µg), and ciprofloxacin (5 µg)], also by the disk diffusion method. Species identification was confirmed by polymerase chain reaction (PCR) using primers and conditions for the different enterococcal species. *E. coli* and enterococci isolates with resistance to one or more antibiotics were selected for the characterization of antibiotic-resistance genes. Their DNA was extracted and tested



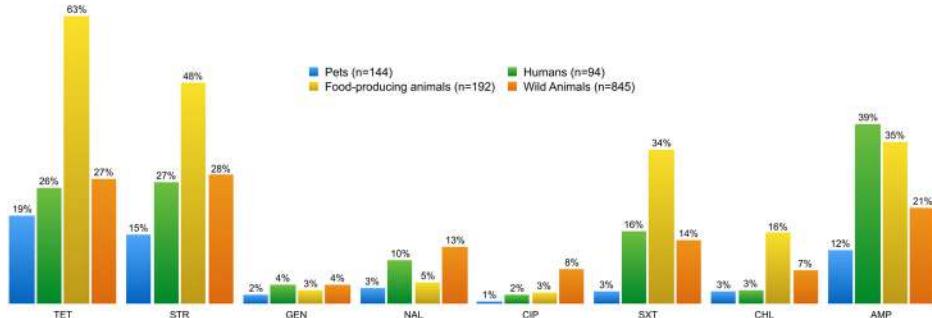
by PCR with specific primers already published. *E. coli* resistant isolates were screened for the following resistance genes: *blaTEM* and *blaSHV* (in ampicillin-resistant isolates); *tetA* and *tetB* (in tetracycline resistant isolates); *aadA*, *aadA5*, *strA*, and *strB* (in streptomycin-resistant isolates); and *sul1*, *sul2*, and *sul3* (in sulfa-methoxazole-trimethoprim-resistant isolates). Also, the presence of *intI*, *intI2* and *qacED + sul1* genes was analyzed by PCR in all sulfamethoxazole-trimethoprim-resistant isolates. On the other hand, enterococci resistant isolates were tested by PCR for detection of the following resistance genes: *erm(B)* and *erm(C)* (in erythromycin-resistant isolates), *tet(M)* and *tet(L)* (in tetracycline-resistant isolates), *aph(3')-IIIA* (in kanamycin-resistant isolates), *aac(6')-aph(2'')* (in gentamicin-resistant isolates), *ant(6)-Ia* (in streptomycin-resistant isolates), *vat(D)* and *vat(E)* (in quinupristin/dalfopristin-resistant isolates), and *van(A)* (in vancomycin-resistant isolates). Positive and negative controls were used in all PCR reactions, from the strain collection of the University of Trás-os-Montes and Alto Douro (Portugal).

In this collation of data, a total of 1,841 samples were collected and screened for *E. coli*. The overall *E. coli* recovery rate was 69.26% (Table 1), but rates were different among the different

sampling groups, ranging from 96.97% in fecal samples from food-producing animals down to 52.08% in fecal samples from pets. These are higher percentages than the *E. coli* recovery rate (51%) from humans, domestic and food-producing animal feces, or sewage and surface water in the USA (Sayah et al., 2005). A total of 2,539 samples were screened for enterococci, and a total of 2,096 enterococci isolates were obtained giving a recovery rate of 78.1% (Table 2). Rates of recovery from the four sampling groups were similar (75.93% for pet fecal samples, 66% for human samples, 82.78% for fecal samples from food-producing animals; and 88.39% for fecal samples from wild animals). These rates are very similar to previously reported rates (77%) of enterococci recovery from human, animal and environmental samples in several European countries (Kuhn et al., 2003).

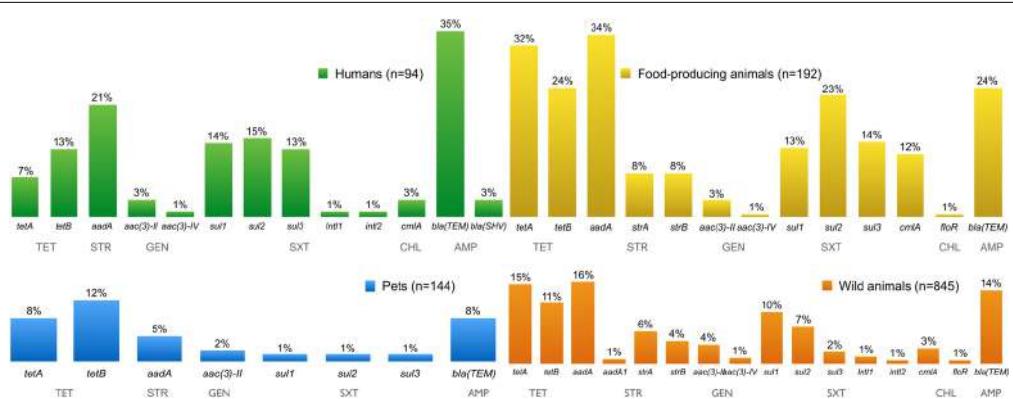
## Escherichia coli DATA DESCRIPTION

Phenotypic and genotypic studies were used to assess the AMR profile of the isolates. Figures 2 and 3 summarize the results of tests for antibiotic resistance and identification of



**FIGURE 2 | Percentage of phenotypic antibiotic resistance detected in enterococci and *E. coli* isolates from each of the four sampling groups.**

The resistance profile of enterococci from food-producing animals is distinct from those of the other sampling groups, showing the highest resistance to tetracycline, streptomycin, trimethoprim-sulfamethoxazole, and chloramphenicol. Enterococci from pets displayed the lowest resistance profile to all tested antibiotics. TET, tetracycline; STR, streptomycin; GEN, gentamicin; NAL, nalidixic acid; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; AMP, ampicillin.

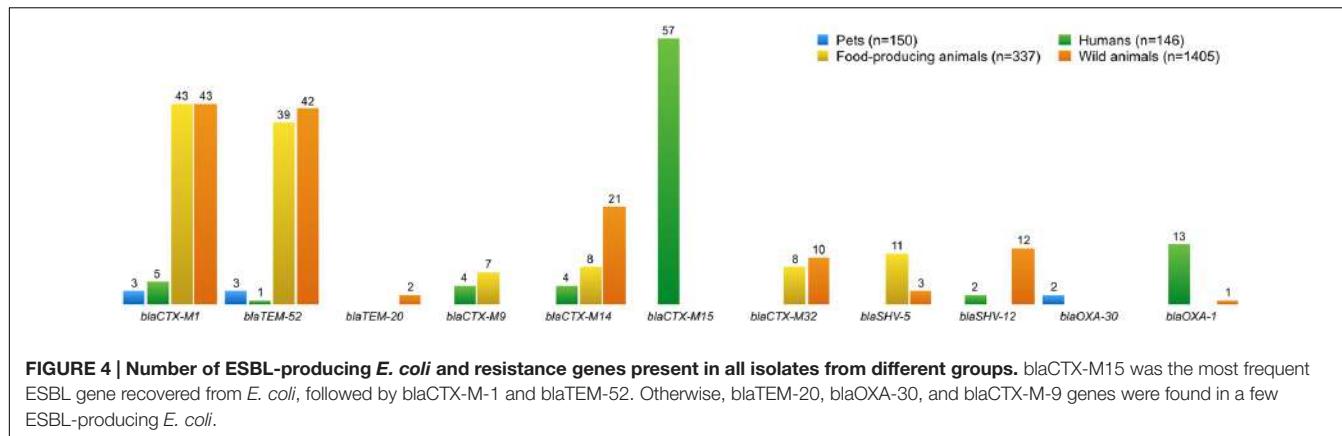


**FIGURE 3 | Percentage of antimicrobial resistance (AMR) genes detected in *E. coli* resistant isolates from all sources over a decade.**

associated resistance genes in *E. coli* isolates, comparing the four different sample groups described in **Table 1**. Resistance to all the antibiotics tested was found among the samples. Remarkably, *E. coli* from food-producing animals showed higher rates of resistance to tetracycline, streptomycin, trimethoprim-sulfamethoxazole, and chloramphenicol than *E. coli* from humans (**Figure 2**). Indeed fecal *E. coli* isolates recovered from food-producing animals all across Europe have shown a high prevalence of resistance to these antimicrobial agents (Guerra et al., 2003; Enne et al., 2008; Lapierre et al., 2008). Despite the ban on chloramphenicol use in farming in Europe since 1994, food-producing animals still carry resistant strains. Regular and multipurpose use of antibiotics, dissemination of resistant bacteria via fecal contamination and intensive animal production have turned husbandry facilities into important reservoirs of AMR bacteria (van den Bogaard and Stobberingh, 2000). High levels of resistance to tetracycline and streptomycin have been found in bacteria from wild animals. The high prevalence of tetracycline resistance in either *E. coli* or enterococci found in different wild animal species might be related to the recurrent use of this antibiotic in veterinary medicine. The same may be true of the prevalence of resistance to erythromycin, ampicillin, aminoglycosides, and quinolones. One of the main routes of

transmission of resistant strains and resistant genes has been shown to be through the food chain (Aarestrup et al., 2008). Antibiotics can be administered to animals to treat (therapy) or prevent (prophylaxis or metaphylaxis) illness and in the past were also used to promote animal growth. Several classes of antimicrobial agents are frequently used in veterinary medicine. A 2013 report showed that in 2011 in 25 countries from the EU/European Economic Area, tetracyclines (37%) were the most sold antimicrobial class for administering to food-producing species, followed by penicillins (23%), sulfonamides (11%), macrolides (8%), lincosamides (2.9%), aminoglycosides (2%), trimethoprim (1.6%), and fluoroquinolones (1.6%) (European Medicines Agency, 2013). Antimicrobial use in veterinary medicine and AMR in commensal *E. coli* from various food-producing animals has indeed been correlated (Chantziaras et al., 2014). Ampicillin is a β-lactam used as a farm animal growth promoter (Sayah et al., 2005). *E. coli* from human samples differ from *E. coli* from other sampling groups in terms of their ampicillin resistance, which is of particular importance considering that ampicillin is a first-line antibiotic commonly effective against a broad spectrum of pathogens.

Comparing AMR *E. coli* from independent sources, several genes conferring AMR were found in resistant isolates (**Figure 3**).



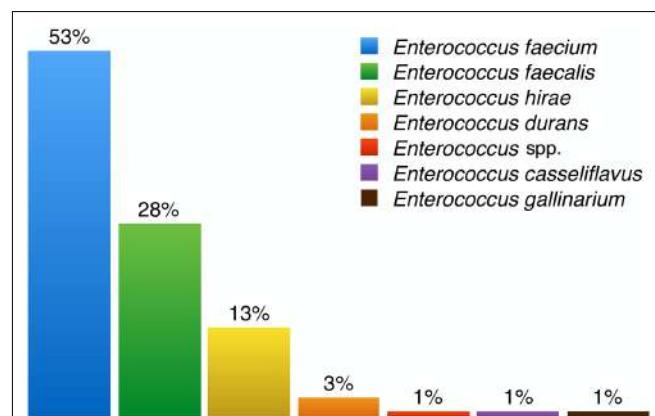
**FIGURE 4 | Number of ESBL-producing *E. coli* and resistance genes present in all isolates from different groups.** *blaCTX-M15* was the most frequent ESBL gene recovered from *E. coli*, followed by *blaCTX-M-1* and *blaTEM-52*. Otherwise, *blaTEM-20*, *blaOXA-30*, and *blaCTX-M-9* genes were found in a few ESBL-producing *E. coli*.

Fourteen different *tet* genes have been reported in Gram-negative bacteria, encoding proteins active in the three mechanisms of tetracycline resistance, namely an efflux pump, ribosomal protection, or direct enzymatic inactivation of the antibiotic. The resistance genes *tetA* and *tetB* confer resistance to tetracycline, and were found at similar frequencies in all different sources of resistant *E. coli*. However, *tetA* and *tetB* are more likely to be associated with tetracycline resistance in *E. coli* isolated from human and animal samples (Bryan et al., 2004).

The streptomycin resistance genes *aadA*, *strA*, *strB*, and *aadA1* were found, in descending order of frequency, in resistant *E. coli*. The *strA* and *strB* genes encode streptomycin-inactivating enzymes which confer streptomycin resistance in many Gram-negative bacteria distributed worldwide. The *aadA* gene encodes an adenyllylation enzyme that modifies streptomycin and, in the *aadA1* gene cassette, is often found in streptomycin-resistant *E. coli* strains from human, animal, and food samples (Saenz et al., 2004).

Gentamicin resistance genes *aac(3)-II* and *aac(3)-IV* were present in resistant *E. coli* isolates. Trimethoprim-sulfamethoxazole-resistant *E. coli* isolates had *sul1*, *sul2* and/or *sul3* genes, and the integrons *intI1* and *intI2* genes, encoding class 1 and class 2 integrases, respectively. The *sul1*, *sul2*, and *sul3* genes each encode dihydropteroate synthases to confer plasmid-mediated resistance to sulphonamides (Perreten and Boerlin, 2003; Skold, 2010). The *sul1* gene is frequently found linked to other resistance genes in class 1 integrons, while *sul2* is normally located on small plasmids of the *incQ* incompatibility group, or other small plasmids represented by pBP1 (Skold, 2010). The *sul1* and *sul2* genes have been regularly found at similar frequencies among sulphonamide resistant Gram-negative clinical isolates. However, an increase in prevalence of the *sul2* gene has been observed. The association between *sul2* and multiresistance plasmids, which may be co-selected through the use of other antimicrobial agents, might explain the increased prevalence (Blahna et al., 2006; Frank et al., 2007).

Chloramphenicol resistant *E. coli* isolates have the resistance genes *cmlA* and *floR*, both coding efflux pumps that reduce the intracellular concentration of antibiotics by exporting different



**FIGURE 5 | Percentages of enterococci species isolated from samples.** *Enterococcus faecium* is the most frequently isolated enterococci species among all recovered samples and *Enterococcus gallinarum* the least.

molecules (Saenz et al., 2004). Some infections are difficult to treat because of bacterial expression of multidrug efflux systems causing resistance. ESBLs confer resistance to penicillins and most cephalosporins, including third-generation cephalosporins, and more than 700 different ESBLs have now been described (van Duijn et al., 2011). A total of 403 isolates of ESBL-producing *E. coli* were obtained from all four sampling groups: humans ( $n = 138$ ), pets ( $n = 8$ ), food-producing animals ( $n = 116$ ), and wild animals ( $n = 141$ ). Figure 4 shows all genes encoding ESBLs that were detected in these isolates. The most prevalent ESBL genes were *blaCTX-M-1* ( $n = 94$ ) and *blaTEM-52* ( $n = 85$ ), specifically isolated from *E. coli* from food-producing and wild animals, followed by the *blaCTX-M-32* ( $n = 57$ ) gene found in isolates from human samples. The mechanisms of resistance against one antibiotic might not be exclusive, and activity may be exhibited against other similar compounds. For instance, it has been proven that mutations in the genes encoding penicillin-inactivating enzymes (e.g., TEM and SHV) can also confer resistance to third-generation cephalosporins or  $\beta$ -lactamase inhibitors (Martinez et al., 2007).

## ENTEROCOCCI DATA DESCRIPTION

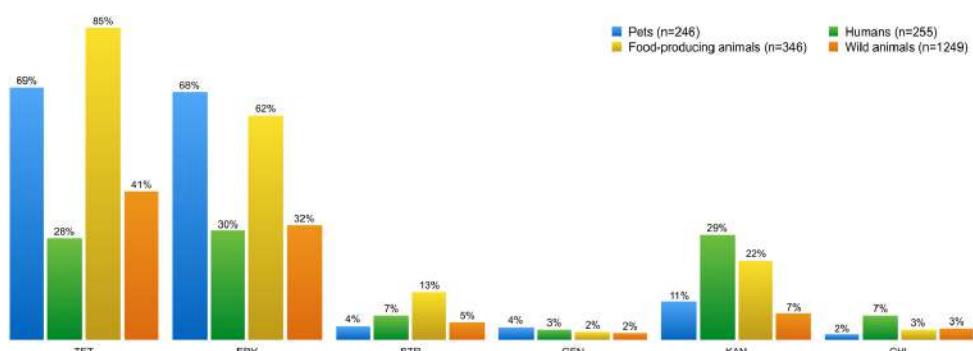
Several species of enterococci were recovered from different environments, with some being more common than others depending on the source of the sample. **Figure 5** summarizes all data on enterococci species prevalence reported in the publications listed in **Table 2**. *E. faecalis* is the most common enterococcal species in human stools and the most frequent cause of clinical infection. The natural resistance of this species to penicillin, cephalosporins, and quinolones has contributed to its emergence as a cause of nosocomial infection. However, *E. faecium* is inherently more antibiotic-resistant and has also gradually become a major hospital pathogen (French, 2010; Radimersky et al., 2010). Overall from all the enterococci species we isolated, *E. faecium* was by far the most frequently recovered (52.9%), followed by *E. faecalis*, *E. hirae*, *E. durans*, *E. casseliflavus*, and *E. gallinarum* (**Figure 5**).

**Figures 6 and 7** show the collated antibiotic resistance data of enterococci isolates from the different sources listed in **Table 2**. Remarkably, more enterococci from the group

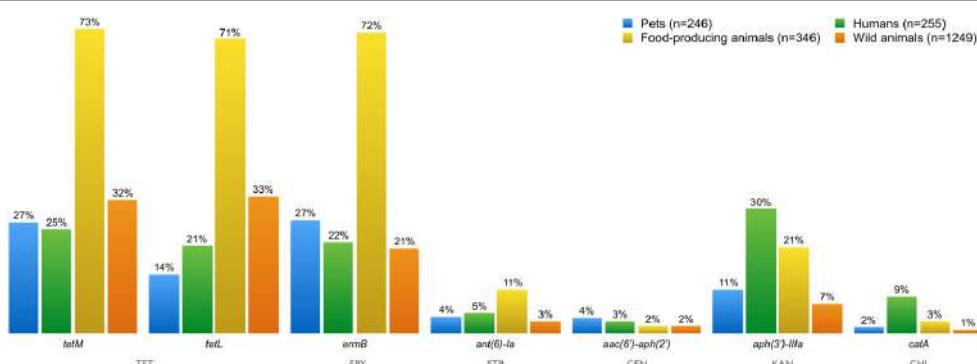
of food-producing animals were resistant to tetracycline and erythromycin than from the human group (**Figure 6**). Many enterococci resistant to tetracycline and erythromycin were also found in the wild animals group. Enterococci resistant to kanamycin and chloramphenicol were more frequent in samples from humans than from other sources.

The distribution of AMR genes in the different sampling groups is illustrated in **Figure 7**. The *tetM* and *tetL* genes are associated with the tetracycline-resistance phenotype in enterococci. Tetracycline resistance due to TetL efflux proteins has been becoming more frequently reported among Gram-positive bacteria from animal sources (Figueiredo et al., 2009; Radhouani et al., 2010).

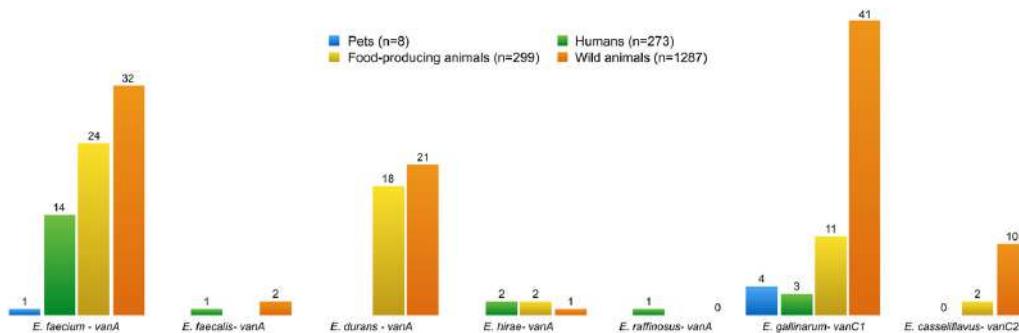
Erythromycin-resistance in enterococci is usually associated with the presence of the *ermB* gene. The *ermB* and *tetM* genes have been frequently identified together in the same isolate, probably because they both occur in the mobile conjugative transposon *Tn1545*, prevalent in clinically important Gram-positive bacteria (De Leener et al., 2004). The resistance genes *ant(6)-Ia*, *aac(6')-aph(2')*, *aph(3')-IIIA* and *catA* were present in enterococci isolates resistant to streptomycin, gentamicin,



**FIGURE 6 | Percentage of phenotypic antibiotic resistance detected in *Enterococcus* spp. isolates from each of the four sampling groups.** Enterococci isolates showed high resistance levels to tetracycline and erythromycin, regardless of the source. However, food-producing animals and pets stand out from the other sampling groups. Streptomycin, kanamycin, and chloramphenicol-resistant enterococci are more frequent in samples from humans than from other sources. TET, tetracycline; ERY, erythromycin; STR, streptomycin; GEN, gentamicin; KAN, kanamycin; CHL, chloramphenicol.



**FIGURE 7 | Percentage of AMR genes detected in resistant Enterococci isolates from all sources over a decade.** Resistance genes *tet(M)/tet(L)*, *erm(B)*, *ant(6)-Ia*, *aac(6')-aph(2')*, *aph(3')-IIIA*, and *catA* were present in enterococci isolates resistant to tetracycline, erythromycin, streptomycin, gentamicin, kanamycin, and chloramphenicol, respectively. TET, tetracycline; ERY, erythromycin; STR, streptomycin; GEN, gentamicin; KAN, kanamycin; CHL, chloramphenicol.



**FIGURE 8 | Number of vancomycin-resistant enterococci species, and resistance genes present in all isolates from different groups.** *E. gallinarum* and *E. casseliflavus* are intrinsically resistant to vancomycin, and harbor the genes vanC1 and vanC2, respectively, in their genome.

kanamycin, and chloramphenicol, respectively. Resistance to all tested antibiotics has been showed in isolates from all groups.

The presence of VRE isolates was noted in all four groups (**Figure 8**): humans ( $n = 21$ ), pets ( $n = 5$ ), food-producing animals ( $n = 57$ ), and wild animals ( $n = 107$ ). The presence of VRE isolates in diverse samples from Portugal is likely to be related to the use of avoparcin to promote growth of food animals in Europe since the 1970s until its prohibition in 2006. VRE was first characterized in Uttley et al. (1988), and has been repeatedly isolated since then from several sources. Many antimicrobials used in food animals belong to the same classes as those used in humans, leading to concerns about cross-resistance (Furuya and Lowy, 2006). For instance, vancomycin is still the most widely used antibiotic to treat serious infections like MRSA. Vancomycin resistance has emerged in *S. aureus* through acquisition of the *vanA* gene from VRE (Chang et al., 2003). It is important therefore to highlight the higher number of vancomycin resistant determinants found in wild animals compared to humans. In Europe, spread from animals to humans appears to have occurred outside hospital facilities, which explains why VRE is more commonly found in the environment than in the community (Furuya and Lowy, 2006).

## CONCLUSION

Generalized use of antibiotics by humans is a recent addition to the natural and ancient process of antibiotic production and resistance evolution that still occurs on a global scale in the soil (Woolhouse et al., 2015). The main attributed cause of the rise and spread of AMR is the extensive clinical use of antibiotics in both human and veterinary medicine (Levy, 2001). However, antibiotic use in veterinary medicine alone does not explain the spread and persistence of AMR bacteria in animal populations in which the antibiotic use is discontinued or else is very sporadic or limited like in wildlife. AMR does not have borders, so it can freely cross through several populations, and homologous resistance genes have been reported in bacteria from pathogens, normal flora and soil. Farm animals are a significant factor in this system and they are still exposed to

large amounts of antibiotics and act as reservoirs of many resistance genes. Other risk factors such as antibiotic residues in the environment and AMR gene dissemination in different human and animal populations also have an impact on the AMR levels (Alali et al., 2009). We are currently facing the potential loss of antimicrobial therapy, so it is essential to continue tracking AMR.

## FUTURE PERSPECTIVES

Human use of antibiotics for medicine and agriculture may have consequences far beyond their intended applications. The environment is a major reservoir of resistant organisms and antibiotic drugs freely circulating; though not much is known about the antibiotic resistome, shading their hypothetical impact on clinically important bacteria (Allen et al., 2010). Several resistance genes have been reported in distinct bacterial species, from diverse sources (Poeta et al., 2007a; Pallecchi et al., 2008; Gillings and Stokes, 2012). Nonetheless, more studies using standardized methods are required to recognize roles and patterns of antibiotic resistance genes in microbial communities. In the meantime, it is important to moderate the use of antimicrobials and restrain the rise and dissemination of AMR bacteria, so the management of emerging zoonotic diseases and their impacts might be forecasted (Allen et al., 2010). The foreseen decline in antibiotics effectiveness and the current lack of new antimicrobials on the horizon to replace those that become ineffective brings added urgency to the protection of the efficacy of existing drugs (Carlet, 2012; WHO, 2014). Meanwhile, omics approaches to generate molecular data on AMR mechanisms will continue to be the foundation of our work at the Functional Genomics and Proteomics Unit at UTAD.

## AUTHOR CONTRIBUTIONS

CM and TS wrote the manuscript. AG, PP, and GI helped interpret compiled data. GI conceived the review. All the authors reviewed and contributed to the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# A Prescription for Resistance: Management of Staphylococcal Skin Abscesses by General Practitioners in Australia

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## OPEN ACCESS

### Edited by:

José Luis Capelo,  
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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 30 January 2016

Accepted: 11 May 2016

Published: 06 June 2016

### Citation:

Parrott C, Wood G, Bogatyreva E, Coombs GW, Johnson PDR and Bennett CM (2016) A Prescription for Resistance: Management of Staphylococcal Skin Abscesses by General Practitioners in Australia. *Front. Microbiol.* 7:802.  
doi: 10.3389/fmicb.2016.00802

**Objectives:** We investigated the management of staphylococcal abscesses (boils) by general practitioners (GPs) in the context of rising antibiotic resistance in community strains of *Staphylococcus aureus*.

**Design, Setting, Participants:** We analyzed patient-reported management of 66 cases of uncomplicated skin abscesses from the frequency matched methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) Community-Onset *Staphylococcus aureus* Household Cohort (COSAHC) study (Melbourne, Australia, 2008–2012). Susceptibilities in all cases were known: 50/66 abscesses were caused by MRSA. In order to investigate GP-reported management of staphylococcal abscesses, we surveyed a random subset of GPs, from the COSAHC study (41), and of GPs (39) who used the same community-based pathology service (December 2011–May 2012).

**Main outcome measures:** Patient outcomes, antibiotics prescribed, antibiotic resistance profiles of infecting strains, rates of incision and drainage (I&D), and attitudes to ordering microbiological cultures.

**Results:** MRSA was three times more likely to be cultured from an abscess than MSSA. Patient-reported management revealed 100% were prescribed antibiotics and only 60.6% had I&D. Of those 85% who remembered their prescription(s), 81% of MRSA cases and 23% of MSSA cases initially received inactive antibiotics. Repeat GP visits where antibiotics were changed occurred in 45 MRSA and 7 MSSA cases, although at least 33% of subsequent prescriptions were inactive for the MRSA infections.

Patients treated with I&D and antibiotics did no better than those treated with only I&D, regardless of the antibiotic activity. In the GP surveys, 89% reported I&D, with or without antibiotics, to be their preferred management. Only 29.9% of GPs would routinely swab abscesses.

**Conclusion:** The recommended management of uncomplicated *Staphylococcus* abscesses is I&D without antibiotics to reduce exposure to unnecessary antibiotics. In our study, I&D was performed in only 60.6% of 66 patients, and antibiotics were always prescribed. The prescribed antibiotics were frequently inactive and often changed, and did not appear to affect patient recovery. Our results show that community GPs can confidently reduce their use of antibiotics for patients with skin abscesses and should be aware that MRSA is a much more common in this type of infection.

**Keywords:** antibiotic resistance, *Staphylococcus aureus*, skin and soft tissue infections, community medicine, boils and abscesses

## INTRODUCTION

Little is known about Australian general practitioners' (GPs) management protocols in practice for skin abscesses and how this might be evolving with the changing epidemiology of *Staphylococcus aureus*. Australian Therapeutic Guidelines: Antibiotic (version 15; Antibiotic Expert Groups, 2014) recommends *uncomplicated* abscesses should be managed with incision and drainage (I&D) alone – without antibiotics. Previous guidelines (Dermatology Expert Groups, 2009) available to GPs at the time of our study, also recommended only I&D for abscesses <5 cm. Antibiotics were only recommended if there was associated spreading cellulitis or systemic symptoms.

After a 2011 review, clinical practice guidelines from the Infectious Diseases Society of America (IDSA) continued to recommend I&D; specifically, that effective treatment requires incision, thorough evacuation of pus, and probing the cavity to break up loculations (Liu et al., 2011; Moran et al., 2013). IDSA practice guidelines are similar to the Australian Therapeutic Guidelines for primary treatment (CDC, AMA, IDSA, 2007; Lowy et al., 2008; Liu et al., 2011; Centers for Disease Control and Prevention [CDC], 2013; Moran et al., 2013). However, they differ in their recommended first line antibiotics in cases where antibiotics are indicated, due to higher rates of community-acquired MRSA (CA-MRSA) in the United States. The IDSA guidelines recommend clindamycin, trimethoprim-sulfamethoxazole, a tetracycline (doxycycline/minocycline) and linezolid (Liu et al., 2011). In contrast, the recommended antibiotics for complicated abscesses in the Australian Therapeutic Guidelines are di-/flucloxacillin or, in penicillin-allergic patients, cephalexin. In penicillin hypersensitive patients, the recommendation is to use clindamycin or trimethoprim-sulfamethoxazole. Antibiotics are to be modified based on culture results (Antibiotic Expert Groups, 2014).

We have calculated that in 2006, 3–5% of patients presenting with community-onset *S. aureus* infections in Melbourne had an MRSA infection (Bennett et al., 2014). The preliminary findings of the COSAHC study suggest this has risen to 8–10% by 2010, and may be higher again when aggressive pyogenic soft tissue *S. aureus* infections have been investigated, showing MRSA the more likely causative organism (Jahamy et al., 2008; del Giudice et al., 2009; Coombs et al., 2014). This study will show that CA-MRSA is three times more likely than MSSA to be the cultured organism in skin abscesses.

With CA-MRSA increasing in prevalence in Australia and elsewhere, there may be an argument for all lesions to be swabbed for microbiological culture and sensitivity (MC&S), not only to tailor treatment (as in complicated infections) but also for MRSA surveillance (CDC, AMA, IDSA, 2007; Lowy et al., 2008; Dermatology Expert Groups, 2009; Liu et al., 2011; Centers for Disease Control and Prevention [CDC], 2013; Antibiotic Expert Groups, 2014). However, for uncomplicated abscesses, if, as is recommended, I&D is routinely and correctly used, (thorough evacuation of pus, and probing the cavity to break up loculations) and antibiotics are *not* used, then swabs may have little impact on individual care (Parnes et al., 2011). Community GPs may not realize that CA-MRSA is the most likely organism to cause uncomplicated abscesses. Further over prescription may occur with the return of a culture and sensitivity report, indicating the prescribed (unnecessary) antibiotic is inactive, which may drive an antibiotic change, resulting in even more ineffective antibiotics as well as unnecessary visits to the GP.

Our study set out to describe the management by community GPs of staphylococcal skin abscesses so we can better understand how often I&D is performed, if and what types of antibiotics are used, and whether antibiotics affect patient recovery. We also wanted to understand how often and for what reason GPs send swabs for MC&S.

## MATERIALS AND METHODS

### Patient Data

Community-Onset *Staphylococcus aureus* Household Cohort is a longitudinal cohort study of 291 index patients with community-onset *S. aureus* infections, and 446 household contacts. Index patients were recruited through a large private pathology provider on the basis of a positive *S. aureus* MC&S result for their infection. The pathology provider serves the entire Melbourne metropolitan area. All eligible patients with community-onset MRSA infections were invited to participate together with a frequency-matched subset of eligible patients with MSSA infections. The COSAHC project was approved by the Deakin University Human Research Ethics Committee (project number 2009-162).

Shared households (204) were followed up at 3-monthly intervals for up to 2 years (recruitment period 2008–2011). At each visit, index patients and household members had swabs obtained from nares and axillae for *S. aureus* carriage

to determine the molecular epidemiology of strains circulating within households. Questionnaires were completed to provide information on the medical management and outcome of the index cases' infection as well as household interactions, infection history, new infections and risks for *S. aureus* transmission and MRSA carriage.

Of the 291 index patients, 137 had MRSA infections and 154 had MSSA infections. The majority were skin and soft tissue infections (86.2%), and 66 of the index patients had skin abscesses (50 MRSA, 16 MSSA) and are included in the present analysis. We extracted data on the doctor-reported data management (when remembered) as well as patient-reported management and outcomes for these 66 skin abscess infections; including whether I&D was performed or if and what type of antibiotics were prescribed. We also established the resistance profile of the infecting strain, and the number of days off normal activities and timing of infection resolution.

## Doctor Survey Data

To understand the clinical decisions behind treatment practices, we conducted a cross-sectional survey of community GPs on their management protocols for *S. aureus* abscesses using a constructed case study (see below). Participating doctors were from one of two groups, recruited by telephone and fax from December 2011 to May 2012. The survey tool asked a series of questions on their treatment of this hypothetical case, and decisions to swab.

### Case Study Extract

A patient presents to your clinic with a boil in their right armpit. Over the course of 3 days, the area has become increasingly reddened, tender and the center is raised and now forms a pus-filled head. There are no signs of systemic infection but there is evidence of localized infection. The patient has no other health problems and is not on any medication.

1. With this history, how would you treat the patient? (Select 1 or more)
  - (a) No treatment
  - (b) Incision and drainage (I&D)
  - (c) I&D and antibiotics
  - (d) Antibiotics only
  - (e) Other (please specify)
2. If this boil was weeping or lanced\*, would you swab this boil for culture and if so why?

Followed by a series of multiple choice and open ended questions on treatment choices and motivators for, and frequency of, collecting swabs.

\*Lanced used interchangeably with incision

## Group 1: Doctors Who Ordered the Baseline Tests on Patients Who Were Recruited to the COSAHC Study

We surveyed a random sample of 41 doctors treating community-onset *S. aureus* infections that had previously been studied in COSAHC. A subset of these doctors had treated eight COSAHC- patients who had an abscess as their index infection, allowing us the additional opportunity to directly compare GP-reported management protocols from the survey to the real life patient-reported management in these cases.

## Group 2: Comparative GP Sample

To determine how representative COSAHC doctors' patient management was, we compared their survey responses to those of a random sample of 39 GPs practicing within metropolitan Melbourne who had ordered routine blood tests (Full blood count and Urea and Electrolytes) in the same period from the same pathology provider.

Both groups of doctors were invited to return a survey tool by fax or complete over the telephone. The exclusion criteria were being on leave for more than one month at the time approached and/or no longer working at the practice. A sample size of 40 per group was recruited to provide 80% power ( $\alpha = 0.05$ ) to detect a 30% difference in antibiotic use between GPs.

The survey tool was developed to assess management protocols and swabbing practice in the context of a short case study of a patient with an axillary skin abscess. We also asked participants to provide information on any change to their practice over the previous three years.

## Analysis

Descriptive statistics were applied, including computing differences between proportions and the associated  $p$ -values and 95% confidence intervals (CIs) using StataSE12. We report median and inter-quartile range (IQR) for all non-parametric data.

## RESULTS

### COSAHC Participants Report of GP Management of Their Lesion

Abscess management and outcome information was obtained from the 66 index patients at the first COSAHC household visit. The susceptibilities of the organisms were known as the patients were recruited on the basis of a positive *S. aureus* MC&S result. The first household visit occurred at an average of 4 months from the onset of index infection. For the total COSAHC study, the ratio of MSSA:MRSA in the index patients' infections was 154:137. Within this abscess subset, the ratio was 16:50. This indicates that in the setting of an abscess, MRSA is much more common than background rates in community onset infections. The average age of the abscess patients was 34.4 years (95% CI 29.3, 39.5), 48% were female (95% CI 37, 60), with an average household size of 2.7 (95% CI 2.25, 3.14). Nearly half (44% (95% CI 33, 56)) reported a history of previous skin abscesses. Abscess site varied (Table 1), however, leg/foot was the most common (over 30%). Torso (24%) ranked second for MRSA abscesses, while torso and arm/hand were equal second for MSSA abscesses (19%).

Abscess management by the GPs, as reported by COSAHC study patients, are shown in Table 2 with index infections stratified by methicillin resistance. I&D was performed in 60.6% of cases, but never in isolation. Antibiotics were prescribed for 100% of cases, however, two patients reported not taking the antibiotics.

Patient-reported infection resolution is shown in Table 3. Of those 40 patients who had I&D as part of their treatment, the

**TABLE 1 | Infection sites.**

Site of infection	MSSA (n = 16) (%, CI)	MRSA (n = 50) (%, CI)
Leg/foot	31% (11, 59)	34% (21, 49)
Torso (front/back)	19% (4, 46)	24% (13, 38)
Arm/hand	19% (4, 46)	12% (5, 24)
Axilla	13% (2, 38)	12% (5, 24)
Head/neck	13% (2, 38)	8% (2, 19)
Groin	6% (0.2, 30)	10% (3, 22)

**TABLE 2 | Patient-reported GP abscess management by methicillin resistance (causal organism).**

Treatment	MSSA (n = 16) (%, CI)	MRSA (n = 50) (%, CI)	Total (n = 66) (%, CI)
I&D and antibiotics	38% (15, 65)	36% (25, 53)	36% (25, 49)
I&D only	0%	0%	0%
Antibiotics only	25% (7, 52)	30% (18, 45)	29% (18, 41)
I&D, antibiotics and other*	25% (7, 52)	24% (13, 38)	24% (15, 36)
Antibiotics and other*	13% (2, 38)	10% (3, 22)	11% (4, 21)

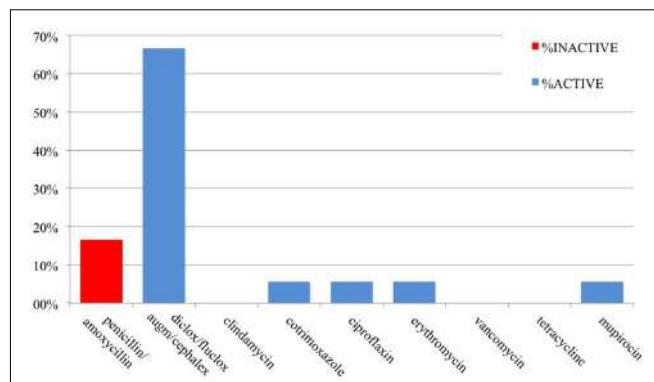
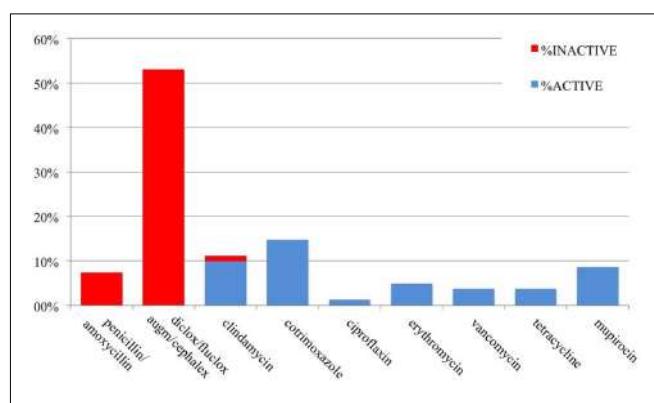
\*Other treatments include: abscess burst on its own, dressings, hospital admission, IV antibiotics, syringing, topical creams (non-antibiotic or unspecified), and surgical removal.

infection resolved by the first home visit in 85% of cases, (95% CI 70, 94). Where I&D was not used (26) the infection was resolved in 73% of cases (95% CI 52, 88) by the first visit.

## Analysis of Antibiotic Use

The antibiotics prescribed were not known for all patients, meaning the patient could recall the actual name of the antibiotic(s). However, the majority (85%) could remember the name of at least one of their prescribed antibiotics: 43 patients with MRSA and 13 patients with MSSA. We analyzed the antibiotic susceptibility profiles of the clinical isolates and the activity of the known antibiotics that were prescribed for these 56 patients (Figures 1 and 2). Antibiotics are described as *active* when the organism was susceptible to the prescribed antibiotic and *inactive* when the organism was resistant.

In total, over the course of their infections, there were 18 prescriptions of known antibiotics given to 13 patients with MSSA, of which three (16.7%) were inactive. Of the 81 known antibiotics prescribed for 43 patients with MRSA, 50 (62%) were

**FIGURE 1 | Antibiotic susceptibility by prescription (n = 18) for MSSA infections.****FIGURE 2 | Antibiotic susceptibility by prescription (n = 81) for MRSA infections.**

inactive. Overall, approximately 53.5% of prescribed antibiotics were inactive: these included penicillin/amoxicillin for both MSSA and MRSA, and cephalexin, flucloxacillin, augmentin, or dicloxacillin for MRSA infections.

Prescriptions for MSSA infections were dominated by penicillin 16.7% and flucloxacillin, dicloxacillin, cephalexin, and augmentin (66.7%); However, augmentin and penicillin are not recommended first line of treatment for MSSA infections: augmentin because of its unnecessarily broad spectrum activity

**TABLE 3 | Patient-reported infection resolution by first home visit (by management protocol).**

Treatment	MSSA (N = 16)		MRSA (N = 50)		Total (N = 66)	
	Resolved	95% CI	Resolved	95% CI	Resolved	95% CI
I&D and antibiotics	5/6 (83%)	(36, 100)	15/18 (83%)	(59, 96)	20/24 (83%)	(63, 95)
I&D only	0 (0%)	—	0 (0%)	—	0 (0%)	—
Antibiotics only	3/4 (75%)	(19, 99)	12/15 (80%)	(52, 96)	15/19 (79%)	(54, 94)
I&D, antibiotics and other*	3/4 (75%)	(19, 99)	11/12 (92%)	(62, 100)	14/16 (88%)	(62, 98)
Antibiotics and other*	2/2 (100%)	(13, 99)	2/5 (40%)	(5, 85)	4/7 (57%)	(18, 90)

\*Other treatments include: abscess burst on its own, dressings, hospital admission, IV antibiotics, syringing, topical creams (non-antibiotic or unspecified), and surgical removal.

and penicillin/amoxicillin because only 5–10% of MSSA are susceptible (Turnidge et al., 2016).

## Antibiotic Changes and Repeat GP Visits

Of the 43 patients with MRSA infections who recalled at least one of their specific antibiotic(s), 35 (81%) were initially prescribed an inactive drug. Of these, 23% never received an active antibiotic, whilst the rest were subsequently prescribed active (58%) or unknown (19%) antibiotic(s) at follow-up visits. Three (23.1%) of 13 patients with MSSA who recalled the specific antibiotic(s) were also prescribed an inactive drug, with one subsequently prescribed an active antibiotic.

In our study, 56 patients were able to remember a total of 99 antibiotics (an additional 19 were remembered as a prescription but not by name). Thirty-seven patients (6 with MSSA and 31 with MRSA infections) were prescribed more than one antibiotic and, of those, thirteen (1 MSSA patients and 12 MRSA) were prescribed more than two antibiotics. In addition, eight were prescribed Mupirocin (site of use unspecified but all organisms susceptible). We presume that for every new antibiotic prescribed the patients required a repeat visit to their GP for assessment, although we do not know if the subsequent prescription changes were due to treatment failure or antibiotic change based on lab results showing resistance.

## GP Surveys of Abscess Management

One hundred and seventy GPs were approached and 81 completed surveys (48%; 41 COSAHC GPs, 39 comparison GP and one unlabeled form that could not be ascribed to a particular GP group). The response rate was higher in the COSAHC GP group (60% compared with 38%). The average number of years in practice of those participating was 23.1 years. No differences were found with treatment preferences between the two cohorts (**Table 4**).

Overall, GPs report 'I&D and antibiotics' as their preferred management of abscesses (70%), followed by 'I&D only' (19%) and 'Antibiotics only' (10%). Some specified I&D included additions; one would incise with review and another would prescribe antibiotic cream alongside. Other selections included 'no initial treatment, but subsequent patient review' (indicated as 'other' in **Table 4**).

## Decision to Swab Wound

When we stratified treatment choices according to the GPs decision to swab, we found abscess management did not differ

**TABLE 4 | Self-reported management practices by doctor group.**

Treatment	COSAHC GPs 41 n (%) <sup>*</sup>	Non-COSAHC GPs (39) n (%)	Total GPs (81) n (%)
I&D and antibiotics	29 (71%)	27 (69%)	57 (70%)
I&D	8 (20%)	7 (18%)	15 (19%)
Antibiotics only	3 (7%)	5 (13%)	8 (10%)
Other	1 (2%)	0 (0%)	1 (1%)

\*All percentages are column percentages.

significantly between the two surveyed doctor groups, with 70% of both groups reporting their preferred management protocols as 'I&D and antibiotics.' This indicates we have not introduced patient-management bias via the recruitment method used, other than including a greater proportion of patients treated by GPs who swab. More COSAHC doctors did indicate they would swab if the abscess was weeping or incised (76.9% vs. 56.4%;  $p = 0.049$ ), but this was not shown to be associated with differences in patient management, so we pooled the two GP groups for further analyses.

Overall, a minority (29.9%) of GPs reported that they would routinely swab abscesses for culture. Doctors reported they would swab for definitive diagnostic purposes (68.8%) or for persistent abscesses (97.4%). About half of doctors (48.6%) report being influenced by abscess size and a perceived increased risk of systemic infection or spread to adjacent tissues. When abscess size was identified as important, thresholds of size varied from >1 cm to >3 cm. Swabbing practice was not related to treatment choice or the commencement of antibiotics.

The rise of MRSA in the community was identified as reason for changing practice (23%). Some doctors report they are now more likely to wait for results to prescribe antibiotics and choose to swab based on clinical site and severity of abscess.

## COSAHC Patient Management – GP-Reported Practice versus Actual Patient-Reported

Eight doctors of COSAHC patients with abscesses also completed the GP survey. Whilst the numbers are small, it does provide us the added opportunity of direct comparison between a patient' reported abscess treatment and their doctor's survey responses (**Table 5**).

None of the eight responded with the recommended first line treatment for an abscess, regardless of size. Two reported that they would not perform I&D and would solely prescribe antibiotics, which corresponds to the patients' report. The remaining six doctors who selected 'I&D and Antibiotics' in the survey varied in their actual clinical response – three out of six (50%) did not perform I&D and only prescribed antibiotics. Interestingly, two out of six (33%) administered an antibiotic

**TABLE 5 | Community GP-reported management for abscesses versus patient-reported management.**

GP survey response	Patients' report of treatment by same GP
Treatment of patient in case study	Treatment given for abscess
Antibiotics	Antibiotics
Antibiotics	Antibiotics
I&D and Antibiotics	I&D and Antibiotics
I&D and Antibiotics	Self I&D, Antibiotics
I&D and Antibiotics	I&D attempt, penicillin injection, oral antibiotics
I&D and Antibiotics	I&D ×3, antibiotic injection, oral antibiotics
I&D and Antibiotics	Antibiotics (I&D of previous boil)
I&D and Antibiotics	Antibiotics

injection as well as oral antibiotics. This is particularly surprising because there are no intramuscularly injectable antibiotics that are active against most *S. aureus* organisms.

## DISCUSSION

We live in an era of rising antibiotic resistance. Conservation of antibiotics is of critical importance, and in response antibiotic stewardship programs are being progressively implemented. Antibiotic stewardship originated to address the problems of antibiotic overuse in hospitals, but is now being extended to the community as it is increasingly realized most antibiotics for human use are prescribed and consumed in the community.

Australian GP compliance with clinical guidelines for abscess treatment has not previously been researched. In the US the management of community-acquired abscesses have been investigated both in general practice and in hospital emergency departments (Odenholt et al., 2002; Daly et al., 2011; Forcade et al., 2011; Kemper et al., 2011; Parnes et al., 2011; May et al., 2012; Schmitz et al., 2013). Pre/post intervention studies in primary care have been conducted to evaluate abscess management. For example, the IRENE study looked at antibiotic prescription coverage – pre-intervention cephalosporins were frequently prescribed (43%), dropping to 18% of prescriptions post-intervention. In this cohort, 84% of patients received I&D and antibiotics pre-intervention. Post-intervention, 97% received antibiotics and I&D, but there was a demonstrated increase in MRSA susceptible antibiotics. The option to not prescribe antibiotics was discussed but did not translate into practice (Daly et al., 2011).

Similarly, in the SNOCAP-USA and DARTnet study (Parnes et al., 2011), procedure rates for I&D were initially low. A practical intervention had no significant effect on the number of I&D completed or for microbiological cultures, but again, did result in increased use of MRSA-susceptible antibiotics.

STARnet (Forcade et al., 2011) conducted a prospective community based observational study of suspected MRSA abscesses. Only 7% of this cohort had I&D alone, while 64% received both antibiotics and I&D. Twenty-eight percent of the cohort received only antibiotics with trimethoprim-sulfamethoxazole used frequently as monotherapy. These studies also demonstrate the reluctance to stop providing antibiotics alongside I&D, or at all, despite recommendations.

Hospital emergency department research in the US showed only 17–19% of abscesses were treated with I&D alone and treatment with antibiotics alone ranged from 4 to 17%, while a combination of I&D and antibiotics was the most common practice (66–79.9%; Moran et al., 2006; Talan et al., 2011; May et al., 2012; Schmitz et al., 2013). A quality improvement project (Kemper et al., 2011) was conducted amongst American pediatricians to develop an intervention to increase adherence to best practice of abscesses; 83% of those interviewed responded that they could perform I&D, however, 34% stated it was too time consuming to perform. The challenge exists then to emphasize the importance of I&D in treating patients with abscesses and encouraging best practice.

Our study focuses on community-onset abscess infections where MRSA is over-represented as a deliberate part of our sampling strategy. The data drawn from the prospective cohort of community-onset staphylococcal infections (COSAHC) identified via a private community-based pathology service, and the cross-sectional survey of doctors ordering tests through the same pathology service, has provided the opportunity to explore doctors' attitudes and practices in the context of the changing epidemiology of *S. aureus* in an Australian urban community.

The antibiotic susceptibility of isolates for all the COSAHC participants were known and as this was the criteria for entry into the study. In the COSAHC study, we found that 85% of community-onset *S. aureus* infections were skin and soft tissue infections and in 66 the infection were skin abscesses and used in this study. In the management of the 66 participants with abscesses: 100% were prescribed antibiotics but only 60% had their abscess incised and drained. Contrary to this, a larger percentage of doctors (75%) surveyed indicated I&D with antibiotics was their management of choice for community-acquired abscesses. That is, more community GPs indicated that I&D with antibiotics was their management of choice than appeared to perform it.

Furthermore, where the antibiotic was known, the majority of patients with abscesses caused by MRSA (81%) were initially prescribed inactive antibiotics, compared with 23% of those with MSSA. Antibiotics were changed for 37 patients, resulting in nearly all MSSA (85%), but only 60.5% of MRSA infections ever being prescribed an active antibiotic. Overall, 53.5% of antibiotic choices were inactive against the targeted pathogen.

We have previously estimated that about 8% of infections caused by *S. aureus* in the community of metropolitan Melbourne are MRSA (Bennett et al., 2014), but this is likely to be much higher for abscesses based on our findings here. Given the proportion of COSAHC community-onset infections that are abscesses (66/291, 23%), and the high rate due to MRSA infections (78% compared with 47% of all infections among the frequency-matched COSAHC index cases), we estimate the prevalence of MRSA infections might be as high as 24% (three times higher) in community-onset infections presenting as abscesses in metropolitan Melbourne 2010–2012.

From our survey we found that only 30% of community GPs swab for MC&S. Therefore, many doctors would be unaware of the proportion of antibiotics prescribed that were ineffective and they are unlikely to modify their prescribing practice. According to both contemporary and Australian guidelines, almost all of the prescriptions for uncomplicated abscesses could be considered unnecessary. In support of these guidelines, our study showed there was little difference in infection resolution whether the antibiotics were active or not. This was true for 'I&D + antibiotics' and 'antibiotics' alone ( $p = 0.71$ ), whether the antibiotics were active ( $p = 1.0$ ) or inactive ( $p = 0.84$ ). However, there was a trend to greater resolution at first household visit for those patients who had I&D performed, with 85% of infections resolved (95% CI 70, 94) compared with 73.1% (95%

CI 52.2, 88.4) of patients who did not have I&D performed. A 2007 literature review (Hankin and Everett, 2007) also showed that those patients treated with I&D alone had the same rate of resolution as those with I&D and antibiotics.

If an antibiotic is prescribed it is usually at first presentation. The swab will not help in the initial antibiotic prescription but a MC&S may be useful in the case of treatment failure and MRSA surveillance. If I&D is performed correctly, our findings support guidelines and no antibiotics are needed and can be stopped. Patients' expectations regarding treatment is a challenge for GPs. Patient understanding of best practice can be narrow and limited to their own situation. There is a need to increase the health literacy of patients and to make them aware of the population wider risks associated with antibiotic over prescription.

Although I&D was consistently reported by GPs to be their preferred treatment option for uncomplicated abscesses, the COSAHC study observed only 60% of patients with abscesses were treated with I&D in practice. Furthermore, I&D was always accompanied by antibiotics. This suggests that whilst there may be an understanding of the importance of I&D, there may be a lack of confidence in performing the procedure without antibiotic cover.

Given the number of antibiotic changes (37 patients requiring additional antibiotics) and therefore the presumed increased number of visits to the GP, it is clear that skin abscesses are difficult to treat and many different antibiotics are used.

Our study has a number of limitations. The response rate of 48% in the GP survey has the potential for sampling bias. However, we believe that any selection bias that may have been introduced would act to favor doctors who are more aware of community MRSA and therefore more keen to participate. Therefore, it is possible that these results underestimate the actual deviation from recommended practice in the wider GP community. If this were the case, then the true situation regarding GP practices may be even more removed from therapeutic practice guidelines than captured here. It would be useful to conduct a larger study to more comprehensively examine GP practice, and gain more detailed insight into GPs' ability to perform I&D correctly. Incision type, use of pain control, irrigation, wound cultures, and packing, would inform targeted strategies (Forcade et al., 2011; Centers for Disease Control and Prevention [CDC], 2013).

## CONCLUSION

Our findings demonstrate many GPs are not following guideline recommended practice when it comes to treating patients with staphylococcal skin abscesses. Our findings support the

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recommendations in the current antibiotic guidelines that would act to curb this trend (Antibiotic Expert Groups, 2014). In particular:

- (1) Antibiotics not indicated unless spreading cellulitis with systemic symptoms.
- (2) Perform I&D correctly on all abscesses, even if antibiotic therapy is considered.
- (3) Modify therapy based on clinical response to initial therapy and the results of cultures and susceptibility testing. If *S. aureus* is isolated, and lesion is responding to drainage, stop any antibiotics, inactive and active. If antibiotics are necessary: treatment for 5 days is generally sufficient, but a longer duration of therapy may be required for patients who are slow to respond or have a more severe infection.

Overall, the doctors we surveyed demonstrated awareness of the changing epidemiology of *S. aureus* infections in Melbourne. This study provides a basis for developing new programs to assist GPs to better understand and reduce their use of antibiotics, and potentially the number of return patient visits. There is also vital need to improve confidence and proficiency of surgical techniques such as I&D, which are more effective than antibiotics in such cases.

## AUTHOR CONTRIBUTIONS

CP initiated this work, lead the study design and ethics approval, and managed the recruitment and oversaw the data analysis, and instigated the writing of this manuscript. GW was responsible for managing the partnership with Dorevitch laboratory and GP recruitment and contributed to the design of the survey tool, interpretation of data, and manuscript preparation. EB assisted in data analysis and preparing the figures for this paper. GC was responsible for the *S. aureus* laboratory typing and determination of resistance, and contributed to these elements of the paper. PJ also contributed to the development of the survey tool and to the writing of the manuscript, especially the clinical relevance aspects in the discussion and conclusions of this manuscript. CB developed the concept for this research with CP, lead the COSAHC study that contributed data to this work, oversaw the data analysis and data reporting processes, and contributed to the initial writing and then polishing of this manuscript.

## FUNDING

This project was funded by the National Health and Medical Research Council [NHMRC 509304], and the Centre for Population Health Research, Deakin University.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Specialty section:**

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 18 June 2015

**Accepted:** 27 August 2015

**Published:** 09 September 2015

**Citation:**

Xu Z, Mkrtchyan HV and Cutler RR  
(2015) Antibiotic resistance and *mecA*  
characterization of coagulase-negative  
staphylococci isolated from three  
hotels in London, UK.  
*Front. Microbiol.* 6:947.

doi: 10.3389/fmicb.2015.00947

# Antibiotic resistance and *mecA* characterization of coagulase-negative staphylococci isolated from three hotels in London, UK

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Antibiotic resistance in bacteria isolated from non-healthcare environments, is a potential problem to public health. In our survey a total of 71 coagulase negative staphylococci (CNS) belonging to 11 different species were isolated from three large hotels in London, UK. The most prevalent species was *Staphylococcus haemolyticus*, with *S. hominis*, *S. warneri*, *S. cohnii*, and *Staphylococcus epidermidis* commonly detected. Antimicrobial susceptibilities and carriage of the *mecA* gene were determined for all of these isolates. Most (85.9%) staphylococci were resistant to multiple antibiotics with all displaying increased susceptibility toward penicillin, fusidic acid, erythromycin, and cefepime. Twenty-one (29.5%) of the isolates were *mecA* positive, however MIC values to oxacillin, normally associated with the carriage of *mecA*, varied widely in this group (from 0.06 to 256 mg/L). Fifteen of the twenty-one *mecA* positive isolates carried SCCmec of these seven were type V, one type I, one type II, and one type IV. Additionally, five of these 15 isolates carried a previously unreported type, 1A, which involves an association between class A *mec* complex and *ccr* type 1. The remaining six of the 21 isolates were non-typeable and carried a combination of class A *mec* complex and *ccrC*. In addition to this, we also report on new MLST types which were assigned for five *S. epidermidis* isolates. Four out of these five isolates had MICs between 0.06 and 256 mg/L to oxacillin and would be regarded as clinically susceptible but one isolate had a high oxacillin MIC of 256 mg/L. We demonstrated widespread multiple drug resistance among different staphylococcal species isolated from non-healthcare environments highlighting the potential for these species to act as a reservoir for methicillin and other forms of drug resistance.

**Keywords:** antibiotic resistance, coagulase-negative staphylococci, *mecA* gene, SCCmec typing, MLST

## Introduction

Coagulase-negative staphylococci (CNS) are opportunistic pathogens that have emerged as a major cause of nosocomial infections often associated with healthcare settings (Bouchami et al., 2011a; Zong et al., 2011). *S. epidermidis*, *S. haemolyticus*, and *S. hominis* are amongst the CNS which can be responsible for a wide range of illnesses from minor skin infections to life threatening diseases (Huebner and Goldmann, 1999; Basaglia et al., 2003). In addition to this, the treatment of CNS infections has become more difficult, as many isolates in hospitals carry multiple drug resistance (Bouchami et al., 2011b) due to an increase of ineffectiveness of a wide range of antibiotics (John and Harvin, 2007). Methicillin resistance is commonly associated with the carriage of the *mecA* gene that encodes for penicillin binding protein PBP2a and has a low affinity for  $\beta$ -lactam antibiotics (Tulinski et al., 2012). The *mecA* gene is located on a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*) (Milheirico et al., 2007). There are 11 SCC*mec* types currently reported and typing is based on different combinations of *mec* types (A, B, C1, C2, and D) and *ccr* types (*ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4*, and *ccrC*) (IWG-SCC, 2009; Shore et al., 2011).

The spread of methicillin resistant *S. aureus* (MRSA) outside of hospital settings, has been reported by several authors and their studies demonstrated that non-healthcare settings, including fire stations, student housing sites/common areas, public buses can be reservoir for MRSA (Roberts et al., 2011a,b; Simões et al., 2011). We have previously reported on the isolation of methicillin resistant coagulase negative staphylococci (MRCNS) from non-hospital restrooms (Mkrtyan et al., 2013). In this current study we report on the wide variety of antibiotic resistance patterns and the molecular characterization of *mecA* positive determinants that we have isolated from non-healthcare environments.

## Materials and Methods

### Screening of Staphylococcal Isolates

With the permission of the hotel management/owners, we sampled three hotels in London, UK. The results from each hotel were reported to each manager/owner for their information. The sampling was of inanimate objects only. Eighty-eight randomly selected sites in 32 different hotel rooms were sampled using COPAN dry swabs (Copan Diagnostics Inc., USA).

In addition to these samples, 12 air samples were also collected from 12 randomly selected rooms using a high volume air sampler (Cherwell SAS Super 100, Cherwell Laboratories, UK). The airflow of sample collection was between 200 and 1000 l per min.

All specimens were inoculated onto Nutrient Agar (Nutrient Agar, Oxoid, Basingstoke, UK) and Mannitol Salt Agar plates (Oxoid Basingstoke, UK). These cultures were incubated aerobically at 37°C for 24–72 h.

### Identification of Environment Staphylococci

All isolates were initially screened using conventional methods including microscopy (Gram film), catalase and coagulase

testing. Prolex™ staph latex kits (ProLab Diagnostics, Neston, UK) were used to identify CNS and *S. aureus*. All potential staphylococcal isolates were then fully identified using a Matrix-assisted laser desorption ionization time flight mass-spectroscopy (MALDI-TOF-MS, Microflex LT, Bruker Daltonics, Coventry, UK) in a positive linear mode (2000–20,000 m/z range). Samples were prepared as described previously (Mkrtyan et al., 2013). In brief, 3–5 colonies of fresh cultures were added into 300 µl distilled water and mixed with 900 µl absolute ethanol. The suspension was centrifuged for 2 min at 13,000 × g and the pellets were re-suspended in 25 µl of 70% formic acid and then mixed with 25 µl pure acetonitrile. The mixture was centrifuge for 2 min at 13,000 × g. One microliter aliquots of the obtained supernatant were spotted on the MALDI target plate and overlaid with 1 µl of  $\alpha$ -Cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker Daltonics, Coventry, UK). The resulting spectra for each isolate was analyzed by MALDI-Biotyper 3.0 software (Bruker Daltonics, Coventry, UK). *Escherichia coli* DH5 $\alpha$  (Bruker Daltonics, Coventry, UK) was used as a standard for calibration and quality control.

### Antimicrobial Susceptibility Testing for All Staphylococci

The susceptibility of 12 antibiotics was tested using standard disk diffusion method as previously described (Andrews and Howe, 2011). This included amoxicillin (10 µg); cefepime (30 µg); chloramphenicol (30 µg); erythromycin (5 µg); fusidic acid (10 µg); gentamicin (10 µg); mupirocin (20 µg); oxacillin (1 µg); penicillin (1 unit); streptomycin (10 µg); tetracycline (10 µg); vancomycin (5 µg). The categories susceptible, intermediate resistant or resistant were assigned on the basis of the Guidelines for Susceptibility Testing (Andrews and Howe, 2011). The Minimum Inhibitory Concentrations (MIC) for oxacillin were additionally evaluated using “M.I.C. evaluators” (Oxoid Ltd., Basingstoke, UK).

### Detection of *mecA* Gene and Staphylococci Cassette Chromosome *mec* (SCC*mec*) Typing

The detection of *mecA* gene was carried out using primers and methods as described previously (Hanssen et al., 2004). SCC*mec* typing was performed to *mecA* positive isolates using PCR as described previously, and the isolates were investigated using primers for *mec* and *ccr* complexes (Kondo et al., 2007).

### MLST Typing of *Staphylococcus epidermidis*

*S. epidermidis* isolates were further analyzed by Multi Locus Sequence Typing (MLST) for seven housekeeping genes, as has been described previously (Thomas et al., 2007). Sequence types were determined by comparing the alleles to those in the *S. epidermidis* database ([www.mlst.net](http://www.mlst.net)).

Amplicons were sequenced by Eurofins MWG GmbH (Ebersberg, Germany) using ABI 3730XL DNA analyser. Sequence similarity searches were carried out using BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST>).

## Southern Blotting of *mecA* Positive Isolates with Low Oxacillin MIC

*S. hominis* which had the lowest MIC to oxacillin was selected for southern blotting. *S. aureus* NCTC6571 was used as a negative control. *Clal*-digested genomic DNA were transferred to a nitrocellulose membrane (Roche Diagnostics Ltd., West Sussex, UK) as recommended by the manufacturers (GE health care UK Ltd., Buckinghamshire, UK). Hybridization was carried out with a DNA probe for *mecA* at 68°C, and using the DIG luminescence detection kit according to the manufacturer's instructions (Roche Diagnostics Limited, Charles Avenue, Burgess Hill, West Sussex, UK).

## Results

### Sample Collection

A total of 71 staphylococcal isolates were recovered in this study, this included 62 from hotel surfaces and nine collected from hotel air samples.

### Identification of Environmental Staphylococci

We identified 71 staphylococcal isolates as belonging to 11 different staphylococcal species. These included *S. haemolyticus* ( $n = 34$ ), *S. hominis* ( $n = 12$ ), *S. warneri* ( $n = 5$ ), *S. cohnii* ( $n = 5$ ), *S. epidermidis* ( $n = 5$ ), *S. lugdunensis* ( $n = 3$ ),

*S. pettenkoferi* ( $n = 2$ ), *S. capititis* ( $n = 2$ ), *S. sciuri* ( $n = 1$ ), *S. pasteuri* ( $n = 1$ ), and *S. equorum* ( $n = 1$ ).

### Antibiotic Susceptibilities for All Staphylococci

All isolates were tested for their susceptibility to 12 commonly used antibiotics. We found that all isolates showed resistance to at least one antibiotic. In addition to this, 61 (86%) out of 71 staphylococcal isolates were resistant to two or more of the 12 antibiotics evaluated (Tables 1, 2). Fifty-two (73%) staphylococcal isolates were resistant to penicillin and 50 (70%) were resistance to fusidic acid, 35 (49%) to erythromycin, 24 (34%) to amoxycillin, 23 (32%) to tetracycline, 20 (28%) to cefepime, 20 (28%) to vancomycin, 18 (25%) to mupirocin, 14 (20%) to oxacillin, 13 (18%) to chloramphenicol, 8 (11%) to gentamicin, and 7 (10%) to streptomycin. Among all staphylococci species *S. haemolyticus* was predominantly resistant to erythromycin (59%), followed by *S. epidermidis* to amoxicillin 60% and tetracycline 60% respectively and *S. cohnii* to erythromycin (80%), whereas *S. haemolyticus* (ID 54) and *S. pasteuri* (ID 68) were susceptible to almost all antibiotics tested except for streptomycin (Tables 1, 2).

### Determination of MICs to Oxacillin and SCCmec Typing

Twenty-one of the *mecA* positive isolates were multiple drug resistant however we found that susceptibility to oxacillin was

**TABLE 1 | Molecular characterisation of antibiotic resistant and *mecA* gene positive coagulase negative staphylococci isolated from hotel and air samples.**

ID	Species	Source	AM	CEF	CHL	ERY	FC	GEN	MUP	PG	STR	TET	VAN	<i>mecA</i>	<i>mec</i>	<i>ccr</i>	SCC <i>mec</i>	MIC/Ox (mg/L)
1	<i>S. cohnii</i>	HAS	S	R	S	S	R	S	R	R	I	S	S	+	Class C	C	V	2
2	<i>S. cohnii</i>	HAS	S	R	R	R	R	S	S	R	R	R	R	+	Class A	C	NT	1
3	<i>S. epidermidis</i>	DSH	R	R	S	R	R	R	R	R	I	R	S	+	Class B	2	IV	256
4	<i>S. epidermidis</i>	DSH	R	S	S	I	S	S	S	R	S	R	S	+	Class A	1	1A	0.12
5	<i>S. haemolyticus</i>	HAS	S	R	S	R	R	S	S	R	R	R	S	+	Class A	C	NT	256
6	<i>S. haemolyticus</i>	DSH	S	R	R	R	R	S	R	R	I	S	R	+	Class C	C	V	256
7	<i>S. haemolyticus</i>	DSH	R	R	R	S	R	R	S	R	R	R	R	+	Class A	2	II	256
8	<i>S. haemolyticus</i>	DSH	R	R	S	R	R	R	S	R	R	S	S	+	Class C	C	V	8
9	<i>S. haemolyticus</i>	DSH	R	R	S	R	R	S	S	R	I	S	S	+	Class C	C	V	8
10	<i>S. haemolyticus</i>	DSH	S	R	R	S	R	S	R	R	I	R	R	+	Class A	1	1A	1
11	<i>S. haemolyticus</i>	HAS	S	S	R	R	R	S	R	R	R	R	R	+	Class C	C	V	0.5
12	<i>S. haemolyticus</i>	DSH	R	S	S	S	R	S	S	R	I	S	R	+	Class C	C	V	0.25
13	<i>S. haemolyticus</i>	DSH	R	S	S	S	S	S	S	R	I	R	S	+	Class B	1	I	0.12
14	<i>S. hominis</i>	DSH	S	S	R	S	R	S	S	R	S	S	R	+	Class A	1	1A	8
15	<i>S. hominis</i>	DSH	R	S	S	R	R	S	R	R	S	R	R	+	Class A	1	1A	0.12
16	<i>S. hominis</i>	DSH	R	R	S	R	R	S	R	R	I	S	S	+	Class A	1	1A	0.06
17	<i>S. lugdunensis</i>	DSH	S	S	S	R	S	S	S	R	I	S	S	+	Class A	C	NT	0.5
18	<i>S. pettenkoferi</i>	DSH	R	R	R	R	R	R	S	R	R	R	R	+	Class A	C	NT	8
19	<i>S. sciuri</i>	DSH	S	R	R	S	R	S	S	R	I	S	R	+	Class A	C	NT	16
20	<i>S. warneri</i>	DSH	S	S	S	R	S	S	S	R	I	S	S	+	Class A	C	NT	0.25
21	<i>S. warneri</i>	DSH	S	S	S	S	R	S	S	R	I	S	R	+	Class C	C	V	0.25

DSH, different sites from hotels; HAS, hotel air samples; AM, amoxicillin (10 µg); CEP, cefepime (30 µg); CHL, chloramphenicol (30 µg); ERY, erythromycin (5 µg); FC, fusidic acid (10 µg); GEN, gentamicin (10 µg); MUP, mupirocin (20 µg); PG, penicillin (1 unit); STR, streptomycin (10 µg); TET, tetracycline (10 µg); VAN, vancomycin (5 µg); R, resistance; S, sensitive; I, intermediate; MIC/Ox, minimum inhibitory concentration Oxacillin; NT, not typeable.

**TABLE 2 | Molecular characterisation of antibiotic resistant but *mecA* gene negative, coagulase negative staphylococci isolated from hotel and air samples.**

ID	Species	Source	AMO	CEF	CHL	ERY	FC	GEN	MUP	PG	STR	TET	VAN	OX
22	<i>S. capitis</i>	DSH	S	S	S	S	S	S	R	R	S	S	S	S
23	<i>S. capitis</i>	DSH	S	S	S	S	R	S	S	R	I	S	S	S
24	<i>S. cohnii</i>	DSH	R	S	S	R	R	S	S	R	S	S	R	S
25	<i>S. cohnii</i>	DSH	R	S	S	R	S	S	R	R	S	S	S	S
26	<i>S. cohnii</i>	DSH	S	S	R	R	R	S	S	R	S	S	S	S
27	<i>S. epidermidis</i>	DSH	S	S	S	S	R	S	S	R	I	S	R	S
28	<i>S. epidermidis</i>	DSH	R	S	R	R	R	S	S	R	I	R	S	S
29	<i>S. epidermidis</i>	DSH	S	R	R	S	R	S	R	R	I	S	R	S
30	<i>S. equorum</i>	DSH	S	S	S	S	S	S	S	R	I	S	S	S
31	<i>S. haemolyticus</i>	DSH	R	S	S	R	S	R	S	S	I	R	S	S
32	<i>S. haemolyticus</i>	DSH	R	R	S	R	R	S	S	R	S	S	S	S
33	<i>S. haemolyticus</i>	DSH	S	S	S	R	R	R	R	R	I	S	R	S
34	<i>S. haemolyticus</i>	DSH	S	S	S	R	S	S	S	S	I	S	S	S
35	<i>S. haemolyticus</i>	DSH	S	S	S	R	R	S	R	R	S	S	S	S
36	<i>S. haemolyticus</i>	DSH	S	S	S	R	R	S	R	R	I	S	S	S
37	<i>S. haemolyticus</i>	DSH	R	S	S	R	R	S	S	R	I	S	S	S
38	<i>S. haemolyticus</i>	DSH	S	R	S	R	R	S	S	S	S	S	R	S
39	<i>S. haemolyticus</i>	DSH	S	S	S	R	R	S	S	S	I	S	S	S
40	<i>S. haemolyticus</i>	DSH	S	R	S	R	S	S	S	S	I	R	S	S
41	<i>S. haemolyticus</i>	DSH	S	S	S	S	R	S	S	R	I	S	R	S
42	<i>S. haemolyticus</i>	DSH	S	S	S	S	R	S	S	R	I	S	S	S
43	<i>S. haemolyticus</i>	DSH	S	R	R	R	S	S	S	S	I	R	S	S
44	<i>S. haemolyticus</i>	DSH	S	S	S	R	S	S	S	S	I	R	R	S
45	<i>S. haemolyticus</i>	DSH	R	R	S	S	R	S	S	R	I	R	S	S
46	<i>S. haemolyticus</i>	DSH	R	S	S	R	S	S	S	R	I	S	S	S
47	<i>S. haemolyticus</i>	DSH	R	S	S	R	R	S	S	R	I	S	R	S
48	<i>S. haemolyticus</i>	DSH	S	S	S	S	S	S	S	S	I	R	S	S
49	<i>S. haemolyticus</i>	DSH	S	S	S	R	S	S	S	S	I	R	S	S
50	<i>S. haemolyticus</i>	DSH	S	S	S	S	R	S	S	R	I	R	S	S
51	<i>S. haemolyticus</i>	DSH	S	S	S	S	R	S	R	S	S	S	S	S
52	<i>S. haemolyticus</i>	HAS	S	S	S	S	S	R	R	S	S	I	R	S
53	<i>S. haemolyticus</i>	HAS	S	R	S	S	R	R	S	S	S	I	S	S
54	<i>S. haemolyticus</i>	HAS	S	S	S	S	S	S	S	S	I	S	S	S
55	<i>S. haemolyticus</i>	HAS	R	R	R	S	R	S	R	R	R	R	S	S
56	<i>S. hominis</i>	DSH	S	S	S	R	R	R	S	S	S	S	S	S
57	<i>S. hominis</i>	DSH	S	S	S	R	R	S	R	R	S	S	S	S
58	<i>S. hominis</i>	DSH	R	S	S	S	R	S	S	R	I	S	S	S
59	<i>S. hominis</i>	DSH	R	S	S	S	S	S	S	R	S	S	S	S
60	<i>S. hominis</i>	DSH	S	S	S	S	S	S	S	S	S	S	S	S
61	<i>S. hominis</i>	DSH	S	S	S	R	S	S	S	S	S	S	S	S
62	<i>S. hominis</i>	DSH	S	S	S	S	R	S	S	R	I	R	S	S
63	<i>S. hominis</i>	DSH	S	S	S	I	R	S	S	S	S	S	S	S
64	<i>S. hominis</i>	DSH	S	S	S	S	R	S	S	S	S	S	S	S
65	<i>S. lugdunensis</i>	DSH	S	S	S	S	R	S	R	R	I	S	S	S
66	<i>S. lugdunensis</i>	HAS	S	S	S	S	R	S	S	R	I	S	S	S
67	<i>S. pettenkoferi</i>	DSH	S	S	S	S	S	S	S	R	I	S	S	S
68	<i>S. pasteurii</i>	DSH	S	S	S	S	S	S	S	S	I	S	S	S
69	<i>S. warneri</i>	DSH	R	S	S	S	R	R	R	S	I	S	S	S
70	<i>S. warneri</i>	DSH	R	S	S	R	R	S	S	R	I	R	S	S
71	<i>S. warneri</i>	DSH	S	S	S	R	S	S	R	I	S	R	S	S

DSH, different sites from hotels; HAS, hotel air samples; AMO, amoxicillin (10 µg); CEP, ceftazidime (30 µg); CHL, chloramphenicol (30 µg); ERY, erythromycin (5 µg); FC, fusidic acid (10 µg); GEN, gentamicin (10 µg); MUP, mupirocin (20 µg); OX, Oxacillin (1 µg); PEN, penicillin (1 unit); STR, streptomycin (10 µg); TET, tetracycline (10 µg); VAN, vancomycin (5 µg); R, resistant; S, sensitive; I, intermediate.

highly variable with MICs ranging from 0.06 to 256 mg/L. Carriage of *mecA* did not always result in isolates demonstrating significant levels of resistance to oxacillin. Seven of the 21 isolates which were *mecA* positive were found to have MICs below 0.5 mg/L to oxacillin (**Table 1**).

*SCCmec* types were assigned in 15 out of the 21 *mecA* positive isolates. Seven isolates harbored *SCCmec* type V ( $n = 7$ ), and one isolate each harbored *SCCmec* type I ( $n = 1$ ), type II ( $n = 1$ ), and type IV ( $n = 1$ ). Five isolates harbored a new *SCCmec* type 1A, which carried combination of class A *mec* complex and *ccr* type 1. The six isolates that were non-typeable, carried a combination of class A *mec* complex and *ccrC* (**Table 1**).

### MLST Typing of *Staphylococcus epidermidis*

MLST typing revealed that all five *S. epidermidis* isolates contained novel MLST types. These types were assigned as ST515, ST516, ST517, ST518, and ST519.

### Southern Blotting of *mecA* Positive Isolates with Low Oxacillin MIC

There were also seven isolates that were *mecA* positive but had low MICs to oxacillin. In order to fully confirm that these isolates were indeed *mecA* positive, we selected the isolate from this group with the lowest oxacillin MIC (*S. hominis*, isolate 16, oxacillin MIC 0.06 mg/L, **Table 1**) and confirmed that it was indeed *mecA* positive using Southern blotting.

## Discussion

The potential threat of antibiotic resistance in environmental/non-healthcare associated bacteria is a concern for public health. Most of the studies on methicillin resistance in staphylococci focus on MRSA and CNS strains isolated from hospital patients (Zong et al., 2011; Brennan et al., 2012; Kinnevey et al., 2013). We have previously reported on the high levels of antibiotic resistance that can be found in bacteria isolated from non-healthcare restrooms (Mkrtyan et al., 2013). The aim of this current study was to evaluate the levels antibiotic resistance, the carriage of *mecA*, and the diversity of *SCCmec* elements in other staphylococci isolated from high-throughput, non-healthcare environments, in this case hotels.

### Bacterial Isolates

A total of 71 staphylococcal isolates belonging to 11 different species recovered from hotel rooms. Within this group, we found that 61 out of 71 isolates (85.9%) were resistant to two or more antibiotics, including one isolate was resistant to 10 antibiotics; 1–9 antibiotics, four isolates were resistant to eight antibiotics; 4–7 antibiotics; 5–6 antibiotics; 7–5 antibiotics; 13–4 antibiotics; 14–3 antibiotics and 12–2 antibiotics (**Tables 1, 2**). In addition to this the MICs to oxacillin varied widely from 0.06 to 256 mg/L, and although *mecA* genes were detected in 21 isolates, seven of these demonstrated only low levels of methicillin resistance. This is the NCCLS standard level for

determining resistance in CNS which are *mecA* positive (Hussain et al., 2000).

### SCCmec Typing

*SCCmec* types were assigned for the 21 isolates mentioned above. Type V was the most common (7 isolates). This is consistent with some previously published works carried out with clinical isolates (Zong et al., 2011). We did however also identify types I, II, and IV in our isolates, whereas types III, VI, VII, VIII, IX, X, and XI were not detected. It has been reported by others that the distribution of *SCCmec* types in MRCNS varies and may depend on the human host and geographical locations of the isolates were obtained from (Oliveira et al., 2006; IWG-SCC, 2009; Zhang et al., 2009; Zong et al., 2011). In addition to this, in previous papers, *SCCmec* types I, II, III, and V were found to be the most common in environmental isolates, such isolates were taken from areas such as public beaches (Soge et al., 2009).

The structural diversity of *SCCmec* has been reported in hospital environments (Ruppé et al., 2009; Barbier et al., 2011; Zong et al., 2011) with high throughputs of patients. It also possible that the *SCCmec* variations we observed were similarly related to the high throughput of people in the hotels tested (Barbier et al., 2011). In addition to this, we found associations between *SCCmec* carriage and certain species, for example *SCCmec* type V was preferentially associated with *S. haemolyticus*, *S. hominis*, *S. warneri*, *S. epidermidis*, and *S. sciuri*. Previously, with clinical isolates, type V *SCCmec* was reported to be associated mainly with *S. haemolyticus* (Zong et al., 2011).

Apart from the variations in the classified *SCCmec* types isolated, we also reported on 11 unclassified *SCCmec* types. Six of these had a combination of class A *mec* complex and *ccrC* and five had a combination of class A *mec* complex and *ccr* type 1. The latter has been reported by other workers to be a new type 1A (Bouchami et al., 2011b).

### MLST Types

Finally to emphasize the wide range of genetic variability that exists among these isolates, we also report new MLST types were assigned for five *S. epidermidis* isolates. Interestingly, four of these were determined to be clinically susceptible to oxacillin, whereas one was highly resistant to oxacillin (MIC of 256 mg/L) (Hussain et al., 2000). To date, studies on *S. epidermidis* have been focused on clinical isolates (Li et al., 2009), we believe that this is the first report to include the molecular characterisation of *S. epidermidis* isolated from non-healthcare sources.

In conclusion, public environments are potential reservoirs of multidrug resistant staphylococci. The characterization of an *S. epidermidis* isolate type with an MIC to oxacillin of 256 mg/L is of particular interest and requires further investigation with regard to its classification within the existing *SCCmec* typing system. We also aim in the future to look into the genetics of these isolates for other 11 antibiotics used in this study. Moreover, we have several other studies on-going which are looking at the bacterial populations in non-healthcare environments that will allow us to compare these samples with those from

the current as well as previous studies. The dissemination of multidrug resistance in non-healthcare environments is evidence that infection control measures in the hospitals and in public

places are ineffective in limiting the spread of such clones and that these environments are a source of antibiotic resistant pathogens.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# *bla*CTX-M-152, a Novel Variant of CTX-M-group-25, Identified in a Study Performed on the Prevalence of Multidrug Resistance among Natural Inhabitants of River Yamuna, India

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## OPEN ACCESS

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equally to this work.

### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 09 October 2015

Accepted: 01 February 2016

Published: 23 February 2016

### Citation:

Azam M, Jan AT and Haq QMR (2016)  
*bla*CTX-M-152, a Novel Variant of  
CTX-M-group-25, Identified in a Study  
Performed on the Prevalence of  
Multidrug Resistance among Natural  
Inhabitants of River Yamuna, India.  
*Front. Microbiol.* 7:176.  
doi: 10.3389/fmicb.2016.00176

Natural environment influenced by anthropogenic activities creates selective pressure for acquisition and spread of resistance genes. In this study, we determined the prevalence of Extended Spectrum β-Lactamases producing gram negative bacteria from the River Yamuna, India, and report the identification and characterization of a novel CTX-M gene variant *bla*CTX-M-152. Of the total 230 non-duplicate isolates obtained from collected water samples, 40 isolates were found positive for ESBL production through Inhibitor-Potentiation Disc Diffusion test. Based on their resistance profile, 3% were found exhibiting pandrug resistance (PDR), 47% extensively drug resistance (XDR), and remaining 50% showing multidrug resistant (MDR). Following screening and antimicrobial profiling, characterization of ESBLs (*bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>), and mercury tolerance determinants (*merP*, *merT*, and *merB*) were performed. In addition to abundance of *bla*<sub>TEM-116</sub> (57.5%) and *bla*<sub>CTX-M-15</sub> (37.5%), bacteria were also found to harbor other variants of ESBLs like *bla*CTX-M-71 (5%), *bla*CTX-M-3 (7.5%), *bla*CTX-M-32 (2.5%), *bla*CTX-M-152 (7.5%), *bla*CTX-M-55 (2.5%), along with some non-ESBLs; *bla*<sub>TEM-1</sub> (25%) and *bla*<sub>OXY</sub> (5%). Additionally, co-occurrence of mercury tolerance genes were observed among 40% of isolates. *In silico* studies of the new variant, *bla*CTX-M-152 were conducted through modeling for the generation of structure followed by docking to determine its catalytic profile. CTX-M-152 was found to be an out-member of CTX-M-group-25 due to Q26H, T154A, G89D, P99S, and D146G substitutions. Five residues Ser70, Asn132, Ser237, Gly238, and Arg273 were found responsible for positioning of cefotaxime into the active site through seven H-bonds with binding energy of -7.6 Kcal/mol. Despite small active site, co-operative interactions of Ser237 and Arg276 were found actively contributing to its high catalytic efficiency. To the best of our knowledge, this is the first report of *bla*CTX-M-152 of CTX-M-group-25 from Indian subcontinent. Taking a note of bacteria harboring such high proportion of multidrug and mercury resistance determinants, their presence in natural water resources employed for human consumption increases the chances of potential risk to human health. Hence, deeper

insights into mechanisms pertaining to resistance development are required to frame out strategies to tackle the situation and prevent acquisition and dissemination of resistance determinants so as to combat the escalating burden of infectious diseases.

**Keywords:** antibiotics, ESBL, mechanisms of resistance, polluted environment, resistance genes

## INTRODUCTION

Extended spectrum  $\beta$ -lactamases (ESBLs), which have emerged in response to the widespread use of cephalosporins, represent the most diverse group of class A  $\beta$ -lactamases. With more than 220 variants of *bla*<sub>TEM</sub> and 172 different *bla*<sub>CTX-M</sub> variants reported to date, these active site serine  $\beta$ -lactamases (*bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>) represent the most prevalent  $\beta$ -Lactamases among members of Enterobacteriaceae (<http://www.lahey.org/studies>). By conferring higher hydrolytic activity, ESBLs exhibit a high level of resistance toward aminopenicillins (ampicillin or amoxicillin), carboxypenicillins (carbenicillin or tricarcillin), ureidopenicillins (piperacillin), cephalosporins (cephalothin, cephaloridine, and cefuroxime), oxi-imino cephalosporins (cefotaxime and ceftriaxone), cefepime, and cefpirome. Based on the broad host range and efficiency of conjugation, *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> genes after several mobilization events via horizontal gene transfer, have now became part of plasmids among different bacterial species (Thomas and Nielsen, 2005; Barlow et al., 2008; Woodford et al., 2009).

The emergence of ESBL producing isolates have been often studied in clinics because they are commonly associated with outbreaks or sporadic infections (Kohlenberg et al., 2012; Walsh, 2013). However, a lot of studies have also been dedicated toward investigation of ESBL producers among microbial inhabitants of aquatic environments that receive a continuous influx of treated and untreated sewage (Prado et al., 2008; Chagas et al., 2011; Korzeniewska and Harnisz, 2013; Wellington et al., 2013). There are reports that suggest an additional selection effect by partially metabolized (10–90%) antibiotics used for human and animal health care in aquatic ecosystems (Kummerer, 2009; Harnisz, 2013). Concomitantly, the persistence of mercury in sewage has enabled bacteria to develop an array of resistance mechanisms based on clustering of genes either on the chromosome or plasmids (Jan et al., 2009; Jackson et al., 2011). Co-selection for metal and drug resistance determinants has resulted in conferring an advantage to bacteria that helps their survival in heavily polluted environments (Seiler and Berendonk, 2012; Zhou et al., 2015). Taken together, these findings highlight the necessity for controlling the emergence, and therefore, the dissemination of multi-drug and mercury resistance in bacteria through aquatic environments.

Antibiotic resistance has worsened in developing countries owing to compromised sanitary conditions, which exacerbate movement of genes via mobile genetic elements (Schlüter et al., 2007; Kelly et al., 2009; Knapp et al., 2010). The disease burden of India is highest among different countries of the world (GARP-India, 2011). In this regard, Study of the Monitoring of Antimicrobial Resistance Trends (SMART) revealed *E. coli* in being the most prevalent pathogen among the top five resistant

gram-negative bacteria, causing 47.8% of intra-abdominal and 44.3% of urinary tract infections worldwide (Morrissey et al., 2013). In another resistance surveillance program carried out in India, Mendes et al. (2013) found that nearly 78% of *E. coli* and 64% of *Klebsiella spp.* carry ESBL determinants imparting resistance (Mendes et al., 2013). Despite these studies, there still lies paucity in the available information regarding prevalence of resistance determinants among bacteria and the pattern of antimicrobial resistance they exhibit. Hence, it becomes necessary to have an understanding of potential genetic variables that lead to acquisition of resistance and information regarding ESBL producers (ESBL<sup>+</sup>) among the microbial inhabitants of aquatic environments. The present study was conducted to investigate the prevalence of ESBL genes (*bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>) and the pattern of antimicrobial resistance among the natural inhabitants of river Yamuna, India, in order to have an insight of the resistance mechanisms that operate against  $\beta$ -lactam antibiotics. A novel variant of *bla*<sub>CTX-M</sub> gene identified in the study was characterized to unravel its catalytic profile through *in silico* studies. As the river receives higher amount of discharges (both treated and untreated), it raises serious concerns as water from the River Yamuna after passing through different stages of treatment processes, is used for several domestic, industrial and agricultural purposes. In studies of Sehgal et al. (2012) and Malik et al. (2014), they report presence of higher amount of mercury along with other metals in the samples collected from river Yamuna, India (Sehgal et al., 2012; Malik et al., 2014). Taking this into account, an investigation of *mer* operon genes as a representation of tolerance to metals was carried out to determine its role in the selection and survival of these isolates in polluted environments. The present study provides useful information regarding occurrence of multidrug resistance among bacterial inhabitants of aquatic environment that exhibit broader risk for community infections. From the study, it becomes evident that polluted water bodies acts as a pool for the emergence of new variants; thereby highlights the need to carry out in depth studies preferably toward understanding the factors that led to transfer and as such acquisition of different determinants among bacteria in their natural habitats.

## METHODOLOGY

### Sample Collection and Screening for Identification of Bacteria

Water samples were collected from the 22 km Delhi stretch of the Yamuna River, starting from upstream of the Wazirabad barrage to downstream of the Okhla barrage. The Yamuna River primarily receives sewage (treated, partially treated, or untreated) from domestic and industrial settings. For this study,

samples were collected aseptically during March 2012–August 2014 from 13 different sites ~100–150 m downstream of major drains discharging into the river. Soon after collection, samples were screened for the presence of bacterial isolates using nutrient agar and nutrient broth. Lactose fermenting Gram negative bacterial colonies were initially assessed based on their characteristic growth on MacConkey agar and Eosin Methylene Blue (EMB) agar followed by the IMViC standard biochemical tests (Clinical and Laboratory Standards Institute, 2010). Isolates from single site that were found to have same phenotypes such as growth characteristics, colony morphology, and resistance phenotypes, were excluded from the study. Further confirmation of all non-duplicate phenotypically identified bacterial isolates was carried out through analysis of the 16S rRNA gene. Subsequent to amplification of the desired (~685 bp) fragment that exhibit maximum variability across different groups of bacteria, sequencing reaction was carried out to get isolates correctly annotated with their respective group members. In case, similarity with more than one group was observed, they were re-sequenced for larger (~1250 bp) fragment size of the 16S rRNA gene for identification.

### Antibiotic Susceptibility Tests

After identification, screening for ESBL production was performed against third generation cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) by the Kirby Bauer disc diffusion method using Mueller Hinton Agar (MHA) plates. Isolates with a zone diameter of ≤22, ≤27, and ≤25 mm corresponding to ceftazidime, cefotaxime, and ceftriaxone, respectively, were considered ESBL producers. An Inhibitor-Potentiation Disc Diffusion (IPDD) test was then performed by placing discs containing ceftazidime (30 µg) and cefotaxime (30 µg) alone and in combination with clavulanic acid (10 µg) 30 mm apart on MHA plates. A ≥5 mm increase in zone diameter around the disc with antibiotic plus clavulanic acid relative to the discs with antibiotics alone was considered positive for ESBL production (Clinical and Laboratory Standards Institute, 2012). *K. pneumonia* ATCC 700603 and *E. coli* ATCC 25922 were used as ESBL positive and negative controls, respectively.

The *in vitro* antimicrobial susceptibilities against 21 antibiotics belonging to 13 different classes (β-lactam, aminoglycosides, fluoroquinolones, polymixins, rifampicins, tetracyclines, and trimethoprim from HiMedia labs., India) were then investigated according to the Clinical Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2012). The minimum inhibitory concentrations (MICs) against ceftazidime (CAZ), ceftazidime + clavulanic acid (CAC), cefotaxime (CTX), and cefotaxime + clavulanic acid (CEC) were determined for all ESBL-producing isolates by the broth micro-dilution method using Luria Bertania broth. Results were interpreted according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2012).

### Determination of Mercury Tolerance among Bacterial Isolates

Screened isolates were checked for tolerance to mercury by streaking on luria agar plates containing 0.02 mg/L mercuric

chloride ( $HgCl_2$ ). This value was ~10 times higher than its permissible limit of 0.002 mg/L for drinking water (US-EPA, <http://water.epa.gov/drink/>). Isolates showing tolerance to mercury were selected for MIC determination against various concentrations of mercuric chloride (0.02–20 mg/L) by the broth micro-dilution method using Luria Bertania broth. The MIC was defined as the lowest concentration of  $HgCl_2$  at which no growth was seen for the isolates in the culture media. *E. coli* ATCC 25922 was used as negative control.

### Detection of Genes Imparting Resistance to Bacteria

Screened bacterial isolates were analyzed for the presence of ESBLs (*bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>) and *mer* operon determinants (*merP*, *merT*, and *merB*) conferring resistance to a broad range of antibiotics and mercury. Overnight grown cultures were used for isolation of genomic DNA by phenol-chloroform-isoamyl (PCI) method and plasmid DNA by alkaline lysis method using Qiagen kit. After retrieving information from NCBI, multiple sequence alignment using ClustalW option of the BioEdit program, was performed for selection of region to design gene specific primers. After procurement of gene specific primers (Table 1), amplification corresponding to full or partial length (*bla*<sub>TEM</sub>, *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-25</sub>, *merB*, *merT*, and *merP*) gene sequences were accomplished under the following cycle conditions: 94°C for 5 min (initial denaturation) followed by 30 cycles of denaturation at 94°C for 1 min, annealing at temperatures specific for each primer set (54.5–62°C) for 30 s and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. Following purification using a QIA quick spin column (Qiagen Inc.), samples corresponding to different gene products were sequenced using an automated sequencer (ABI 1377) at Xcelris Lab (Gujarat, India). Additionally, isolates harboring the new identified variant (*bla*<sub>CTX-M-152</sub>) were analyzed for localization of the gene on chromosomal DNA and/or plasmid DNA by PCR. A reaction of 16S rRNA gene was used as control in case plasmid DNA was used as template for PCR to minimize chances of contamination of chromosomal DNA.

### In silico Analysis of Variants

Sequences of different genes were analyzed for genetic relatedness corresponding to their respective group members followed by identification of variations using ClustalW in the BioEdit 5.0.9 sequence analysis software and the MEGA6 software program. The complete coding sequence (CDS) of novel variant represented here as *bla*<sub>CTX-M-152</sub> of *Kluyvera georgiana* was translated using the Expasy translation tool ([www.expasy.org/translate](http://www.expasy.org/translate)). However, to obtain insight into the relationship between amino acid substitutions and affinity for the substrate (cefotaxime), we conducted modeling and docking studies of the variant *bla*<sub>CTX-M-152</sub> with respect to its ligand, cefotaxime.

### Analysis of Deleterious Substitution by SIFT

Sorting Intolerant from Tolerant (SIFT), which predicts the phenotypic effect of amino acid substitutions on a protein, works

**TABLE 1 | Sequences of primers used for the detection of different genes among ESBL<sup>+</sup> isolates.**

Gene type	Primer	Sequence	Product size (bp)	Reference
16S rDNA	ID-F	5'-GGCGGACGGGTGAGTAATG- 3'	685	Designed primers for this study.
	ID-R	5'-ATCCTGTTGCTCCCCACG- 3'		
	RRF	5'-GGCGGACGGGTGAGTAATG-3'		
	RRR	5'-GAAGTCGGAATCGCTAGTAATCG-3'		
TEM	TEM-F	5'-ATGAGTATTCAACATTCGTG- 3'	861	
	TEM-R	5'-TTA CCA ATG CTT AAT CAG TGA GG- 3'		
CTX-M	CTX-MF	5'-SCVATGTGCAGYACCAGTAA- 3'	480	
	CTX-MR	5'-GCTGCCGGTYTTATCVCC- 3'		
CTX-M-1	CM1F	5'-ATGGTTAAAAAATCACTGCGYCAGTTCACGC- 3'	875	
	CM1R	5'-TTACAAACCGTYGGTGACGATTTAGCCG- 3'		
CTX-M-2	CM2F	5'-ATGATGACTCAGAGCATTGCC- 3'	742	
	CM2R	5'-TCGTTGGTGGTGCATAATCTCC- 3'		
CTX-M-8	CM8F	5'-AACGCACAGACGCTCTACC- 3'	517	
	CM8R	5'-GGGTAGCCCAGCCTGAAT- 3'		
CTX-M-9	CM9F	5'-ATGGTGACAAAGAGAGTGCAACGG- 3'	875	
	CM9R	5'-TTACAGCCCTTCGGCGATGATTG- 3'		
CTX-M-25	CM25F	5'-ATGATGAGAAAAGCGTAAGGCGGG- 3'	876	
	CM25R	5'-TTAATAACCGTCGGTGACAATTCTGGC- 3'		
<i>merP</i>	merP-F	5'-ATGAAGAAACTGTTGCCTCC- 3'	276	
	merP-R	5'-TCACTGCTTGACGCTGGACG- 3'		
<i>merT</i>	merT-F	5'-TTAATAGAAAATGGAACGAC- 3'	351	
	merT-R	5'-ATGTCTGAACCACAAACGGG- 3'		
<i>merB</i>	merB-F	5'-ATGAAGCTGCCCATATATTAG- 3'	667	
	merB-R	5'-TCAACGGTGTCTAGATGACATGG- 3'		

on the principle of correlation of protein evolution with protein function (Ng and Henikoff, 2001, 2003). Here, we submitted a query in the form of protein sequences to detect the nature of substitutions. SIFT analysis was conducted by allowing the algorithm to search for homologous sequences using the default settings (SWISS-PROT 45 and TrEMBL 28 databases, median conservation score 3.00, remove sequences >90% identical to query sequence). This generated alignments with homologous sequences and assigned scores to each residue between 0 and 1 for evolutionary conservation and intolerance and tolerance to substitution. SIFT scores <0.05 are predicted by the algorithm to be intolerant (deleterious amino acid substitutions), whereas scores >0.05 are considered tolerant (Ng and Henikoff, 2003). A higher tolerance index of a particular amino acid substitution is associated with a lower probable impact.

## Homology Modeling for Structure Prediction of CTX-M-152

To retrieve potential structural templates for homology modeling of *bla*<sub>CTX-M-152</sub>, *bla*<sub>CTX-M</sub> sequences were searched using the protein data bank (PDB). Rather than finding a close associate, we found that crystal structures for PDB ID 1YLJ (CTX-M-9) and 1IYQ (Toho-1) showed the highest homology (85 and 86%) with

that of *bla*<sub>CTX-M-152</sub>. Using these as a template for modeling of our protein (KJ461948) by I-TASSER (an online server for modeling), the best model was selected based on the highest C-score and lowest Z-score. Simultaneously, protein modeling was conducted manually using Modeller version 9.12. Upon selection of the best model with the lowest DOPE score, a comparative study of both models (obtained from I-TASSER and Modeller 9.12) with our template was performed. The model with the lowest RMSD was then selected for further studies. Verification of the selected model for structural constraints was conducted using a Ramachandran plot generated with the Rampage server tool (<http://mordred.bioc.cam.ac.uk/~rapper/rampage>). Prior to docking studies, the verified structure was submitted to the DogsSite Server (<http://dogssite.zbh.uni-hamburg.de/>) for potential active site prediction. This tool predicted the existence of 10 different pockets, and the one with the highest P score was selected as the most reliable active site pocket and considered to have potential active site residues.

## Docking of Cefotaxime with CTX-M-152

Following retrieval of the cefotaxime structure from Drug Bank (Accession no. DB00493), it was docked against the modeled structure of CTX-M-152 using Autodock 4.2. Before docking,

the ligand was prepared by adding partial Gasteiger charges and defining free rotatable bonds. Simultaneously, the target structure (CTX-M-152) was prepared by removing solvent (water) molecules and adding non-polar hydrogen atoms. An affinity grid was generated using the Autogrid program of the Autodock package with a defined spacing of 1 Å and grid size of 50 × 50 × 50 Å. PI was calculated using the Compute PI/Mw tool provided by Expasy and crosschecked by ProtParam.

## RESULTS

### Screening for ESBL Producing Bacterial Isolates

Of the total 230 non-duplicate bacterial isolates, 40 isolates belonging to different groups of Gram negative bacteria were found to be ESBL producers based on the Kirby Bauer disc diffusion (**Table S1**) and IPDD tests (**Table S2**). Subsequent analysis of sequences corresponding to 16S rRNA genes from all 40 isolates for their correct representation revealed 34 to be *E. coli*, while the remaining isolates were *Klebsiella pneumonia* (1), *Aeromonas* sps. (2), *Klebsiella oxytoca* (1), *K. georgiana* (1), and *Acinetobacter junii* (1) (**Table 2**). Screening of ESBL<sup>+</sup> isolates revealed MIC values ≥512 mg/L for 25 (63%) isolates against cefotaxime, 17 (43%) isolates against ceftazidime, and 28 (70%) isolates against ceftriaxone (**Table 2**). Among the ESBL<sup>+</sup> isolates, five (MRA11, MRC17, MRE18, MRE31, and MRE44) were resistant to a combination of ceftazidime + clavulanic acid and cefotaxime + clavulanic acid, and showed the highest resistance toward third generation cephalosporins. Resistance to fluoroquinolones was found to be increased with 55% (22/40) of isolates being resistant to ciprofloxacin and 28% (11/40) for levofloxacin and ofloxacin among ESBL<sup>+</sup> isolates. The test isolates were reported to have high resistance level for ampicillin (100%) and rifampicin (93%), while on other hand, the resistance level was low for amikacin (5%) and imipenem (8%) (**Figure 1**).

Based on European Centre for Disease Prevention and Control (ECDC) and the Centre for Disease Control and Prevention (CDC) categorization, 3% of the 40 isolates investigated in this study were PDR, 47% XDR and 50% MDR. Regardless of the origin or mode of action, resistance was observed for all classes of antibiotics among all isolates, with susceptibility slightly higher for polymixin B and tobramycin. Information regarding patterns of resistance for these isolates against different classes of antibiotics is summarized in **Tables S3A,B**. All bacterial isolates in the test library were multidrug resistant against at least four classes of antibiotics, with one strain represented as PDR being resistant to at least one agent of all 13 categories.

### Mercury Tolerance among ESBL<sup>+</sup> Isolates

Screening of ESBL<sup>+</sup> isolates for their ability to tolerate various concentrations of mercury revealed that 40% of isolates were tolerant to 2 mg/L (10<sup>3</sup> times higher than its permissible limit of 0.002 mg/L for drinking water), while the rest were tolerant to 0.2 mg/L of mercury. Around, 23% isolates tolerating high concentrations of mercuric chloride (2 mg/L), were found

positive for presence of all three (*merT*, *merP*, and *merB*) *mer* operon genes. Presence of *mer* operon genes are believed for attributing bacterial isolates with the resistance phenotype. To our surprise, an *E. coli* isolate (MRC3) that tested negative for all three *mer* operon genes, was also found to tolerate high concentration of mercury (2 mg/L), attributed to the presence of resistance determinants other than that used in the study.

### Screening for Determinants Imparting Dual Resistance to Bacteria

β-lactamase genes detected more often among the ESBL<sup>+</sup> isolates include *bla*<sub>TEM-116</sub> (23, 57.5%) and *bla*<sub>CTX-M-15</sub> (15, 37.5%). Additionally, they were found to harbor *bla*<sub>CTX-M-71</sub> (2, 5%), *bla*<sub>CTX-M-3</sub> (3, 7.5%), *bla*<sub>CTX-M-32</sub> (1, 2.5%), *bla*<sub>CTX-M-152</sub> (3, 7.5%), *bla*<sub>CTX-M-55</sub> (1, 2.5%), and some non-ESBL genes like *bla*<sub>TEM-1</sub> (10, 25%) and *bla*<sub>OXY</sub> (2, 5%) (**Table 2**). All of the CTX-M encoding isolates harbored genes with sequence homology to members of group 1 (i.e., CTX-M-15, 3, 32, 55, and 71), except for one sequence [later denoted *bla*<sub>CTX-M-152</sub> by lahey's organization (www.lahey.org)] that showed maximum similarity to a CTX-M-group-25 member, *bla*<sub>CTX-M-78</sub> (Rodríguez et al., 2010). The *mer* operon consist of genes mostly associated with functions such as transport (*merT* and *merP*), regulation (*merR* and *merD*), and reduction (*merA* and *merB*; Jan et al., 2009). Of 40 ESBL<sup>+</sup> isolates, 26 (65%) and 28 (70%) were positive for *merP* and *merT* genes respectively that encodes for membrane transport proteins. The *merB* gene encoding organomercurial lyase, which catalyzes protonolytic cleavage of the carbon-mercury bond of organomercurials, was amplified from 20 (50%) isolates (**Table 2**). Taken together, 14 bacterial isolates were found harboring broad spectrum *mer* operon genes (*merT*, *merP*, and *merB*), known for their significant contribution in achieving tolerant phenotype against mercury. One isolate was found to have only *merB* gene. In comparison to isolate MRC24 which was found negative for all genes, five isolates were found to harbor both ESBL genes (*bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>) and determinants of the *mer* operon (*merP*, *merT*, and *merB*) responsible for imparting resistance to a broad range of antibiotics and mercury.

### Comparative Studies of CTX-M Variants

Sequences of *bla*<sub>TEM</sub> were found to be highly homologous to TEM-1 and TEM-116, while those corresponding to the *bla*<sub>CTX-M</sub> gene displayed a disparity in their homology with five different members of the CTX-M-group-1 viz. CTX-M-3, CTX-M-15, CTX-M-32, CTX-M-55, and CTX-M-71 (**Figure 2**). The conserved motifs involved in cefotaxime hydrolysis found in CTX-M-1, were also conserved among variants obtained in the study (**Figure S1**). The nucleotide sequence of one variant of CTX-M gene denoted CTX-M-152 was found to be highly homologous with members of the CTX-M-group-25. Unlike CTX-M-1, CTX-M-25 like β-lactamases has been observed less frequently worldwide. Similar to our study, two other novel variants, CTX-M-94 and CTX-M-100 belonging to group-25 were reported in Israel (Vervoort et al., 2012). The *bla*<sub>CTX-M-152</sub> variant identified in *K. georgiana* isolate was found localized on chromosomal DNA while those identified

**TABLE 2 | Characterization of gene sequences along with their MIC values for different antibiotics and heavy metals among ESBL<sup>+</sup> bacterial isolates.**

	<b>β-Lactamases genes</b>		<b>GenBank Accession nos.</b>		<b>mer operon genes</b>			<b>MIC values (μg/ml)</b>					
	<b>ESBL</b>	<b>Non-ESBL</b>	<b>16S rRNA gene</b>	<b>β-lactamases genes</b>	<b>merP</b>	<b>merT</b>	<b>merB</b>	<b>CAZ</b>	<b>CAC</b>	<b>CTX</b>	<b>CEC</b>	<b>CTR</b>	<b>HgCl<sub>2</sub></b>
<i>Klebsiella pneumoniae</i> MRA3	TEM-116	–	KJ906614		–	+	–	>512	0.38	512	0.125	>512	2
<i>Aeromonas</i> sps MRA5	TEM-116, CTX-M-15	–	KJ957158	KJ923000, KM873149	+	+	+	>512	0.75	512	0.25	128	20
<i>Aeromonas</i> sps MRA10	TEM-116, CTX-M-15	–	KJ957159	KJ923001, KM873161	–	–	–	4	1	16	0.75	4	2
<i>E. coli</i> MRA11	TEM-116	TEM-1	KJ957160	KJ923003, KM923002	+	+	+	512	>4	512	>1	>512	2
<i>Klebsiella oxytoca</i> MRA13	TEM-116	–	KJ957161	KJ923004	+	+	+	64	1.5	32	0.25	4	20
<i>E. coli</i> MRB2	TEM-116	–	KJ906615	KJ923005	+	+	–	256	2	64	0.5	64	2
<i>E. coli</i> MRB6	TEM-116, CTX-M-71	–	KJ906619	KJ923006, KM873170	+	+	+	32	0.5	>512	0.75	>512	20
<i>E. coli</i> MRC2	TEM-116, CTX-M-15	–	KJ906616	KJ923008, KM873150	+	+	–	16	2	128	0.5	512	20
<i>E. coli</i> MRC3	TEM-116, CTX-M-15	–	KJ906617	KJ923009, KM873151	–	–	–	32	1	512	0.5	>512	20
<i>E. coli</i> MRC6	TEM-116, CTX-M-71	–	KJ906618	KJ939551, KM873171	+	+	+	16	3	512	0.75	>512	2
<i>E. coli</i> MRC7	–	–	KM822763		+	–	–	32	0.25	16	0.125	16	2
<i>E. coli</i> MRC13	TEM-116	–	KM822764	KM873145	–	–	–	4	2	512	0.094	>512	2
<i>E. coli</i> MRC17	CTX-M-55	TEM-1	KJ906623	KM873174, KJ939552	+	+	–	256	>4	128	>1	256	20
<i>E. coli</i> MRC24	–	–	KM822765		–	–	–	>512	0.125	>512	0.094	128	2
<i>E. coli</i> MRE2	TEM-116, CTX-M-15	–	KC963022	KJ939553, KM873162	+	+	–	512	3	>512	0.125	512	2
<i>E. coli</i> MRF6	–	TEM-1	KC963027	KJ939560	+	+	+	>512	0.25	>512	0.032	512	20
<i>Acinetobacter</i> <i>junii</i> MRH8	CTX-M-15	TEM-1	KC963028	KM873163, KM593699	–	–	+	>512	3	256	0.023	16	2
<i>E. coli</i> MRK28	TEM-116	OXY	KJ923019	KM593700	+	+	–	512	1	128	0.125	512	2
<i>E. coli</i> MROB6	CTX-M-3	TEM-1	KC963015	KM873169, KM593701	–	+	+	64	0.5	16	0.125	16	20
<i>E. coli</i> MROB11	CTX-M-15	TEM-1	KC963018	KM873164, KM593702	+	+	+	512	0.5	128	0.125	16	20
<i>E. coli</i> MROB16	CTX-M-15	TEM-1	KC963017	KM873165, KM593703	–	–	+	256	0.38	128	0.19	64	20
<i>E. coli</i> MRAE2	TEM-116, CTX-M-32	–	KM822766	KM593704, KR560052	–	–	–	16	2	>512	0.25	>512	2
<i>E. coli</i> MRAE5	TEM-116, CTX-M-15	–	KJ906620	KJ939554, KM873152	–	–	+	8	0.5	512	0.032	>512	2
<i>E. coli</i> MRAE6	TEM-116, CTX-M-15	–	KJ906624	KJ939555, KM873153	–	+	–	16	3	>512	0.19	>512	2
<i>E. coli</i> MRAE9	CTX-M-15	–	KJ923010	KM873154	+	+	–	16	0.5	128	0.094	>512	20
<i>E. coli</i> MRAE14	TEM-116	–	KM822767	KM873146	+	–	–	16	0.75	512	0.125	>512	2
<i>E. coli</i> MRAE17	CTX-M-3	–	KJ906621	KM873166	+	+	+	16	2	512	0.094	>512	2
<i>E. coli</i> MRAE18	TEM-116, CTX-M-3	–	KJ906622	KJ939556, KM873167	+	+	+	>512	>4	>512	>1	>512	2

(Continued)

**TABLE 2 | Continued**

	$\beta$ -Lactamases genes		GenBank Accession nos.		<i>mer</i> operon genes			MIC values ( $\mu\text{g/ml}$ )					
	ESBL	Non-ESBL	16S rRNA gene	$\beta$ -lactamases genes	<i>merP</i>	<i>merT</i>	<i>merB</i>	CAZ	CAC	CTX	CEC	CTR	HgCl <sub>2</sub>
<i>E. coli</i> MRAE21	TEM-116, CTX-M-152	–	KJ923011	KM873147, KM873172	+	+	–	>512	2	>512	0.064	>512	2
<i>E. coli</i> MRAE23	CTX-M-15	TEM-1	KJ923012	KM873155, KM593705	–	+	+	>512	0.38	>512	0.125	>512	20
<i>E. coli</i> MRAE25	–	–	KJ923013		+	+	+	>512	1	>512	0.094	>512	2
<i>E. coli</i> MRAE26	TEM-116	–	KJ923014	KJ939557	+	+	+	>512	0.094	>512	0.125	>512	20
<i>E. coli</i> MRAE27	TEM-116, CTX-M-15	–	KJ923015	KJ939558, KM873156	–	+	–	512	0.38	256	0.125	512	2
<i>E. coli</i> MRAE31	CTX-M-15	–	KJ923016	KM873157	+	+	+	>512	>4	>512	>1	>512	20
<i>E. coli</i> MRAE32	TEM-116, CTX-M-3, CTX-M-15	–	KJ923017	KJ939559, KM873168, KM873158	+	+	+	8	0.5	512	0.125	>512	2
<i>E. coli</i> MRAE33	TEM-116	–	KM822768	KM873148	+	+	+	64	0.38	512	0.047	>512	20
<i>E. coli</i> MRAE36	CTX-M-15	–	KJ957162	KM873159	+	+	+	16	0.25	256	0.125	>512	20
<i>E. coli</i> MRAE42	CTX-M-15	–	KJ957163	KM873160	–	–	–	64	1	512	0.094	>512	2
<i>E. coli</i> MRAE44	TEM-116, CTX-M-152	–	KJ923018	KM593706, KM873173	+	+	–	512	>4	256	>1	512	2
<i>Kluvera georgiana</i> MRB7	TEM-116, CTX-M-152	–	KM822769	KJ923007, KJ461948	+	–	–	256	1.5	>512	0.38	256	2
<i>Klebsiella pneumoniae</i> ATCC 700603	–	–	–	–	–	–	–	>32	2	8	0.75	16	0.02
<i>E. coli</i> ATCC 25922	–	–	–	–	–	–	–	2	0.094	1	0.023	0	0.02

CAZ, Ceftazidime; CAC, Ceftazidime + clavulanic acid; CTX, Cefotaxime; CEC, Cefotaxime + clavulanic acid; CTR, Ceftriaxone; HgCl<sub>2</sub>, Mercuric chloride.

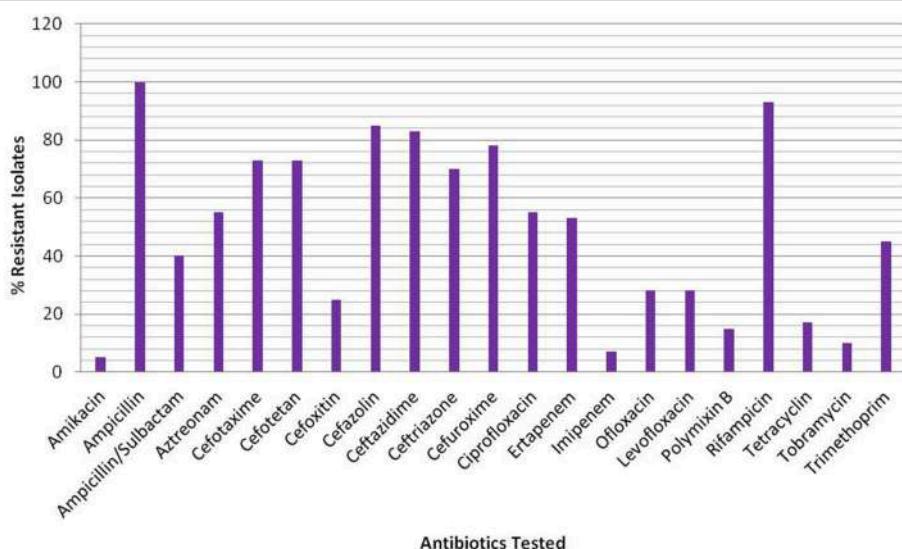
in *E. coli* isolates screened from samples collected from downstream region of river Yamuna were found localized on plasmid. It strongly depicts their mobilization in the aquatic habitat. *K. georgiana* isolate harboring *bla*<sub>CTX-M-152</sub> was found to have high MIC values against penicillins, cephalosporins, monobactams, and rifampicins (Table 3).

Sequence of *bla*<sub>CTX-M-152</sub> was found to be 98.6% similar with *bla*<sub>CTX-M-78</sub> (CTX-M-group-25 member first identified in *K. georgiana*; Rodríguez et al., 2010). *bla*<sub>CTX-M-152</sub> also showed 97.3% homology with the *bla*<sub>CTX-M-25</sub> and *bla*<sub>CTX-M-94</sub> sequences, 97.5% homology with *bla*<sub>CTX-M-39</sub>, and 97.4% homology with *bla*<sub>CTX-M-26</sub>, *bla*<sub>CTX-M-89</sub>, and *bla*<sub>CTX-M-100</sub>. Both variants (CTX-M-152 and CTX-M-78) differed from other members of the CTX-M-group-25 by amino acids substitutions M7I, C24Y, G81D, P99S, and G146D. CTX-M-152 is the only variant in the CTX-M-group-25 with a T154A substitution. This is the first report of any CTX-M variant

containing histidine substituted with glutamine at position 26 (Figure S1). The genetic relatedness of the identified variant to members of group-25 obtained by MEGA6 suggests earlier branching of CTX-M-152, possibly due to Q26H, T154A, G89D, P99S, and D146G amino acid substitutions.

### SIFT Analysis for Deleterious Substitutions

A homology check of the sequence revealed 10 individual substitutions (Q26H, Q89D, N92S, P99S, V103I, A120G, T189A, H197N, T209M, P266S) in sequence of CTX-M-152 in comparison to the most relevant CTX-M-9 with respect to sequence homology. To predict the functional importance of amino acid substitutions, all 10 substitutions were submitted independently to the SIFT programme to check their tolerance index with respect to CTX-M-9. Of the 10 substitutions, one having the T189A substitution was found to be deleterious, with a tolerance index score of 0.02, while others nine viz. Q29H,



**FIGURE 1 | Percentage of resistant isolates against the  $\beta$ -lactam and non  $\beta$ -lactam classes of antibiotics.**

Q92D, N95S, P102S, V106I, A123G, H200N, T212M, and P269S are tolerable having tolerance index score of 0.12, 0.32, 0.64, 0.08, 0.17, 0.15, 0.50, 0.10, and 0.37, respectively.

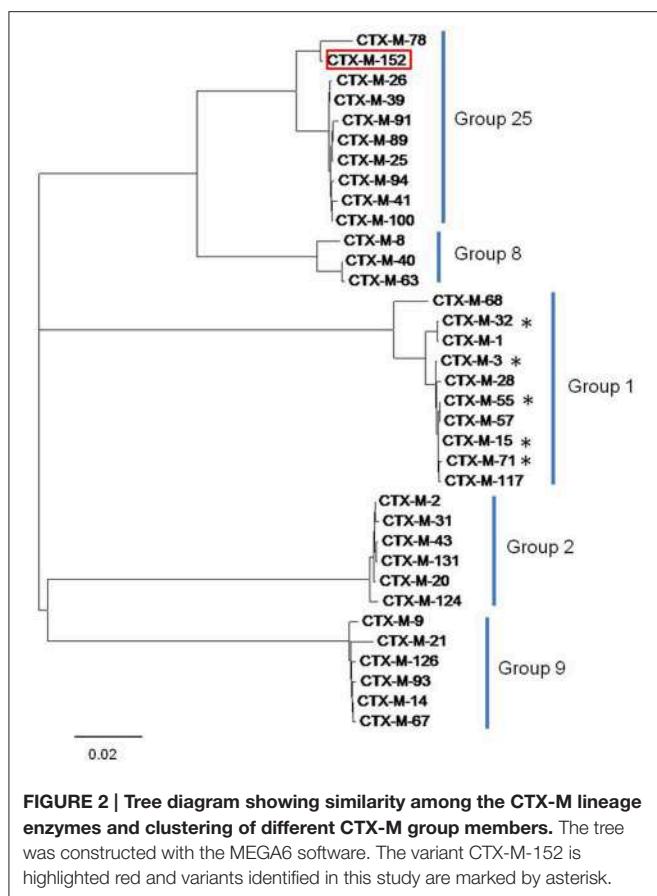
## Homology Modeling of CTX-M-152

When compared to the well-studied TEM, SHV and CTX-M-1 group members, there have been no structural studies of CTX-M-group-25 members to best of our knowledge. The nucleotide sequence of *bla*<sub>CTX-M-152</sub> contains several point mutations (87G→C, 368C→G, 369A→G, 574A→G, and 576G→T), which lead to Q26H, A120G, and T189A amino acid substitutions, respectively. Upon finding close relatedness to CTX-M-9 (PDB 1YLJ), the backbone was used to generate the structural model of CTX-M-152. The structure generated by I-TASSER was monitored for stereochemical quality of the models using the PROCHECK and Rampage programs. As revealed by the Ramachandran contour plot obtained using the Rampage software, over 84% of the amino acid residues in the modeled structure were present in the most favored region, while another 10% were in the allowed region. The modeled structure contains an  $\alpha$  and  $\alpha/\beta$  domain, with the active site residing at the interface of two domains. Despite differences in orientation that include length of the helix and pleated regions, resemblance in the backbone structure of the two variants (CTX-M-9 and -152) shows an RMSD value ( $C^{\alpha}$ ) of 0.412 Å (Figure 3). The pI value as calculated by ExPasy tool was reported to be 8.81.

## Docking with Cefotaxime

For docking purpose, a maximum of nine different conformations corresponding to cefotaxime (DB00497) were taken into account to estimate ligand binding conformations using the Lamarckian Genetic Algorithm (LGA) in the Auto dock. The conformation of the ligand with least binding energy indicates high affinity of  $\beta$ -lactamases for cefotaxime. Therefore, ligands showing a binding energy of -7.6 Kcal/mol that depict

a more stable and effective interaction for facilitating the enzyme activity, was selected for further analysis. Although the docking results revealed interactions via seven H-bonds with five proposed active site residues (Ser70, Asn132, Ser237, Gly238, and Arg273), there were other surrounding residues that were also found to contribute toward hydrophobicity at the active site (Figures 4, 5). The active sites residues in association with the surrounding interacting residues are known to have four conserved regions that are critical to catalyze the substrate. The first conserved element (Ser70-Xxx-Xxx-Lys73) contains active serine70 and one helix turn with downstream Lys73, pointing to the bottom of the active site. Accordingly, we found that residues were similar, even in the structure of CTX-M-152. Drawz and Bonomo (2010) showed that CTX-M enzymes use this reactive serine (Ser70), a catalytic water molecule and an activator residue (Glu166) to hydrolyze the  $\beta$ -lactam ring through an acid-base catalytic mechanism (Drawz and Bonomo, 2010). Complying with the results of Chen et al. (2005a) regarding substitution of histidine for proline at position 99 in CTX-M-27, Pro99Ser substitution in CTX-M-152 was found to be in the tolerable range, thereby conferring no change in stability and function of CTX-M-152 (Chen et al., 2005a). The second motif, Ser130-Asp131-Asn132 situated on the short loop in the alpha domain forms the left side of the catalytic cavity. Among Ser130, Asp131, and Asn132 residues in the structure of CTX-M-152, only Asn132 interacted with cefotaxime. These findings are in accordance with studies suggesting residues in helices and pleated regions (in the case of CTX-M-9) are favored over linker region residues for interaction with the substrate. Similarly, residue Asn104 and Tyr105 forms a bend in the binding site as observed in the structure of both CTX-M-9 and CTX-M-152. While working with the Toho-1-cefotaxime complex, Shimamura et al. (2002) reported involvement of the N104 side chain residue in hydrogen bonding with the side chain carbonyl of cefotaxime (Shimamura et al., 2002). However,

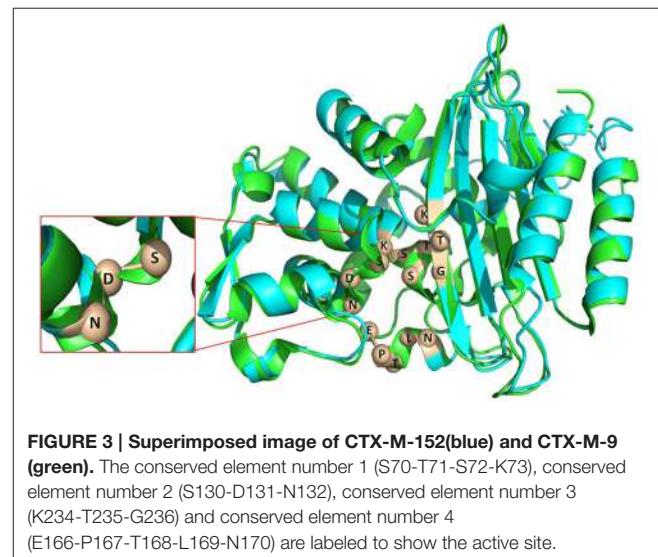


**FIGURE 2 |** Tree diagram showing similarity among the CTX-M lineage enzymes and clustering of different CTX-M group members. The tree was constructed with the MEGA6 software. The variant CTX-M-152 is highlighted red and variants identified in this study are marked by asterisk.

changes in the hydrolytic pocket resulting from substitution of isoleucine for valine (V103I) render N104 residue out of range to form a hydrogen bond. The V103 residue conserved in CTX-M-1, -2, and -14 was replaced by isoleucine among members of CTX-M-group-25 in a similar method as reported for CTX-M-group-8. The conformational change influencing the positioning of Asn104 and/or Tyr105 greatly affects the interaction between enzymes and substrates. Based on the location of the third conserved element (Lys234-Thr235-Gly236) on the  $\beta$ 3 strand of the  $\beta$ -sheet in the  $\alpha/\beta$  domain, it forms the opposite wall of the catalytic cavity. Shakil and Khan (2010) reported the same type of interaction between side chains of cefotaxime and the backbone oxygen of Ser237 (Shakil and Khan, 2010). Upon docking of cefotaxime against the modeled CTX-M-152, only Ser237 of the Ser237 and Asn104 residues was found to interact with the carboxylate group of cefotaxime. This interaction might induce rotation of the carboxylate group in the acyl-intermediate structure of CTX-M, bringing the carbonyl group of  $\beta$ -lactam to a suitable position in the oxyanion hole, thereby promoting drug-hydrolysis. The last conserved element (Glu166-Pro167-Thr168-Leu169-Asn170) located on the 19-residue loop (positions 161–179), which is referred to as the omega ( $\Omega$ ) loop, forms the floor of active site. Both the  $\beta$ 3 strand and the  $\Omega$ -loop are important constituents of the active site cleft.

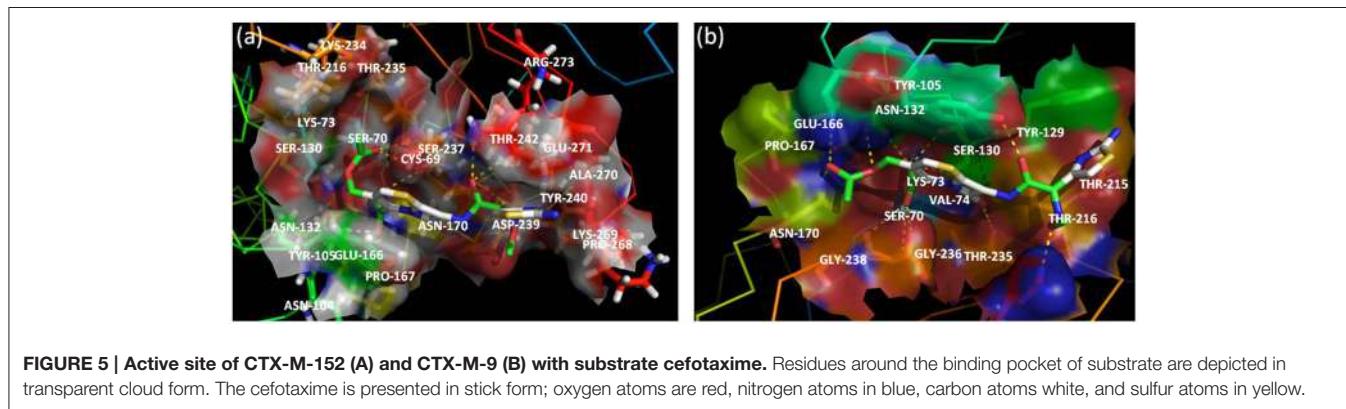
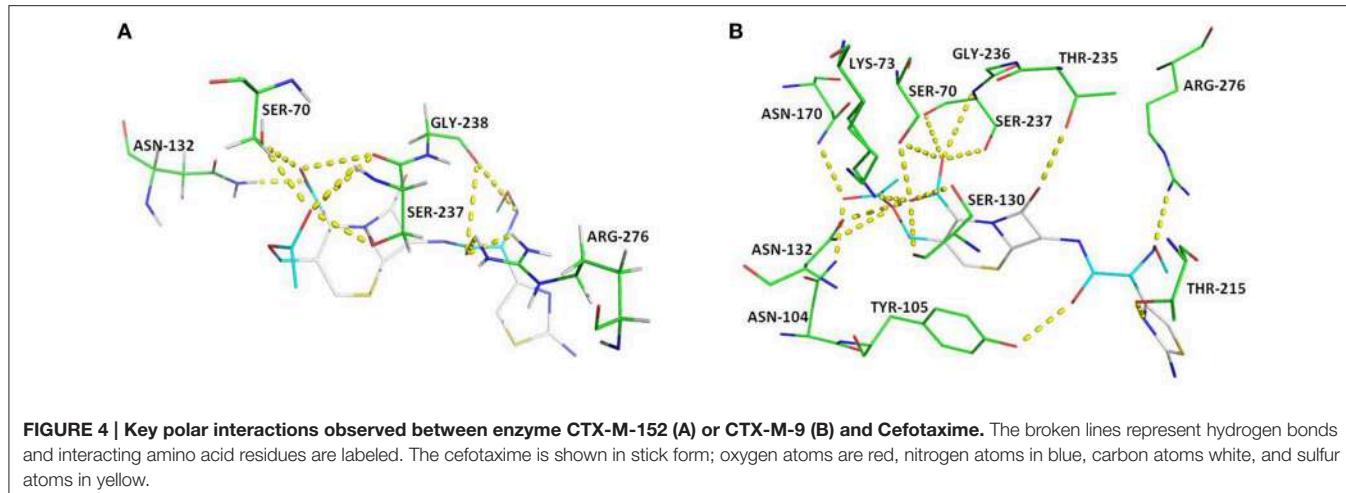
**TABLE 3 | MIC values corresponding to different antibiotics for *K. georgiana* MRB7 isolate.**

Antibiotic(s)	MIC (mg/L)
Amikacin	0.5
Amoxycillin	>240
Aztreonam	120
Carbenicillin	>512
Cefotaxime	>512
Cefotaxime + Clavulanic Acid	0.38
Ceftazidime	256
Ceftazidime + Clavulanic Acid	1.5
Ceftriaxon	256
Ciprofloxacin	2
Kanamycin	3
Ofloxacin	0.1
Rifampicin	32
Tetracycline	12



**FIGURE 3 |** Superimposed image of CTX-M-152(blue) and CTX-M-9 (green). The conserved element number 1 (S70-T71-S72-K73), conserved element number 2 (S130-D131-N132), conserved element number 3 (K234-T235-G236) and conserved element number 4 (E166-P167-T168-L169-N170) are labeled to show the active site.

Among other residues that contribute to effective interaction between enzymes and antibiotics, Asn104, Ser237, Asp240, and Arg276, which form the flexible arms of the  $\beta$ 3 strand and  $\Omega$ -loop, were found to be involved in cefotaxime hydrolysing activity of CTX-M enzymes. As reported by Perez-Llarena et al. (2011), Ala219 in the loop that connects  $\alpha$  and  $\alpha/\beta$  domains (occupying top of active site), and are critical to the flexibility and breathing dynamics of  $\beta$ -lactamases, was also found conserved in CTX-M-152 (Perez-Llarena et al., 2011). In another study, Delmas et al. (2008) found that Val180, Arg191, Ala247, and Val260 constituted different hydrophobic clusters, thereby affecting the dynamics and flexibility of enzymes important to the hydrolysis of substrates (Delmas et al., 2008). Frequent among class A members, Arg274 residue increase substrate specificity because the side chain point toward the active site cavity. Similarly, residues Cys69, Ser72, Met135, Phe160 Thr165, and Ser237, which were conserved in CTX-M-152, are considered important with respect to substrate specificity (Péduzzi et al.,



1997). In comparison to Asn270 residue of CTX-M-14 (along with other members of group-9) involved in establishing hydrogen bond with Asp240, CTX-M-152 similar to other members of group-25 possesses Lys270 to interact with residue Asp240 for correct positioning of  $\beta$ 3 strand residues during catalytic process. CTX-M-152 containing Asp240 rather than Gly240 that increases its catalytic efficacy against ceftazidime, helped the variant to retain the high stability of the enzyme in the activity stability trade-off.

## DISCUSSION

The escalating problem of multidrug resistance among infection causing organisms represents one of the greatest challenges worldwide. With increased antimicrobial usage, complexities in the resistance mechanisms have become more advanced. Densely populated centers with improper water supplies and inconsistent sanitation contribute significantly to acquisition and dissemination of resistance determinants among microbial inhabitants of water bodies. The Yamuna River, which originates from the Yamnotri glacier in the lower Himalayas ( $38^{\circ}59'N$   $78^{\circ}27'E$ ), is the major source of water to urban areas in Delhi. Although the proportion of the river catchment area in Delhi is small (~2%), this area contributes more than 50% of pollutants

that it receives through sewage from urban effluents, with high levels of antimicrobials in addition to toxic compounds being discharged by industries (Sharma and Kansal, 2011; Sehgal et al., 2012; Mutiyar and Mittal, 2014). The acquisition and transmission of resistance genes from microflora of human and animal origin discharged as part of sewage can substantially influence the pattern of resistance among the microbial inhabitants of the aquatic ecosystem (Amos et al., 2014).

Increasing incidences of ESBL-producing bacteria that showed a drastic shift in recent years in environmental settings are of serious concern. Contribution of selection to acquisition and as such spread of resistance among bacteria against major classes of antibiotics is alarming due to their higher dissemination rate. As such, high prevalence of ESBL producing isolates in natural water bodies like ponds, lakes, rivers, and tap water has drawn concern regarding increased spread of resistance in the environment (Upadhyay and Joshi, 2015). Recently, Bajaj et al. (2015) and Ahammad et al. (2014) also reported high prevalence of the several  $\beta$ -lactamase genes (TEM, SHV, CTX-M, AmpC, and NDM-1) among *E. coli* and other coliform bacterial species screened in collected water samples from upper ranges of Ganges River till its tributary Yamuna that stretches in Delhi and beyond. In our study, 93% of ESBL<sup>+</sup> Gram negative isolates were observed to harbor *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, and/or *bla*<sub>OXY</sub>. These

variants were showing similarity to those reported by Wattal et al. (2010) and Rastogi et al. (2010), during their studies on ESBL production among clinical isolates (Rastogi et al., 2010; Wattal et al., 2010). In our study, we found ESBL producing bacterial isolates to have co-resistance to five others non- $\beta$ -lactam classes of antibiotics in addition of exhibiting resistant phenotype to aztreonam (55%), ceftazidime (83%), cefazolin (85%), cefotaxime (73%), cefotetan (73%), and ertapenem (53%). The resistance of 21 isolates to ertapenem alerts for the decrease of carbapenem activity in the treatment of potential infections caused by these bacteria. These results are in concordance with the study of Datta et al. (2012), which reported steady increase in the percentage of carbapenem resistance among *E. coli* (40% in 2002 to 61% in 2009) and *K. pneumonia* (2% in 2002 to 52% in 2009) in a span of 10 years in tertiary-care hospital in New Delhi. In an another study, Center for Disease Dynamics, Economics & Policy (CDDEP) reported an increase in the percentage of carbapenems resistance from 10% in 2008 to 13% in 2013 among *E. coli* and 29% in 2008 to 57% in 2014 in isolates of *K. pneumonia* from India (Datta et al., 2012; Center for Disease Dynamics Economics and Policy (CDDEP), 2015). Besides strengthening the existing knowledge of their prevalence, presence of MDR, XDR, and PDR bacteria in the natural environment endorses them for the potent threat that they posses for the mankind. Mercury tolerance among the tested isolates appears to be an adaptation of bacteria that is correlated with their ability to live in mercury polluted environments. Accordingly, investigations of ESBL genes (*bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>) among bacteria that harbor both multidrug resistance and mercury tolerance are thought to provide useful information regarding their epidemiology in human influenced polluted environments.

Identification of *bla*<sub>CTX-M-152</sub> in *K. georgiana* as part of this study is the first report of identification of any CTX-M-group-25 member from India. Docking studies of cefotaxime against modeled CTX-M-152 revealed that formation of a hydrogen bond between Ser237 and a carboxylate oxygen of cefotaxime induced rotation of the carboxylate group in the acyl-intermediate structure of CTX-M. Interaction of Ser237 with cefotaxime helps bring the carbonyl group of  $\beta$ -lactam to a suitable position in the oxyanion hole, thereby promoting hydrolysis of the drug. This was confirmed by the mutant S70G:S237A:R276A-cefotaxime complex, which displayed a significant loss of activity as a cefotaximase (Adamski et al., 2015). Simultaneously, Ser237 and Arg276, which are responsible for high substrate specificity, act cooperatively to promote cefotaxime hydrolysis through structural alterations in the active site to accommodate the larger cefotaxime molecule (Delmas et al., 2008, 2010). In a recent study, Adamski et al. (2015) revealed that co-operative interactions of Ser237 and Arg276 for cefotaxime enhance hydrolysis (~30-fold) relative to TEM-1/SHV-1 (Adamski et al., 2015). Hence, the presence of Ser237 and Arg276 in CTX-M-152 supports its high catalytic efficacy, even though it has a small active site similar to classical TEM-1 and SHV-1. Amino acid substitutions are of considerable significance in that they have direct or indirect involvement in changing enzyme activity. The hydrogen bond between side chains of Lys234 and Ser130 connecting  $\alpha$  domain and  $\alpha/\beta$

domain in class A enzymes was not observed in CTX-M-152. Conversely, substitutions such as N92S, P99S, A121G, and H197N, which represent changes with bulkier to light side chains, are thought to be involved in increasing the flexibility of protein, which is responsible for its high catalytic efficiency.

Delmas et al. (2008, 2010) reported binding of cefotaxime to CTX-M-9 that results in conformational changes at active sites though breakage of the hydrogen bond between Asn170 and Asp240 connecting the omega loop to the  $\beta$ 3 strand (Delmas et al., 2008, 2010). This expands the active site to allow adequate positioning of the cefotaxime substrate for catalysis. The Asp240Gly substitution, which is known to increase the activity toward ceftazidime, was missing in CTX-M-152. Similar to Asp240Gly, the Val231Ala substitution on the  $\beta$ 3 strand responsible for the stability-activity trade-off in the evolution of resistance enzymes, was absent from CTX-M-152. Although the two substitutions (Val231Ala and Asp240Gly) in the CTX-M enzymes did not alter the active site configuration, both have been reported to cause decreased protein stability, presumably through loss of favorable packing and polar interactions (Chen et al., 2005b). Hence, CTX-M-152 with residues having higher stability obtained from the Yamuna River isolate of *K. georgiana* is believed to be the progenitor of CTX-M genes. It is in concordance with the previous reports that documented the possible emergence of *bla*<sub>CTX-M</sub> genes from *Kluyvera sp.* (Sarria et al., 2001; Humeniuk et al., 2002; Bonnet, 2004; Munday et al., 2004; McGgettigan et al., 2009; Zhang et al., 2009).

Continuous threat posed by resistant organisms to human health has necessitated the need for further studies to improve understanding of their resistance mechanisms. Presence of CTX-M-152 with high stability and hydrolytic efficacy in an isolate of *K. georgiana* from the river Yamuna goes hand-on-hand with the generalization regarding natural environment acting as a source of resistance genes from which newer variants of enzymes evolve. Alongside, the presence of CTX-M-152 variant on the plasmid of *E. coli* isolates collected from downstream region of river Yamuna possibly demonstrate the mobilization of resistance genes through recombination events in bacteria. High incidence of CTX-M family member's warrant additional studies to be performed that might provide deeper insight into prevalence and information about the factors that led to spread of the resistant determinants, thereby can help in adopting strategies that can prevent selection, expansion, and transmission of resistance genes among bacteria associated with multiple human complications.

## AUTHOR CONTRIBUTIONS

QH conceived the topic. MA and AT contributed equally to this work. MA, AJ, and QH together contributed equally to the writing.

## ACKNOWLEDGMENTS

Mudsseer Azam acknowledge Council of Scientific & Industrial Research (CSIR), India for financial support as Senior Research Fellowship (09/466(0136)/2011-EMR-I).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00176>

**Table S1 |** *In vitro* susceptibility of ESBL<sup>+</sup> bacterial isolates to 3<sup>rd</sup> generation cephalosporins.

**Table S2 |** Phenotypic disc confirmatory test of ESBL<sup>+</sup> bacterial isolates.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Breaking the Spell: Combating Multidrug Resistant ‘Superbugs’

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Multidrug-resistant (MDR) bacteria have become a severe threat to community wellbeing. Conventional antibiotics are getting progressively more ineffective as a consequence of resistance, making it imperative to realize improved antimicrobial options. In this review we emphasized the microorganisms primarily reported of being resistance, referred as ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*) accentuating their capacity to “escape” from routine antimicrobial regimes. The upcoming antimicrobial agents showing great potential and can serve as alternative therapeutic options are discussed. We also provided succinct overview of two evolving technologies; specifically network pharmacology and functional genomics profiling. Furthermore, *In vivo* imaging techniques can provide novel targets and a real time tool for potential lead molecule assessment. The employment of such approaches at prelude of a drug development process, will enables more informed decisions on candidate drug selection and will maximize or predict therapeutic potential before clinical testing.

## OPEN ACCESS

### Edited by:

Gilberto Igrelas,  
University of Trás-os-Montes and Alto Douro, Portugal

### Reviewed by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 30 October 2015

**Accepted:** 01 February 2016

**Published:** 18 February 2016

### Citation:

Khan SN and Khan AU (2016)  
Breaking the Spell: Combating Multidrug Resistant ‘Superbugs’.  
*Front. Microbiol.* 7:174.  
doi: 10.3389/fmicb.2016.00174

## INTRODUCTION

A change in the pattern of serious hospital infection after the introduction of antibiotics was noticed early in the antibiotic era. Furthermore with the triumph of the penicillin discovery and the synthesis of antibacterial sulfonamides in the initial half of the 20th century, the modern antimicrobial revolution started. There onward, newer antimicrobial were reported and several semi-synthetic and synthetic antibacterial agents came into existence. Antimicrobial drugs have cured plenty of cases with life-threatening bacterial infections and relieved patients’ agony. Unfortunately, unrestrained use of antibacterial past 50 years has wielded selection pressure on susceptible bacteria stains, which attributed to the endurance of drug resistance (Levy and Marshall, 2004; Tacconelli, 2009), among them some are resistant to more than one antibiotic. Presently, the treatment of these infections has once again becomes increasingly complicated as microorganisms are becoming resistance to the available antimicrobial options (Pitout and Laupland, 2008; Nordmann et al., 2011; Khan and Nordmann, 2012a; Labro and Bryskier, 2014). With course of time, sustained selective pressure by various antibiotics has culminated into organisms augmenting ancillary resistance mechanisms that led to multidrug resistance (MDR)—novel penicillin-binding proteins (PBPs), enzyme dependant drug alteration, altered membrane permeability, mutated drug targets and increased efflux pump expression. Further to mention few most challenging MDR organisms presently being encountered includes the so called ESKAPE pathogens like *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, and *Klebsiella pneumoniae* with

extended-spectrum  $\beta$ -lactamases (ESBL), vancomycin resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant MRSA strains, extensively drug-resistant (XDR) *Mycobacterium tuberculosis* and newly identified transmissible carbapenamase, New Delhi metallo-beta-lactamases (NDM) in Enterobacteriaceae (Alekshun and Levy, 2007; Gootz, 2010; Khan and Nordmann, 2012b). Carbapenems were the only sensitive antibiotics for the treatment of MDR coliforms but the development of carbapenem resistance recently is a matter of great concern. Efforts directed toward identifying newer antibiotics were formerly an exquisite research area and development priority among pharmaceutical giants but poor success rate has dampens the interest. Further studies comprehending resistance illustrates that the evolution and spread of antimicrobial resistance (AMR) is in fact, a very convoluted issue. Hence, a sole target will not ensure eradication of AMR; rather a coordinated multidisciplinary approach is needed to tackle this problem (Smith et al., 2009; Cantas et al., 2013). Mortality rates and length of hospital stay associated with the treatment of drug resistant infections are about twice as big when patients infected with drug sensitive bacteria of the same species, thereby ensuing inflation in healthcare costs.

The aim of this article is to emphasize the ever growing problem of antimicrobial resistance, counting in the present approaches to limit the spread of MDR. We specifically highlighted how the emerging technologies could be a great promise for new antimicrobial discovery.

## FAILURE OF PRESENT MEASURES TO COMBAT MDR

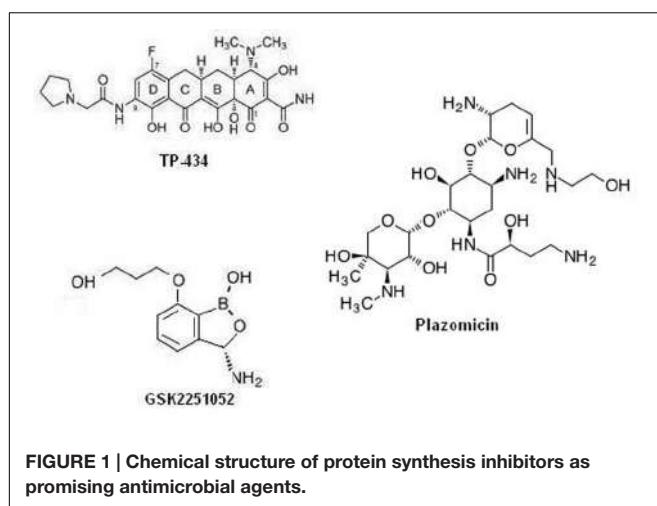
Multidrug-resistant is prevalent in nature, and the strategy of eliminating resistance genes makes no sense, as the natural function of most resistance genes is not primarily confirming MDR (Morar and Wright, 2010). Most probably, there is a huge “intrinsic resistome” in bacterial organisms, composed of various genes with diverse phylogeny which contribute to resistance only on interaction with the antibiotic (Fajardo et al., 2008; Grgis et al., 2009; Sommer et al., 2009). Possible strategy to combat with emerging drug resistance is to control the emergence, selection, and spread of MDR strains of bacteria from hospital settings and community (Wright, 2009). The conventional strategies of combating the emergence and spread of MDR atypically relies on the discovery of newer drugs (Wright, 2009; Nordmann et al., 2012), reduction in antibiotic induced bacterial mutation, genetics dependant recombination and horizontal-transfer at lower concentration of drugs (Couce and Blázquez, 2009), suppression of phenotypic traits of resistance (Udekwu et al., 2009), use of combinations therapy (De Cristóbal et al., 2008), including antagonistic drugs (Drew, 2009), early intensive (frontline) therapy, maintaining a low bacterial density (Motter, 2010), and lately, surveillance of hypermutable organisms (Oliver et al., 2000; Carattoli, 2009) and targeting regulating functions necessary for infection (Dandekar and Dandekar, 2010; Greenberg et al., 2010). In essence, these preventive measures are proving increasingly inadequate in the existing global scenario

of MDR (Boucher et al., 2009). Averting the spread of resistance can apparently be significant for the person, but exhibits weak impact on the community (Durante-Mangoni and Zarrilli, 2011). Procedures that might work in the initial phase of the development of resistance in hospitals or nations with low rates of MDR, may not be competent enough in regions with prevailing higher resistance frequency (De Gelder et al., 2007). Reports from regions with low levels of MDR such as Sweden, revealed that discontinuance of trimethoprim use for 2 years had no impact on the resistance rate of *Escherichia coli*. That was further linked to the extensive dissemination of trimethoprim resistance genes (*dfr*) along with other resistance determinants, thus encouraging co-selection of *dfr* with other resistance genes as well (Brolund et al., 2010). In a global scenario, eventually, resistance commencing in these heavily contaminated “sources of resistance” will infest areas that are still cleaner (Thaller et al., 2010). Thus, antibiotic resistance keep evolving to attain higher antibiotic resistance exhibiting elevated bacterial genetic evolvability, a phenomenon known as “genetic capitalism”, where the rich tends to become richer. It refers to further adaptive potential of an organism to enrich their resistance mechanism either via mutational or gene acquisition events.

## POTENTIAL THERAPEUTIC INTERVENTIONS

### Molecules Targeting Protein Synthesis

Among the many exploited targets the one, which has been frequently approved by nature and by many successful clinical trial is the ribosome. Many antibiotics targeting protein synthesis are in development or in different phases of clinical trials as a prospective remedy of temperate-to-serious community acquired bacterial infections: solithromycin, cethromycin, omadacycline, CEM-102, GSK1322322, radezolid, and tedizolid. Interestingly, antibiotics targeting the cellular protein synthesis, TP-434, GSK2251052, and plazomicin, are reported to have a range that covers drug resistant gram negative class of bacteria. Among which, TP-434 (Figure 1) is a C7, C9 di-substituted broad-spectrum tetracycline based antibiotic was discovered and being developed by Tetraphase Pharmaceuticals, MA, showed effective mechanism-driven impediment of protein synthesis in a coupled transcription/translation assay [half maximal inhibitory concentration ( $IC_{50}$ ) = 0.29  $\mu$ g/mL] and a competition assay employing radio labeled tetracycline (Grossman et al., 2012). Furthermore, TP-434 activity is also reported against tetracycline-specific efflux proteins (including *mepA*) genes bearing stains, ribosomal defense mechanisms and for enzymes capable of destructing tetracycline antibiotics (Sutcliffe et al., 2013). Further, TP-434 is insusceptible to *blaNDM-1* positive carbapenemase that has recently been reported in enterobacteriaceae. Plazomicin (ACHN-490, Figure 1), a “neoglycoside,” a semisynthetic drug evolved from sisomicin with considerably enhanced efficacy toward amikacin and gentamicin resistant bacteria (Aggen et al., 2010; Armstrong and Miller, 2010). The minimum inhibitory concentration (MIC) 50/90 reported for plazomicin ranges in 0.5–1  $\mu$ g/mL against resistant *K. pneumoniae*, including the serine



**FIGURE 1 |** Chemical structure of protein synthesis inhibitors as promising antimicrobial agents.

carbapenemase KPC strains and 1/2  $\mu\text{g}/\text{mL}$  against 493 strains of MRSA positive bacteria (Tenover et al., 2011). Whereas, MICs tested against 65 carbapenem resistant enterobacteriaceae was found to be  $\leq 2 \mu\text{g}/\text{mL}$  except for NDM metallo- $\beta$ -lactamase producers (Livermore et al., 2011). Plazomicin has successfully concluded a level 2 in global multilocation indiscriminate double blind trial evaluating its efficacy relative to levofloxacin, a gold standard for complex urinary tract infection (cUTI) and acute pyelonephritis (AP) and now registered in a level 3 trial stage ([ClinicalTrials.gov](#) identifier: NCT01970371). GSK2251052 (Figure 1), is a novel boron derivative that interacts with the leucyl-tRNA synthetase at adenosine ribose (A76) termini with an  $\text{IC}_{50}$  of  $0.31 \mu\text{M}$ , thereby aborting protein synthesis (Goldstein et al., 2013). The compound has shown variable range of  $\text{MIC}_{50/90}$  for various rampant gram negative aerobic and anaerobic bacteria and anaerobic gram-positive strains (Mendes et al., 2013). Further, in an *in vitro* potency test against blaNDM-1 producing enterobacteriaceae isolates,  $\text{MIC}_{90}$  of the compound was reported to be  $1 \mu\text{g}/\text{mL}$ , with a range of  $0.5\text{--}2 \mu\text{g}/\text{mL}$ . This novel antibiotic has also shown activity toward category A and B bacterial biothreat infections, with  $\text{MIC}_{90}$  concentration in the range of  $0.12\text{--}2 \mu\text{g}/\text{ml}$  (Heine et al., 2011). When tested on healthy cohort as single IV doses of 200, 400, 900, 2,000, or 3,000 mg (1-hour infusion) and as multiple ascending doses (MADs) of 500, 750, 1,200, or 2,000 mg two times a day for 8 days (500 mg) or 14 days (all other cohorts) was well taken, with no severe implications leading to patients withdrawal but a recent article reports development of some resistance toward it due to specific mutations in the LeuRS editing domain (O'Dwyer et al., 2015). A review by Sutcliffe on antibiotics targeting protein synthesis describes them in detail (Sutcliffe, 2011). Specifically, Dewan et al. (2014) describes in detail the importance aminoacyl-tRNA synthetases as a therapeutic target for novel antibiotic development.

## Peptides

Combinational administration of antimicrobial peptides (AMP's) with other antimicrobials has been recommended as an

alternative strategy for enhanced therapeutic outcomes. They act by affecting cell membrane integrity, inhibiting protein and cell wall synthesis, altering enzyme activity, and others (Guilhelmelli et al., 2013). Their discrete therapeutic potency and less side effects makes AMP's interesting candidates for concurrent or subsequent use in diverse bacterial infections. AMP's are recently regarded as a viable option for eradicating the multidrug resistant micro-organisms, based on their distinct mechanism of action than that of the presently used antibiotics (Fjell et al., 2011; Nan et al., 2012). Till date, more than 3700 AMP's have already been reported and characterized from flora, fauna, and other sources (Tossi and Sandri, 2002). AMP's can also be produced synthetically and moreover, recombinant AMP's can be yielded by various recombinant cell and/or organisms (Yu et al., 2010). A good number of synthetic AMPs are being tested in clinical trials and in excess of 15 peptides or mimetics are in (or have completed) clinical testing as antimicrobial agents. In a phase 1/2 of clinical testing, the AMP hLF1-11 (1–11 amino acids long amino-terminal of human lactoferrin) was found to be risk free with no after effects when delivered intravenously (Velden et al., 2009). Two peptides, pexiganan and omiganan have exhibited efficacy in Phase III clinical trials but waiting for approval. Pexiganan (Lipsky et al., 2008), a derived from magainin, demonstrated similar effectiveness to an oral fluoroquinolone for treating patients with diabetes foot ulcer but was not approved by the US Food and Drug Administration, although there is indication that it might resurface in clinical trials. Omiganan (MBI226), a derivative of indolicidin, proved competent in drastically limiting catheter colonization and microbial tunnel infections during catheterization (ClinicalTrials.gov identifier: NCT00608959). AMP's when tested for treating UTI, the most common infections in hospital settings and well documented in general population, seems to work best due to the optimum environment where peptides naturally, exhibit their potency (Lupetti et al., 2003; Melendez-Alafort et al., 2004). Cudic et al. (2003), illustrated that higher salt concentrations decreases the antimicrobial activity of various AMP's, however, lactoferricins and derivates thereof, are quite active against UTI, regardless of limited antimicrobial activity at higher salt concentration as suggested in *in vitro* settings. Probably, the peptide is effective in eradicating urinary tract infections at remote sites, likely through passive or active transfer of the peptide to the contagion site via renal discharge (Del Olmo et al., 2009; Fornili et al., 2010).

Administration of antimicrobial peptides treated equipment offers potential microbial protection, although the attrition in antimicrobial attributes due to cross linking of the peptide to solid supports must be addressed (Onaizi and Leong, 2011). There are satisfying instances for cationic peptides possessing clinical efficacy (Gordon et al., 2005); polymyxin a cationic lipopeptide is the last resort treatment for multi-drug resistant *Pseudomonas* and *Acinetobacter* infections, the gramicidin S a cyclic cationic peptide is frequently prescribed in topical and eye infections, and the cationic nisin antibiotic is already an approved food supplement in Europe. As per our inference, the increasing accessibility and exploitation of high throughput involved strategies has shown substantial prospects to improve the search for the next generation therapeutic peptides and

peptide mimetic as antimicrobials not only specific for bacteria that are resistant to existing antibiotics but also for eliminating other disease causing bugs like protozoa, helminthes, insects and fungi (Bommarius and Kalman, 2009; Tavares et al., 2013).

## Vaccines

Rational designing of vaccines have markedly improved with the advancement of recombinant DNA technology against complex human pathologies. In spite of extraordinary performances in pre-clinical and clinical testing, only few recombinant vaccines are deemed safe for humans use till date. Wherein, extensive screening procedures for its production and speculations about their use might be accountable. Recent application of new approaches like systems biology and advanced immunology to better understand mechanism of vaccines is propelling the evolution of vaccinology. Moreover, the outcomes of synthetic biology in vaccine design as evident in the first synthetic bacterium, demonstrates its distinction and rational attributes. Over the last few years there have been several excellent research articles describing strategies of creating the ‘optimal’ vaccine, as well as highlighting the significant challenges of developing an effective vaccines (Otto, 2010; Wu et al., 2012). Inactivated bacterial strains have shown to be extremely immunogenic against Gram-negative bacteria and exhibit protective immunity against several other bacterial infections. However, its applicability in human is limited due to the presence of lipopolysaccharide induced endotoxin levels. Nevertheless, in a recent report it was suggested that lipopolysaccharide impaired and inactivated *A. baumannii* whole cells can induce protection against *A. baumannii* infections. In which, the endotoxins levels were found to be reduced ( $<1.0$  toxin unit/ $10^6$  cells) relative to the wild type strain, as on inactivation of lipopolysaccharide due to a mutation in the lpxD gene (García-Quintanilla et al., 2014). Vaccines, intended at combat *P. aeruginosa* infections in cystic fibrosis have been developed and are under different clinical phases, though none has been recommended for use (Johansen and Gøtzsche, 2015). Besides the ongoing improvement in whole-cell enterotoxigenic *Escherichia coli* (ETEC) vaccine development the research on subunit or polypeptide vaccines, offers great therapeutic promise. Recent article by Zhang and Sack (2015), has critically summarizes the progress in the development of ETEC vaccine.

Despite the daunting odds of success, there are at least seven active vaccine efforts are ongoing in different stages of clinical development (GlaxoSmithKline/Nabi, Pfizer/Inhibitex, Sanofi Pasteur/Syntiron, Novartis, Novadigm, Integrated Biotherapeutics, and Vaccine Research International). Several studies have been conducted to evaluate the potential of vaccination with microbial surface components illustrating adhesive matrix molecules (MSCRAMMs) whole protein or subunit offers superior protection against staphylococcal infection. Earlier reported, recombinant parts of collagen adhesin (CNA) were shown to provide immunity in a murine model of sepsis (Kuklin et al., 2006). Shkreta et al. (2004) illustrated the protective potential of both clumping factor A (ClfA) and Fibronectin binding protein (FnBP) as an optimal vaccination strategy in bovine mastitis model, eliciting cellular

and humoral immune response providing partial protection against *Staphylococcal mastitis*. Recently in an *In silico* analysis, potential multisubunit vaccine targets like ClfA, ron-regulated surface determinant (IsdB) and gamma hemolysin (Hlg) were predicted against *S. aureus* (Delfani et al., 2015). Likewise, several extracellular and surface protein factors in combination with aluminum hydroxide have shown to induce high and broad protection against *S. aureus* (Bagnoli et al., 2015).

Another promising alternative to vaccine prophylaxis against bacterial pathogenesis is the ‘DNA-vaccine’, wherein DNA sequence encodes the antigen(s) against which an immune response is sought. It will be less labor intensive and may offer a unique method for vaccination (Tang et al., 1992). Though to obtain an improved immunogenic response of DNA vaccines entails detailed familiarity of underlying mechanism through which DNA vaccines provide immunity to the patient and needs the right combination adjuvants as crucial molecules for strengthening the response (Coban et al., 2008). In implant infections caused by *S. aureus*, MSCRAMM is reported to act as an excellent immunogen for an anti-*Staphylococcus* vaccine. A survey comprising multiple studies in where MSCRAMMs of staphylococcal are exploited as the immunogenic entity, which can implemented following the start of infection as presented in a recent review by Arciola et al., where the authors suggests a “Decalogue” for a rational selection of specific target in constructing DNA vaccines to impede infections in implants (Arciola et al., 2009). Another proposed strategy to induce protective immunity against infectious diseases is the application of live attenuated vaccines. Recent studies illustrates that nasopharyngeal colonization with *Streptococcus pneumoniae* induces protective immunity against subsequent invasive infection, suggesting live vaccination through attenuated bacteria could be a treatment of choice in future. However the bacterial attributes influencing the strength of this adaptive immune response are still to be explored (Cohen et al., 2012).

## THE SEARCH OF NOVEL ANTIMICROBIALS

### Concept of Network Pharmacology

The conception of directed “magic bullets” has long been a therapeutic goal to achieve since Paul Ehrlich (Strehardt and Ullrich, 2008) and a realistic standard in drug development for past three decades. The central concept of drug development is the notion of developing maximally selective molecules directed to discrete drug targets. Conversely, current therapeutics affects multiple proteins rather than discrete target to execute its effect. Development in the area of systems biology further indicates phenotypic robustness and a network structure implying that specific ligands, compared to multi-target ones, exhibit inferior to expected clinical potency. This revised paradigm about polypharmacology offers improved strategy to rise upon the major pitfalls in new antibiotic development, i.e., efficacy and toxicity. Combining system biology with polypharmacology brings in the opportunity of increasing the existing druggable targets. Nevertheless, the coherent design of polypharmacology

needs to fulfill the requirement for new procedures to confirm target associations and augment structure-activity interactions while preserving drug like attributes. Progresses in similar avenues are paving the path toward the next paradigm in novel antibiotic discovery as ‘network pharmacology’.

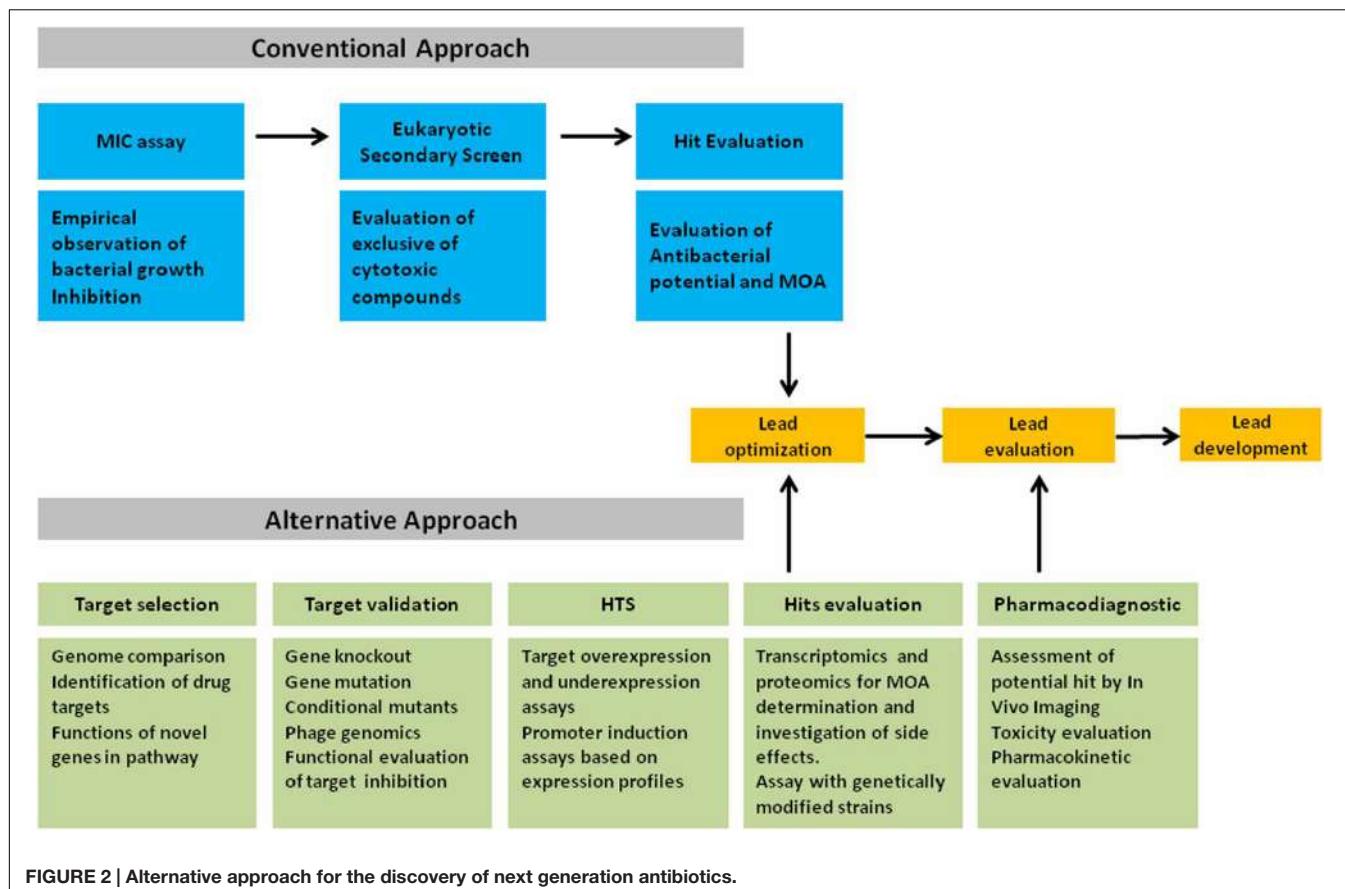
The notion about ‘one drug for one target’ has affected various facets of drug development approach, like disease categorization, target validation, drug design, and planning of clinical testing. Though, a growing data in post-genomics research is suggesting a more complicated scenario in drug action. An interesting research by Yildirim et al. (2007), illustrates not only the existence of several keys for each lock but also a single key for multiple locks. Employing network analysis of combined profiles and a network distance metric, can differentiate between soothing drugs, which ease symptoms, and drugs affecting directly the concern disease genes. Furthermore, the trends in recent years toward the drugs that target the genes associated with disease were emphasized by the network distance metric. It has been well accepted that many effective drugs in therapeutic areas as varied as oncology, psychiatry and antimicrobials affects multiple rather than single targets (Paolini et al., 2006; Tian and Liu, 2012). Many efficient antibiotics are directed toward multiple proteins concurrently rather than individual protein (Lange et al., 2007). For instance, the antimicrobial property of beta-lactams is reliant on the inhibition of more than one PBPs. Indeed, as multiple PBPs can be eliminated with least effect on phenotype (Denome et al., 1999), the approach of lone target essentiality may not have revealed this significant group of antimicrobial drug targets. Likewise, fluoroquinolone based antibiotics can inhibit more than one target proteins ParC and GyrA (Janoir et al., 1996). D-Cycloserine is reported to inhibit four proteins, dimer of alanine racemases and D-Ala-D-Ala ligases. Similarly, fosfomycin circumvent the excess of UDP N-acetylglucosamine enolpyruvyl transferases by restraining them both. Consequently, any effort in the direction of drug designing for combating drug resistance, could consider the development of procedures to identify and evaluate which group of targets can be repressed by one single drug and are indispensable, either alone (‘dualessentials’) or when taken together (‘synthetic lethals’).

Network biology is suggested to play imperative role in drug target characterization. Is it feasible to recognize drug targets by tracing their location in a biological network? When the researchers mapped drug targets against human protein interaction data, result suggests that drug targets tend to have more interactions than average proteins but fewer interactions than crucial proteins to a statistically significant degree (Hopkins, 2007, 2008). The findings about the linkage among drug targets that are not exclusively essential, opens up the avenues for statistical analysis to serve as a vital tool for scoring them. Conventionally, medicinal chemists considered the design of ligands with multiple activities with apprehension, skeptical about conjugated ligands with high molecular weights complex and/or their structure-activity relationships (Hopkins et al., 2006; Morphy and Rankovic, 2006). Though, polypharmacology of standard drugs illustrates that a more opportunistic plan exploring multi-target action could be more efficient. Employing chemogenomics along with network biology will bring in a

efficient network-pharmacology approach to drug design (Keiser et al., 2007; Morphy and Rankovic, 2007). Developing strategy to assist polypharmacology will further help to predict better efficacy and redundant off-target properties.

## Functional Genomics in Drug Designing

The tremendous advancement in the technology for genome screening had a substantial impact on all biological sciences, including antimicrobial research. Since the decoding of the *Haemophilus influenza* genome (Fleischmann et al., 1995), various other bacterial genomes were deduced. Complete genomes of >180 bacteria were sequenced and are now freely accessible, including various imperative disease causing pathogens (e.g., [http://www.genomenewsnetwork.org/resources/sequenced\\_genomes/genome\\_guide\\_p1.shtml](http://www.genomenewsnetwork.org/resources/sequenced_genomes/genome_guide_p1.shtml)). Therefore, the drug development industry exploited the sequencing data as the basis for a rational, target-specific novel drug designing approach to complement the conventional strategy. Central to the paradigm shift was the idea of the occurrence of the so-far unexploited targets with the prospective for effective and discerning antibiotics for broad spectrum of bacterial infections (McDevitt et al., 2002; Gwynn et al., 2010; Brunschweiger and Hall, 2012). An array of genomics platforms are employed in the present antiinfectious drug development process to lay more proficient procedure compared to conventional strategy. Herein, we proposed a more robust strategy as an alternative for the discovery of next generation antibiotics (**Figure 2**), which includes all possible coverage for better output. Including knockout analyses and mutation studies in the strategy will enhance the prioritization and validation of prospective novel drug target by questioning its importance for bacterial existence. Applications of genetically manipulated cell, along with holistic profiling strategies like ‘OMICS’, i.e., transcriptomic and proteomic are contributory in verifying the mode of action (MOA) while screening lead molecules from target assays. Functional genomics strategies also offers vital clues on the mechanism of lead molecules screened through the empiric approach for novel antibacterial discovery. Furthermore, comparing genomes facilitates the identification of proteins that are conserved across the clinically critical pathogens, assisting functional characterization of new genes and identification of novel targets. Genome wide gene silencing studies facilitate an approximation of the number of genes necessary for survival of various bacterial species. The inference on the role of same gene product essentiality (‘ortholog’) in different species will augment the feasibility of such targets. While selecting a target diverse criterion, such as screening competence, drug like attributes and metabolical context are of a great significance. Although, data on these features are often not accessible and even some targets are not completely characterized. The number of uncharacterized targets typically increases when focusing on attributes preserved in a selective group of pathogens (narrowed range of bacteria). Growth studies with different supplements, cytological assessment, transcriptome, proteome analyses, and metabolic labeling experiments of conditional mutants may assist in the evaluation of corresponding functional genes. Further interesting strategy to explore novel drug is to evaluate the



molecular action of bacteriophages to arrest critical cellular pathways. As done by Liu et al. (2004), on sequencing 26 *S. aureus* phages thirty one (31) novel polypeptide were identified that on expression exhibit cell death in *S. aureus*. Affinity chromatography illustrates the role of DnaI as a target protein, a requisite protein for primosome activity on the initiation of DNA replication. Such screening techniques can be further extended to other species as well and could be instrumental in proving imperative information and as screening tools for novel or unexplored targets. In addition functional genomics applications have proved to be indispensable for expediting the characterization of mechanism of action for novel compounds with antifungal activity and offers novel mechanism based screening options by generating and characterizing point mutations that confers drug resistance. In general conditional mutants with target gene repression illustrate an increase in sensitivity toward that target specific inhibitor. When analyzed, the relative growth suppression of these mutants to the wild type enables an easy validation confirming the mechanism of novel drugs/hits, as reported by DeVito et al. (2002) and Forsyth et al. (2002).

An approach of global expression profiling can revolutionize the analysis of drug targets holistically as never before. As whole genome profiling offers the static blueprint of cells attributes, as being highly dynamic in nature the transcriptome and proteome are appropriate to obtain snapshots of that cell physiology

when challenged by environmental alterations, exposure to stress or antibiotic administration. As application of a DNA microarray provides direct assessment of the mRNA of the gene of interest, the proteomic profiling by 2D gel electrophoresis involves an implicit protein atlas needed initially to confirm the identity of each protein (as determined by mass spectrometry) to its spot location on the 2D gel (Brötz-Oesterhelt et al., 2005). The expression profiles resulted from these techniques illustrates the molecular insight of the adaptive physiology of bacteria like the regulatory mechanism, signal transduction and the underlying metabolism that influence the phenotypic outcomes. Transcriptome as well as proteome profiling were widely used to study the physiology of bacteria in response to various environmental stresses provided particularly useful information about the underlying network of adaptive responses. As a proof-of-concept is the *B. subtilis* mutant down regulating the peptide deformylase, wherein the proteomic profiling of this strain matched adequately well with the wild-type strain exposed to the deformylase inhibitor actinonin (Bandow et al., 2003). Furthermore, bacterial acetyl-CoA carboxylase (ACC) is recently been suggested as the target of moiramide B based on a reference compilation of transcriptome profiles (Freiberg et al., 2004, 2005). In a recent interesting work Murray et al. (2015) has employed expression- and fitness-based genomic strategies to validate role of genetic loci exhibiting drug resistance in human pathogen *P. aeruginosa*. Signifying that gene expression

standalone is not a reliable predictor of resistance determinants. Moreover when combine together, whole genome expression and fitness correlation may offer better mechanistic perceptions about multidrug resistance.

## In Vivo Imaging Technologies

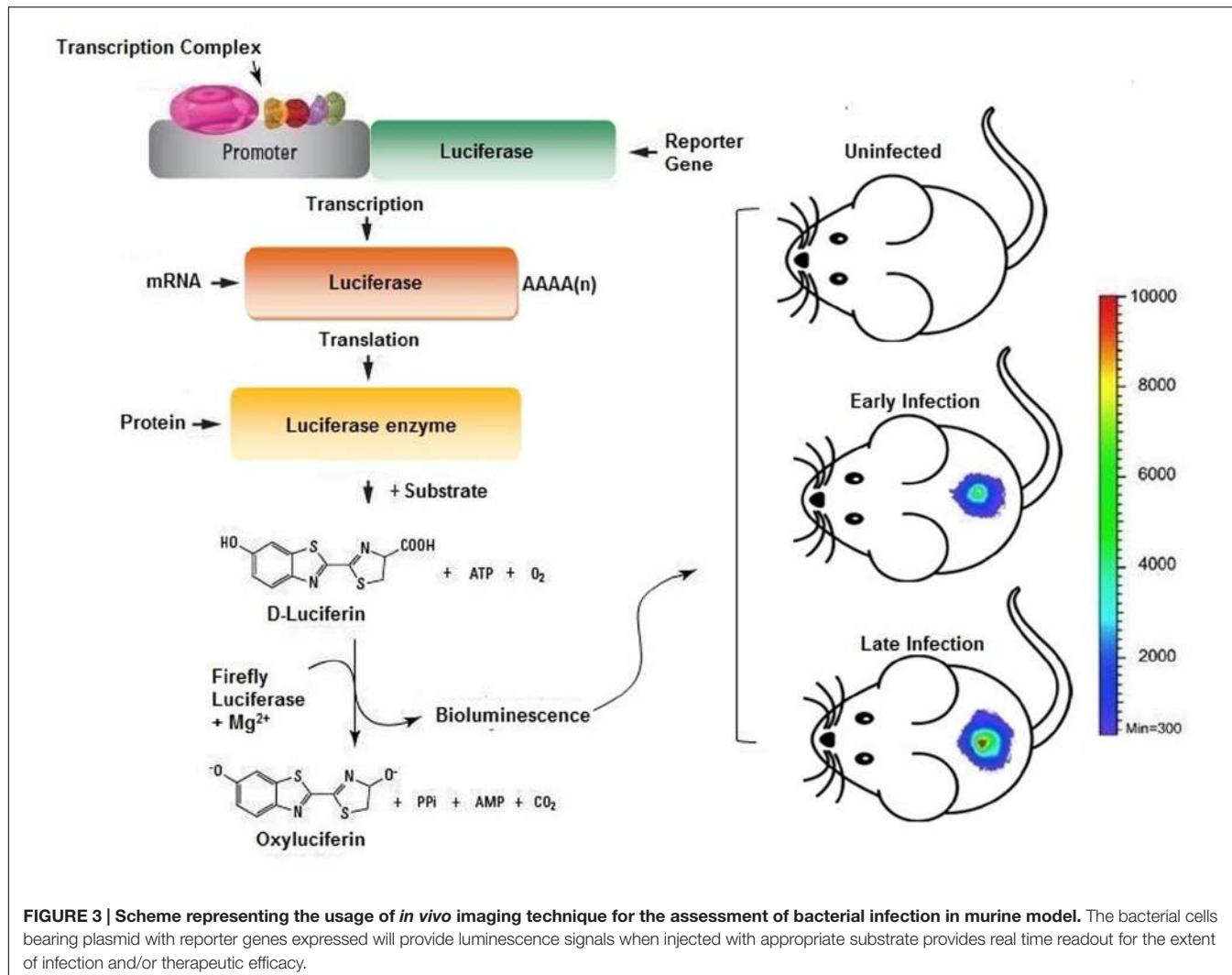
Recently, *in vivo* imaging techniques are being proved to be a powerful complement to various platforms of antimicrobial resistance and drug discovery (Probst et al., 2012; Roda and Guardigli, 2012). It may not only minimize the use of histopathological examination of autopsies but also be helpful in limiting the animal sacrifice and cost for *in vivo* drug-kinetic studies. Hence, we propose that improvement of intravital dynamic monitoring strategies to probe cellular phenotypes will reflect as wise drug development investment. Real time monitoring enables a faster readout of drug action in the complex model organisms, thus facilitating comprehensive assessment of drug profiling *in vivo*. Moreover, this could enable lead optimization and medicinal chemistry can be directed by instructive functional *in vivo* response data. The clinical reliability of hightthroughput screens and *in vivo* monitoring strategies are yet to be fully comprehended. In particular, the progress in bioluminescence based monitoring of disease progression *in vivo* is ever increasing. Further identifies underlying physiology, such as signal transduction, proliferation and cell renewal to be examined in the purview of an intact organism. Two commonly employed strategies for incorporating bioluminescent marker in a model organism are: First is the *lux* operon from *Photorhabdus luminescens* or *Xenorhabdus luminescens*, encodes genes to produce luciferase and its substrate luciferin constitutively, and glow even absence of any exogenous substrate. The *lux* operon gets stably incorporated to other bacterial species but fail to integrate in higher species, though continuous efforts are make to use it as a reporter in mammalian system. The second strategy for bioluminescent probes uses enzyme luciferase from firefly (*Photinus pyralis*) or sea pansy (*Renilla reniformis*), as firefly and Renilla luciferase require two distinct substrates, which make it possible to link them with two different biological processes in the same research model (Bhaumik and Gambhir, 2002; Ng et al., 2011). However, each reporter differs in the pharmacokinetic of their substrate, as on intraperitoneal administration of luciferin, firefly luciferase bioluminescence attain saturation in about 5–10 min and stays almost constant for 30 min (Paroo et al., 2004), however, renilla luciferase reach to its maxima in 1 min on intravenous coelenterazine administration and declines back in 10 min (Bhaumik and Gambhir, 2002). This technology can be successfully employed to study a variety of infection and their progression in animal models (Figure 3). Also manifested by the work of other researchers, that bioluminescence imaging can be used to validate new features in host-pathogen interactions. Furthermore developments in the live cell-fluorescent reporter molecules, brings in the opportunity to adopt *in vivo* monitoring applications in drug development (Carragher et al., 2012).

Past few years, array of nanoparticles have emerged with a promise of superior attributes for *in vivo* fluorescence imaging technology. Besides providing dynamic size range, these

particles offers diverse structural and optical properties with superior signals strength compared to organic fluorophores, providing more sensitive visualization of even sparse biomarkers. Furthermore, nanoparticles can incorporate multiple probes due larger surface area, facilitating multivalent targeting. Above mentioned attributes make these nanocarriers fit for therapeutic applications, biomedical imaging or a combination of both (theranostics). Quantum dots (QDs) are the best characterized and most studied nanocrystals (Mandal and Parvin, 2011; Li et al., 2012). These minute nanomaterials are semiconductive in nature and have an adjustable emission fluorescence that can be adjusted by regulating their synthesis (Bentolila et al., 2009). Furthermore can be visualized in a wide, from the ultraviolet to the near infrared exhibit higher intensity and photoresistant attributes. Recently, QDs treated with coordination complexes were used to specifically visualize a rough *Escherichia coli* mutant facilitating optical detection in murine infection model (Leevy et al., 2008). Various other nanoparticles that have been developed for *in vivo* fluorescence imaging applications are metallic, hybrid, C dots, polymeric or biodegradable in nature (Coto-García et al., 2011). Recent development in capturing detailed images and image based analysis softwares are beginning to impact the design of algorithms that can be customized to fit intricate and pertinent biological models and tissue specimen (Jones et al., 2009). Amendable image analyzing software's can leverage quantitative data from complex biological systems, integrating varied cell types, fresh biopsies, or material from cell lines or primary cells for efficient mimicking of the cellular environment. Therefore, high-resolution *in vivo* imaging should facilitate drug discovery per se and understanding of the drug therapeutic effectiveness (Table 1), and will apparently increase the success rate in clinical testing by providing pertinent biological perspective to genomic and proteomic approaches.

## CONCLUSION AND FUTURE PERSPECTIVE

The yin and yang of novel antibiotic discovery swings around from a serious need for antibiotics to eradicate drug resistant bacteria to antimicrobial agents. Ever increasing resistance despite the rigorous attempts for control use of antibiotics and sanitize hospitals routinely is the driver for the need of next generation antibiotics. These bacteria “superbugs” have escaped the clinical settings and attain the status of community acquired pathogens. Besides the upcoming promising antiinfective agents as discussed in this article earlier, there is the need to adopt alternative approaches for the discovery of next generation of antibiotics. The strategies discussed in this review are network pharmacology and functional genomics in complement with *in vivo* imaging platforms. The network pharmacology encompasses systems biology, network analysis, connectivity, redundancy, and pleiotropy to improve clinical efficacy and understanding side effects and toxicity. Functional genomics techniques are proved to be indispensable for *in vitro* target authentication and elucidating mechanism of action of novel antibacterial. In addition, the



**FIGURE 3 | Scheme representing the usage of *in vivo* imaging technique for the assessment of bacterial infection in murine model.** The bacterial cells bearing plasmid with reporter genes expressed will provide luminescence signals when injected with appropriate substrate provides real time readout for the extent of infection and/or therapeutic efficacy.

**TABLE 1 | Potential applications of *in vivo* imaging in drug discovery.**

Assessment of disease model efficiency	Disease model can be assessed for the optimal engraftment or infection from orthotopic cells before treatment regimes
Potential hit screening	Evaluation of MIC values across multiple pathways to determine the response of target activity
Biomarker discovery	Detection of post-translational markers of therapeutic relevance from clinical biopsy or body fluids
Identify drug-target mechanisms	Identification and validation of drug combination strategies and synergism of drugs
Predictive <i>in vivo</i> pharmacodynamics	Monitor organ-specific response and correlate with functional drug response
Drug candidate profiling <i>in vitro</i>	Establish broad pathway-activity mechanisms
Confirming functional genomics screens and pathways	Characterize impact of knockdown of genes on key pathways

approach of functional genomics not only relies on target-based screening but also supports the traditional screening strategy by predicting novel mechanism for hits and natural products with potential antibacterial activity and unknown targets. Complementary to approach of drug discovery, *in vivo* imaging is an economical, user friendly and radioactive free tool that can extract quick semi-qualitative or quantitative information representing physiological milieu from the cellular to the computable form. Key for best realization of imaging and post-translational pathway modeling approaches into routine

antibiotic discovery relies on its early integration with target specific drug development efforts. Validating efficiency and safety profiles of hit molecules in pathological models, before cost intensive medicinal approaches, offer a perfect solution for reducing the unsustainable expense. In summary, there is strong likelihood that the combination of both the traditional and genomics-based approaches, together with the live cell visualization techniques, will lead to better strategies for the discovery of newer antibiotics to combat the emergence of bacterial resistance.

## AUTHOR CONTRIBUTIONS

SNK wrote review article. AUK guided and gave idea of writing.

## ACKNOWLEDGMENTS

Author acknowledges DBT, government of India for the support and internal facilities of the department. This

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Characterization of antibiotic resistance in commensal bacteria from an aquaculture ecosystem

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### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 29 May 2015

Accepted: 20 August 2015

Published: 08 September 2015

### Citation:

Huang Y, Zhang L, Tiu L and Wang HH (2015) Characterization of antibiotic resistance in commensal bacteria from an aquaculture ecosystem. *Front. Microbiol.* 6:914.  
doi: 10.3389/fmicb.2015.00914

The objective of the study was to improve the understanding of antibiotic resistance (AR) ecology through characterization of antibiotic-resistant commensal isolates associated with an aquaculture production system. A total of 4767 isolates non-susceptible to sulfamethoxazole/trimethoprim (Sul/Tri), tetracycline (Tet), erythromycin (Erm), or cefotaxime (Ctx), originated from fish, feed, and environmental samples of an aquaculture farm with no known history of antibiotic applications were examined. Close to 80% of the isolates exhibited multi-drug resistance in media containing the corresponding antibiotics, and representative AR genes were detected in various isolates by PCR, with feed isolates had the highest positive rate detected. Identified AR gene carriers involved 18 bacterial genera. Selected AR genes led to acquired resistance in other bacteria by transformation. The AR traits in many isolates were stable in the absence of selective pressure. AR-rich feed and possibly environmental factors may contribute to AR in the aquaculture ecosystem. For minimum inhibitory concentration test, brain heart infusion medium was found more suitable for majority of the bacteria examined than cation-adjusted Mueller Hinton broth, with latter being the recommended medium for clinical isolates by standard protocol. The data indicated a need to update the methodology due to genetic diversity of microbiota for better understanding of the AR ecology.

**Keywords:** antibiotic resistance, aquaculture ecosystem, multiple risk factors, commensal bacteria

## Introduction

The rapid emergence of antibiotic resistance (AR) in the global ecosystem has become a major public health concern. In the past decade, with a broadened scope of investigation, both population- and organism-based studies have illustrated that commensal bacteria likely have played a key role in AR ecology (Andremont, 2003; Wang et al., 2006; Wang and Schaffner, 2011). With the expansion of research territory, AR has been found prevalent across the ecosystem, from clinical samples, animal and human hosts, retail foods, to waste water, soil, and other natural environment (Zhanel et al., 2000; Österblad et al., 2001; Aubry-Damon et al., 2004; Wang et al., 2006; Koike et al., 2007; Sommer et al., 2009; Li and Wang, 2010; D'Costa et al., 2011; Zhang, 2011; Ye et al., 2013). For instance, using non-specific culturing methods and culture-independent real-time PCR, the abundance of AR in the food chain was revealed and a broad-spectrum of

non-pathogenic and even beneficial bacteria were found carrying transmissible AR genes (Duran and Marshall, 2005; Wang et al., 2006; Manuzon et al., 2007). Characterization of antibiotic-resistant (ART) isolates associated with food animals, ready-to-eat food products, human, and environmental samples has led to the discovery of various mechanisms involved in AR evolution (Sommer et al., 2009; Bhullar et al., 2012), enrichment (Zhang et al., 2011), and persistence (Sørum et al., 2006; Rosvoll et al., 2010; Li et al., 2011). It becomes evident that commensal flora provides a particular window of vulnerability of AR in the ecosystem, and prompt response to the early indication can be critically important for preventive AR management (Andremont, 2003; Wang et al., 2006; Wang and Schaffner, 2011). Due to the advancement in metagenomics and other molecular techniques, the complexity of microbiome associated with both hosts and environment has been gradually uncovered (Venter et al., 2004; Sommer et al., 2009; Zhang et al., 2011). However, the extremely large size and diverse distribution of the commensal population, as well as genetic diversity of the subpopulations presented several major challenges to researchers. There is no single way of recovering, culturing, or characterizing all the cultures, making proper interpretation of data a daunting task.

Aquatic creatures are susceptible to infectious diseases. Use of antibiotics in aquaculture production systems is considered a major risk factor contributing to AR in aquaculture products and the ecosystem. ART pathogens, such as *Aeromonas* (Akinbowale et al., 2007; Penders and Stobberingh, 2008), *Vibrio* (Oh et al., 2011; Rebouças et al., 2011), and *Salmonella* (Ribeiro et al., 2010; Budati et al., 2013), have been reported to be associated with the aquaculture system. Particularly, certain AR determinants have been found in both aquaculture and clinical isolates, indicating potential AR dissemination between food and humans. For instance, one gene cassette containing *bla<sub>CMY-2</sub>*, *sugE*, and *blc* detected in *Aeromonas salmonicida* ssp. *salmonicida* isolates from Atlantic Canadian salmon farm was identical to a transposon-like element widely distributed among clinical and food-borne *Salmonella* and other Enterobacteriaceae throughout Asia and the United States (McIntosh et al., 2008). Rhodes et al. (2000) reported the dissemination of *tet(A)*-associated Tn1721 and Tn1721-like elements among different *Aeromonas* species and *Escherichia coli*, and between the human and aquaculture environment in distinct geographical locations (including Norway, Scotland, England, and Germany). In addition, various AR genes were found in a broad spectrum of commensal bacteria associated with aquaculture products and the environment (Ye et al., 2013; Shah et al., 2014). However, these data are still insufficient in elucidating AR risk factors in the aquaculture ecosystem.

In a recent study, we have uncovered a rich profile of ART bacteria in samples from a domestic aquaculture farm with no known history of antibiotic application, by culture-dependent and -independent methods (Huang, 2014). The objective of this study was to characterize the genotypic and phenotypic features of the corresponding ART isolates, as well as AR persistence and dissemination, for an improved understanding of

the AR ecology associated with aquaculture production. During the evaluation of minimum inhibitory concentration (MIC) for representative antibiotics of the isolates, we have compared the data using both standard approach for pathogens (CLSI, 2013) and a modified method (Wang et al., 2006), to collect baseline information for further methodology improvement, to address the needs for proper assessment of commensal bacteria.

## Materials and methods

### Bacterial Strains and Culture Condition

A total of 4767 isolates recovered from Sul/Tri<sup>r</sup>, Tet<sup>r</sup>, Erm<sup>r</sup>, or Ctx-containing agar plates were examined in the study. These isolates originated from fish intestine (1045 isolates), fish surface rinsing water (850 isolates), fish feed (150 isolates), pond mud (1293 isolates), and pond water (1429 isolates) from an aquaculture farm with no known history of antibiotic applications. All isolates were cultured in brain heart infusion (BHI) media containing the corresponding antibiotics, including 152 µg/ml of sulfamethoxazole (Sigma-Aldrich, St. Louis, MO, USA) with 8 µg/ml of trimethoprim (Sigma-Aldrich), 16 µg/ml of tetracycline (Sigma-Aldrich), 100 µg/ml of erythromycin (Fisher Scientific, Waltham, MA, USA), or 4 µg/ml of cefotaxime (Sigma-Aldrich; Ye et al., 2013).

### Determination of Drug Resistance Profiles, AR Genes, and Identification of AR Gene Carriers

Recovered Sul/Tri<sup>r</sup>, Tet<sup>r</sup>, Erm<sup>r</sup>, and Ctx<sup>r</sup> isolates were spotted on BHI agar plates containing each of the four antibiotics for rapid assessment of their phenotypic resistance profile.

Approximately one-fourth of the ART isolates were randomly selected for conventional PCR screening for representative AR genes (Li and Wang, 2010). AR gene primers used in the study were listed in Table 1. Approximately 10% of the positive PCR products were confirmed by DNA sequence assessment using an ABI Prism 3700 sequencer (Applied Biosystems, Foster City, CA, USA) at the Plant Microbe Genomics Facility, The Ohio State University, and the obtained DNA sequences were compared with published AR gene sequences deposited in the NCBI database. Approximately 50% of the AR gene carriers were further identified by PCR and partial 16S rRNA gene sequence analysis, as described previously (Wang et al., 2006).

### MIC Assessments

Twenty-nine ART isolates carrying AR genes were subjected to the MIC test. The assessments were conducted using microbroth dilution method as suggested by CLSI (2013) protocol. BHI and cation-adjusted Mueller Hinton (CAMH) broth were employed as basic medium independently. ART isolates were grown in the broth containing the corresponding antibiotic (including up to 608 µg/ml Sul with 32 µg/ml Tri, 512 µg/ml Tet, 512 µg/ml Erm, or 512 µg/ml Ctx). Reference strains *Staphylococcus aureus* ATCC®29213, *Enterococcus faecalis* ATCC®29212, *E. coli* ATCC®25922, and *Pseudomonas aeruginosa* ATCC®27853 were examined in parallel as controls.

**TABLE 1 |** Primers used for conventional PCR.

Primer	Sequence (5'-3')	Reference
sul1 F	CGGGCGTGGGCTACCTGAACG	Kerr (2002)
sul1 R	GCCGATCGCGTGAAGTCCG	
sul2 F	GCAGGCGCGTAAGCTGA	Zhang et al. (2011)
sul2 R	GGCTCGTGTGCGGATG	
tetS F	GAACGCCAGAGAGGTATT	Zhang et al. (2011)
tetS R	TACCTCATTGGACCTCAC	
tetL F	TTGGATCGATACTAGGCC	Zhang et al. (2011)
tetL R	GTAACCAGCCAACAAATGAC	
tetM F	CGAACAAAGAGGAAAGCATAAG	Zhang et al. (2011)
tetM R	CAATACAATAGGAGCAAGC	
ermB F	TGGTATTCCAATGCGTAAATG	Zhang et al. (2011)
ermB R	CTGTGGTATGGCGGGTAAGT	
ermC F	GCTAAATTGTTAACATCGTCAAT	Wang et al. (2006)
ermC R	TCAAAACATAATATAGATAAA	
bla <sub>TEM</sub> F	CATTCGGTGTGCGCCCTTATT	Dallenne et al. (2010)
bla <sub>TEM</sub> R	CGTTCATCCATAGTTGCCTGAC	
bla <sub>SHV</sub> F	AGCCGCTTGAGCAAATTAAAC	Dallenne et al. (2010)
bla <sub>SHV</sub> R	ATCCCGCAGATAAATCACAC	
bla <sub>OXA</sub> F	GGCACCAAGATTCAACTTCAAG	Dallenne et al. (2010)
bla <sub>OXA</sub> R	GACCCCAAGTTCTGTAAAGTG	
bla <sub>CMY-2</sub> F	GACAGCCTCTTCTCCACA	Zhang et al. (2011)
bla <sub>CMY-2</sub> R	TGGAACGAAGGCTACGTA	
bla <sub>CTX MU1</sub>	ATGTGCAGYACCAGTAARGT	Zhang et al. (2011)
bla <sub>CTX MU2</sub>	TGGGTRAARTARGTSACCAGA	

## Persistence of AR

AR stability in resistant isolates was determined according to the published procedures (Li et al., 2011) with slight modifications. Basically, after consecutive transfer every 12 h for 30 days, the cultures were serially diluted and plated on BHI agar plates. One hundred colonies were randomly picked from each sample, and spotted onto BHI agar plates with and without the corresponding antibiotic. The ratio of resistant to total colonies was used to describe the resistance persistence in ART isolates at the absence of the antibiotic selective pressure.

## Transformation

Chemical transformation was conducted using plasmids from nine Gram-negative ART isolates carrying AR genes and *E. coli* DH5 $\alpha$  as a recipient by the calcium chloride transformation method (Dagert and Ehrlich, 1979). Meanwhile, electroporation was employed when introducing plasmids from five Gram-positive ART isolates carrying AR genes to *Lactococcus lactis* LM 2301 following the procedures described previously (Mcintyre and Harlander, 1989).

## Results

### MIC Assessment

MIC analysis showed that the MIC results of Tet, Erm, or Ctx for the four reference strains were comparable in BHI and CAMH broth. However, culture medium had varied but clear impact

on MIC results of Sul/Tri. For instance, while the MIC values of Sul/Tri for *P. aeruginosa* ATCC®27853 in both media were comparable (608/32  $\mu$ g/ml), the other three reference strains showed 4- to 64-fold higher MIC values in BHI than that of in CAMH. It is worth noting that *E. faecalis* ATCC®29212 didn't grow as well in CAMH as in BHI broth.

As illustrated in **Table 2**, twenty-nine isolates used in the MIC assessment were identified and they belonged to 15 genera. MIC values for Tet, Erm, or Ctx in BHI were the same as, or twofold to fourfold higher than the results from CAMH. Among the strains examined, 9 out of 20 (45%) *Ter<sup>r</sup>*, 6 out of 6 (100%) *Erm<sup>r</sup>*, and 2 out of 5 (40%) *Ctx<sup>r</sup>* isolates exhibited high and consistent MIC values (no less than 128  $\mu$ g/ml) in both BHI and CAMH for Tet, Erm, or Ctx, respectively. But the MIC values for Sul/Tri in BHI and CAMH varied significantly in close to 70% of the isolates (21 out of 29). Some Sul/Tri<sup>r</sup> isolates showed very high value (no less than 608/32  $\mu$ g/ml) in both BHI and CAMH, including *Plesiomonas* sp., *Aeromonas* sp., and *Psychrobacter* sp. Bacteria from genera such as *Enterobacter* sp., *Bacillus* sp., and *Kurthia* sp. showed higher MIC value in BHI (no less than 304/16  $\mu$ g/ml) than that in CAMH (no more than 9.5/0.5  $\mu$ g/ml). Moreover, isolates of *Vagococcus* sp., *Aerococcus* sp., *Corynebacterium* sp., and *Enterococcus* sp. didn't grow or had poor growth in CAMH, but exhibited higher MIC value in BHI (no less than 608/32  $\mu$ g/ml).

### Phenotypic-Resistant Profiles of the ART Isolates

Of the recovered Sul/Tri<sup>r</sup>, *Tet<sup>r</sup>*, *Erm<sup>r</sup>*, and *Ctx<sup>r</sup>* isolates (from BHI plates) associated with fish intestine, surface rinsing water, fish feed, pond mud, and pond water samples, 3772 of 4767 total isolates (79.1%) showed resistance to more than one antibiotic, and 824 isolates (17.4%) were resistant to all four antibiotics tested. As shown in **Table 3**, multi-drug-resistant bacteria were common in all samples.

### Prevalence of the AR Genes and Identification of the Isolates

As shown in **Table 4**, more AR genes were detected in isolates associated with fish feed and fish intestine than surface rinsing water, pond mud, and pond water samples. For instance, a high percentage and broad spectrum of AR genes were found in isolates from fish feed samples, including *sul1* (10.2%), *sul2* (2.0%), *tetL* (13.3%), *tetM* (9.2%), *tetS* (6.1%), and *ermB* (1.0%). Within the 13 AR determinants examined, *tetM* had the highest detection rate (8.1%) in the isolates.

As illustrated in **Table 4**, identified AR gene carriers belonged to 18 genera. *Aeromonas* sp., *Enterococcus* sp., *Enterobacter* sp., and *Plesiomonas* sp. identified in fish intestine samples were commonly isolated from fish (Austin, 2002). Some of the isolates examined, including *Bacillus* sp., *Carnobacterium* sp., *Corynebacterium* sp., *Enterococcus* sp., *Plesiomonas* sp., *Lactococcus* sp., and *Psychrobacter* sp. were found to carry multiple resistance encoding genes. Fish feed contained various AR genes in a broad spectrum of organisms, though total count of ART bacteria was relatively low.

**TABLE 2 | Comparison of MIC results between BHI and CAMH medium.**

Isolates	Identity	AR genes carried	Resistance phenotype and MIC ( $\mu\text{g/ml}$ )							
			Sul/Tri <sup>r</sup>		Tet <sup>r</sup>		Erm <sup>r</sup>		Ctx <sup>r</sup>	
			BHI	CAMH	BHI	CAMH	BHI	CAMH	BHI	CAMH
17iT21	<i>Plesiomonas</i> sp.	<i>sul1</i>	>608/32	>608/32	256	128	—	—	—	—
17wT9	<i>Plesiomonas</i> sp.	<i>tetM</i>	—	—	256	128	—	—	—	—
18iS4	<i>Plesiomonas</i> sp.	<i>sul2</i>	>608/32	>608/32	—	—	—	—	—	—
18iS31	<i>Plesiomonas</i> sp.	<i>sul2</i>	>608/32	>608/32	—	—	—	—	—	—
17fS1	<i>Enterococcus</i> sp.	<i>tetM, tetL</i>	>608/32	Weak growth $\leq 9.5/0.5$	256	128	—	—	—	—
17fS3	<i>Enterococcus</i> sp.	<i>sul1</i>	>608/32	Weak growth $\leq 9.5/0.5$	16	16	—	—	—	—
18iX15	<i>Enterococcus</i> sp.	<i>tetS, tetL</i>	>608/32	Weak growth $\leq 9.5/0.5$	256	128	—	—	512	512
18fT19	<i>Enterococcus</i> sp.	<i>tetS</i>	>608/32	Weak growth $\leq 9.5/0.5$	256	128	—	—	—	—
17iE3	<i>Enterobacter</i> sp.	<i>sul2</i>	304/16	$\leq 9.5/0.5$	—	—	512	256	—	—
17iE10	<i>Enterobacter</i> sp.	<i>sul2</i>	>608/32	$\leq 9.5/0.5$	—	—	512	256	—	—
17iE11	<i>Enterobacter</i> sp.	<i>sul2</i>	304/16	$\leq 9.5/0.5$	—	—	512	256	16	8
17iE26	<i>Enterobacter</i> sp.	<i>sul2</i>	>608/32	$\leq 9.5/0.5$	—	—	512	256	—	—
17fT6	<i>Kocuria</i> sp.	<i>sul1</i>	>608/32	$\leq 9.5/0.5$	64	64	—	—	—	—
17fT2	<i>Corynebacterium</i> sp.	<i>sul1</i>	>608/32	Weak growth	256	128	—	—	—	—
18fT26	<i>Corynebacterium</i> sp.	<i>tetL</i>	—	—	32	32	—	—	—	—
18fT27	<i>Corynebacterium</i> sp.	<i>tetL</i>	—	—	32	32	—	—	—	—
18iS7	<i>Aeromonas</i> sp.	<i>sul1</i>	>608/32	>608/32	—	—	—	—	—	—
18fE1	<i>Bacillus</i> sp.	<i>ermB</i>	>608/32	$\leq 9.5/0.5$	256	256	512	512	4	4
17fT12	<i>Aerococcus</i> sp.	<i>tetM</i>	>608/32	Weak growth	256	128	—	—	—	—
17fS5	<i>Aerococcus</i> sp.	<i>tetM</i>	>608/32	Weak growth	32	32	—	—	—	—
18fS13	<i>Psychrobacter</i> sp.	<i>sul2</i>	>608/32	>608/32	32	32	—	—	—	—
18fS19	<i>Psychrobacter</i> sp.	<i>sul1, sul2</i>	>608/32	>608/32	32	32	—	—	—	—
18pwE5	<i>Staphylococcus</i> sp.	<i>ermC</i>	>608/32	$\leq 9.5/0.5$	—	—	>512	>512	>512	>512
18fT47	<i>Carnobacterium</i> sp.	<i>tetS, tetL</i>	—	—	64	64	—	—	—	—
18fT5	<i>Kurthia</i> sp.	<i>tetM</i>	>608/32	$\leq 9.5/0.5$	256	256	—	—	64	16
18fT29	<i>Lactobacillus</i> sp.	<i>tetL</i>	—	—	32	32	—	—	—	—
18fS10	<i>Pseudoclavibacter</i> sp.	<i>sul1</i>	608/32	608/32	32	32	—	—	—	—
18fT12	<i>Vagococcus</i> sp.	<i>tetL</i>	>608/32	No growth	128	No growth	—	—	—	—

BHI, Brain heart infusion; CAMH, cation-adjusted Mueller Hinton.

**TABLE 3 | Multi-drug-resistant isolates from aquaculture samples.**

Sample	Isolates resistant to antibiotic			
	1AR <sup>a</sup>	2AR	3AR	4AR
Surface rinsing water ( $n = 850$ )	18.7% (159/850)	35.6% (303/850)	32.2% (274/850)	13.4% (114/850)
Fish intestine ( $n = 1045$ )	19.8% (207/1045)	58.5% (611/1045)	12.1% (126/1045)	9.7% (101/1045)
Pond mud ( $n = 1293$ )	13.4% (173/1293)	34.9% (451/1293)	34.0% (440/1293)	17.7% (229/1293)
Pond water ( $n = 1429$ )	8.7% (125/1429)	15.2% (217/1429)	50.6% (723/1429)	25.5% (364/1429)
Fish feed ( $n = 150$ )	36.0% (54/150)	31.3% (47/150)	22.0% (33/150)	10.7% (16/150)

<sup>a</sup>Number of antibiotic(s) the isolates were resistant to.

## Stability of the AR

Results of persistence assessment showed that 90–100% of the progenies from nine ART isolates examined retained their original AR traits, indicating that the AR determinants are generally stable in the resistant isolates without the corresponding antibiotic selective pressure. Progenies of an *Enterococcus* strain from the intestine sample retained resistance to Sur/Tri but became susceptible to Tet and Ctx.

## Functionality of the AR Determinants

Plasmids from two out of nine Gram-negative isolates (*sul1*<sup>+</sup> *Aeromonas* sp. from the fish intestine and *sul2*<sup>+</sup> *Psychrobacter* sp. from the fish feed) and one out of five Gram-positive isolate (*tetL*<sup>+</sup> *Vagococcus* sp. from fish feed) were successfully transferred to *E. coli* and *L. lactis*, respectively, resulting in acquired resistance in transformants. The transformants exhibited comparable MIC for the corresponding antibiotic with

**TABLE 4 | The identity of AR gene carriers.**

Sample (# isolates tested)	Detected AR genes (# positive isolates)	Gene carrier [AR gene (#isolates identified)]
Surface rinsing water (209)	sul1 (8), sul2 (1), tetM (30), sul1+tetM (5)	<i>Plesiomonas</i> sp. [sul1 (3), sul2 (1), tetM(3), sul1+tetM (2)]
Fish intestine (357)	sul1 (7), sul2 (15), tetL (1), tetM (9), sul1+tetM (1), tetL+ tetS(1),	<i>Aeromonas</i> sp. [ sul1 (1)]; <i>Enterobacter</i> sp., [sul2 (7)]; <i>Enterococcus</i> sp. [tetL (1), tetL+ tetS (1)]; <i>Plesiomonas</i> sp. [sul1 (3), sul2 (6), sul1+ tetM (1)]; <i>Aeromonas</i> sp. [sul1 (1)]; <i>Plesiomonas</i> sp. [sul1 (1)]
Pond Mud (269)	sul1 (3), sul2 (1)	<i>Exiguobacterium</i> sp. [sul1 (2)]; <i>Staphylococcus</i> sp. [ermC (1)];
Pond water (244)	sul1 (5), tetS (1), tetL+tetS (3), ermC(1)	<i>Lactococcus</i> sp. [tetS (1), tetL+tetS (2)];
Fish feed (98)	sul1 (9), sul2 (1), sul1+sul2 (1), tetL (4), tetM (2), tetS (3), tetL+ tetM (6), tetL+ tetS (2), tetL+tetM+tetS (1), ermB (1)	<i>Aerococcus</i> sp. [tetM (2)]; <i>Bacillus</i> sp. [tetS (1), ermB (1)]; <i>Carnobacterium</i> sp. [tetL+tetS (1)]; <i>Corynebacterium</i> sp. [sul1 (1), tetL (2)]; <i>Kurthia</i> sp. [tetM (1)]; <i>Enterococcus</i> sp. [sul1 (1), tetS (2), tetL+tetM (5), tetL+tetS (1), tetL+tetM+tetS (1)]; <i>Kocuria</i> sp. [sul1 (4)]; <i>Lactobacillus</i> sp. [tetL (1)]; <i>Pseudoclavibacter</i> sp. [sul1 (1)]; <i>Psychrobacter</i> sp. [sul2 (1), sul1+sul2 (1)]; <i>Staphylococcus</i> sp. [sul1 (1)]; <i>Vagococcus</i> sp. [ tetL (1)]

the donors, indicating the resistance genes were functional in other bacteria if acquired via horizontal gene transfer events.

## Discussion

It is recognized that AR ecology, of the emergence, persistence, and dissemination of ART bacteria and AR genes in the microbial ecosystem, is much more complicated than previously thought. Up to  $10^7$  CFU/g ART bacteria, representing about 1% of the total bacterial population, were detected in the samples from this aquaculture farm, despite that the farm has no known history of antibiotic applications during production (Huang, 2014). Here, we report that majority of the ART isolates from the fish and aquaculture environment were resistant to two or more antibiotics.

MIC is a relatively precise measure of resistance to antibiotics. The measurement is essential for AR assessment not just because of the need to measure resistance trend, but more importantly, the MIC value is correlated to the dosage required for effective therapy. It is important to recognize that bacterial strains carrying the same resistance gene may have different MIC values, even for those from the same genus (Miranda et al., 2003). Understanding the molecular mechanisms contributing to the phenotype would be of great value to properly evaluate AR risk and control its dissemination. However, it has been difficult to compare antibiotic susceptibility results because of variation in testing methods in published studies. Most studies in the United States used CAMH broth, following NCCLS-recommended procedure (now it is CLSI), while Iso-Sensitest broth is widely used in Europe (Koeth et al., 2000). Koeth et al. (2000) conducted a study using 124 Centers for Disease Control and Prevention (CDC) reference strains compared the medium effect on MIC results, and concluded that data from the above two broth were comparable.

Due to genetic diversity of microbiota associated with both host and natural environment, bacterial media used in the studies likely have an impact on the results. Sul/Tri<sup>r</sup> population was

found abundant in microbiota across the host and environmental samples. Considering the potential effect of cultural medium on MIC results, in this study we have examined MIC for representative strains in both CAMH and BHI. The isolates had different MIC values in the two media, especially for Sul/Tri<sup>r</sup> isolates. Approximately 67% Sul/Tri<sup>r</sup> isolates recovered from BHI medium exhibited resistance against Sul/Tri in CAMH medium (data no shown), and some of them had much higher MIC values in BHI than that in CAMH. As shown in Table 2, some isolates such as *Enterococcus* sp. (17fS1, 17fS3, 18iX15, and 18fT19) and *Enterobacter* sp. (17iE3, 17iE10, 17iE11, and 17iE26) were classified as susceptible to Sul/Tri using CAMH, but showed high MIC values in BHI. In fact, the corresponding resistance determinants sul1 or sul2 were identified in the above strains with positive detection rate comparable to other AR genes, suggesting their MIC results in CAMH-Sul/Tri broth cannot represent their real resistance status against the antibiotics. As a matter of fact, CLSI has acknowledged the mismatch between MIC results of *Enterococcus* sp. using CAMH-Sul/Tri medium and resistant patterns in clinical treatments (CLSI, 2013). Moreover, although CAMH medium is widely used for MIC test, bacteria from certain genera didn't grow well in CAMH. As shown in Table 2, one tetL bearing isolate *Vagococcus* sp. could not grow in CAMH-Tet, but showed high MIC value (128 µg/ml) in BHI-Tet. These data indicated the limitations of CAMH as the medium for MIC assessment. While we have found that BHI serves the need for MIC analysis for most of the commensal isolates in this study, further validation using more reference strains and supplementation of media for cultures discriminated by BHI will be essential for methodology improvement. Moreover, ART isolates with higher MIC values are more difficult to eliminate with antibiotics. Whether the high MICs of the above strains are due to new molecular determinants or established resistance genes but with additional enhancement mechanisms are worth further investigation.

As illustrated in Table 4, 30 of 98 ART isolates (30.6%) from fish feed samples were found to carry one or more of the 12 AR genes examined, and the positive detection rate was much

higher than those of the ART isolates from other samples, being 9.5% (34 of 357) in fish intestine, 21% (44 of 209) in surface rinsing water associated with skin microbiota, 4.1% (10 of 244) in pond water, and 1.5% (4 of 269) in pond mud sample. This result was consistent with the finding that multiple AR gene pools in high levels were detected in fish feed samples by real-time PCR (Huang, 2014). In addition, fish skin and intestine samples also contained a large number of ART bacteria and AR genes which also is in agreement with the results reported by Ye et al. (2013). Because animal and fish by-products, often rich in ART bacteria, are used in fish feed as an important protein source, it is inevitable that there is a large pool of AR genes and potentially AR gene carrying bacteria in fish feed. ART bacteria even multi-drug-resistant bacteria were reported in various types of animal feeds, such as poultry feeds (Schwalbe et al., 1999), cattle feed ingredients (Dargatz et al., 2005), and rendered animal protein products originating from poultry, cattle, and fish (Hofacre et al., 2001). Without proper treatment, fish feed rich in AR gene-carrying bacteria survived the feed manufacturing process could be a potential risk factor spreading ART bacteria in the aquaculture production system and subsequently the food chain. In fact, several genera of ART bacteria were found in multiple types of samples. For example, *Plesiomonas* sp. were detected in surface rinsing water, fish intestine, and pond mud samples, while *Aeromonas* sp. were present in fish intestine and pond mud samples. The data suggested that these microorganisms were present in both the host and farm environment, and may have been circulating within the aquaculture ecosystem.

As illustrated in **Table 4**, there was no significant correlation of the types of AR encoding genes and their carriers between fish intestine and feed, indicating that hosts (fish) potentially also have a role in the selective enrichment of or breeding certain resistant bacteria in the population. In this study, the *sul1* gene of *Aeromonas* sp. from the fish intestine, the *sul2* gene of

*Psychrobacter* sp. and the *tet* (L) gene of *Vagococcus* sp. from the fish feed were found functional after being transferred to the corresponding recipients, suggesting that they can serve as a source of AR genes if involved in horizontal gene transfer events. Finally, representative AR genes were only found in a small percent of the ART isolates. A functional genomic analysis has already discovered a new Tet<sup>r</sup>-encoding gene, *tet47*, from a Tet<sup>r</sup> isolate associated with a fish intestine sample (Huang et al., 2015).

In summary, ART commensal bacteria associated with this aquaculture system with no known history of antibiotics application exhibited multi-drug-resistance (MDR). Various AR determinants were detected and 18 bacterial genera were identified among those AR gene carrying isolates. The data suggested that the aquaculture system is a rich reservoir of AR, risk factors other than direct antibiotic application, such as AR-rich feed or even environmental factors, may have played important role(s) disseminating AR in this ecosystem. Follow-up studies are needed to reveal a more comprehensive picture of AR in aquaculture production, and for targeted and effective AR mitigation in the ecosystem. Moreover, BHI medium was found more suitable for majority of the commensal bacteria examined than CAMH broth for MIC assessment.

## Acknowledgments

This study is supported by Ohio Soybean Council (RF project 60031678), U.S.-UK Global Innovation Initiative Award by Institute of International Education, U.S. Department of State (RF project 60043259), and State and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center (project OHOA1006). Chinese Scholarship Council provided stipend support for YH.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Correlation of PK/PD Indices with Resistance Selection for Cefquinome against *Staphylococcus aureus* in an In Vitro Model

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equally to this work.

### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 30 August 2015

**Accepted:** 21 March 2016

**Published:** 12 April 2016

### Citation:

Li Y, Feng B, Gu X, Yang D, Zeng Z,  
Zhang B and Ding H (2016)  
Correlation of PK/PD Indices with  
Resistance Selection for Cefquinome  
against *Staphylococcus aureus*  
in an In Vitro Model.  
*Front. Microbiol.* 7:466.  
doi: 10.3389/fmicb.2016.00466

Cefquinome is a fourth-generation Cephalosporin approved for use in animals exclusively. The objective of this study was to explore the relationship of cefquinome pharmacokinetic/pharmacodynamic (PK/PD) indices with resistance selection of *Staphylococcus aureus* ATCC25923 in an *in vitro* model. Six dosing regimens of cefquinome at an interval of 24 h for three consecutive times were simulated, resulting in maximum concentrations ( $C_{max}$ ) from 1/2 to 16MIC and terminal half-lives ( $t_{1/2\beta}$ ) of 3 and 6 h, respectively. The *in vitro* sensitivity of *S. aureus* was monitored by bacterial susceptibility and dynamic time-kill curve experiments over the six cefquinome concentrations. The correlation between changes in bacterial susceptibility (MIC<sub>72</sub>/MIC<sub>0</sub>) and the percentage of time within mutant selection window versus dosing interval (T<sub>MSW</sub> %) was subjected to the Gaussian function and regression analysis. Our results favored the consensus that time above MIC ( $T > MIC$ ) was recognized as an important PK/PD parameter of cephalosporins for antibacterial efficiency. Cefquinome reached the maximum killing effect when  $T > MIC$  attained approximately 40~60%. The subsequent correlation analysis demonstrated that resistant *S. aureus* ATCC25923 was easy to occur when T<sub>MSW</sub> % attained an index of about 20% with  $t_{1/2\beta}$  of 3 h after multiple dosing, and 40% with  $t_{1/2\beta}$  of 6 h after multiple dosing, respectively.

**Keywords:** cefquinome, *Staphylococcus aureus*, PK/PD, MPC, *in vitro* model

## INTRODUCTION

*Staphylococcus aureus* is a major pathogen for animals and humans, which contributes to a variety of severe infections, including bacteremia, meningitis, endocarditis, skin and wood infection or other diseases for animals and humans (Archer, 1998; Lowy, 1998; Azizoglu et al., 2013). More importantly, *S. aureus* plays an important role in the food contamination by foodborne pathogens. Food poisoning caused by staphylococcal enterotoxin (SE) is a pressing worldwide health problem (Tauxe, 2002; Le Loir et al., 2003). In spite of the progress in antimicrobial therapy, treatment of *S. aureus* infection has become more and more challenging

because drug-resistance of *S. aureus* has increased globally over the past decade. The methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) were particularly reported and well documented (Harris et al., 2010; Mohammed Fayaz et al., 2011).

In veterinary medicine, cefquinome (CEQ) has been licensed in European countries by virtue of its broad antibacterial spectrums and remarkable antibacterial activities. Despite of this, the cephalosporins should be in prudent use considering the escalating antimicrobial resistance. Evidence has showed that different animal species may harbor the same resistance determinant and are recognized as possible reservoirs of antimicrobial-resistant bacteria (Guardabassi et al., 2004). It has been also observed that cefquinome exerted a selective effect on *bla*<sub>CTX-M</sub> producing *Escherichia coli* strains (Cavaco et al., 2008). Therefore, further information concerning the ability of cefquinome to prevent occurring of resistant strains seems to be investigated.

Since mutant prevention concentration (MPC) of antibiotics was first described by Dong et al. (1999), it has been successfully applied to evaluate the ability of antibiotics that restrict the selection of resistant strains (Allen et al., 2004; Allen and Hankins, 2009; Wang et al., 2010; Blondeau et al., 2012) and optimize the current dosing regimens of antibiotics to slow the emergence of resistant strains. MPC was taken as the lowest doubling dilution drug concentration that prevented a population of  $10^{10}$  colony forming unit (CFU)/mL or even more microorganisms from first-step mutation. Zone of the drug concentrations between minimum inhibitory concentration (MIC) and MPC was defined as the mutant selection window (MSW). MPC and MSW concepts, representing the ability of antibiotics to select resistant strains, have been tested in various *in vitro* studies (Allen et al., 2004; Croisier et al., 2004b; Ferran et al., 2007), in *ex vivo* pharmacodynamic studies (Bronner et al., 2002) and in *in vivo* studies (Croisier et al., 2004a; Cui et al., 2006). Here, we developed an *in vitro* kinetic model to investigate the relationship between pharmacokinetic/pharmacodynamic (PK/PD) indices of cefquinome with terminal half-lives ( $t_{1/2\beta}$ ) of 3 or 6 h and resistance development of *S. aureus* ATCC25923.

## MATERIALS AND METHODS

### Antimicrobial Agents, Medium, and Bacterial Strains

Raw material of cefquinome (purity of 84.1%) was obtained from Hebei yuanzheng Pharmaceutical Enterprise, Co., Ltd. Mueller-Hinton broth (MHB) and agar were purchased from Guangzhou huankai Comapany. *S. aureus* ATCC 25923 was purchased from the China Institute of Veterinary Drug Control (Beijing, China).

### In Vitro Susceptibility Testing

The MIC of CEQ against *S. aureus* with an inoculum of  $5 \times 10^5$  CFU/mL was determined by standard agar dilution method established by the Clinical and Laboratory Standards Institute (CLSI).

### Measurement of MIC<sub>99</sub>, Mutant Prevention Concentration (MPC) and Selection Index (SI)

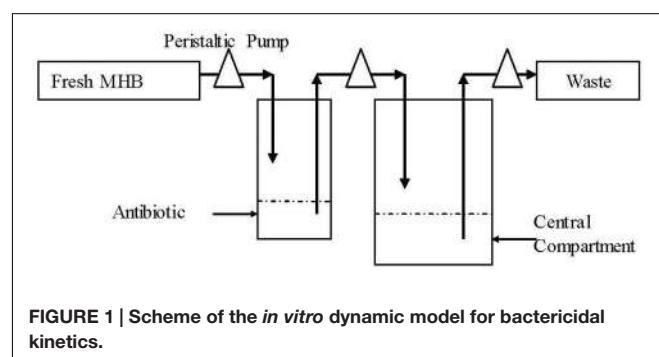
The MIC<sub>99</sub> was defined as the drug concentration that inhibited 99% of bacteria colony formation. MPC was reckoned as the lowest cefquinome concentration blocking  $\geq 10^{10}$  CFU/mL inoculants growth. The measurements of MIC<sub>99</sub> and MPC were mainly based on the method reported by Zhao and Drlica (2002) with slight modification. For MIC<sub>99</sub>, agar plates containing a series of cefquinome (concentrations ranging from 0.5 to 0.164 µg/mL) at 20% per sequential decrease were prepared.  $3 \times 10^7$  CFU/mL *S. aureus* suspension in logarithmic phase of growth was then subjected to serial 10-fold dilutions with MHB to  $3 \times 10^2$  CFU/mL bacteria. Again, 100 µL of each dilution was plated onto the agar plates containing the series of CEQ concentrations mentioned above and incubated at 37°C overnight. Bacterial colonies that recovered growth in each dilution were counted. Drug concentrations versus the percentages of colony recovery were plotted and the interpolation method was adopted to calculate the cefquinome concentration blocking 99% bacteria growth.

The determination of MPC was similar to that of MIC<sub>99</sub>. Instead, 100 µL of  $10^{10}$  CFU/mL inoculants was spread on agar plates containing a series of antimicrobial concentrations. The lowest antimicrobial concentration preventing bacterial colony formation at 72 h after incubation was measured as provisional mutant prevention concentration (MPC<sub>pr</sub>). A second determination that utilized linear MPC<sub>pr</sub> decreases (about 20% per sequential decrease) was performed. MPC was the lowest CEQ concentration preventing 100% growth of *S. aureus* colony.

The calculated ratio of MPC/MIC<sub>99</sub> for *S. aureus* was defined as selection index (SI) of cefquinome.

### In Vitro Dynamic PK/PD Model Simulation

A previous described dynamic model (Grasso et al., 1978) with modification was developed in this study. One-compartment open model with first-order absorption of pharmacokinetics for cefquinome was simulated. The schematic representation of *in vitro* PK/PD model used is depicted in Figure 1. Briefly, the system was mainly composed of three sealed containers



(compartments) and they were connected with peristaltic pumps in line, each containing sterile MHB and a magnetic stirrer to ensure adequate mixing. One 500 mL sealed compartment containing 300 mL MHB, provided with either a bacterial culture alone (control growth experiments) or a bacterial culture plus antibiotic (killing/re-growth experiments), acted as the central chamber. Another 100 mL sealed container charged with 60 mL of sterile MHB, acted as the absorption chamber, with desired calculated drug concentrations. The third one was used to provide fresh MHB. Waste was also collected. Peristaltic pumps circulated fresh broth to central and absorption compartments at the desired flow rate. The flow rate of the pump was set on the basis of the terminal half-life being simulated. A series of dosage regimens were designed to generate different CEQ concentration profiles with terminal half-lives of 3 and 6 h for three consecutive administrations in this apparatus.

### In Vitro PK/PD Model and CEQ Dosing Regimens

The apparatus ran under 37°C. The elimination terminal half-lives of cefquinome reported in the literature varied from 0.5 to 10 h (Limbert et al., 1991; Li et al., 2008; Al-Taher, 2010; Tohamy, 2011; Yuan et al., 2011; Zonca et al., 2011; Dumka et al., 2013), therefore, 3 and 6 h were selected in our study. Peristaltic pumps circulated fresh MHB medium to and from the central compartment at a flow rate of 15.75 r/min for  $t_{1/2\beta}$  of 3 h or 7.86 r/min for  $t_{1/2\beta}$  of 6 h, respectively. 3 mL bacterial suspensions ( $10^8$  CFU/mL) at the logarithmic phase were injected into the central compartment container. The bacteria were incubated in the model for 2 h to result in exponentially growing cultures before addition of cefquinome. Antibiotic doses were calculated to generate CEQ initial concentrations of 1/2MIC, MIC, 2MIC, 4MIC, 8MIC, and 16MIC levels, respectively. The *in vitro* pharmacokinetics of cefquinome for all doses after three consecutive administrations with 24 h interval for terminal half-lives of 3 and 6 h were simulated.

### Quantification of Cefquinome in MHB

Samples were collected from the central compartment immediately before and at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 24.0 h after the first and second administration, and 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0, 30.0, 36.0, and 48.0 h after the third administration. These samples were stored at -20°C until analysis.

1.5 mL of MHB was extracted for the determination of cefquinome. The concentrations of cefquinome in MHB were analyzed by high-performance liquid chromatography-MS/MS (HPLC-MS/MS) with an assay range of 0.005–0.5 µg/mL. The protein in MHB sample (1 mL) was precipitated by 2 mL acetonitrile, and the supernatant was directly injected into HPLC-MS/MS after high speed centrifugation. Analysis of quality control (QC) samples at three levels (0.01, 0.05, 0.2 µg/mL) showed that the recoveries of the method were above 70%; the intra-day and inter-day coefficients of variation were within 15%. The pharmacokinetic parameters were calculated

by a WinNonlin software (version 5.2.1; Pharsight Corporation, USA).

### In Vitro Time-Kill Experiments and Susceptibility Testing of *S. aureus*

To measure the antimicrobial effect of cefquinome with different terminal half-lives against *S. aureus*, the colony count and susceptibility of bacteria in each time point were performed after treatment. Half of each sample was subjected to time-kill kinetic assays. Samples (100 µL) in series of 10-fold dilution with sterile saline (0.9% NaCl) were spread onto Mueller-Hinton agar to determine the number of total or resistant cells. The  $\log_{10}$  of surviving *S. aureus* cells (CFU/mL) was plotted against each time point. The other half of each sample was used for the susceptibility test. MIC values of bacteria post-exposed in each time point was conducted using tube dilution method according to the criteria established by the Clinical Laboratory Standards Institute [CLSI] (2013).

### Pharmacokinetic/Pharmacodynamic (PK/PD) Integration Analysis

By using the individual pharmacokinetic result from each *in vitro* dosage regimen, the following PK/PD parameters were obtained: ratio of area under the curve of cefquinome concentration versus time to MIC ( $AUC_{0-\infty}/MIC$ ) or ( $AUC_{0-24\text{ h}}/MIC$ ), ratio of area under the curve of cefquinome concentration exceeding MIC to MIC ( $AUC_{C > MIC}/MIC$ ), time of concentration above MIC ( $T > MIC$ ) or MPC ( $T > MPC$ ), time of concentration within MPC and MIC ( $T_{MSW}$ ) or expressed as percentage ( $T_{MSW}\%$ ), ratio of  $MIC_{72}$  to  $MIC_{initial}$  ( $MIC_{72}/MIC_{initial}$ ).

Gaussian function was used to simulate the correlation of  $MIC_{72\text{ h}}/MIC_{initial}$  with  $T_{MSW}\%$  (percentage of the time during which cefquinome concentrations were inside the MSW). The formula was as follows:

$$y = y_0 + \frac{A}{\sqrt{2\pi}\sigma} e^{-\frac{(x-x_0)^2}{2\sigma^2}}$$

where A was the area under curve and upper baseline;  $x_0$  was the minimum value of  $T_{MSW}\%$  which resulted in the maximum  $MIC_{72\text{ h}}/MIC_{initial}$ .

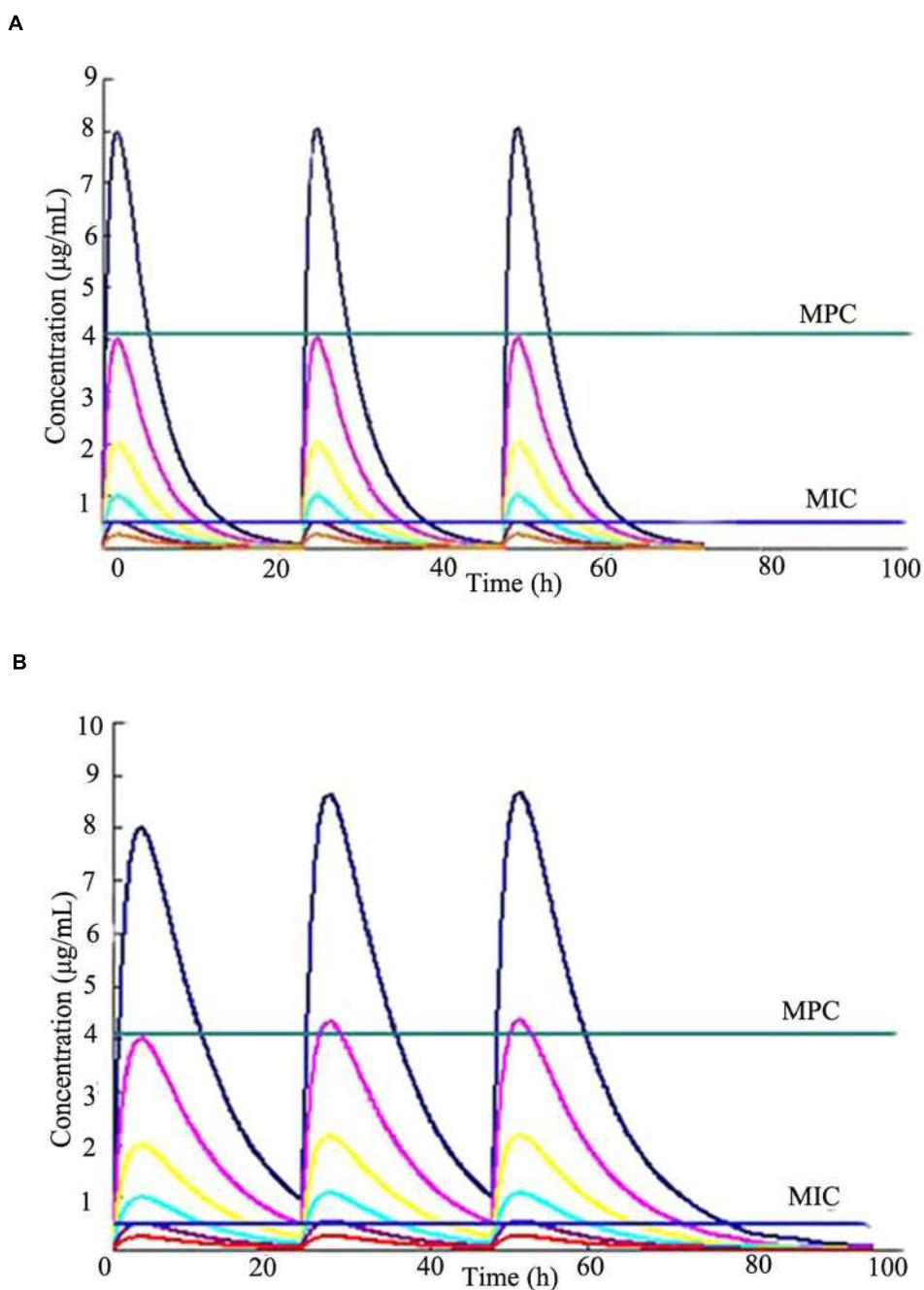
## RESULTS

### $MIC_{99}$ and MPC

The  $MIC_{99}$  and MPC of cefquinome against *S. aureus* ATCC 25923 were 0.4 and 4.096 µg/mL, respectively. So the SI was 10.24 (MPC/MIC<sub>99</sub>).

### In Vitro Simulated Pharmacokinetics

The *in vitro* simulated time-concentration curves for cefquinome with terminal half-lives of 3 and 6 h are shown in Figure 2. This study used time-concentration curves of the unbound fractions of cefquinome in Mueller-Hinton broth. The maximum concentrations were approximately equal to 1/2MIC to 16-fold MIC of cefquinome against *S. aureus* ATCC 25923 after three



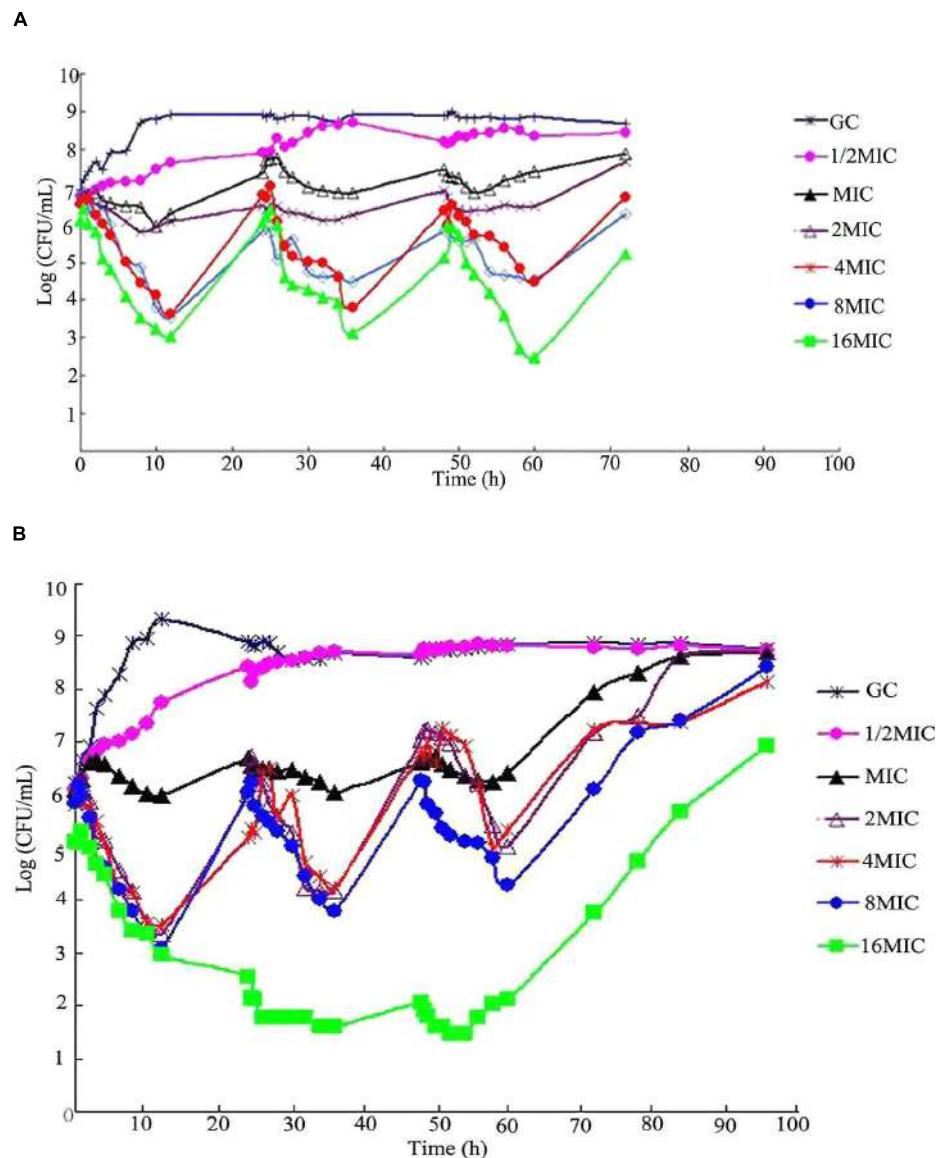
**FIGURE 2 |** Concentration-time curves of cefquinome in the *in vitro* pharmacokinetics model of two terminal half-lives ( $t_{1/2\beta}$ ). The horizontal lines indicate the MPC ( $4.096\text{ }\mu\text{g/mL}$ ) and MIC ( $0.4\text{ }\mu\text{g/mL}$ ) for *Staphylococcus aureus* ATCC 25923, respectively. **(A)**  $t_{1/2\beta} = 3\text{ h}$ , **(B)**  $t_{1/2\beta} = 6\text{ h}$ .

consecutive administrations. The MPC and MIC levels were also indicated in the simulated time-concentration curves.

### The Bacteria Killing Curves in *In Vitro* Models

Time-killing curves of *S. aureus* ATCC 25923 in the *in vitro* model under different dosing regimens are shown in the following Figure 3. For a  $t_{1/2\beta}$  of 3 h multiple dosages,

bacteriostatic or bactericidal action was observed when maximum cefquinome concentrations were equal to MIC or over MIC levels (dosages ranged from 0.672 to 10.767 mg). For a  $t_{1/2\beta}$  of 6 h multiple dosages, continuous bactericidal action was observed when maximum cefquinome concentrations were at 4MIC, 8MIC, 16MIC (dose ranged from 2.691 to 10.767 mg), and the continuous time of bactericidal action at 16MIC was the longest among three concentrations.



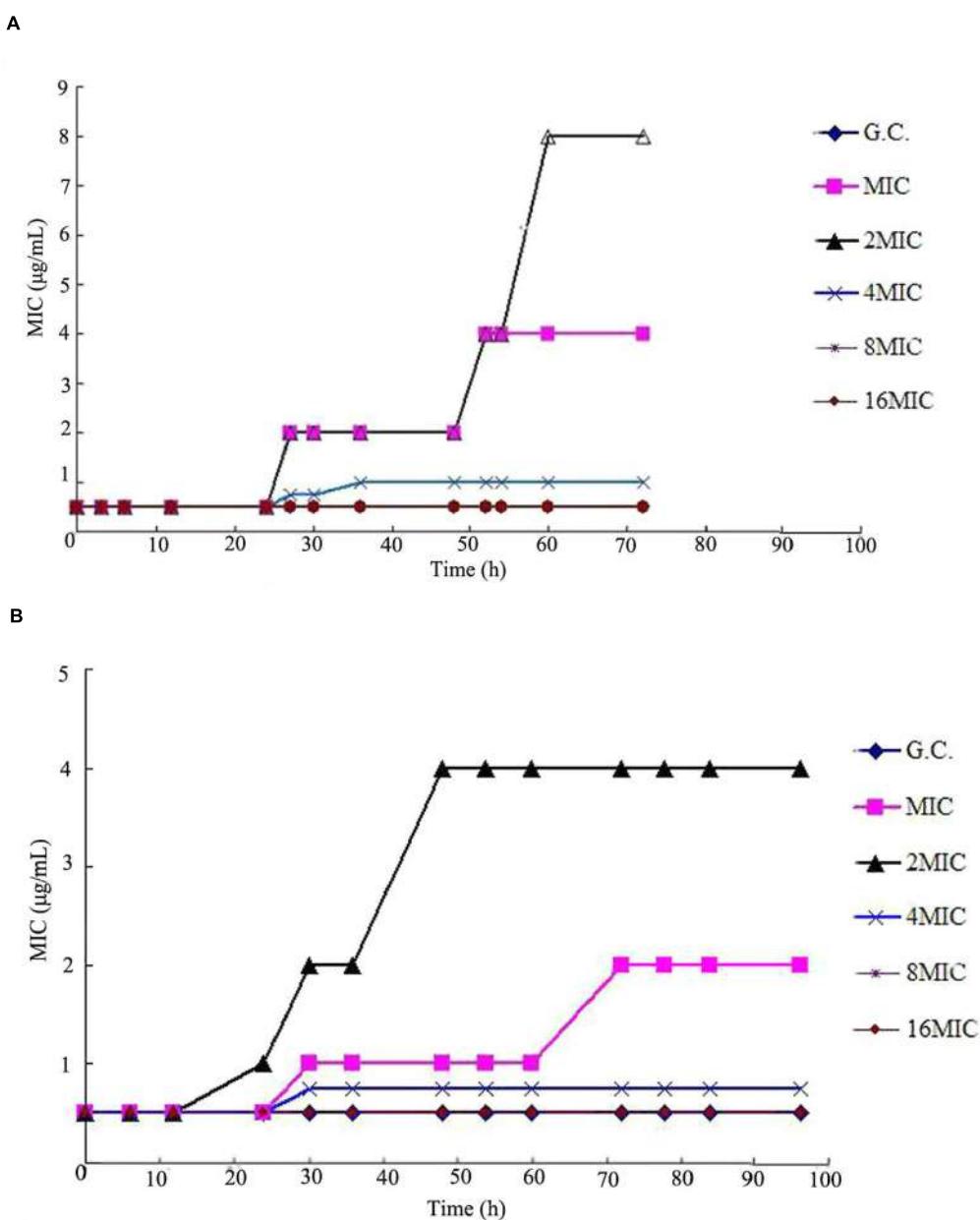
**FIGURE 3 |** Viable counts of *S. aureus* ATCCC 25923 in time-kill assays with cefquinome in different terminal half-lives ( $t_{1/2\beta}$ ) at cefquinome concentrations from a half to 16-fold the minimum inhibitory concentration (MIC), in comparison with a cefquinome-free control. (A)  $t_{1/2\beta} = 3$  h, (B)  $t_{1/2\beta} = 6$  h.

## Loss of Susceptibility to Cefquinome

Compared to drug-free group, loss of susceptibility was observed in MIC, 2MIC, and 4MIC concentration groups after administrations, as shown in **Figure 4**. For multiple dosages with  $t_{1/2\beta}$  of 3 h, the bacterial MICs after administration increased to 4.0, 8.0, and 1.0  $\mu\text{g}/\text{mL}$  for MIC, 2MIC, and 4MIC groups, respectively. For multiple dosages with  $t_{1/2\beta}$  of 6 h, the bacterial MICs after administration increased to 2.0, 4.0, and 0.8  $\mu\text{g}/\text{mL}$  for MIC, and 2MIC, and 4MIC concentration administrations, respectively. The MIC values did not change through the whole experiment period in 8MIC and 16MIC administration groups with two different terminal half-lives.

## Correlation of PK/PD Indices with Resistance Selection

Pharmacokinetic/pharmacodynamic indices, such as  $\text{AUC}_{24\text{ h}}/\text{MIC}$  (where  $\text{AUC}_{24\text{ h}}$  is the area under the drug concentration time curve in a 24 h interval) and time above the MIC, provide an empirical way to relate antimicrobial dose to favor the treatment effect of bactericidal agents. Relationships between PK/PD indices and loss of susceptibility are shown in **Table 1**. For cephalosporin,  $T > \text{MIC}$  was the index most commonly associated with restricting susceptible cell growth. When  $T > \text{MIC}$  attained 17.14 h or  $T > \text{MIC}\%$  attained 24%, the MIC value of cefquinome against *S. aureus* increased to



**FIGURE 4 |** Typical susceptibility responses of *S. aureus* ATCCC 25923 observed in the *in vitro* PK/PD model simulating the pharmacokinetics of cefquinome with different terminal half-lives ( $t_{1/2\beta}$ ). (A)  $t_{1/2\beta} = 3$  h, (B)  $t_{1/2\beta} = 6$  h.

16-fold of initial value at 72 h after multiple administrations in groups with  $t_{1/2\beta}$  of 3 h. Similarly, in groups with  $t_{1/2\beta}$  of 6 h, when  $T > \text{MIC} = 34.28$  h or  $T > \text{MIC}\% = 48\%$ , the value of cefquinome MIC increased to eightfold of initial value after multiple dosages.

Other PK/PD indices also showed correlation with the selection of resistance (Table 1). When  $T_{MSW}$  and  $T_{MSW\%}$  were 17.14 h and 24%, the MIC increased to 16-fold of initial value in groups with  $t_{1/2\beta}$  of 3 h after multiple dosages, and the MIC increased to eightfold of initial value when  $T_{MSW}$  and  $T_{MSW\%}$

were 34.28 h and 48% with  $t_{1/2\beta}$  of 6 h after multiple dosages, respectively.

### Correlation Analysis of MIC Increase with $T_{MSW\%}$

According to Gaussian function and regression analysis results, the bacteria was prone to develop resistance when  $T_{MSW\%}$  was about 20% ( $x_0 = 0.2027$ ) in groups with  $t_{1/2\beta}$  of 3 h after multiple dosages ( $R^2 = 0.9989$ ), and  $T_{MSW\%}$  was

**TABLE 1 | Pharmacokinetic/pharmacodynamic (PK/PD) indices of antimicrobial efficacy and risk of resistance selection amongst *Staphylococcus aureus* ATCC 25923 over the complete dosing interval following PK simulations of three consecutive administrations of cefquinome in  $t_{1/2\beta}$  of 3 and 6 h.**

$t_{1/2\beta}$ (h)	D (mg)	$\frac{AUC_{C>MIC}}{MIC}$ (h)	$\frac{AUC_{24}}{MIC}$ (h)	T > MIC (h)	T > MPC (h)	T <sub>MSW</sub> (h)	T <sub>MSW%</sub>	T > MIC%	$\frac{MIC_{T>MIC}}{MIC_{initial}}$
3	1/2MIC	0	3.22	0	0	0	0	0	1
	MIC	6.41	6.43	8.11	0	8.11	11	11	8
	2MIC	25.89	12.89	17.14	0	17.14	24	24	16
	4MIC	64.68	25.76	26.13	0	26.13	36	36	2
	8MIC	142.34	51.52	35.13	7.81	27.32	38	49	1
	16MIC	297.75	103.07	44.14	16.82	27.32	38	61	1
6	1/2MIC	0	5.96	0	0	0	0	0	1
	MIC	12.81	11.92	16.21	0	16.21	23	23	4
	2MIC	51.77	23.89	34.28	0	34.28	48	48	8
	4MIC	129.35	47.73	52.26	0	52.26	73	73	1.5
	8MIC	284.68	95.45	70.26	15.63	54.64	76	98	1
	16MIC	595.50	190.96	88.28	33.64	54.64	76	123	1

about 40% ( $x_0 = 0.4102$ ) in groups with  $t_{1/2\beta}$  of 6 h after multiple dosages ( $R^2 = 0.9986$ ), respectively, as shown in Figure 5. Those results were consistent with the data from Table 1.

## DISCUSSION

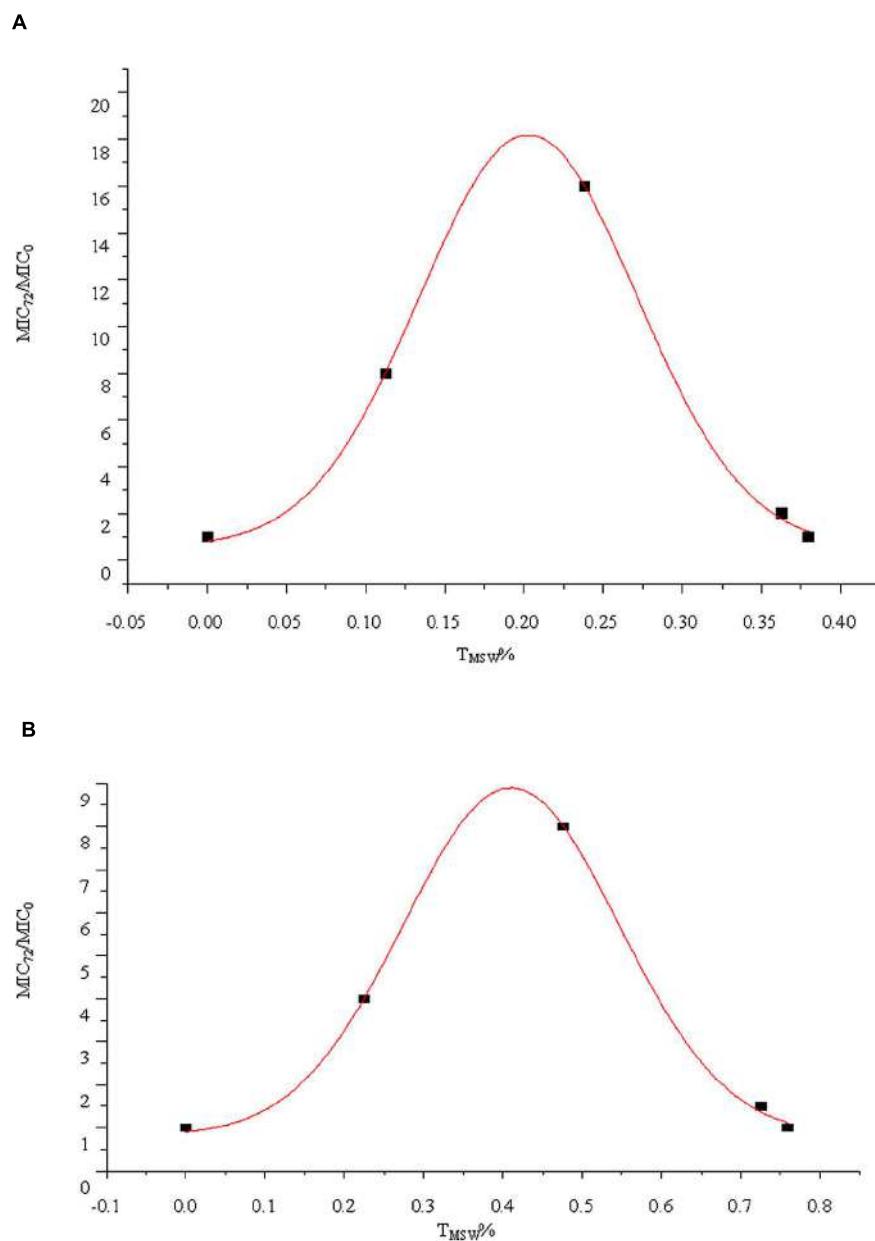
Antimicrobial resistance has been a global problem and a great number of strategies have been proposed to slow the emergence of resistance (Stratton, 2003; Levy and Marshall, 2004). *In vivo* or *in vitro* PK/PD models have previously been applied to optimize the dosage regimen of various antibiotics in the literature (Croisier et al., 2004b; Ferran et al., 2009; Andraud et al., 2011; Gebru et al., 2012). However, PK/PD model has been renewably used to investigate relationship of bacterial resistance with AUC/MIC,  $C_{max}/MIC$  and T > MIC, recently. In the present study, we simulated the cefquinome pharmacokinetic profiles with different terminal half-lives using this dynamic model and tried to predict the selection of resistant *S. aureus* with some indices, such as T > MIC and T<sub>MSW%</sub>.

It is believed that integration of drug pharmacokinetics, mutant prevention concentration is helpful in slowing the emergence of resistance. In this experiment, the MPC and MSW of cefquinome against *S. aureus* ATCC25923 were determined. The SI, ratio of MPC to  $MIC_{99}$ , is the ability of a drug to select resistant mutants (Zhao and Drlica, 2001). In other word, the bigger the SI is, the easier the drug is to induce resistance. The SI of cefquinome against *S. aureus* ATCC25923 was 10.24 in our study, which suggested that cefquinome could induce *S. aureus* resistant mutation easily and effectively.

T > MIC was regarded as a promising predictor for the efficacy of cephalosporins. Previous pharmacodynamic studies have addressed separate issues around the relationships between T > MIC and efficacy for cephalosporins. Craig (1995) has reviewed the data of efficacy for cephalosporins against *Enterbacteriaceae*, *streptococci* and *S. aureus* in several animal infection models and found that the time above MIC required for a bacteriostatic effect against strains of *Enterbacteriaceae*,

*Streptococci* were generally 60–70%, and 40–50% for *S. aureus*, respectively. In our study, the value of T > MIC for maximal killing was 40~60% for cefquinome against *S. aureus* reference strain. In consistent with the widespread idea that antibacterial efficacy of  $\beta$ -lactams depend on T > MIC, the effect of cefquinome against *S. aureus* in our study exhibited similar correlation. As observed in Figure 3, killing curves of *S. aureus* exposed to each dose of cefquinome showed the typical pattern of time-dependent bactericidal action. The similar observation was also reported for other  $\beta$ -lactam drugs in previous studies (Lindecrona et al., 2000; Burgess and Hall, 2004). Considering that the T > MIC was one of the most important parameters for optimal dosage regimen, T > MIC was selected as a parameter to evaluate resistant mutation in this article. In the present study, the values of T > MIC ranging from 8.11 to 17.14 h were dangerous zones for inducing resistant mutation in groups with  $t_{1/2\beta}$  of 3 h after multiple dosages, and values ranging from 16.21 to 34.28 h were dangerous zones for inducing resistant mutation in groups with  $t_{1/2\beta}$  of 6 h multiple dosages. Compared these values with previously reported study data that T > MIC value ranged from 42 to 54 h for cefquinome in dairy cows (Zonca et al., 2011), the prevention of selecting resistant *S. aureus* strains seems to be achieved by the present dosage regimens approved by the European Medicines Agency (EMA) for the treatment of dairy cow mastitis.

T<sub>MSW</sub> is another important parameter used for evaluating resistant mutation. A previous study conducted in a rabbit lung infection model with *Streptococcus pneumoniae* showed that the selection of resistant bacteria occurred systematically when concentrations of gatifloxacin were within the MSW (T<sub>MSW</sub>) for more than 45% of the treatment duration (Croisier et al., 2004b). Another experiment in rabbits infected by *S. aureus* also showed that time in the MSW > 33% was preferable to select mutants (Cui et al., 2006). When the abilities of the indices T<sub>MSW</sub>, AUC<sub>24</sub> h/MIC,  $C_{max}/MIC$  to predict the selection of resistant bacteria were compared, only T<sub>MSW</sub> appeared to be a good predictor of the prevention of resistance (Ferran et al., 2009). In this study, T<sub>MSW</sub> was also used to predict selection of



**FIGURE 5 |** Relationship between the T<sub>MSW</sub>% and MIC<sub>72</sub>/MIC<sub>0</sub> for *S. aureus* ATCC 25293 exposed to cefquinome in the *in vitro* model with two terminal half-lives (t<sub>1/2β</sub>). (A) t<sub>1/2β</sub> = 3 h, (B) t<sub>1/2β</sub> = 6 h.

resistant *S. aureus* against cefquinome. It was clearly determined that when T<sub>MSW</sub> was above 36% in groups with t<sub>1/2β</sub> of 3 h after multiple dosages or above 73% in groups with t<sub>1/2β</sub> of 6 h after multiple dosages, cefquinome could restrict the resistant mutation.

In the present investigation, the bacterial resistant mutation was affected by different terminal half-lives and dosages. Within the group of the same terminal half-life (3 or 6 h), the bacterial resistant mutation happened under the condition of relative low dosage (maximum concentrations were 2MIC and 4MIC). When the maximum concentration was over 8MIC, resistant mutation

was prevented. In the same dosages (maximum concentration was 2MIC or 4MIC), the MIC<sub>72 h</sub> increased from 8- to 16-fold in groups with t<sub>1/2β</sub> of 3 h after multiple dosages and increased from 4- to 8-fold in groups with t<sub>1/2β</sub> of 6 h after multiple dosages, respectively. Those results suggested that the bacterial resistant mutation might happen at a low concentration. In high dosages or long terminal half-lives, the drug concentrations might exceed the MPC value, the upper boundary of MSW, and the resistant mutation could be inhibited. The conclusion from this study was consistent with other similar studies (Cui et al., 2006; Ferran et al., 2009; Zhu et al., 2012).

The limitation of the study was that only one reference strain was used to simulate the efficacy of cefquinome in this *in vitro* model; however, the multi-resistance is becoming an urgent trend all over the world. It may be reasonable that more clinical isolates of *S. aureus* could be used for further study. Firsov et al. (2008) used two methicillin-resistant strains of *S. aureus*, ATCC 6538 and ATCC 43300, to study the enrichment of ciprofloxacin resistant mutation in an *in vitro* dynamic model. Liang et al. (2011), chose three clinical isolates of *S. aureus*, SA99, RN450 and RN450-A1, to analyze the enrichment of levofloxacin resistant mutation with *in vitro* dynamic model. From the results obtained in the dynamic model, more clinical isolates could be used to study resistant mutation easily based on the substantial method. Moreover, to our knowledge, evaluation of antimicrobial activity for cefquinome with different terminal half-lives is scarce. Considering the fact that cefquinome is the time-dependent drug, it is positive to study the correlation resistant mutation with different terminal half-life conditions. Another limitation of the current study was the absence of resistance mechanisms of *S. aureus* observed *in vitro*. Therefore, the pattern of *S. aureus* resistant to cefquinome, such as production of  $\beta$ -lactamases, alteration of drug targets, porin-mediated resistance, and/or efflux-mediated resistance, needs confirmation in the further study.

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## CONCLUSION

The present study of resistant mutation with *S. aureus* ATCC 25923 exposed to cefquinome with two different terminal half-lives in *in vitro* dynamic models supports the MSW hypothesis and provides some useful parameters to predict the resistant mutation, especially  $T > \text{MIC}$  and  $T_{\text{MSW}}\%$ . Further study will concentrate on more clinical isolates which could be used for verification of these results and resistance patterns of *S. aureus* resistant to cefquinome.

## AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

## ACKNOWLEDGMENTS

The authors would like to thank the financial support by the Natural Science Foundation of China (grant number 31172366) and 973 program (grant number 2013CB127200/2013CB127203).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# CTX-M-27 Producing *Salmonella enterica* Serotypes Typhimurium and Indiana Are Prevalent among Food-Producing Animals in China

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 08 January 2016

Accepted: 17 March 2016

Published: 31 March 2016

### Citation:

Zhang W-H, Lin X-Y, Xu L, Gu X-X, Yang L, Li W, Ren S-Q, Liu Y-H, Zeng Z-L and Jiang H-X (2016) CTX-M-27 Producing *Salmonella enterica* Serotypes Typhimurium and Indiana Are Prevalent among Food-Producing Animals in China. *Front. Microbiol.* 7:436.  
doi: 10.3389/fmicb.2016.00436

*Salmonella* spp. is one of the most important food-borne pathogens causing digestive tract and invasive infections in both humans and animals. Extended-spectrum β-lactamases (ESBLs) especially the CTX-M-type ESBLs are increasingly being reported worldwide and in China. These studies seldom focused on *Salmonella* isolates from food-producing animals. The aim of this study was to characterize the antimicrobial resistance profiles, serotypes and ESBLs and in particular, CTX-M producing *Salmonella* isolates from chickens and pigs in China. *Salmonella* isolates were identified by API20E system and polymerase chain reaction (PCR) assay; serotypes were determined using slide agglutination with hyperimmune sera; antimicrobial susceptibility was tested using the agar dilution method; the prevalence of ESBLs and PMQR genes were screened by PCR; CTX-M-producing isolates were further characterized by conjugation along with genetic relatedness and plasmid replicon type. In total, 159 *Salmonella* strains were identified, among which 95 strains were *Salmonella enterica* serovar Typhimurium, 63 strains were *S. enterica* serovar Indiana, and 1 strain was *S. enterica* serovar Enteritidis. All of these isolates presented multi-drug resistant phenotypes. Forty-five isolates carried *bla*<sub>CTX-M</sub> genes, the most common subtype was CTX-M-27(34), followed by CTX-M-65(7) and CTX-M-14(4). Most *bla*<sub>CTX-M</sub> genes were transmitted by non-typeable or IncN/IncFIB/IncP/IncA/C/IncHI2 plasmids with sizes ranging from 80 to 280 kb. In particular, all the 14 non-typeable plasmids were carrying *bla*<sub>CTX-M-27</sub> gene and had a similar size. PFGE profiles indicated that CTX-M-positive isolates were clonally related among the same serotype, whilst the isolates of different serotypes were genetically divergent. This suggested that both clonal spread of resistant strains and horizontal transmission of the resistance plasmids contributed to the dissemination of *bla*<sub>CTX-M-9G</sub>-positive *Salmonella* isolates. The presence and spread of CTX-M, especially the CTX-M-27 in *S. enterica* serovars Typhimurium and Indiana from food-producing animals poses a potential threat for public health. Control strategies to limit the dissemination of these strains through the food chain are necessary.

**Keywords:** *Salmonella*, food-producing animals, serotype, antimicrobial resistance, CTX-M

## INTRODUCTION

The emergence of antibiotic-resistant bacteria has become a serious challenge in human and veterinary medicine globally and poses a serious public health threat. Non-typhoidal *Salmonella* (NTS) are a leading cause of bacterial diarrhea worldwide and one of the most important foodborne pathogens. The majorities of human infections by NTS are associated with food product consumption of meat, eggs, milk, seafood, and other fresh products derived from animals (Foley and Lynne, 2008). In the animal husbandry industry, antibiotics are used both for therapy and as growth promoters. Additionally, animals are always a large repository of resistant bacteria. Domestic farm animals, especially poultry and pigs have been shown to be major environmental reservoirs of food-borne NTS (Vo et al., 2006).

Serotyping is a useful classification scheme that allows for trends in *Salmonella* surveillance data to be followed over time. These patterns are also related to the ability to cause disease and with the associated antibiotic resistance profile. Over 2,600 serotypes of *Salmonella* have been identified based on the reactivity of antisera to O and H antigens (Stevens et al., 2009), and there are numerous overlaps between animal and human *Salmonella* serotypes (Alcaine et al., 2006). This suggests that *Salmonella* transmission from animals to humans occurs via the food chain. *Salmonella enterica* serovars Enteritidis and Typhimurium are the most common serotypes in Europe (European Centre for Disease Prevention and Control, 2013). However, serotype distribution is varied in different provinces of China and in total the most prevalent serovars were also Typhimurium and Enteritidis with a rising rate of reports of *S. enterica* serovar Indiana in isolates obtained from animals (Xia et al., 2009; Yang et al., 2010; Lu et al., 2014; Zhang et al., 2014).

It is estimated that between 1994 and 2005 roughly 22% of foodborne diseases in China were caused by *Salmonella* though there was no official surveillance data (Wang et al., 2007). In 2013, unpublished data from the China CDC surveillance system showed that the carriage rate of human salmonellosis was 549 per 100,000 people. This is more than 33 times higher than human infections in the USA in 2012 (16.4 per 100,000; Centers for Disease Control and Prevention, 2013; Sun et al., 2014). There is also an increasing risk of *Salmonella* spread from animals to humans via the food chain due to worldwide distribution of animal food products from China. Extended spectrum cephalosporins (ESCs) and fluoroquinolones are the drugs of choice for treatment of invasive *Salmonella* infections in food animals and for people at risk of such infections (Harrois et al., 2014). This is the focus of current concerns on the emergence of resistance to these drugs. ESCs- and fluoroquinolone-resistant *Salmonella* populations have increased dramatically worldwide (Arlet et al., 2006; Hendriksen et al., 2009; Jiang et al., 2014; Lu et al., 2014). Resistance to cephalosporins is mainly due to the acquisition of extended-spectrum β-lactamases (ESBLs) genes especially the CTX-M type that is primarily carried by transferable plasmids and transposons (Liebana et al., 2013). These plasmids and transposons, in many cases, also carry resistance genes for other antimicrobial classes such as the fluoroquinolones

which sometimes limits treatment options (Harrois et al., 2014). Plasmid-mediated quinolone resistance (PMQR) has recently been categorized as containing three distinct resistance mechanisms: (1) Qnr proteins that mediate target protection, (2) A variant of an aminoglycoside acetyltransferase designated AAC(6')-Ib-cr that acetylates ciprofloxacin and norfloxacin and (3) Drug efflux mediated by OqxAB and QepA (Strahilevitz et al., 2009). Although these PMQR determinants confer only low-level fluoroquinolone resistance, their presence can provide a selective advantage for bacteria to develop to high-level quinolone resistance. This occurs by chromosomal mutations in the quinolone resistance-determining regions (QRDRs) of genes encoding target enzymes (Strahilevitz et al., 2009). The situation is especially troubling when ESBL genes and PMQR genes are co-transmitted through transferable plasmids.

Indiscriminate use of antibiotics in both human and veterinary medicine make the ESBLs especially the CTX-M-producing bacteria including *Salmonella* increase rapidly accelerating the spread of antibiotic resistance. There have been several reports regarding characteristics of CTX-M-producing *Salmonella* isolated from food-producing animals in China. However, data on distribution of serotypes and antimicrobial susceptibility of *Salmonella* recovered from animals are available in most of these reports (Yan et al., 2010; Yang et al., 2010; Li R. et al., 2013; Li et al., 2014; Lu et al., 2014; Kuang et al., 2015). The aim of the present study was to investigate the prevalence of antibiotic resistance, serotypes and ESBLs among *Salmonella* isolates from chickens and pigs in China. Plasmids that carried bla<sub>CTX-M</sub> genes were also further studied to characterize the mechanism of transfer and dissemination of β-lactamases.

## MATERIALS AND METHODS

### *Salmonella* Isolation, Identification, and Serotyping

In 2014, a total of 3850 non-repetitive fecal swabs were collected from healthy chickens and pigs in Guangdong, Shandong, Henan, Hubei provinces of China. Of these samples, 2090 samples were from chickens and 1760 samples were from pigs.

Cotton swabs of feces were inoculated into sterile selenite cystine broth for 24 h at 37°C, and then streaked onto chromogenic medium selective for *Salmonella* (CHROMagar Microbiology, France) and incubated for another 24 h at 37°C. One purple colony was selected from each plate and then confirmed using the API20E system (bioMérieux, Marcy l'Étoile, France) and a PCR assay targeting the *invA* gene (Rahn et al., 1992). *Salmonella* isolates were serotyped using slide agglutination with hyperimmune sera (S&A Company, Bangkok, Thailand) and the results were interpreted according to the Kauffmann–White scheme. All identified isolates were stored at -80°C in Luria–Bertani (LB) broth containing 30% glycerol.

### Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of *Salmonella* isolates were determined using the agar dilution method

following Clinical and Laboratory Standards Institution guidelines. Sixteen antimicrobials were tested: ampicillin (AMP), cefotaxime (CTX), cefoxitin (CXT), ceftiofur (CTF), ceftazidime (CAZ), ceftriaxone (CTR), nalidixic acid (NAL), ciprofloxacin (CIP), enrofloxacin (ENR), kanamycin (KAN), gentamycin (GEN), amikacin (AMK), tetracycline (TET), chloramphenicol (CHL), florfenicol (FFC) and olaquindox (OLA). The results were interpreted according to the standards described by CLSI (M100-S25) (Clinical and Laboratory Standards Institute [CLSI], 2015) (ampicillin, cefotaxime, ceftiofur, ceftriaxone, cefoxitin, ceftazidime, nalidixic acid, ciprofloxacin, kanamycin, amikacin) and VET01-A4/VET01-S2 (Clinical and Laboratory Standards Institute [CLSI], 2013) (gentamicin, enrofloxacin, tetracycline, chloramphenicol, florfenicol) and the DANMAP 98 (olaquindox). *Escherichia coli* ATCC 25922 was used as quality control.

## Detection of $\beta$ -Lactamase Genes and PMQR Genes

The *Salmonella* isolates harvested from Mueller Hinton (MH) agar plates supplied with 2 mg/L cefotaxime were subjected to screening for ESBL-genes using PCR as described previously (Jiang et al., 2012). The DNA sequences and deduced amino acid sequences were compared with the reported sequences from GenBank<sup>1</sup>. The presence of PMQR determinants [*qnrA*, *qnrB*, *qnrS*, *qnrD*, *qnrC*, *aac(6')-Ib-cr*, *qepA*, and *oqxAB*] was also analyzed using PCR primers and conditions described previously (Jiang et al., 2012). Mutations in QRDR of target genes (*gyrA* and *parC*) were further analyzed in both PMQR and CTX-M-positive isolates as described previously (O'Regan et al., 2009).

## Molecular Typing

Genetic relatedness of all *bla*<sub>CTX-M</sub>-positive isolates was analyzed by pulsed-field gel electrophoresis (PFGE) after *Xba*I-digested genomic DNA using a CHEF-MAPPER System (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (Jiang et al., 2014). The resulting PFGE patterns were compared using the Dice similarity coefficient of BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

## Conjugation and Transformation Experiments

Conjugation experiments were conducted in *bla*<sub>CTX-M</sub>-positive strains by liquid mating-out assay in LB-medium using a sodium azide-resistant *E. coli* J53 as the recipient. Transconjugants were selected on MacConkey agar containing cefotaxime (2 mg/L) and sodium azide (300 mg/L). For transformation experiments, plasmid DNA from the *bla*<sub>CTX-M</sub>-positive strains were extracted using Qiagen Plasmid Midi Kit according to the manufacturer's instructions (Qiagen, Germany). Purified plasmids were transformed into *E. coli* DH5a (TaKaRa Biotechnology, Dalian, China). Selection of transformants was performed on MacConkey agar containing 2 mg/L cefotaxime. MICs and the

presence of *bla*<sub>CTX-M</sub> gene of transconjugants and transformants were determined as described above.

## Plasmid Characterization

PCR-based replicon typing (PBRT) was performed on transconjugants and transformants using primers as described previously (Carattoli et al., 2005). Then PFGE with S1 nuclease (Takara Biotechnology, Dalian, China) digestion of whole genomic DNA was carried out as described previously (Barton et al., 1995). The resulting gels were analyzed by Southern transfer and probing with a DIG-labeled *bla*<sub>CTX-M</sub> gene fragment according to the manufacturer's instructions (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Applied Science, Mannheim, Germany).

## RESULTS

### *Salmonella* Isolation, Identification, and Serotyping

A total of 159 *Salmonella* strains were obtained among which 90 (56.6%) were isolated from pigs and 69 (43.4%) were recovered from chickens. In all, three serotypes were identified in the 159 *Salmonella* isolates and this accounted for 95 strains of *S. enterica* serovar Typhimurium, 63 strains of *S. enterica* serovar Indiana, and 1 strain of *S. enterica* serovar Enteritidis.

The *S. enterica* serovar Typhimurium isolates were distributed as follows: 73.7% from pigs and 26.3% from chickens while the Indiana isolates were almost evenly distributed (50.8% from chickens and 49.2% from pigs, relatively).

### Antimicrobial Susceptibility Phenotypes

Among the 159 *Salmonella* isolates, resistance was most frequently observed to tetracycline (89.9%), ampicillin (84.9%), and gentamicin (81.1%). Resistance rates of the quinolone antibiotics were all above 50% including resistance to nalidixic acid (77.3%), ciprofloxacin (64.8%), and enrofloxacin (62.9%). Cephalosporin resistance rates ranged from 17.6 to 76.7% with high resistance rates to ceftriaxone (76.7%), ceftiofur (52.8%), and cefotaxime (47.2%). Resistance rates to ceftazidime and cefoxitin were 30.8 and 17.6%, respectively. The presence of resistance to olaquindox, a growth promoter used extensively in pigs and recently prohibited for poultry use, was detected in 65.4% of all isolates (Table 1).

All the 159 *Salmonella* isolates were multi-drug resistant displaying resistance to three or more classes of antimicrobials. Higher resistance frequencies were found among *S. enterica* serovar Indiana isolates compared with *S. enterica* serovar Typhimurium isolates. For example, cefotaxime resistance in Indiana and Typhimurium isolates were 71.4 and 30.5%, respectively, while the ciprofloxacin resistance rates were 73.0 and 43.2%, respectively (Table 2).

## ESBL Characterization

Seventy-five isolates displayed non-wild type MICs for cefotaxime and 45 of these carried *bla*<sub>CTX-M</sub> genes, three

<sup>1</sup>[www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)

**TABLE 1 |** Antimicrobial resistance of *Salmonella enterica* isolates from food-producing animals in China.

Antimicrobial agents	Number(%) of resistant isolates (n = 159)
<b>β-Lactams</b>	
Ampicillin	135 (84.9)
Cefotaxime	75 (47.2)
Cefoxitin	28 (17.6)
Ceftiofur	84 (52.8)
Ceftazidime	49 (30.8)
Ceftriaxone	122 (76.7)
<b>Quinolones</b>	
Nalidixic acid	123 (77.4)
Ciprofloxacin	103 (64.8)
Enrofloxacin	100 (62.9)
Aminoglycosides	
Kanamycin	124 (78.0)
Gentamicin	129 (81.1)
Amikacin	9 (5.7)
<b>Other Antibiotics</b>	
Tetracycline	143 (89.9)
Chloramphenicol	126 (79.2)
Florfenicol	124 (78.0)
Olaquindox	104 (65.4)

harbored *bla*<sub>CMY-2</sub> one of which coexisted with the CTX-M-encoding gene. For other β-lactamase genes, narrow-spectrum β-lactamase *bla*<sub>TEM-1</sub> was present in 86 of the 159 isolates, *bla*<sub>SHV-1</sub> and *bla*<sub>OXA-1</sub> genes were found in 62 and 55 of these isolates, respectively. No other type of ESBLs gene was found.

For the PMQR genes, three classes of resistance mechanisms were represented: *oqxAB* (78.0%, n = 124); *aac(6')-Ib-cr*(69.2%, n = 110) and *qnr*(17.6%, n = 28). For 28 *qnr* genes, 12 were *qnrD*, 7 were *qnrB*, 5 were *qnrS*, and the remaining 4 were *qnrA*. All *qnr*-positive strains also carried *oqxAB* and/or *aac(6')-Ib-cr*. At least one PMQR gene was found in each CTX-M-producing strains, especially the *aac(6')-Ib-cr +oqxAB* and *qnr+aac(6')-Ib-cr +oqxAB* were found in 17 and 4 of all 45 CTX-M-producing strains, respectively. Mutations in the QRDRs of *gyrA* and *parC* were analyzed among the 45 PMQR and CTX-M-positive isolates. A combination of mutations in *gyrA*83 (S83F) and 87 (D87G) and in *parC* 80(S80R) were found in 35 (77.8%) isolates, a signal mutation in *gyrA* 87 (D87N) without mutation in *parC* was detected in the remaining 10 isolates (Table 3).

A total of three different CTX-M ESBLs were detected in these isolates and they all belonged to the CTX-M-9 group with the CTX-M-encoding genes distributed as 34 *bla*<sub>CTX-M-27</sub>, 7 *bla*<sub>CTX-M-65</sub>, and 4 *bla*<sub>CTX-M-14</sub>. The distribution of these three CTX-M subtypes showed no specificity between serotypes and animals and were found in both Indiana and Typhimurium isolates from pigs and chickens. Antimicrobial susceptibility testing demonstrated that CTX-M-27-positive isolates had significantly higher resistance levels than the CTX-M-14 or CTX-M-65 -positive isolates.

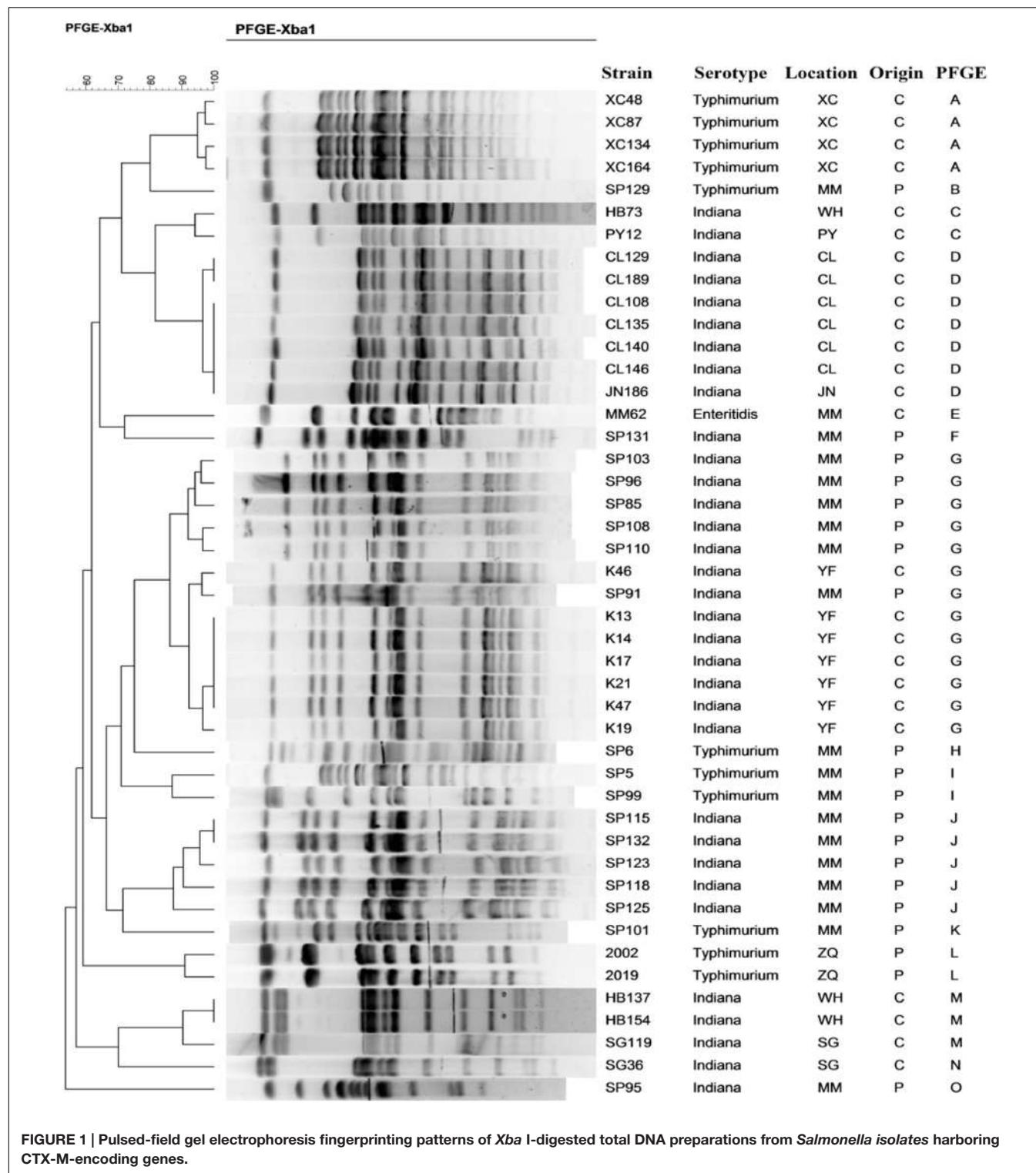
**TABLE 2 |** Antibiotic resistance rate of *Salmonella enterica* serotype Typhimurium and Indiana isolates (%).

Serotype \ Antibiotics	AMP	CTF	CTX	CTZ	CTR	GEN	KAN	AMI	CIP	ENR	NAL	TET	CHL	FFC	OQX
Indiana (63)	93.7	76.2	71.4	19.0	33.3	82.5	88.9	84.1	6.3	73.0	77.8	87.3	96.8	90.5	84.1
Typhimurium (95)	89.5	37.9	30.5	11.6	29.5	73.7	76.8	74.7	5.3	60.0	52.6	70.5	86.3	72.6	64.2

**TABLE 3 | Characteristics of CTX-M-producing *Salmonella enterica* isolates from food-producing animals in China.**

Strain	Source	Serotype	ESBL gene	PMQR	Amino acid substitutions		Co-transferred resistant gene	Plasmid replicon type	Plasmid approx. size(kb)
					<i>gyrA</i>	<i>parC</i>			
SP5	Pig	Typhimurium	<i>bla</i> <sub>CTX-M-14</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	D87N	—	<i>bla</i> <sub>SHV-1</sub> , <i>aac</i> -(6')- <i>lb-cr</i>	FIB	100
SP85	Pig	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>OXA-1</sub> , <i>aac</i> -(6')- <i>lb-cr</i>	NT	100
SP96	Pig	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub> , <i>oqxAB</i>	P	150
SP99	Pig	Typhimurium	<i>bla</i> <sub>CTX-M-27</sub>	<i>qnrB, aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr</i> <i>oqxAB</i>	NT	100
SP101	Pig	Typhimurium	<i>bla</i> <sub>CTX-M-65</sub>	<i>oqxAB</i>	D87N	—	<i>oqxAB</i>	FIB	150
SP108	Pig	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub> , <i>aac</i> -(6')- <i>lb-cr</i>	A/C	150
<u>SP110</u>	Pig	Indiana	<i>bla</i> <sub>CTX-M-65</sub>	<i>oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub>	N	100
<u>SP115</u>	Pig	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr</i>	NT	100
SP123	Pig	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	D87N	—	—	FIB	150
SP125	Pig	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	D87N	—	—	FIB	150
SP129	Pig	Typhimurium	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub> , <i>aac</i> -(6')- <i>lb-cr</i>	P	80
SP131	Pig	Indiana	<i>bla</i> <sub>CTX-M-65</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub> , <i>aac</i> -(6')- <i>lb-cr</i>	N	150
SP132	Pig	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub> , <i>oqxAB</i>	N	150
<u>SP2019</u>	Pig	Typhimurium	<i>bla</i> <sub>CTX-M-14</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	D87N	—	<i>bla</i> <sub>OXA-1</sub> , <i>oqxAB</i>	HII2	280
<u>CL108</u>	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	—	—	NT	100
CL129	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub> , <i>aac</i> -(6')- <i>lb-cr</i>	FIB	150
CL135	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	S80R	—	NT	100
CL140	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr</i>	S83F,D87G	S80R	—	NT	100
CL146	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>qnrD, aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub> , <i>aac</i> -(6')- <i>lb-cr</i>	NT	100
CL189	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	S80R	<i>oqxAB</i>	HII2	200
XC48	Chicken	Typhimurium	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr</i>	N	150
XC87	Chicken	Typhimurium	<i>bla</i> <sub>CTX-M-65</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr</i> <i>oqxAB</i>	N,	150
XC134	Chicken	Typhimurium	<i>bla</i> <sub>CTX-M-14</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>oqxAB</i>	N,A/C	150
XC164	Chicken	Typhimurium	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>TEM-1b, oqxAB</sub>	HII2	194
HB73	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	S80R	—	A/C	130
HB137	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>oqxAB</i>	NT	100
HB154	Chicken	Indiana	<i>bla</i> <sub>CTX-M-65</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr</i> <i>oqxAB</i>	N,	130
SG36	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	S80R	<i>bla</i> <sub>SHV-1</sub>	NT	100
SG119	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr</i>	S83F,D87G	S80R	—	NT	100
MM62	Chicken	Enteritidis	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr</i>	D87N	—	<i>aac</i> -(6')- <i>lb-cr</i>	P	80
PY12	Chicken	Indiana	<i>bla</i> <sub>CTX-M-14</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	D87N	—	<i>bla</i> <sub>SHV-1</sub>	N,A/C	130
K13	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	—	NT	100
<u>K14</u>	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>oqxAB</i>	S83F,D87G	S80R	—	NT	100
K17	Chicken	Typhimurium	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr</i>	NT	100
<u>K19</u>	Chicken	Indiana	<i>bla</i> <sub>CTX-M-65</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	N	150
K21	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr</i>	D87N	—	<i>aac</i> -(6')- <i>lb-cr</i>	FIB	150
K46	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr, oqxAB</i>	P	100
K47	Chicken	Indiana	<i>bla</i> <sub>CTX-M-27</sub>	<i>aac</i> -(6')- <i>lb-cr</i>	S83F,D87G	S80R	<i>aac</i> -(6')- <i>lb-cr</i>	NT	100

NT, non-typeable incompatibility (Inc) group plasmid. The underline isolates were transconjugants, the others were transformants.



**FIGURE 1 |** Pulsed-field gel electrophoresis fingerprinting patterns of *Xba*I-digested total DNA preparations from *Salmonella* isolates harboring CTX-M-encoding genes.

## Plasmid Characterization

Six transconjugants and 32 transformants were successfully obtained from 45 *bla*<sub>CTX-M</sub>-positive isolates by conjugation/transformation experiments with frequencies of 10<sup>-8</sup> to 10<sup>-3</sup> per donor cell. PMQR genes were found to

be co-transferred with *bla*<sub>CTX-M</sub> genes in 26 (68.4%) transconjugants/transformants and the most common co-transferring pattern was *bla*<sub>CTX-M</sub> + *aac*(6')-Ib-cr accounting for 13 of 26 transconjugants/transformants, *bla*<sub>CTX-M</sub> + *oqxAB* and *bla*<sub>CTX-M</sub> + *aac*(6')-Ib-cr + *oqxAB* accounting for eight and

five transconjugants/transformants, respectively. For narrow-spectrum  $\beta$ -lactamases, *bla<sub>SHV-1</sub>*, *bla<sub>OXA-1</sub>*, and *bla<sub>Tem-1b</sub>* were co-transferred with *bla<sub>CTX-M</sub>* in 11, 2, and 1 isolates, respectively.

Plasmid replicon typing of transconjugants/transformants revealed that IncN, IncFIB, IncP, IncA/C, and IncHI2 were detected in 9, 6, 4, 4, and 3 transconjugants/transformants, respectively. Two plasmid replicons (IncN in combination with IncA/C) were simultaneously present in 2 CTX-M-14-producing transformants. Surprisingly, the replicon types could not be determined by PBRT in 14 transconjugants/transformants all of which carried the *bla<sub>CTX-M-27</sub>* gene (**Table 3**).

Southern blot hybridization suggested that the plasmid sizes of *bla<sub>CTX-M</sub>* genes varied between 80 and 280 kb among which the 100 kb ( $n = 17$ ) and 150 kb ( $n = 13$ ) were the most common sizes. Interestingly, the size of all the 14 non-typeable *bla<sub>CTX-M-27</sub>* plasmids was  $\sim$ 100 kb.

### Pulsed-Field Gel Electrophoresis (PFGE)

A total of 34 different PFGE profiles were obtained from the 45 *bla<sub>CTX-M</sub>*-positive isolates (**Figure 1**). These could be grouped into 15 PFGE clusters designated A–O having 85% genetic similarity. Clusters G, D, J, and A accounted for 28.9, 15.6, 11.1, and 8.9% of the isolates, respectively. In addition, all isolates in each cluster had the same serotype and were also isolated from the same province (same or different cities). The exceptions were clusters C and M both of which were comprised of two isolates each from different provinces (Hubei, Henan, or Shandong provinces).

In the most predominant cluster G, a high similarity of PFGE profiles of the isolates could be found and the isolates were recovered from pigs or chickens in two cities of Guangdong province. Seven isolates in this cluster were recovered from chickens and six were from pigs. In the remaining 14 PFGE clusters, isolates were all recovered from the same origin; either chickens or pigs.

## DISCUSSION

In this study, we examined *Salmonella* isolates recovered from food-producing animals to determine their serotypes, antimicrobial susceptibility phenotypes, and genotypes. The results showed that *S. enterica* serovars Typhimurium and Indiana were the prevailing serotypes of *Salmonella* in pigs and chickens. Both serovars were resistant to multiple antimicrobials including ESCs and fluoroquinolones which are often antimicrobials of choice for treating salmonellosis. Resistance to cefotaxime could be explained by the presence of non-typeable and IncN/IncFIB/IncP/IncA/C/IncHI2 plasmids carrying *bla<sub>CTX-M-9G</sub>* genes. The high detection rate of PMQR determinants especially *aac(6')-Ib-cr* and *oqxAB* and mutations in QRDR of target genes made an important contribution to the non-wild type MICs of the fluoroquinolones.

*Salmonella* has various serotypes, and some serotypes may also relate to multidrug-resistant phenotypes (Meunier et al., 2002). The predominant serotypes change over time and

differ from one geographical area to another. Additionally, a particular serovar can prevail and emerge within a region for a certain period while being completely absent in other regions. *S. enterica* serovar Indiana is rarely reported worldwide but there have been large-scale food poisoning outbreaks caused by this serotype in both the USA and Europe (Price and Carter, 1967; Campbell and Eckman, 1975). In China, little information about *S. enterica* serovar Indiana was available until this serotype was obtained from a food animal (Yan et al., 2010) and showed resistance to multiple antimicrobial agents. Following this, isolation of *S. enterica* serovar Indiana increased rapidly and it became more and more prevalent in China especially in isolates from veterinary clinics and animal foods. The characteristics of multidrug-resistant Indiana in China were also reported (Xia et al., 2009; Yang et al., 2010; Lu et al., 2014). In the present study, all of the 159 *Salmonella* isolates displayed resistance to three or more classes of antimicrobials, but serovar Indiana isolates showed higher resistance levels than serovar Typhimurium isolates. Our results indicate that the multi-resistant serotype Indiana along with Typhimurium isolates are of high prevalence among food-producing animals in China.

CTX-M type ESBLs have been widely reported globally for many years and there is a strong linkage between their emergence and increasing quinolone resistance in the Enterobacteriaceae (Lavilla et al., 2008). The co-existence or co-transfer of PMQR genes in ESBLs and especially in CTX-M-producing isolates has been also previously described (Liu et al., 2013; Jiang et al., 2014; Li et al., 2014; Yang et al., 2015). These may promote the development of multidrug-resistant isolates under selective pressure of the quinolone and/or cephalosporin (Liu et al., 2013). We found all CTX-M-encoding strains contained at least one PMQR genes especially the *aac(6')-Ib-cr* and *oqxAB* genes. These two PMQR genes have been shown to accelerate the development of fluoroquinolone resistance in *S. typhimurium* (Wong et al., 2014a) and were frequently co-transferred with *bla<sub>CTX-M</sub>* genes. Previous reports showed that *aac(6')-Ib-cr* and *oqxAB* were also prevalent PMQR genes in *E. coli* isolates from humans, animals and the environment (Chen et al., 2012). In general, detection rates of PMQR genes in *Salmonella* were less than those observed in *E. coli*. However, these rates in *Salmonella* increased quickly in China especially *aac(6')-Ib-cr* and *oqxAB*. In the present study, 78.0 and 69.2% harbored *oqxAB* and *aac(6')-Ib-cr*, which is higher than levels previously reported in 2009–2010 (32.3%, 25.8%), 2007–2011 (30.8%, 30.8%) and in 2012–2013 (31.7%, 36.5%) in China (Li et al., 2013; Jiang et al., 2014; Li et al., 2014). Our results may indicate that ESBL genes along with PMQR genes are increasing among *Salmonella* strains in China.

The CTX-M type  $\beta$ -lactamases are the most prevalent ESBLs among the Enterobacteriaceae recovered from both animals and humans worldwide (D'Andrea et al., 2013). The spread and characteristics of CTX-M type ESBLs producing pathogenic bacteria had been widely studied previously in China, but works seldom focused on food-borne *Salmonella*. In the present study, 60% of cefotaxime-resistant isolates produced CTX-M type ESBLs and three different CTX-M-9 group variants were detected among which the CTX-M-27 enzyme was the most

dominant. Consistent with the results in this study, the CTX-M-27 enzyme was also the main type of ESBL in our previous study with *Salmonella* (Jiang et al., 2014) and we also previously found the CTX-M-27 was the only CTX-M-type ESBLs detected in *Salmonella* isolates of retail pork (unpublished data). However, only two reports were found about CTX-M-27-producing human clinical *Salmonella* strains. One study showed that CTX-M-27-producing isolates of *S. enterica* serotype Livingstone were the cause of a nosocomial outbreak in the neonatal ward, the other study showed *bla*<sub>CTX-M-27</sub> along with *oqxAB* on the IncHI2 plasmid which was similar to that of the strain CL189 in our study (Bouallegue-Godet et al., 2005; Wong et al., 2014b). Many reports suggested that CTX-M-14 and CTX-M-55 enzymes were the two most dominant types in *E. coli* obtained from food animals in China (Zheng et al., 2012; Rao et al., 2014). CTX-M-27 in *E. coli* was only sporadically detected in several reports (Liu et al., 2007; Lu et al., 2010; Ho et al., 2011; Ma et al., 2012; Zheng et al., 2012; Rao et al., 2014) among which two studies found its high presence in *E. coli* isolated from duck and environmental samples and aquatic sediment samples (Lu et al., 2010; Ma et al., 2012). Study on isolates from human beings and environmental samples suggested that most CTX-M-27-positive *E. coli* from different hospitals and from rivers and lakes belonged to the O25b-ST131-B2 or B2:ST131 which was strongly associated with potentially severe infections in both humans and animals (Matsumura et al., 2012; Zurfluh et al., 2013; Micenková et al., 2014). The high prevalence of *bla*<sub>CTX-M-27</sub> in *Salmonella* obtained from food animals and in *E. coli* isolates from different sources (human beings, environment, and animals) should be a cause for concern which may be a potential threat to public health.

CTX-M-27 which was first found in a clinical isolate from a French hospital differed from its ancestor-CTX-M-14 only by the substitution D240G, but had significant higher hydrolytic activity against ceftazidime (Bonnet et al., 2003). Previous studies (Rao et al., 2014) suggested that CTX-M-1G-positive isolates had significantly higher resistance rates to cefquinome, ceftazidime, amikacin and fosfomycin when compared to the CTX-M-9G-positive isolates. These authors also compared the activity of different CTX-M variants on cephalosporin resistance and proved that CTX-M-1 group enzymes, especially CTX-M-55, have higher hydrolytic activities against cefquinome than CTX-M-9 group enzymes (except CTX-M-27). All the *bla*<sub>CTX-M</sub> genes detected in this study belonged to CTX-M-9 group. When we analyzed the MICs of cephalosporin among different CTX-M-9G variants, we concluded that *bla*<sub>CTX-M-27</sub>-positive isolates had a higher MIC for cephalosporin in particular to ceftazidime than that in CTX-M-14 and CTX-M-65-producing isolates. There were few differences in the resistance phenotypes of different variants of the CTX-M-9G family of  $\beta$ -lactamases isolates. This may also explain the prevalence of different variants.

In this study, 38 *bla*<sub>CTX-M</sub>-positive transconjugants/transformants were obtained, among which 68.4% co-transferred *bla*<sub>CTX-M</sub> with *aac(6')-Ib-cr* and/or *oqxAB*, indicating that these fluoroquinolone resistant determinants can be transferred horizontally simultaneous with the transfer of CTX-M-encoding genes. Characterization of *bla*<sub>CTX-M</sub>

plasmids in the transconjugants/transformants revealed that *bla*<sub>CTX-M</sub> genes were located on plasmids of un-typeable and IncN/IncFIB/IncP/IncA/C/IncHI2 replicon types with different plasmid sizes. In particular, we observed that plasmids carrying *bla*<sub>CTX-M-27</sub> from epidemiologically unrelated strains (from different geographic regions and sources) had surprisingly similar properties (replicon types and sizes) indicating the presence of epidemic *bla*<sub>CTX-M-27</sub> plasmids in China. It's worth noting that the replicon types were untypeable using the current PBRT scheme in half (14/28) of the transconjugants/transformants carrying *bla*<sub>CTX-M-27</sub>. Previous studies on *bla*<sub>CTX-M-27</sub> also found some plasmids could not be determined by PBRT (Ma et al., 2012). Therefore, there may be a new type of plasmid-carrying *bla*<sub>CTX-M-27</sub>. We have sequenced two *bla*<sub>CTX-M-27</sub> plasmids from different sources to further study their characteristics (unpublished data).

Among the five detected plasmid replicons in present study, the IncP type plasmid carrying *bla*<sub>CTX-M</sub> in *Salmonella* isolates was reported for the first time. In general, most *bla*<sub>CTX-M</sub> genes can be horizontally transferred. Untypeable plasmids and IncN/IncFIB/IncP/IncA/C/IncHI2 plasmids can drive such increased dissemination in *Salmonella* isolates from food animals. To the best of our knowledge, the current study is also the first report about the spread of non-typeable or IncN/IncFIB/IncP/IncA/C/IncHI2 plasmids carrying *bla*<sub>CTX-M</sub> genes in *S. enterica* serovars Typhimurium and Indiana isolated from pigs and chickens in China.

PFGE is a gold-standard for *Salmonella* subtyping and has been widely used to determine the relatedness of pathogens to confirm the outbreaks and trace the source of the isolates (White et al., 2007). Fifteen different PFGE clusters (85% genetic similarity) were obtained in this study and these represented a wide variety of genotypes. However, isolates recovered from the same or even different provinces in each identified serovars were highly genetically similar (cluster A, D, G, J and cluster C, M). These may have derived from a specific clone, so the horizontal transmission of the resistance plasmids along with clonal spread of resistant strains were both responsible for dissemination of *bla*<sub>CTX-M-9G</sub>-positive strains.

All of the 45 *bla*<sub>CTX-M-9G</sub>-positive *Salmonella* strains were isolated from food animals from different provinces. Therefore, humans can be infected through the consumption of food of animal origin (Thorns, 2000), accelerating the transmission of such *Salmonella* strains. Along with this, food animals trading may be an important source of *Salmonella* colonization as has been previously assumed (Wegener et al., 2003). Though studies proving definitive evidence of ESC-R bacterial transmission from food animals to humans are limited; differences can always be shown when comparing *Salmonella* isolates from humans and the animals. Importantly, previous studies have shown that the same IncN plasmids harboring *bla*<sub>CTX-M</sub> are simultaneously present in isolates from pigs and farm workers (Moodley and Guardabassi, 2009). Therefore, further study monitoring and comparing antimicrobial susceptibility, serotypes and ESBL-producing *Salmonella* isolates from both veterinary settings (pets, wild animals, and livestock) and humans (communities and

hospitals) in the same geographic region could lead to the discovery of dissemination routes.

## CONCLUSION

Multi-drug resistant *S. enterica* serotype Typhimurium and Indiana and especially CTX-M-producing isolates were predominant among food-producing animals in China. Untypeable and IncN/IncFIB/IncP/IncA/C/IncHI2 resistant plasmids harboring *bla*<sub>CTX-M</sub> genes and clonal spread of strains were both responsible for dissemination of resistant *Salmonella* isolates. The presence and spread of *bla*<sub>CTX-M</sub>, especially the *bla*<sub>CTX-M-27</sub> on non-typeable plasmids in *Salmonella* isolates from food-producing animals may pose a potential threat for public health. Proper molecular approaches and hygienic practices are urgently needed to control the dissemination of resistant *Salmonella* strains through food chain. As ceftiofur and cefquinome have been widely used as therapeutic antibiotic

in veterinary medicine, the third- and fourth-generation cephalosporins should be used more prudently in animal husbandry in China.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Z-LZ, H-XJ, and Y-HL. Performed the experiments: W-HZ, X-YL, LX, and X-XG. Analyzed the data: W-HZ and X-YL. Contributed reagents/materials/analysis tools: LY, WL, and S-QR. Wrote the paper: W-HZ and H-XJ.

## ACKNOWLEDGMENT

This work was supported in-part by the National Natural Science Foundation of China (31272602) and 973 Program (2013CB127203).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Development of Cefotaxime Impregnated Chitosan as Nano-antibiotics: *De Novo* Strategy to Combat Biofilm Forming Multi-drug Resistant Pathogens

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 02 December 2015

Accepted: 02 March 2016

Published: 18 March 2016

### Citation:

Jamil B, Habib H, Abbasi SA, Ihsan A, Nasir H and Imran M (2016) Development of Cefotaxime Impregnated Chitosan as Nano-antibiotics: *De Novo* Strategy to Combat Biofilm Forming Multi-drug Resistant Pathogens. *Front. Microbiol.* 7:330. doi: 10.3389/fmicb.2016.00330

Frequent incidents of antibiotic-resistant biofilm forming pathogens in community-associated and hospital-acquired infections have become a global concern owing to failure of conventional therapies. Nano-antibiotics (NABs) are *de novo* tools to overcome the multi-drug resistant mechanisms employed by the superbugs. Inhibition of biofilm formation is one of those strategies to curb multi drug resistance phenomenon. In the current study, the anti-biofilm and antibacterial potential of newly synthesized cefotaxime loaded chitosan based NABs have been investigated. Both bare and cefotaxime loaded NABs were prepared by ionotropic gelation method. They were found carrying positive zeta potential of more than +50 mV, indicating highly stable nano-dispersion. Moreover, microscopic studies revealed their size as less than 100 nm. NABs were tested against clinical isolates of multi drug resistant *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and methicillin resistant *Staphylococcus aureus* and wherein they demonstrated broad-spectrum anti-biofilm and anti-pathogenic activity. Thus, *in vitro* synergistic action of cephalosporin drugs and chitosan polymer at nano-scale in contrast to free antibiotics can be an improved broad-spectrum strategy to thwart resistance mechanisms in both Gram-positive and Gram-negative resistant pathogens.

**Keywords:** biofilm, chitosan nano-carriers, cephalosporins, drug resistance, growth kinetics, zeta potential

## INTRODUCTION

Though, it is a mutually agreeable fact that bacteria has no definite nervous system but it is also conceded that they evolve certain survival mechanisms that allow them to exist in any environment. Biofilm formation is one of such survival mechanisms. Most of the bacterial species prefer to live in a well-developed community (biofilm) rather than planktonic form (isolated individual cells; Dheilly et al., 2010; Sayem et al., 2011). Along with numerous other benefits, biofilms also provide the basic mechanism of resistance to antibiotics, antibodies, bacteriophages, disinfectants

and other host defense systems (Dheilly et al., 2010) by constituting a multi-layered protection mechanisms (Stewart, 2002). Extracellular polymeric substance (EPS) covering the biofilms make them impermeable to many antibiotics. Likewise, in biofilms microbes exhibit slow growth rate which further makes them resistant to therapeutic agents. Furthermore, biofilm forming microbes develop adaptive stress responses which collectively evince another defense mechanism. The formation of persister cells are further addition to this menace and is the main cause of recurrent and chronic infections (Lewis, 2005). Biofilms also facilitate the horizontal or lateral gene transfer (Madsen et al., 2012) which is considered a significant feature in the evolutionary process of acquiring antibiotic resistance genes between species. Biofilms also provide a wide variety of genetic elements (plasmids) to be transferred among unrelated bacterial species including genes that promote biofilm formation and responsible for resistance (Donlan, 2002).

Extracellular DNA (eDNA) which is formed by the autolysis of a microbial subpopulation also makes an integral part of microbial biofilms and plays a foremost role in the stability of the biofilms (Watnick and Kolter, 2000). These biofilms accumulate large quantity of antibiotic degrading enzymes too (Delcour, 2009). In addition, biofilm formation is associated with the virulence of pathogenic bacteria, and cells included within a biofilm are generally 1000 times more resistant. National Institutes of Health (NIH) has estimated that 70% of all microbial infections in the world are associated with biofilms (Perumal and Mahmud, 2013). It is, therefore, a major concern not only in health care systems but in certain other domains as well. It has also been a major source of contamination in the food industry, water supply pipes, medical implants, catheters and equipment etc (Dheilly et al., 2010; Perumal and Mahmud, 2013).

The effectiveness of many antimicrobial agents is currently decreasing owing to increasing prevalence of multidrug-resistant (MDR) pathogens (Fabbretti et al., 2011). The emergence of these MDR pathogens remains a serious challenge to medicine and healthcare systems (Carlet et al., 2012). One of the mechanisms for such resistance is the formation of biofilms. If antibiotics cannot traverse the biofilm they fail to eradicate other defense strategies. Therefore, it is important to search for alternative therapeutics to control biofilm-associated MDR infections (Perumal and Mahmud, 2013; Kirker et al., 2015).

The development of anti-biofilm and anti-MDR strategies is therefore a major area of interest and currently constitutes an important field of investigation. Albeit, various naturally occurring compounds (including plant extracts) have been investigated in this regard (Salta et al., 2013). However, nano-antimicrobials (NAMs) are offering more promising future to beat MDR phenomenon (Allaker and Memarzadeh, 2014; Jamil et al., 2015). NAMs can also overcome resistance caused by biofilm formation and can prevent biofilm's further growth as well (Jamil et al., 2015). Recently, various NAMs have displayed their anti-biofilm potential to combat resistance mechanisms. Liposomes help thrashing biofilms by promoting adsorption at the outer surfaces of biofilms (Smith, 2005). Besides, silica nanoparticles (NPs) act by releasing large amount of Nitric oxide (NO; Hetrick et al., 2009). Analogously, ZnO, TiO<sub>2</sub>, MgF<sub>2</sub> NPs and

super paramagnetic iron oxide NPs (SPIONs) have also inhibited biofilm formation (Pelgrift and Friedman, 2013). The rationale behind successful eradication of biofilms lies in strong interaction between biofilms and NAMs. The majority of NAMs bear positive charge that attaches firmly to biofilm matrixes carrying negative charge (Hajipour et al., 2012). However, due to metallic NPs associated toxicity issues, nano-medicine research focus has been shifted toward bio-based NPs or nano-carrier systems (NCS). In general, NCS protects the drug from both endogenous and exogenous factors and provide a sustained release and more enhanced bioactivity.

Cefotaxime is a third generation broad spectrum cephalosporins for parenteral administration with a short half-life of 0.8–1.4 h, it is bactericidal and mainly used in the treatment of infections caused by Gram-positive and Gram-negative microorganisms. Very recent and frequent emergence of extended spectrum beta lactamases (ESBL) and metallo beta lactamases (MBL) are reducing the susceptibility of all cephalosporins including cefotaxime. Therefore, present investigation has been carried out in an attempt to enhance the bactericidal activity of this  $\beta$ -lactam antibiotic synergistically with antimicrobial bio polymer chitosan to render it more effective against biofilm producing MDR pathogens.

## MATERIALS AND METHODS

Chitosan-medium molecular weight was purchased from Sigma-Aldrich (Product number 448877, 75–85% deacetylation, 200–800 cP viscosity of 1% w/v in 1% v/v acetic acid). Pentasodium triphosphate (TPP) and glacial acetic acid were also procured from Sigma-Aldrich. All antibiotic disks were obtained from Oxoid. Nutrient agar and nutrient broth were purchased from Oxoid. Cefotaxime sodium for injection (0.5 g) was procured from Sanofi Aventis Pakistan Limited. Standard stock solution of cefotaxime sodium was prepared by dissolving 1 mg/mL in sterilized water.

## Culture Collection

The clinical isolates were obtained from Al-Sayed Hospital (Pvt) Ltd, Rawalpindi and stored at –80°C in nutrient broth containing 20% glycerol. Their identities were confirmed by biochemical test using API (analytical profile index) kits. For the purpose of this study, we had collected pathogenic bacteria which were capable of biofilm formation and were documented as causative agents for severe infections. Clinically resistant pathogens used in this study includes *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa*, *Escherichia coli* (*E. coli*), and Methicillin resistant *Staphylococcus aureus* (MRSA). We could not get clinical isolates of *Listeria monocytogenes*, so *L. monocytogenes* ATCC 13932 had been employed for this study.

Inoculum was prepared by the method described in literature with slight modifications. All the bacterial strains were recovered on a fresh nutrient agar (Oxoid) plate 24 h prior to antimicrobial test. To prepare the inoculum, colonies from fresh agar were transferred into sterile Mueller Hinton (MH) liquid growth medium and incubated at 37°C overnight. The optical density

was adjusted to 0.1 at 600 nm wavelength (Perumal and Mahmud, 2013).

## Resistance Spectra by Disk Diffusion Method

Selected microbes were subjected to antibiotic disks by standard Kirby Bauer method and their resistance patterns were studied and compared to CLSI guidelines. Following antibiotic disks were used including Ceftazidime (CAZ), Cefotaxime (CTX), Imipenem (IPM), Cefepime (FEP), Ceftriaxone (CRO), Aztreonam (ATM), Ampicillin (AMP), Vancomycin (VA), Augmentin (AMC), Colistin (CT), Cefoxitin (FOX), and Minocycline (MH). All selected bacterial cultures were marked as resistant or susceptible based upon their zones of inhibition.

## Quantification of Antibiotics by Nanophotometer

UV-Vis spectrophotometer (Nanophotometer Implen) was used for this purpose (El-Shaboury et al., 2007; Bushra et al., 2014). Volumetric dilutions of cefotaxime were prepared in triplicate from stock solution.  $\lambda_{max}$  was obtained after the wave scan for 200–900 nm range. Calibration curve was constructed with the average absorbance of three replicates. The trend line was obtained by linear regression with standard equation and R squared value.

## Chitosan Nanoparticles (CSNPs)

### Fabrication

Ionotropic gelation method was used to fabricate Chitosan Nanoparticles (CSNPs; Du et al., 2009; Jamil et al., 2016). Ionotropic gelation is based on the ability of poly electrolytes to cross link in the presence of counter-ions to form hydrogel beads also called as gelispheres (Patil et al., 2012). These gelispheres have the ability to control the release of drug. Cefotaxime solution was prepared in tripolyphosphate (TPP) and it was then added dropwise in chitosan (CS) solution with constant stirring. The TPP diffuses slowly into the CS solution forming a three dimensional lattice of ionically crosslinked moiety that has entrapped drug in it (Patil et al., 2012). It was then subjected to sonication for another 30 min by using Sonozap ultrasonic homogenizer 25 kHz. These NPs were then centrifuged at 12000 g for 10 min.

## Nanoparticles Characterizations

The detection and characterization of nanoparticles entail particular challenges. As particle size is a key criterion in nanotechnology, maintaining particle size both *in vitro* and *in vivo* is crucial. Stability of NPs is determined mainly by their size maintenance. Flocculation and aggregation due to van der Waals forces should be avoided as the behavior of NPs is remarkably different from the bulk material of the same matter. Bulk material does not change its physical properties as the size of matter changes, while the NPs display size dependent properties. As a general rule, extent of size reduction is directly proportional to the surface area. Increased surface area contributes toward greater activity.

## Scanning Electron Microscopy (SEM)

Scanning electron microscopy uses electrons instead of light to form an image and is one of the most widely used techniques for the characterization of nanostructures. Jeol JSM 6490A analytical scanning electron microscope was used for this purpose. Sample was prepared by placing a small drop of formulation on glass slide. Gold coating by Jeol Quick Auto Coater (JFC-1500) ion sputtering device was done for 6 s only. Analyses of both empty and drug loaded nanoparticles were carried out at the resolution of 20 kV and 3000–50000 $\times$  magnification by the procedure already published (Jamil et al., 2016).

## Atomic Force Microscopy (AFM)

SEM gives a 2D image while AFM can provide a 3D topographic picture of prepared nano-systems. Agilent Pico Plus was used for this purpose. The AFM probe has a very sharp tip, often less than 100 Å diameter, at the end of a small cantilever beam. The probe is attached to a piezoelectric scanner tube. Inter-atomic forces between the probe tip and the sample surface cause the cantilever to deflect as the sample's surface topography or other properties change. A laser light reflected from the back of the cantilever measures the deflection of the cantilever. Based on the type of application, different operation modes of AFM are used like the contact mode, semi contact mode and tapping mode (Suresh, 2015). However, for this study, all the images were taken in tapping mode at ambient conditions.

## Fourier Transform Infrared Spectroscopy (FTIR) Spectra

Fourier Transform Infrared Spectroscopy was done for qualitative analysis of prepared formulations. Infrared spectroscopy is associated with vibrational energy of atoms or group of atoms in a material. It gives peaks for each functional groups and also gives a clue about bonding and interactions. Perkin Elmer FTIR spectroscope was used to analyze dry sample after preparing their pallet with KBr (FTIR grade Merck) while liquids were analyzed directly. The spectral resolution was 4 cm<sup>-1</sup>, with 96 scans, and an aperture of 4 mm. The optimum beam incidence angle was 45° and all the spectra were acquired in a range between 4000 and 600 cm<sup>-1</sup>.

## Determination of Zeta Potential

Zeta potential was measured to get an indication about long term stability of nano-formulations. Zetasizer Nano ZS (Malvern Instruments, UK) using Doppler electrophoresis as the basic principle of operation was used in this study. The data collected were then imported to Excel and the means and standard deviations of the replicate measurements were calculated.

## Determination of Encapsulation Efficiency

The encapsulation efficiency (EE) of nanoparticles was calculated by the method earlier mentioned (Rotar et al., 2014). Drug loaded CSNPs were isolated from the free drug by centrifugation (12,000 g for 15 min, Eppendorf 5415D, Germany). Free drug in the supernatant was quantified by spectrophotometer at  $\lambda_{max}$

(298) obtained from wave scan. The concentration of drug in the supernatant was estimated by the equation obtained from standard curve. Experiments were performed in triplicate and the EE was calculated as follows:

$$\text{EE} = (\text{total drug} - \text{un encapsulated drug}) \div \text{total drug} \times 100$$

## Antimicrobial Potential of the Prepared Nano-antibiotics (NABs)

Antibacterial activity of blank and  $\beta$ -lactam antibiotic loaded CSNPs were evaluated according to standard liquid micro-dilution susceptibility assays. To this aim, selected microbes were grown in nutrient broth until the exponential growth phase at 37°C while shaking. Bacterial turbidity was compared to McFarland solution. Ten microliters of the bacterial suspension were added to 9 mL broth and similar concentration of antibiotic and drug loaded CSNP were added in inoculated broth. Test tubes were incubated in shaker incubator at 37°C overnight. Optical densities values were taken after every 24 h for 144 h at 595 nm on Elisa Multi-plate reader (Jamil et al., 2016). Both positive and negative controls were added in this assay. Negative control contains only the nutrient broth without culture and served as control of any possible contamination while the positive control contains nutrient broth and inoculum to serve as a control of cell-viability.

At the end of experiment colony forming unit (CFU) assay was done to confirm the results. Serially diluted bacterial suspensions were plated on Nutrient agar surface and incubated for 48 h at 37°C. Results were expressed as log<sub>10</sub> CFU/ml.

## Anti-Biofilm Potential of the Prepared Nano-antibiotics

To assess the anti-biofilm activity of cephalosporin loaded CSNPs against clinically important pathogens; MicroTiter Plate (MTP) assay was carried out using 96-well flat bottom polystyrene titer plates (Kirker et al., 2015).

Biofilms were formed on ELISA plate by the method of George with few modifications (George, 2011). Briefly, all selected pathogens were cultured on fresh Petri plate from stocks preserved at -80°C and from that pure media inoculum was prepared. Turbidity was compared to that of McFarland turbidity standard and 10  $\mu\text{L}$  inoculum was added in test tube containing 9 mL of freshly sterilized nutrient broth. Nano-antibiotic and simple antibiotic solution prepared at same concentration were then added in each test tube. Approximately 200  $\mu\text{L}$  of each sample was added in each well of 96 well ELISA dish. Each sample was prepared in triplicate and incubated at 37°C. After incubation, planktonic cells were removed by turning the plate upside down. It was then submerge gently in a small tub of water and water was removed. Washing was done thrice to remove all the unattached cells and also to lower background staining. Afterwards 200  $\mu\text{L}$  of a 0.1% solution of crystal violet was added to each well of the microtiter plate. It was left in incubator for 10–15 min. Crystal violet solution was removed by tilting the plate and by rinsing with water 3–4 times. It was then blot dried.

To quantify the biofilms, 200  $\mu\text{L}$  of 30% v/v acetic acid solution was added to each well to completely solubilize

the crystal violet. The microtiter plate was incubated at room temperature for 10–15 min. The resultant solution was transferred to a new microtiter dish and absorbance reading was taken in a micro-plate reader at 595 nm (George, 2011; Perumal and Mahmud, 2013).

## RESULTS

### Resistance Spectra by Disk Diffusion Method

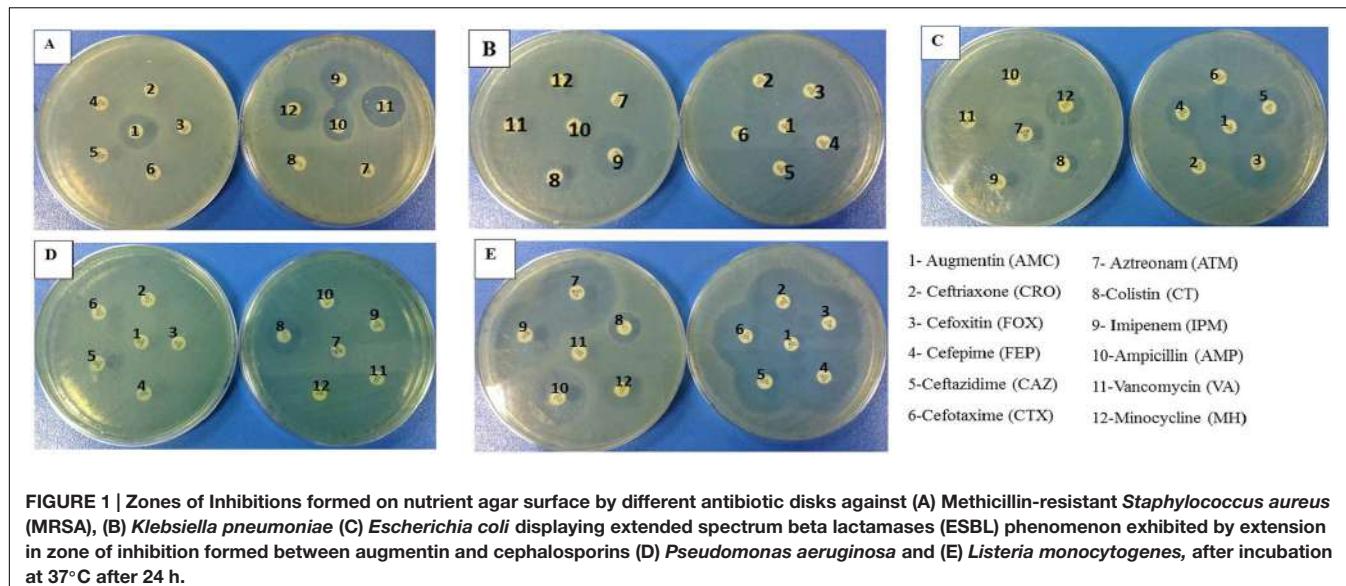
Resistance spectra of selected microbes were determined by standard disk diffusion method. Results were compared to CLSI guidelines to categorize organisms either as susceptible or resistant. Cefoxitin was used as an indicator of methicillin susceptibility disks and an inhibition zone diameter of  $\leq 14$  mm was reported as methicillin resistant. According to our results, *S. aureus* was resistant to cefoxitin (FOX) that is why we marked it as Methicillin Resistant *S. aureus* (Figure 1). It was also resistant to whole range of tested cephalosporins (Table 1). Likewise, *K. pneumoniae* was also resistant to all cephalosporins and augmentin as well, but was susceptible to Imipenem (IPM), Colistin (CT) and Minocycline (MH). *E. coli* was found to be ESBL positive as the inhibition zone expansion was observed with Augmentin (Figure 1C). In case of *P. aeruginosa*, it was susceptible to Colistin (CT) only otherwise it was observed to be highly resistant to all other tested antibiotics. So all the clinical pathogens were found to be highly resistant to the available therapeutic options. However, *L. monocytogenes* (ATCC 13932) was susceptible to all tested antibiotic disks except vancomycin.

### Characterization of Nano-Particles

Ultramicroscopic characterization of nanoparticles was done to gather a firsthand information on particles morphology, size and distribution. AFM was performed to get surface topography and size distribution images of nano-formulations. 3D AFM images of both nano-systems revealed that both nano-formulations were homogeneously dispersed and a maximum height of 50 nm was observed for empty CSNPs and 51 nm for cefotaxime loaded CSNPs (Figure 2). Both empty and loaded nano-formulations were found to be quite uniform. However, cefotaxime loaded CSNPs were displaying more dense population. SEM results revealed that empty and cefotaxime loaded NPs were in the size range of less than 100 nm. Both AFM and SEM analysis demonstrated that there was more concentrated and more dense population of drug loaded NPs as compare to blank NPs (Figure 3).

### FTIR Spectra

Fourier Transform Infrared Spectroscopy analysis of chitosan raw material, cefotaxime powder empty CSNPs and drug loaded CSNPs are shown in Figure 4. FTIR spectra of CS raw material revealed major peaks at 3401, 2878, 1651, 1380, 1080, and at 607  $\text{cm}^{-1}$  that corresponded to O-H stretching peak, C-H stretching, N-H stretch, CH<sub>2</sub> bending, C-O-C stretch and -C≡C-H, respectively (Figure 4A).



In case of CSNPs more broad, strong and characteristic peak for CS was observed at 3406 cm<sup>-1</sup> representing O-H stretching or H-bonding for alcohol or phenols instead of 3401 cm<sup>-1</sup> (for CS) indicating that H bonding is enhanced during NPs formation while the amide peak shifted from 1651 to 1644 cm<sup>-1</sup>. A new sorption bands at 2089 cm<sup>-1</sup> appear, which shows that the ammonium groups are crosslinked with tripolyphosphate molecules.

Analysis of FTIR spectra of CSNPs showed that it was very similar to that of cefotaxime loaded CSNPs (Figures 4C,D). This fact was predictable because the amount of drug loaded in nano-scaffold was very small as compare to the amount of building blocks of nano-structures, that is why no peaks of cefotaxime was observed in the drug loaded nanoparticles.

### Determination of Zeta Potential

According to our results, both the blank CSNPs and drug loaded CSNPs had demonstrated a zeta potential of more than +50 mV (Figures 5A,B). This can be predicted from the data that  $\beta$ -lactam loaded nano-systems are offering long term stability.

### Determination of Encapsulation Efficiency

Encapsulation efficiency was determined at different drug concentrations. It was observed that EE augmented with increase in concentration of drug. It was calculated to be 60% at 100  $\mu$ g/mL; 71% at 800  $\mu$ g/mL and 90% at 2000  $\mu$ g/mL concentration.

### Antimicrobial Potential of the Prepared Nano-antibiotics

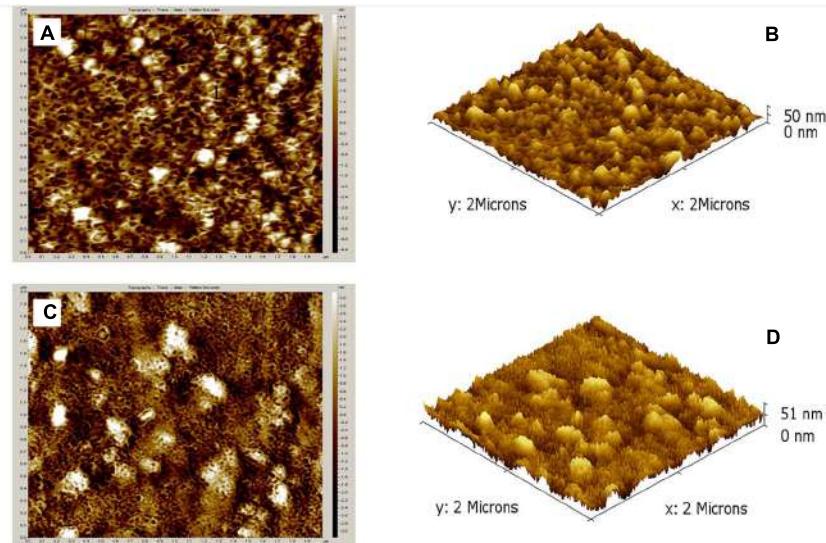
Drug loaded CSNPs were prepared with 1000  $\mu$ g/mL of drug concentration and tested against above-mentioned pathogens by broth dilution assay (Figure 6). It was compared to both plain antibiotic solution and bare CSNPs for 7 days (144 h). It was observed that nano-cefotaxime was highly effective against all MDR pathogens while the simple antibiotic could not control them at all. However, when we compare efficiency of drug loaded CSNPs to bare CSNPs, the difference seems to be low. In case of *P. aeruginosa* the activity was equal. However, for other pathogens bare nano-systems were effective till first 48 h.

**TABLE 1 |** Zones of Inhibition formed by clinical isolates against different tested antibiotic disks.

Pathogens	AMC	CRO	FOX	FEP	CAZ	CTX	ATM	CT	IPM	AMP	VA	MH
	Zones of inhibition (mm)											
MRSA	18	—	—	—	—	—	—	—	17	15	20	20
<i>Klebsiella pneumoniae</i>	—	—	—	—	—	—	—	15	23	—	—	15
<i>Escherichia coli</i>	—	—	24*	16*	19*	—	15	14	28	—	—	18
<i>Pseudomonas aeruginosa</i>	—	—	—	—	—	—	—	16	12	—	—	—
<i>Listeria monocytogenes</i> (ATCC 13932)	30	29	26	29	29	30	33	15	30	25	—	16

Where Augmentin (AMC), Ceftriaxone (CRO), Cefoxitin (FOX) Cefepime (FEP), Ceftazidime (CAZ), Cefotaxime (CTX), Aztreonam (ATM), Colistin (CT), Imipenem (IPM), Ampicillin (AMP), Vancomycin (VA), and Minocycline (MH). ESBL phenomenon exhibited by increase in zone of inhibition. Whereas (—) means 'no zone of inhibition'.

\*indicates ESBL phenomenon marked by increase in zone of inhibition caused by AMC and cephalosporin discs.



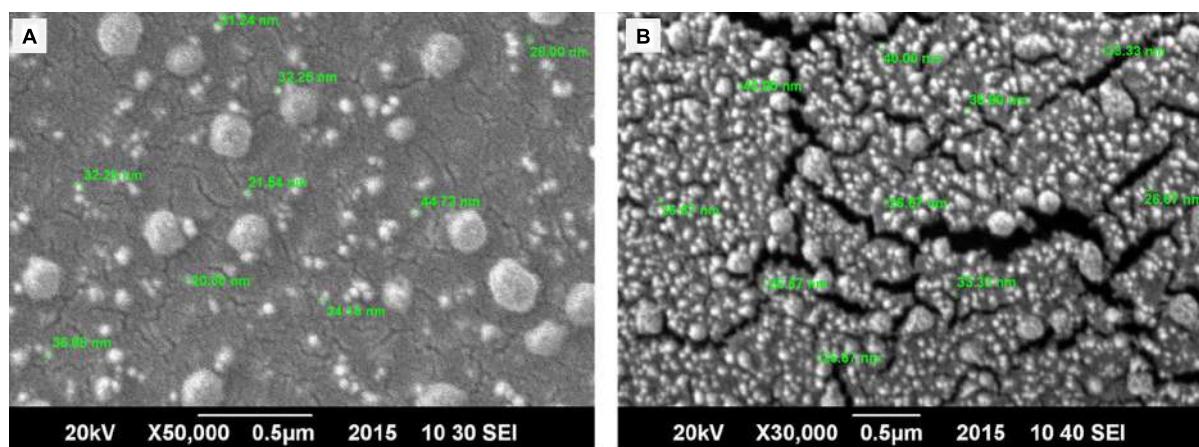
**FIGURE 2 |** Atomic force microscopic images of surface topography and 3-Dimensional (3D) structures of empty CSNPs (A,B) and cefotaxime loaded CSNPs (C,D) respectively. A small drop of sample was dried on glass slide and all images were taken at room temperature without any sample treatment. Results obtained depict height of 50 and 51 nm.

Nonetheless, the drug loaded CSNPs were proved to be more effective in contending the other four pathogens including ATCC *L. monocytogenes* for more prolonged period of time. At the end of experiment CFU count was done to confirm the hypothesis (**Figure 7**). It also endorse the above statement as no colonies were observed in case of *P. aeruginosa*, however, in other pathogens growth was observed only in case of CSNPs.

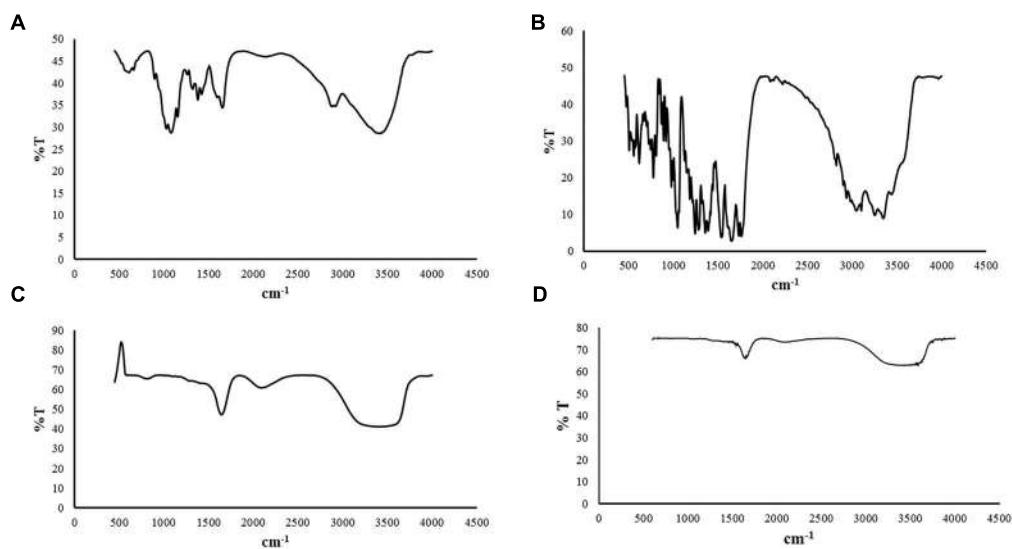
### Anti-Biofilm Activity of the Fabricated Nano-antibiotics

It was observed from the present study that the formation and intensity of biofilm increase with the intensity of pathogenicity

and resistance spectrum of pathogens. The most resistant Gram-negative pathogens were *K. pneumoniae* and *P. aeruginosa* and they formed more intense biofilms. Whereas, *L. monocytogenes* (ATCC) was most susceptible to antibiotic disks and was the weakest in term of biofilm intensity. Antibiotic alone fails to eradicate the biofilm, however, nano-cefotaxime was found to be highly effective in controlling the formation of biofilms (**Figure 8**). CSNPs were also found to be efficient in controlling the biofilms but the drug loaded CSNPs were more effective in every case. It may be concluded that bare CSNPs can effectively kill the planktonic cells, however, cannot efficiently control biofilm formation. Whereas drug loaded CSNPs were more



**FIGURE 3 |** Scanning electron microscopy (SEM) of bare (A) and drug loaded CSNPs (B). SEM images were taken by placing a tiny droplet of sample on 1 × 1 cm glass slide and it was spreaded evenly. After gold sputtering, images were taken at ambient conditions. SEM images depicted spherical particles having diameter of less than 100 nm.



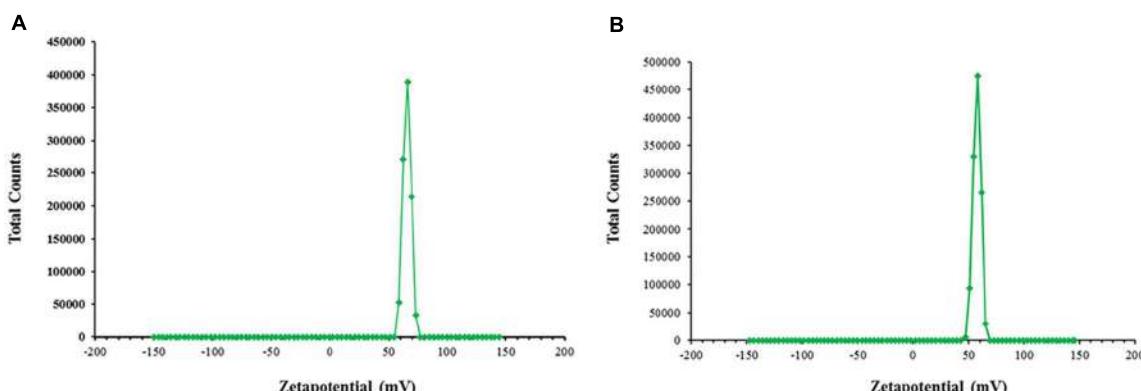
**FIGURE 4 | Fourier Transform Infrared Spectroscopy spectra (A) Chitosan raw material (B) Cefotaxime raw material (C) bare CSNPs (D) cefotaxime loaded CSNPs.** FTIR spectrum of powders were taken after mixing raw material with KBr. Whereas liquid samples were analyzed directly by placing a tiny drop over glass assembly. FTIR spectra indicated no new bond formation so the drug is not reacting chemically with nano-scaffolds.

effective as a therapeutic agent to prevent the formation of biofilm and also in terms of killing pathogens for prolonged period of time.

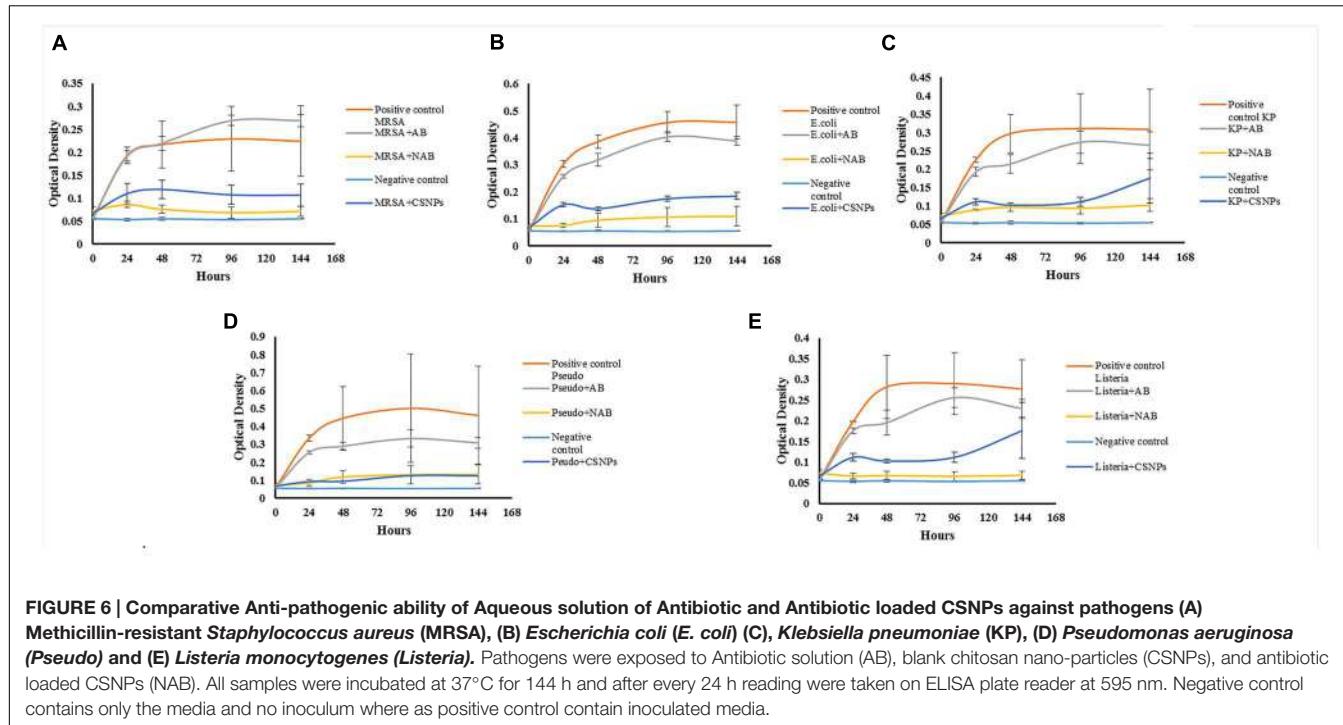
## DISCUSSION

Biofilms are defined as microbial communities of cells that are irreversibly attached to a substratum or to an interface or to each other, and are embedded into a matrix of EPSs that they have produced. There are as many different types of biofilms as are bacteria, and that a single bacterium may even make several different types of biofilms under different environmental conditions (Karatan and Watnick, 2009). These biofilms are offering inherent barrier to the penetration of conventional antibiotics. Though anti-biofilm potential of plant

extracts was reported by many others (Perumal and Mahmud, 2013), yet this study has been designed to sort out the potential of antibiotic loaded chitosan NPs as an antibacterial and anti-biofilm agents. Cefotaxime has been used for this purpose. Albeit, numerous methods are available for qualitative and quantitative assays of antibiotics, nevertheless the UV-Vis Spectrophotometric method has proved to be simple, effective, fast, economical and reproducible for quantification of cefotaxime sodium in pharmaceutical form powder for injectable solution. Resultantly,  $\lambda_{max}$  was obtained at 298 nm. Similar method was also employed by other authors though different  $\lambda_{max}$  values were achieved by them. According to Bushra et al. (2014)  $\lambda_{max}$  value for cefotaxime was obtained at 260 nm, at 228 nm (El-Shaboury et al., 2007), and 253 nm (Hammod et al., 2011). NPs were formulated by ionic gelation method that has proved to be most efficient



**FIGURE 5 | Zeta potential.** Measured by Malvern Zeta sizer at ambient conditions (A) empty CSNPs (B)  $\beta$ -lactam drug loaded CSNPs. Both empty and drug loaded CSNPs are displaying zeta potential values greater than 50 mV. It indicates highly stable colloidal dispersion.

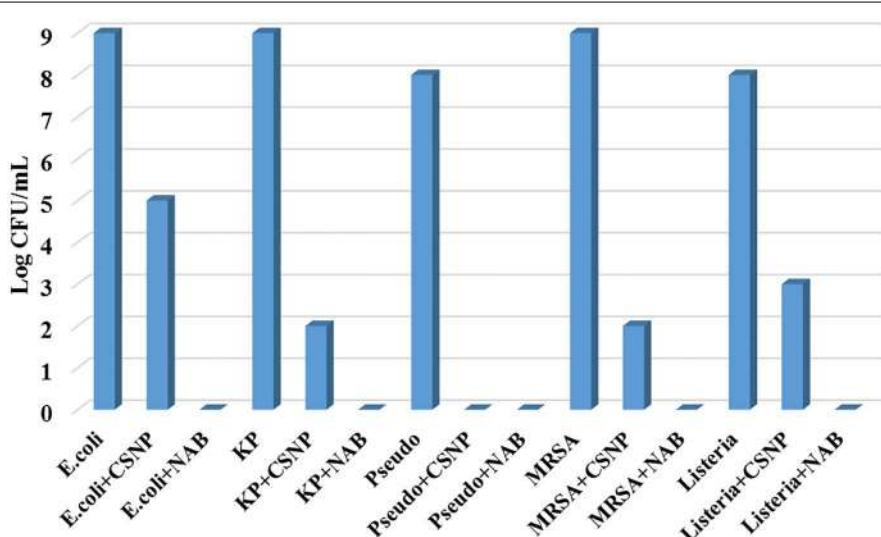


**FIGURE 6 | Comparative Anti-pathogenic ability of Aqueous solution of Antibiotic and Antibiotic loaded CSNPs against pathogens (A) Methicillin-resistant *Staphylococcus aureus* (MRSAs), (B) *Escherichia coli* (E. coli) (C), *Klebsiella pneumoniae* (KP), (D) *Pseudomonas aeruginosa* (Pseudo) and (E) *Listeria monocytogenes* (Listeria).** Pathogens were exposed to Antibiotic solution (AB), blank chitosan nano-particles (CSNPs), and antibiotic loaded CSNPs (NAB). All samples were incubated at 37°C for 144 h and after every 24 h reading were taken on ELISA plate reader at 595 nm. Negative control contains only the media and no inoculum where as positive control contain inoculated media.

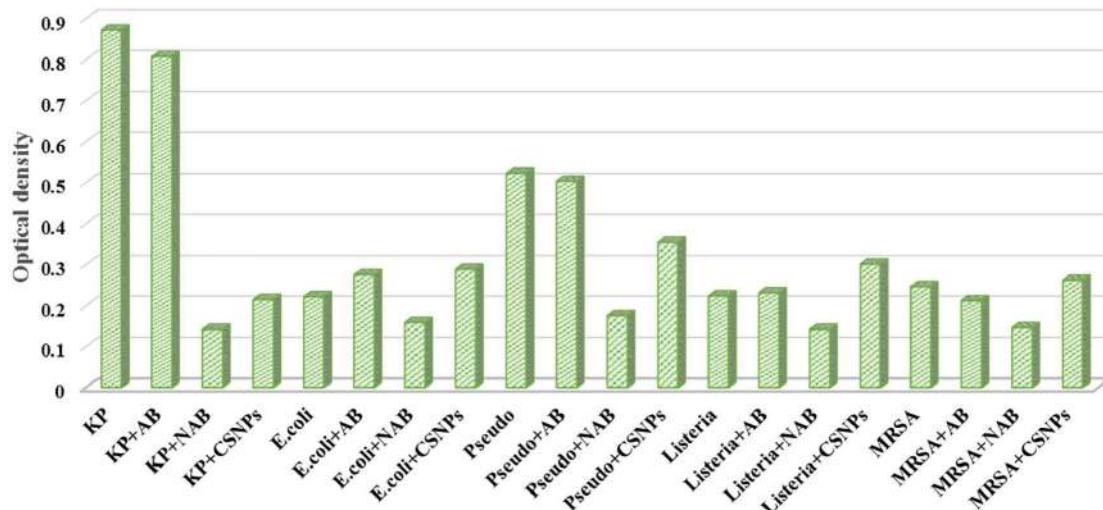
and convenient method. Engineered and naturally forming NPs can vary widely in their physicochemical characteristics such as shape, size, and charge. These characteristics have been reported to impact their interactions with biofilm-coated surfaces (Ikuma et al., 2015). Microscopic attributes were studied via AFM and SEM wherein the results displayed much smaller size of NPs as compared to previous studies. Previous studies have reported the

size of CSNPs in the range of 140–250 nm (Gan et al., 2005; Nesalin and Smith, 2013; Chaubey and Mishra, 2014; Miladi et al., 2015) while in this research size less than 100 nm was achieved.

Fourier Transform Infrared Spectroscopy spectroscopic studies were carried out in order to confirm the formation of NPs and type of interactions between antibiotic and polymer. FTIR has proved to be a powerful and useful characterization



**FIGURE 7 | Colony forming unit (CFU) assay.** CFU assay was performed to count the viable bacteria after reaction of CSNPs and drug loaded CSNPs with pathogens. CFU was done by plating 10 $\mu$ L of sample from each test tube after serial dilutions in normal saline (0.9% NaCl). All plates were then incubated for 48 h and colonies were counted manually. CSNP, Chitosan nano-particles, NAB, Nano-antibiotic; KP, *Klebsiella pneumoniae*; Pseudo, *Pseudomonas aeruginosa* and MRSA for Methicillin-resistant *S. aureus*.



**FIGURE 8 | Anti-biofilm activity of antibiotic suspension (AB) Chitosan nano-particles (CSNPs) and Cefotaxime loaded CSNPs (NAB) against pathogens.** Methicillin-resistant *S. aureus* (MRSA), *Klebsiella pneumoniae* (KP), *Escherichia coli* (E. coli), *Pseudomonas aeruginosa* (Pseudo), and *Listeria monocytogenes* (Listeria) were incubated on ELISA plates at 37°C for 24 h. Biofilms were stained by crystal violet stain and removed by 30% acetic acid solution. This solution was then measured on ELISA plate reader at 595 nm.

method for polymers, and materials in general. This is quite an economical, short time characterization that allows to establish the chemical composition, microstructure, chemical interactions and follow variation of specific functional groups with the passage of time during reactions (Barrios et al., 2012).

Fourier Transform Infrared Spectroscopy spectrum of chitosan showed resemblance with an already reported spectra for this biopolymer (Khan et al., 2002; Qi et al., 2004; Vitali et al., 2006). Spectrum of cefotaxime raw material was also in accordance with previously reported spectrum for this cephalosporin drug (Hammond et al., 2011). Likewise, FTIR spectrum of empty CSNPs was similar to already reported spectra, however, it was quite different from the spectrum of bulk material that indicated the formation of NPs (Hosseini et al., 2013; de Pinho Neves et al., 2014; Antoniou et al., 2015). The spectrum for drug loaded NPs was almost superimposable to that of empty nano-formulation indicating that there was no chemical bonding between the drug and polymers (Figures 4C and 3B).

Zeta potential is an indicator of long term stability of colloidal dispersions. Nanoparticles with a zeta potential between  $-10$  and  $+10$  mV are considered approximately neutral, while nanoparticles with zeta potentials of greater than  $+30$  mV or less than  $-30$  mV are considered strongly cationic and strongly anionic, respectively (Clogston and Patri, 2011) and represent a stable formulation as there would be less aggregation and flocculation as the repulsive forces become more strong (Gazori et al., 2009). According to results, both the blank CSNPs and drug loaded CSNPs were displaying highly positive zeta potential of more than  $+50$  mV (Figures 5A,B). This can be predicted from the data that  $\beta$ -lactam loaded nano-systems are offering long term stability. Positive zeta potential in the range of 21–45 was already reported by many authors (De Campos et al., 2001; Miladi et al., 2015; Zhang et al., 2016).

Encapsulation efficiency was determined at various concentrations of cefotaxime to check the influence of drug concentration on EE. It was observed that EE augmented with increase in concentration of drug. However, the findings indicated that if the drug was dissolved in CS solution and TPP was added afterward it had resulted in very low EE (<3%). Honary et al. (2014) encapsulated vancomycin in CSNPs and reported 60–69% EE (Honary et al., 2014) while 42–55% after encapsulation of 5-Fluorouracil. The well -characterized CSNPs were further investigated for their antipathogenic and anti-biofilm potential.

Bacterial biofilms were first described in 1943. Biofilm formation is an intricate process and colloquially the biofilms are also referred to as microbial cities. Preliminary step to this phenomenon is the attachment to any solid surface termed as ‘adsorption.’ These biofilms are sheltered by a self-secretory matrix called EPS. This EPS hold the cells organized and safeguard them against extraneous agents because of its composition. Biofilms also aid in quorum sensing thus facilitating microbes to communicate with each other. The composition of EPS is protein, polysaccharides and extracellular DNA. Biofilms can ensure maximum intake of nutrients from outside environment. The organization of microbes in these biofilms is such that ancient and dead cell recline in the center where the metabolites get accumulated while the fresh and new cells lie near the outer surface where the flow of materials is maximum. These biofilms are offering an innate resistance mechanism to conventional antibiotics, however, NAMs can encounter biofilms successfully.

The interactions between NPs and the biofilm can be viewed as a three-step process: (1) transport of NPs to the vicinity of the biofilm; (2) attachment to the biofilm surface; and (3) migration within the biofilms (Ikuma et al., 2015).

It has been observed from the present study that the nanocefotaxime was highly effective in controlling the formation of biofilms (**Figure 8**). Though anti-biofilm potential of plant extracts has been reported by many others (Perumal and Mahmud, 2013), however, this is the first report to prove the anti-biofilm activity of cefotaxime loaded CSNPs. It has been mentioned earlier that more than 70% pathogenic microbes are biofilm forming and that is the main mechanism of resistance in pathogens. Blank CSNPs also exhibited good potential in lowering the biofilm formation intensity but fails to eradicate it completely. Therefore it can be concluded that chitosan NPs causes the clumping of bacterial mass and help to slow the growth of pathogens, however, cannot control the biofilm formation efficiently. Less intensity of biofilm is the direct result of less number of pathogens in culture media. The drug loaded CSNPs totally eradicated the pathogens and therefore biofilm formation.

## CONCLUSION

Foregoing in view, it can be positively concluded that the main mechanism of action by which NAMs control MDR pathogens is inhibition of biofilm formation. The results in this research have confirmed that chitosan can be a potential carrier system

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- for cefotaxime to target Gram-negative and Gram-positive multi drug resistant microorganisms that have the tendency to form microbial biofilms. The prepared NPs have been perceived to be stable as carrying positive zeta of more than +50 mV and were displaying homogeneity in both size and shape.

## AUTHOR CONTRIBUTIONS

BJ designed, developed, and performed all the experimental work, analysed and interpreted the data and wrote the manuscript. HH performed and helped in analyzing the Atomic Force Microscopy (AFM) Assays. SA helped in collection of clinical isolates and their characterization. AI provided technical assistance for Zeta sizer analysis. HN done the FTIR analysis and its interpretation. MI supervised the work at each step, provided the budget and scientific assistance for the manuscript write-up.

## FUNDING

This article and research was funded by COMSATS Institute of Information Technology, Islamabad.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 30 October 2015

Accepted: 01 February 2016

Published: 19 February 2016

### Citation:

von Wintersdorff CJH, Penders J, van Nieuwkerk JM, Mills ND, Majumder S, van Alphen LB, Savelkoul PHM and Wolfs PFG (2016) Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Front. Microbiol.* 7:173.  
doi: 10.3389/fmicb.2016.00173

The emergence and spread of antibiotic resistance among pathogenic bacteria has been a rising problem for public health in recent decades. It is becoming increasingly recognized that not only antibiotic resistance genes (ARGs) encountered in clinical pathogens are of relevance, but rather, all pathogenic, commensal as well as environmental bacteria—and also mobile genetic elements and bacteriophages—form a reservoir of ARGs (the resistome) from which pathogenic bacteria can acquire resistance via horizontal gene transfer (HGT). HGT has caused antibiotic resistance to spread from commensal and environmental species to pathogenic ones, as has been shown for some clinically important ARGs. Of the three canonical mechanisms of HGT, conjugation is thought to have the greatest influence on the dissemination of ARGs. While transformation and transduction are deemed less important, recent discoveries suggest their role may be larger than previously thought. Understanding the extent of the resistome and how its mobilization to pathogenic bacteria takes place is essential for efforts to control the dissemination of these genes. Here, we will discuss the concept of the resistome, provide examples of HGT of clinically relevant ARGs and present an overview of the current knowledge of the contributions the various HGT mechanisms make to the spread of antibiotic resistance.

**Keywords:** antibiotic resistance, resistome, transformation, conjugation, transduction, gene transfer agents, GTA, lateral gene transfer

## INTRODUCTION

The ever-increasing magnitude of antimicrobial resistance (AMR) encountered in human pathogens is a huge concern for public health worldwide, limiting treatment options for bacterial infections and thereby reducing clinical efficacy while increasing treatment costs and mortality. With a lack of development of new antibiotics, and increasing resistance even to last-resort antibiotics (Nordmann et al., 2012), there is a need to conserve the ones available.

Natural antibiotics have existed for billions of years (Barlow and Hall, 2002; Hall and Barlow, 2004; Bhullar et al., 2012; Wright and Poinar, 2012), providing a selective benefit for the producing strains by inhibiting or eliminating other bacteria competing for resources (Martinez, 2008; Aminov, 2009). Additionally, their function as cell-cell signaling molecules has been described

(Davies, 2006; Linares et al., 2006). Just as antibiotics are ancient, so are antibiotic resistance genes (ARGs), as evidenced by studies identifying various ARGs in ancient permafrost samples (D'Costa et al., 2011; Perron et al., 2015) and isolated cave microbiomes (Bhullar et al., 2012). Resistance to antibiotics can occur either by mutations or by acquisition of resistance conferring genes via horizontal gene transfer (HGT), of which the latter is considered to be the most important factor in the current pandemic of AMR.

The HGT of ARGs also far predates the production and use of antibiotics by humans. For example, OXA-type  $\beta$ -lactamases were found to be plasmid-borne and able to transfer between bacterial species millions of years ago (Barlow and Hall, 2002). However, while antibiotic resistance and its spread by HGT are ancient mechanisms, the rate at which these processes occur and the number of resistant strains has increased tremendously over the past few decades because of selective pressure through human antibiotic use.

## THE GLOBAL ANTIBIOTIC SELECTION PRESSURE

While the discovery of antibiotics revolutionized the field of medicine, their increasingly large-scale production and consumption has had widespread effects on the microbial biosphere. In their analysis, Van Boeckel et al. showed that global human antibiotic consumption amounted to 54,083,964,813 standard units (pills, capsules, or ampoules) in 2000 and had increased by 36% to 73,620,748,816 standard units by 2010 (Van Boeckel et al., 2014). The same authors estimated that antibiotic consumption in food animals, which is assumed to be larger than that of humans, was over 63 million kg in 2010 and will also drastically increase in the coming years (Van Boeckel et al., 2015) despite recent initiatives to reduce antibiotic use in animals. Such high and continuously increasing amounts of antibiotics overwhelm the natural production, causing a constantly increasing selection pressure on bacterial populations in all exposed environments.

The use and misuse of antibiotics in medicine, agriculture, and aquaculture has been linked to the emergence of resistant bacteria in these settings (Cabello, 2006; Penders and Stobberingh, 2008; Economou and Gousia, 2015). However, the impact of antibiotic usage extends further, as antibiotic residues, resistant bacteria, and genetic resistance elements subsequently spread to adjacent environments. The majority of consumed antibiotics are excreted unchanged (Sarmah et al., 2006) and are then introduced into the environment directly or through waste streams. Such waste streams, as well as wastewater treatment plants, are considered to be hotspots for the dissemination of AMR, since resistance genes, mobile genetic elements (MGEs), and (sub-inhibitory) antibiotic selection pressure from various sources are introduced to commensals and pathogens (Tennstedt et al., 2003; Martinez, 2009; Zhang et al., 2009; Graham et al., 2011). Moreover, the antibiotic compounds are often not completely removed in treatment plants (Watkinson et al., 2007; Le-Minh et al., 2010), from where they then disseminate further. A study by Larsson et al. showed that a treatment plant in India, receiving water from

drug manufacturing sites, exposes its direct environment to very high levels of antibiotics, discharging ~45 kg of ciprofloxacin per day (Larsson et al., 2007), contaminating surface, ground, and drinking water in the area (Fick et al., 2009). As a result, not only were highly multiresistant bacteria found to be common within the treatment plant (Marathe et al., 2013), but high levels of ARGs and MGEs were also detected in nearby river sediments (Kristiansson et al., 2011).

How environmental exposure to antibiotics contributes to the selection of resistant strains and the increase of resistome elements is illustrated by a study comparing soil samples taken between 1940 and 2008, which shows that ARGs from all classes of antibiotics tested ( $\beta$ -lactams, tetracyclines, erythromycins, and glycopeptides) significantly increased since 1940, with a tetracycline ARG being over 15 times more abundant than in the 1970s (Knapp et al., 2010). Moreover, the increasing selection pressure has altered bacterial HGT processes, increasing the number of resistome elements which reside on mobile DNA compared to the pre-antibiotic era (Datta and Hughes, 1983).

## RESERVOIRS OF RESISTANCE

In order to understand the dissemination of antibiotic resistance, it is necessary to map the resistome of various environments, and to unravel to what extend these environments can act as a reservoir for the dissemination of ARGs to bacterial pathogens. In recent years there has been increasing interest in this matter, as many studies have used various techniques to sample the resistome of environments such as, but not limited to, soil, wastewater, and human and animal gut microbiota (Pehrsson et al., 2013; Penders et al., 2013; Rizzo et al., 2013; von Wintersdorff et al., 2014). It has since become clear that ARGs, including clinically relevant ones, are widespread in such environments (Wright, 2010). Studies applying a metagenomic approach directly recover DNA from all microorganisms in a biological sample, thereby avoiding the bias that is introduced when selecting certain organisms, and allowing for the investigation of the resistome of microbial ecosystems. The sequencing of metagenomes from various environments has led to a wealth of data which is often publicly available in databases. Such databases can be mined for the presence of resistance genes, even when the initial studies did not focus specifically on ARG content in these metagenomes. For example, mining such metagenomic databases for the plasmid-mediated colistin resistance gene *mcr-1*, which has recently been discovered in clinical and commensal isolates (Arcilla et al., 2015; Liu et al., 2015), has revealed that this gene had already spread to the human gut microbiome of Chinese subjects several years ago (Hu et al., 2015). While sequence based studies provide huge amounts of data, they are limited to either identifying genes that are already known, or to predicting novel sequence functions based on high homology to known sequences. Annotation by sequence-based studies will keep increasing however, as studies using functional metagenomics keep identifying novel ARGs. An increasing number of such studies have revealed a huge number of previously unknown ARGs present in environments such as soil (Riesenfeld et al., 2004; D'Costa et al., 2006; Allen et al., 2009;

Donato et al., 2010; Torres-Cortes et al., 2011; Perron et al., 2015) or activated sludge (Mori et al., 2008; Parsley et al., 2010), as well as in the microbiota of animals (Kazimierczak et al., 2009; Wichmann et al., 2014) and humans (Sommer et al., 2009; Cheng et al., 2012; Moore et al., 2013, 2015; Card et al., 2014; Fouhy et al., 2014; Clemente et al., 2015).

Recent metagenomic studies have also uncovered that ARGs predominantly cluster by ecology, implying that the resistome in soils, and wastewater treatment plants differ significantly from that of human pathogens (Gibson et al., 2015; Munck et al., 2015). Nonetheless, the authors of these works note that parts of these resistomes are shared (Forsberg et al., 2012) and stress the importance of continuing the exploration of the resistome of such environments.

That commensals and the environment are important reservoirs for resistance is supported by several examples of ARGs on MGEs in human pathogens that appear to have originated from those reservoirs. A well-known example is that of the *bla<sub>CTX-M</sub>* genes, which have become the most prevalent cause of extended-spectrum  $\beta$ -lactamases (ESBLs) in Enterobacteriaceae worldwide and a major cause of clinical treatment problems (Hawkey and Jones, 2009). The potential origin of these genes was identified as the chromosomal DNA of various environmental *Kluyvera* species, from where they spread very successfully to different bacterial species (Canton and Coque, 2006). *Shewanella algae*, a marine and freshwater species, was found to be the origin of plasmid-encoded *qnrA* genes, conferring quinolone resistance (Poirel et al., 2005a), and different *Vibrionaceae* species might be the reservoir for other plasmid-encoded *qnr* genes (Poirel et al., 2005b), which have disseminated globally in various Enterobacteriaceae species, with exceptionally high prevalence rates in some areas (Vien le et al., 2012). The OXA-48-type carbapenem-hydrolyzing  $\beta$ -lactamase genes, which are increasingly reported in enterobacterial species worldwide, were also found to originate from the chromosomes of waterborne, environmental *Shewanella* species (Poirel et al., 2012). As with these few examples, many clinically relevant resistance genes are believed to have originated from non-pathogenic bacteria, highlighting the immense potential of HGT for these pathogens in overcoming human use of antibiotics.

## CONTRIBUTION OF THE VARIOUS HGT MECHANISMS TO THE SPREAD OF ARGs

### Conjugation

Conjugation is the transfer of DNA through a multi-step process requiring cell to cell contact via cell surface pili or adhesins. It is facilitated by the conjugative machinery which is encoded either by genes on autonomously replicating plasmids or by integrative conjugative elements in the chromosome (Smillie et al., 2010; Wozniak and Waldor, 2010). Additionally, this conjugative machinery may enable the mobilization of plasmids that are non-conjugative, as observed for e.g., the exceptionally broad host range IncQ plasmids (Meyer, 2009). Of the various mechanisms that may facilitate HGT (Figure 1), conjugation

is certainly the most commonly studied (Norman et al., 2009; Guglielmini et al., 2013).

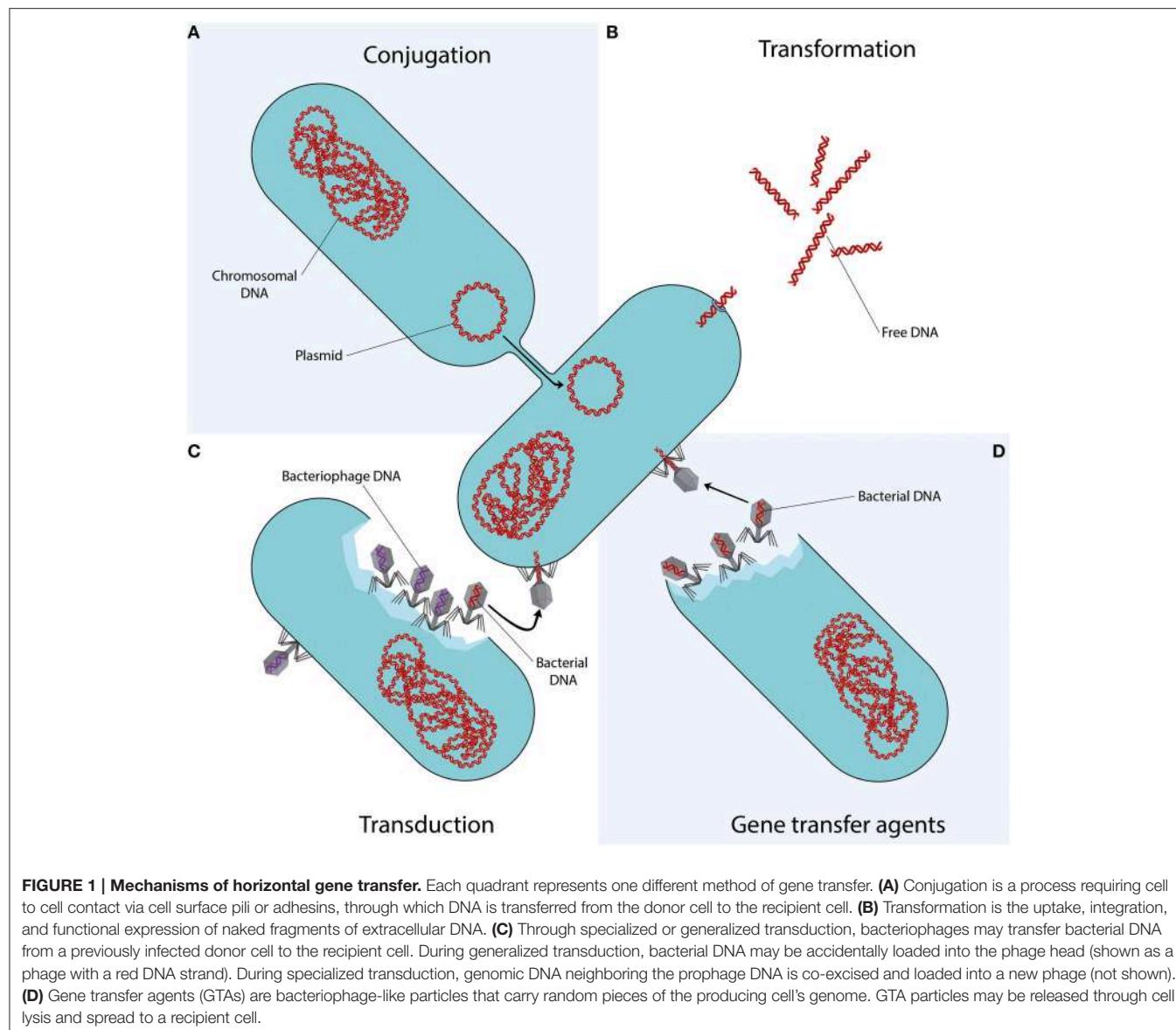
ARGs are in many cases associated with conjugative elements such as plasmids or transposons. While the transfer of these elements may also occur through transformation or transduction, conjugation is often considered as the most likely responsible mechanism. This is due to the fact that it provides better protection from the surrounding environment and a more efficient means of entering the host cell than transformation, while often having a broader host range than bacteriophage transduction. Moreover, while conjugation is a process directed toward the transfer of bacterial genes, transfer of bacterial DNA by transduction is a side-effect of erroneous bacteriophage replication (Norman et al., 2009).

The conjugation of MGEs conferring AMR has been observed in many types of ecosystems, ranging from transfer between bacteria in insects, soil, and water environments to various food and healthcare associated pathogens (Davison, 1999). Importantly, transfer of plasmids and conjugative transposons, such as those of the Tn916 family, between unrelated bacteria over large taxonomic distances has been described (Shoemaker et al., 2001; Musovic et al., 2006; Roberts and Mullany, 2009; Tamminen et al., 2012), indicating that this mechanism contributes significantly to the dissemination of ARGs between different reservoirs via such broad host range MGEs.

The spread of antibiotic resistance plasmids in human pathogens is especially well studied, and shows that once resistance genes have become established on successful plasmids, they may rapidly spread across different strains, species, or even genera. This is well demonstrated by the *bla<sub>CTX-M</sub>* ESBL genes, which have disseminated to various narrow and broad host range plasmids within Enterobacteriaceae, as well as to other opportunistic human pathogens (Canton et al., 2012). These genes are now ubiquitous in humans (Woerther et al., 2013), animals, and the environment (Hartmann et al., 2012). Furthermore, the transfer of plasmids in pathogens has led to the worldwide spread of numerous ARGs encoding resistance to  $\beta$ -lactams, quinolones, aminoglycosides, tetracyclines, sulfonamides, and many other drug classes (Huddleston, 2014). Of particular concern is the increasingly reported spread of plasmids harboring carbapenem resistance (Carattoli, 2013) and the recent discovery of plasmid-encoded colistin resistance in China (Liu et al., 2015), which has now already been identified at multiple continents (Arcilla et al., 2015) and may cause Enterobacteriaceae to truly become pan-drug resistant. Moreover, multiple ARGs are often co-localized on the same plasmid, which allows for the relatively easy spread of multidrug resistance.

### Transformation

In 1928, Griffith became the first to demonstrate direct genetic exchange between different strains of *Streptococcus pneumoniae* (Griffith, 1928). Certain bacteria appeared to be capable of uptake, integration, and functional expression of naked fragments of extracellular DNA, a process called (natural) transformation. It soon became clear that bacteria could use transformation to evade antibiotics, by exchanging ARGs. In



1951, Hotchkiss induced penicillin and streptomycin resistance in previously sensitive strains of *S. pneumoniae* by exposing them to DNA from resistant strains (Hotchkiss, 1951). Alexander et al. furthered this work by demonstrating intra- and inter-species transfer of streptomycin resistance between *H. influenzae*, *H. parainfluenzae*, and *H. suis* (Alexander and Leidy, 1953; Alexander et al., 1956).

In order for transformation to take place, several conditions have to be met. There must be DNA present in the extracellular environment; the recipient bacteria must be in a state of competence; and the translocated DNA must be stabilized, either by integration into the recipient genome, or by recircularisation (in the case of plasmid DNA) (Thomas and Nielsen, 2005). Whereas *Neisseria* spp. are considered to be constitutively competent (Sparling, 1966; Johnston et al., 2014), other bacterial species capable of natural transformation may develop

competence only under certain conditions, such as the presence of peptides or autoinducers, nutritional status, or other stressful conditions, as reviewed in more detail by Johnston et al. (2014). Importantly, studies have shown that exposure to antibiotics can induce competence in many species of bacteria, meaning that antibiotics would not only select for resistant strains, but also stimulate transformation of their ARGs (Prudhomme et al., 2006; Charpentier et al., 2011, 2012).

*In vitro* experiments have done much to elucidate transformation of ARGs. Early work proved that ARGs could be transformed; to this end, streptomycin, rifampicin, erythromycin, nalidixic acid, and kanamycin resistance have variously been transformed into *Neisseria gonorrhoeae* (Sparling, 1966), *Bacillus* spp. (Harford and Mergeay, 1973), *Gallibacterium anatis* (Kristensen et al., 2012), and *S. pneumoniae* (Prudhomme et al., 2006). The introduction of molecular techniques allowed

for the identification of the ARGs being transformed. *In vitro* studies have shown that the genes *parC* and *gyrA* are involved in the transformation of fluoroquinolone resistance between *S. pneumoniae* (Ferrandiz et al., 2000) and several viridans streptococci (Gonzalez et al., 1998; Janoir et al., 1999), and that transformation of *penA* confers penicillin resistance in commensal *Neisseria* species (*N. flavescens* and *N. cinerea*) and *N. meningitidis* (Bowler et al., 1994).

Molecular techniques have also made it possible to look for evidence of transformation outside of the laboratory. Spratt et al. identified the *penA* variant responsible for penicillin resistance in clinical isolates of *N. gonorrhoeae* (Spratt, 1988); sequence analysis revealed a mosaic structure, with blocks homologous to susceptible-type *penA* and blocks that diverge significantly (Spratt et al., 1992). These “resistant blocks” could be traced back to a strain of *N. flavescens* that had been isolated in the pre-antibiotic era, suggesting that such commensal species could have been the original source for the now ubiquitous resistance to penicillin (Spratt et al., 1989; Lujan et al., 1991). Mosaic genes are formed when sections of foreign DNA are incorporated into a recipient genome, as is the case in transformation. Their presence implies that HGT has taken place (Hakenbeck, 1998). In streptococci, the mosaic penicillin-binding protein (PBP) genes that encode PBPs with decreased affinity for  $\beta$ -lactam antibiotics are believed to be the result of gene transfer from related penicillin-resistant species (Sibold et al., 1994) and have disseminated penicillin resistance between various streptococci species (Dowson et al., 1990). Studies of fluoroquinolone resistance have demonstrated that mosaic variants of the genes *parC*, *parE*, and *gyrA* are readily transformed between *S. pneumoniae*, *Streptococcus mitis* and *Streptococcus oralis* (Balsalobre et al., 2003), and between *Streptococcus pyogenes* and *Streptococcus dysgalactiae* (Pletz et al., 2006).

Mao et al. developed a technique to extract intra- and extracellular DNA separately from environmental samples, and applied it to samples from a river basin in China. The result—a greater abundance of DNA outside of cells than inside—implies that in certain environments, extracellular DNA is a large reservoir for genes which may be accessible via transformation (Mao et al., 2014). Furthermore, Domingues et al. demonstrated that MGEs such as transposons, integrons and gene cassettes can be disseminated efficiently between species, regardless of their level of genetic relatedness (Domingues et al., 2012). Similarly, streptococcal species have been shown to exchange conjugative transposons via transformation in addition to conjugation (Chancey et al., 2015). All of this indicates that transformation provides a broad capacity for the horizontal spread of resistance elements between divergent species.

## Transduction

Bacteriophages play an important role in shaping the bacterial microbiome in any environment. Through specialized or generalized transduction, bacteriophages can transfer genes that are advantageous to their microbial hosts, in turn promoting their own survival and dissemination (Modi et al., 2013). The transferable DNA sequences range from chromosomal DNA

to MGEs such as plasmids, transposons and genomic islands (Brown-Jaque et al., 2015).

The mobilization or transfer of ARGs by bacteriophages has been documented for various bacterial species: the transduction of erythromycin (Hyder and Streifeld, 1978), tetracycline or multiple resistances between strains of *S. pyogenes* (Urukata et al., 1975); the transfer of tetracycline and gentamicin resistance between enterococci (Mazaheri Nezhad Fard et al., 2011); the carriage of  $\beta$ -lactamase genes by bacteriophages in *Escherichia coli* (Billard-Pomares et al., 2014) and *Salmonella* (Schmieger and Schicklmaier, 1999); or the transfer of antibiotic resistance plasmids in MRSA (Varga et al., 2012).

Recent studies applying metagenomic approaches to samples from various environments have suggested that bacteriophages may play a bigger part in the spread of ARGs than previously recognized. Colomer-Lluch et al. used qPCR to show that the  $\beta$ -lactam ARGs *blaTEM*, *blaCTX-M* and *mecA* were present in bacteriophages from river and urban sewage water samples. Additionally, cloning of the phage DNA into ampicillin susceptible *E. coli* hosts resulted in resistant transformants, harboring either the *blaTEM*, *blaCTX-M*, or undetermined ARGs (Colomer-Lluch et al., 2011a). In another study, the presence of ARGs in bacteriophages was detected in respiratory tract DNA of cystic fibrosis patients (Rolain et al., 2011). Modi et al. demonstrated that treatment with antibiotics increased the number of ARGs in the intestinal phageome of mice and expanded the interactions between phage and bacterial species (Modi et al., 2013), which is an important observation considering the increased environmental exposure to antibiotics discussed earlier. Furthermore, several studies have used qPCR to detect ARGs in bacteriophages from wastewater samples (Colomer-Lluch et al., 2014a,b), animal and human fecal samples (Colomer-Lluch et al., 2011b; Quiros et al., 2014), wastewater and sludge derived from wastewater treatment plants (Calero-Caceres et al., 2014), and hospital and wastewater treatment plant effluents (Marti et al., 2014), indicating that bacteriophages are significant reservoirs of ARGs. Shousha et al. investigated bacteriophages isolated from chicken meat and found that about a quarter of the randomly isolated bacteriophages were able to transduce resistance to one or more antibiotics into an *E. coli* host. Moreover, they found a significant relationship between the presence of bacteriophages transducing kanamycin ARGs, and *E. coli* isolates resistant to kanamycin, implying a possible role of this mechanism in the spread of AMR (Shousha et al., 2015).

Considering certain bacteriophages have been reported to have a wide host range that crosses between different species (Mazaheri Nezhad Fard et al., 2011) or even different taxonomic classes (Jensen et al., 1998), the observation of the plethora of ARGs carried by bacteriophages in various bacterial communities and environments provides renewed insights into the role of transduction in the dissemination of ARGs in microbial ecosystems.

## Gene Transfer Agents

Gene transfer agents (GTAs), first identified in *Rhodobacter capsulatus* (RcGTA) in 1974 (Marrs, 1974), are host-cell produced particles that resemble bacteriophage structures, capable of

transferring genetic content. GTAs have several characteristic features: (i) rather than carrying DNA encoding their own machinery (as with self-propagating bacteriophages), GTAs carry random pieces of the producing cell's genome (Marrs, 1974; Humphrey et al., 1997; Stanton, 2007; Hynes et al., 2012); (ii) the amount of DNA packaged by the GTAs is insufficient to encode all of their protein components, making them unable to self-propagate (Lang and Beatty, 2000, 2001, 2007); (iii) GTA production is controlled by cell regulatory mechanisms (Lang and Beatty, 2000; Leung et al., 2012; Mercer et al., 2012; Brimacombe et al., 2014); (iv) GTA particles are released through cell lysis (Hynes et al., 2012; Westbye et al., 2013) although cultures do not display observable lysis (Marrs, 1974) as only a small subpopulation of GTA-producing cultures (~3%) is responsible for ~95% of GTA release (Fogg et al., 2012; Hynes et al., 2012); (v) recently, it has been proposed that GTAs combine key aspects of transduction and transformation for cell entry, requiring proteins involved in natural transformation (Brimacombe et al., 2015).

Although GTA particles do not necessarily carry any GTA-encoding genes (Lang et al., 2012), RcGTA-like gene clusters are widespread in alphaproteobacteria, especially in the *Rhodobacterales*: a complete set of RcGTA-like structural genes has been demonstrated in nearly every sequenced member of the *Rhodobacterales* (Lang and Beatty, 2007; Lang et al., 2012). Moreover, two species in the order of *Rhodobacterales*, *Roseovarius nubinhibens* and *Ruegeria mobilis*, are known to produce GTAs, and there is evidence of GTA production in *Ruegeria pomeroyi* (Biers et al., 2008; McDaniel et al., 2010; Lang et al., 2012). Other known GTAs are VSH-1 in the spirochaete *Brachyspira hyodesenteriae*, Dd1 in the delta-proteobacterium *Desulfovibrio desulfuricans*, and VTA in the archaeon *Methanococcus voltae* (Lang and Beatty, 2007; Lang et al., 2012). The genes required for GTA-production are contained within the host genome and appear to have been propagated through vertical transmission (Lang and Beatty, 2007).

It has been suggested that GTAs have several advantages over the previously described mechanisms of HGT (Stanton, 2007): GTA particles afford DNA protection from damaging environmental factors, as opposed to the naked DNA involved in natural transformation; compared to conjugation, the transfer ability of GTAs is likely maintained after conditions killing the host cell, and is moreover not constrained by cell-to-cell contact; lastly, compared to transduction, GTA particles predominantly carry random pieces of host genome, rather than mostly bacteriophage DNA. In the marine environment, GTA-mediated transfer events have been reported to be remarkably high; up to several million times higher than previous estimates

of HGT in marine environments, exceeding previously described transformation and transduction frequencies (McDaniel et al., 2010). Moreover, genes can be exchanged between bacterial phyla (McDaniel et al., 2010; Lang et al., 2012), suggesting the possible widespread contribution of GTAs in shaping and driving adaptation of the natural environment.

In culture, GTA mediated transfer of antibiotic resistance markers has been readily demonstrated in *R. capsulatus* (Marrs, 1974; Solioz et al., 1975; Wall et al., 1975) and the spirochaete *Brachyspira hyodesenteriae* (Stanton et al., 2001, 2008). Moreover, GTAs have been used to transfer traits from plasmids (Scolnik and Haselkorn, 1984). In addition, the *B. hyodesenteriae* GTA VSH-1 can be induced by certain antibiotics (Stanton et al., 2008), which points out its possible impact in its natural environment, the swine intestinal tract. Other *Brachyspira* spp. occur in the intestinal tract of other species, including humans and chickens (Hampson and Ahmed, 2009), in which VSH-1 genes have been described (Motro et al., 2009). However, interspecies GTA-mediated transfer remains to be demonstrated (Motro et al., 2009).

The impact of GTAs on human health has yet to be established, but given the high frequency of transfer events in certain environments, and their ability to exchange genes between phyla, their potential to act as vehicles of resistance traits in the environment, and within the microbiota of humans and farmed animals is an area worthy of further study.

## CONCLUSION

The increase in environmental levels of antibiotics, driven by medical and agricultural demand, is unprecedented and has disrupted the natural balance between microbes and antimicrobials. The effects this has on microbial communities are wide-ranging, and the result is an increasingly tangible threat to healthcare, as resistance to all known antibiotics disseminates rapidly around the globe. Our knowledge of the interactions between antimicrobials and resistance against it, observed not only in the clinic but across different ecosystems around the world, is rapidly increasing and has provided valuable insights. However, it is vital that we continue to unravel the extent of, and dissemination between resistomes of these microbial ecosystems, as any attempt at coming to terms with the AMR problem will have to account for these vast reservoirs of ARGs.

## AUTHOR CONTRIBUTIONS

CW, JN, SM wrote the article. CW, JN made the Figure. JP, LA, PS, PW provided feedback and discussion on the article.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Drug Resistance Mechanisms in Bacteria Causing Sexually Transmitted Diseases and Associated with Vaginosis

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 07 September 2015

Accepted: 03 May 2016

Published: 18 May 2016

### Citation:

Shaskolskiy B, Dementieva E, Leinsoo A, Runina A, Vorobyev D, Plakhova X, Kubanov A, Deryabin D and Gryadunov D (2016) Drug Resistance Mechanisms in Bacteria Causing Sexually Transmitted Diseases and Associated with Vaginosis. *Front. Microbiol.* 7:747.  
doi: 10.3389/fmicb.2016.00747

Here, we review sexually transmitted diseases (STDs) caused by pathogenic bacteria and vaginal infections which result from an overgrowth of opportunistic bacterial microflora. First, we describe the STDs, the corresponding pathogens and the antimicrobials used for their treatment. In addition to the well-known diseases caused by single pathogens (i.e., syphilis, gonococcal infections, and chlamydiosis), we consider polymicrobial reproductive tract infections (especially those that are difficult to effectively clinically manage). Then, we summarize the biochemical mechanisms that lead to antimicrobial resistance and the most recent data on the emergence of drug resistance in STD pathogens and bacteria associated with vaginosis. A large amount of research performed in the last 10–15 years has shed light on the enormous diversity of mechanisms of resistance developed by bacteria. A detailed understanding of the mechanisms of antimicrobials action and the emergence of resistance is necessary to modify existing drugs and to develop new ones directed against new targets.

**Keywords:** human reproductive system, sexually transmitted diseases, bacterial vaginosis, antimicrobials, antimicrobial resistance

## INTRODUCTION

The infections of human reproductive system include the sexually transmitted diseases (STDs) that are defined as infections that spread primarily through person-to-person sexual contact, and non-STDs which are endogenous infections of the genital organs such as bacterial vaginosis (BV). Both STDs and non-STDs are the major concern for public health systems worldwide.

According to the WHO, each year there are approximately half a billion new cases of STDs worldwide (WHO, 2014a), of which 105.7 million are chlamydial infections, 106.1 million are gonorrhea, and 10.6 million are syphilis (WHO, 2013). STDs lead to a decrease in fertility and are harmful to the offspring. Up to 40% of women with untreated gonococcal and chlamydial infections develop inflammatory diseases of the pelvic organs that lead to infertility in 25% of cases; pregnancy in women with the untreated early forms of syphilis results in stillbirth in 25% of cases and neonatal death in 14% (CDC, 2015). Obviously, the timely detection of STDs and adequate antimicrobial therapy are crucial for the successful treatment and prevention of the spread of the disease.

STDs are often polymicrobial and are caused not only by obligate pathogens (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum*, and *Mycoplasma genitalium*) but also by a variety of opportunistic microorganisms which are mostly not sexually transmitted and can occur without sexual relationship. The polymicrobial nature of the reproductive tract pathologies modifies the clinical pattern of the disease, aggravates the inflammatory process, and reduces the efficacy of the determination of their etiologies, which can result in inadequate treatment (Josey and Schwebke, 2008).

A major worldwide problem is the increase in drug resistance of pathogens, which makes it difficult to select proper treatments. In April 2014, the WHO published the report “Antimicrobial resistance: A global report on epidemiological surveillance” (WHO, 2014b). The report emphasized the threat of an increase in the resistance of many pathogens. The greatest concern in addition to tuberculosis, malaria, and staphylococcal infection is the emergence of *N. gonorrhoeae* strains with reduced susceptibility to third-generation cephalosporins. Thus, elucidating the etiology of infections and the concurrent inflammatory processes of the urogenital tract and developing successful therapies require the detection of the primary infectious agents in clinical specimens, the simultaneous identification of a large number of other opportunistic pathogens and an analysis of their susceptibility to common antimicrobial drugs.

This review focuses on the characterization of the most common sexually transmitted bacterial pathogens (excluding viral, fungal, and parasitic infections) and non-STD infections which result from an overgrowth of opportunistic bacterial microflora, and discusses the mechanisms and molecular determinants of their drug resistance.

## BRIEF DESCRIPTION OF THE MOST COMMON BACTERIAL INFECTION OF THE HUMAN REPRODUCTIVE SYSTEM AND THE DRUGS USED TO TREAT THEM

### Syphilis

This disease is caused by the sexually transmitted, Gram-negative, spirochetal bacterium *T. pallidum* and is characterized by lesions of the skin, mucous membranes, nervous system, internal organs, and musculoskeletal system. Syphilis can cause intrauterine infections of the fetus. The disease can occur in both symptomatic and latent forms. Despite significant achievements in the fight against this disease, syphilis remains a challenge for treatment and public health care due to the variations in the course of the disease and the diversity of its clinical manifestations. Syphilis is treated with benzathine penicillin G, tetracycline and its derivative doxycycline, macrolides (e.g., erythromycin and azithromycin), and cephalosporins (e.g., the third generation drug ceftriaxone). The main drug of choice for syphilis treatment in the 2015 CDC recommendations is benzathine penicillin G (Workowski and Bolan, 2015). Patients allergic to penicillin are treated with the other antibiotics mentioned above. In clinical trials, some antibiotics (e.g.,

azithromycin) proved to be more effective than penicillin G for the treatment of early syphilis (Bai et al., 2008).

### Gonococcal Infection

Gonorrhea is one of the most common STDs and is caused by the Gram-negative diplococci *N. gonorrhoeae*. Health complications resulting from the gonococcus disease occur mainly in women and are largely attributed to the predominately asymptomatic nature of lower genital tract, i.e., cervical, infection. Untreated, subclinical infection of the cervix can lead to upper genital tract involvement (e.g., salpingitis) and, potentially, to infertility. The infection primarily affects the genitals, including the cylindrical and glandular epithelium. Although gonococcal vaginitis develops in female children in which menarche has not yet occurred, keratinization occurring with menarche prevents gonococcal vaginitis in the adult female (Edwards and Butler, 2011).

Gonococcal infection is usually treated with ciprofloxacin, ofloxacin, spectinomycin, and  $\beta$ -lactam antibiotics; currently, the most effective recommended drugs are ceftriaxone and azithromycin (Workowski and Bolan, 2015). The widespread occurrence of penicillin-resistant gonorrhea has led to a decrease in the use of first and second generation  $\beta$ -lactams, protected penicillins (clavulanate-potentiated amoxicillin or ticarcillin-clavulanate), and cephalosporins in clinical practice. Ceftriaxone belonging to cephalosporin drugs, that are more resistant to  $\beta$ -lactamases, remains widely used.

Spectinomycin, from the aminocyclitol class of drugs closely related to the aminoglycosides, since 1960s was used for specific treatment of gonorrhea. It was useful in an alternative therapy for gonorrhea resistant to antibiotics of the other groups or in patients allergic to  $\beta$ -lactams. After 10–20 years of treatment, spectinomycin-resistant gonococcal strains were reported in Netherlands, South Korea, United Kingdom, and USA (Unemo and Shafer, 2014). There is a consistent trend of increasing resistance of gonococci to spectinomycin in Russia (up to 15% in 2010) (Kubanova et al., 2010, 2014). Spectinomycin is not used now as a first-line drug for gonorrhea therapy in many countries.

### Chlamydiosis

The infection is caused by the Gram-negative intracellular bacterium *C. trachomatis*. There are 15 identified serovars of *C. trachomatis*. Serotypes Ab, B, Ba, and C are pathogens that cause trachoma, serovars D, E, F, G, H, I, J, and K cause urogenital chlamydiosis, urethritis, prostatitis, vaginitis, and chlamydial cervicitis, and serovars L1, L2, and L3 cause lymphogranuloma venereum and genital ulcers (Workowski and Bolan, 2015). The socio-economic significance of urogenital chlamydial infection is due not only to the high rate of disease but also to a significant percentage of complications, especially the development of secondary infertility in men and women (Vasilevsky et al., 2014).

The drugs of choice for the treatment of chlamydial infections are tetracyclines (doxycycline) and macrolides (josamycin and azithromycin); alternatively, fluoroquinolones (levofloxacin and ofloxacin) can be used (Workowski and Bolan, 2015). Despite the availability of a whole range of drugs against chlamydia, its

treatment is not always successful due to the presence of co-infections with the causative agent of urogenital ureaplasmosis (*Ureaplasma urealyticum*). Thus, adequate therapy requires the accurate identification of additional microorganisms associated with the chlamydia infection and the simultaneous treatment of opportunistic infections.

### Mycoplasmosis and Ureaplasmosis

The diseases are caused by opportunistic microorganisms of the genus *Mycoplasma* (*M. genitalium* and *Mycoplasma hominis*) and *Ureaplasma*. The latter is classified in a separate genus due to its ability to break down urea. Researchers have identified two species of *Ureaplasma* (*U. parvum* and *U. urealyticum*) that are further classified into 14 serovars. Mycoplasma and ureaplasma are detected in more than 40% of patients with inflammatory diseases of the urogenital system; three species have the highest clinical significance (*M. genitalium*, *U. urealyticum*, and *M. hominis*). Mycoplasma and ureaplasma can cause urethritis in both sexes and probably cervicitis, cystitis, and pregnancy complications including post-partum period and post-abortion complications (Larsen and Hwang, 2010). The drugs of choice for the treatment of infections are macrolides (azithromycin and josamycin) and tetracyclines (doxycycline). Alternative medications are fluoroquinolones (levofloxacin and ofloxacin) (Workowski and Bolan, 2015).

### Bacterial Vaginosis (BV)

BV is an infectious non-inflammatory disease of a polymicrobial nature that is predominantly detected in women of reproductive age. The disease is not immediately life-threatening, but it is a risk factor for pregnancy complications and pelvic inflammatory diseases. Moreover, BV is associated with increased risk for acquiring sexually transmitted infections (Martin, 2012). For example, BV can increase a woman's risk of acquiring *N. gonorrhoeae* (gonococcal infection hazard ratio: 1.7; 95% CI: 1.1–2.6), *C. trachomatis* (chlamydial infection odds ratio: 3.4; 95% CI: 1.5–7.8), and *Trichomonas* (trichomonal genital infection hazard ratio: 1.8; 95% CI: 1.3–2.4) (Margolis and Fredricks, 2015).

BV occurs as the result of a drastic imbalance in the normal vaginal microflora rather than as an invasion of a foreign pathogen. In this case, the normal protective lactobacilli are replaced by high quantities of anaerobes (Turovskiy et al., 2012; Margolis and Fredricks, 2015). Among the most commonly detected causative agents of the disease are the microaerophilic bacteria *Gardnerella vaginalis*, obligate anaerobic Gram-positive bacteria (*Mobiluncus* spp. and *Peptostreptococcus* spp.), and the facultative anaerobic bacteria *Atopobium vaginale*. Additionally, the obligate anaerobic Gram-negative bacteria *Prevotella* spp., *Bacteroides* spp., and *Fusobacterium* spp. are sometimes present in clinical samples (Verhelst et al., 2004; Menard, 2011). The list of bacteria that have been shown to be associated with vaginosis is constantly widening (i.e., *Dialister*, *Prevotella*, and *Megasphaera*) (Margolis and Fredricks, 2015).

*G. vaginalis* bacteria are commonly detected at moderately high concentrations in BV-positive women but their presence alone is not specific for BV (Kalra et al., 2007). The frequent detection of genital mycoplasmas together with anaerobic

microflora is due to the ability of *G. vaginalis* to secrete succinic acid, which is used by other microorganisms. In turn, ureaplasma and mycoplasma, which actively consume oxygen during their metabolism, stimulate the proliferation of anaerobic bacteria (Africa et al., 2014). Agents active against *G. vaginalis* include metronidazole and clindamycin.

The proportion of *A. vaginae* (another causative agent of vaginitis) ranges from 50 to 95%. This Gram-positive anaerobic bacillus from the *Coriobacteriaceae* family produces organic acids and can be found together with *G. vaginalis* within the layer of microorganisms on the surface of the vaginal mucosa in bacterial vaginosis patients (Verhelst et al., 2004). The dominant growth of *A. vaginae* is also treated with metronidazole and clindamycin.

*Mobiluncus* is a motile anaerobic microorganism belonging to the family *Actinomycetaceae*. Two species of the genus *Mobiluncus* are usually found in the human genitourinary tract (*M. curtisi* and *M. mulieris*). Both bacteria are susceptible to β-lactam antibiotics, glycopeptide drugs, clindamycin, chloramphenicol, and rifampicin and are primarily resistant to metronidazole (Spiegel, 1987). Currently, infections caused by *Mobiluncus* spp. are commonly treated with clindamycin (Verstraelen and Verhelst, 2009).

*Bacteroides fragilis* is a Gram-negative anaerobic bacterium that is a constituent of the normal microflora of the vagina and vulva. *B. fragilis* may cause pyelonephritis, cystitis, urethritis, prostatitis, and other infectious diseases of the genitourinary system in both sexes. The main drug for the treatment of infections caused by *B. fragilis* is metronidazole (Löfmark et al., 2010); alternative treatments include β-lactam antibiotics in combination with β-lactamase inhibitors, clindamycin and chloramphenicol.

Taking into account the leading role of obligate anaerobic bacteria in BV, the drugs of choice for BV treatment are those with anti-anaerobic activity (e.g., clindamycin and 5-nitroimidazole derivatives). Notably, effective BV treatment must be based on a differential diagnosis with other urogenital diseases caused by pathogens (*N. gonorrhoeae*, *C. trachomatis*, *Trichomonas vaginalis*, and *M. genitalium*) (Menard, 2011; Workowski and Bolan, 2015).

## DRUG RESISTANCE OF STD PATHOGENS

The continuously increasing resistance of STD pathogens to antimicrobial agents is a worldwide problem. Antibiotics that have lost their effectiveness are replaced by new drugs, but new strains appear with new determinants of resistance; this issue applies to all classes of drugs. The increase in the resistance and diversity of the drugs used in clinical practice leads to the emergence of bacteria with multidrug resistance (MDR). Some strains with MDR have the ability to spread rapidly (so-called high-risk clones). Some pathogens acquire simultaneous resistance to most drugs developed for their treatment (extensively drug-resistant pathogens) or even to all drugs (pandrug resistance) (Unemo and Nicholas, 2012;

Rossolini et al., 2014). Currently, multidrug-resistant isolates have been identified for both Gram-positive and Gram-negative species, including *N. gonorrhoeae*.

### **T. pallidum**

Although diagnostic tests for syphilis and antibiotic therapy are now available, the disease remains endemic in many developing countries. Widespread syphilis epidemics occurred in Russia in the 1990s (Stamm, 2010) and more recently in China (Tucker and Cohen, 2011). A recent increase in syphilis rates in women and infants in the USA has also been described (CDC, 2015).

Although penicillins have been used for the treatment of syphilis for 70 years, no natural penicillin-resistant forms have been found. To date, no clinically proven cases of treatment failure using penicillin-related drugs have been described in patients with syphilis. However, the possibility of the emergence of acquired resistance is still discussable (Stamm, 2010). Recent analysis of *T. pallidum* resistance to antibiotics in the Russian Federation revealed no resistance to  $\beta$ -lactam antibiotics. Specifically, no meaningful mutations were found in the *tp47* and *tromp* genes encoding the targets of  $\beta$ -lactams (Kubanova et al., 2013).

Currently, there is no documented resistance of *T. pallidum* to the tetracyclines. Clinically significant resistance of *T. pallidum* to macrolides (a second-line alternative to penicillin) has been demonstrated by many authors, and macrolide-resistant strains are now prevalent in several developed countries (Stamm, 2010). Clinical cases of *T. pallidum* resistance to macrolides (particularly erythromycin; Stamm and Bergen, 2000), azithromycin (Katz and Klausner, 2008; Stamm, 2010), clarithromycin (Stamm, 2010), and spiramycin (Matějková et al., 2009) have been reported. There was a report concerning treatment failure with clindamycin but these data were not confirmed and *T. pallidum* appeared to have intrinsic resistance to this drug (Stamm, 2010).

The first strain resistant to erythromycin and azithromycin was isolated in 1977 (Street strain 14) (Stamm, 2012). Sequencing of the *T. pallidum* genome has shown that it lacks the genetic elements responsible for horizontal gene transfer (plasmids and transposons); thus, it was concluded that the resistance to macrolides in Street strain 14 emerged endogenously by a spontaneous chromosomal mutation in the 23S rRNA gene. The mutations responsible for macrolide resistance were shown to be A2058G and A2059G (23S rRNA). Mutation A2058G was reported in several areas of the USA, Canada, Europe, and China (Stamm, 2012), whereas the replacement of A2059G was less common but was not geographically isolated (Matějková et al., 2009; Cruz et al., 2013). There were reports concerning patients with *T. pallidum* bearing the A2059G mutation in the Czech Republic (Matějková et al., 2009), Columbia (Cruz et al., 2013), and the UK (Tipple et al., 2011). The proportion of isolates resistant to macrolides has been growing, indicating a need for further drug development and surveillance for resistance in *T. pallidum* (Tipple et al., 2011).

### **N. gonorrhoeae**

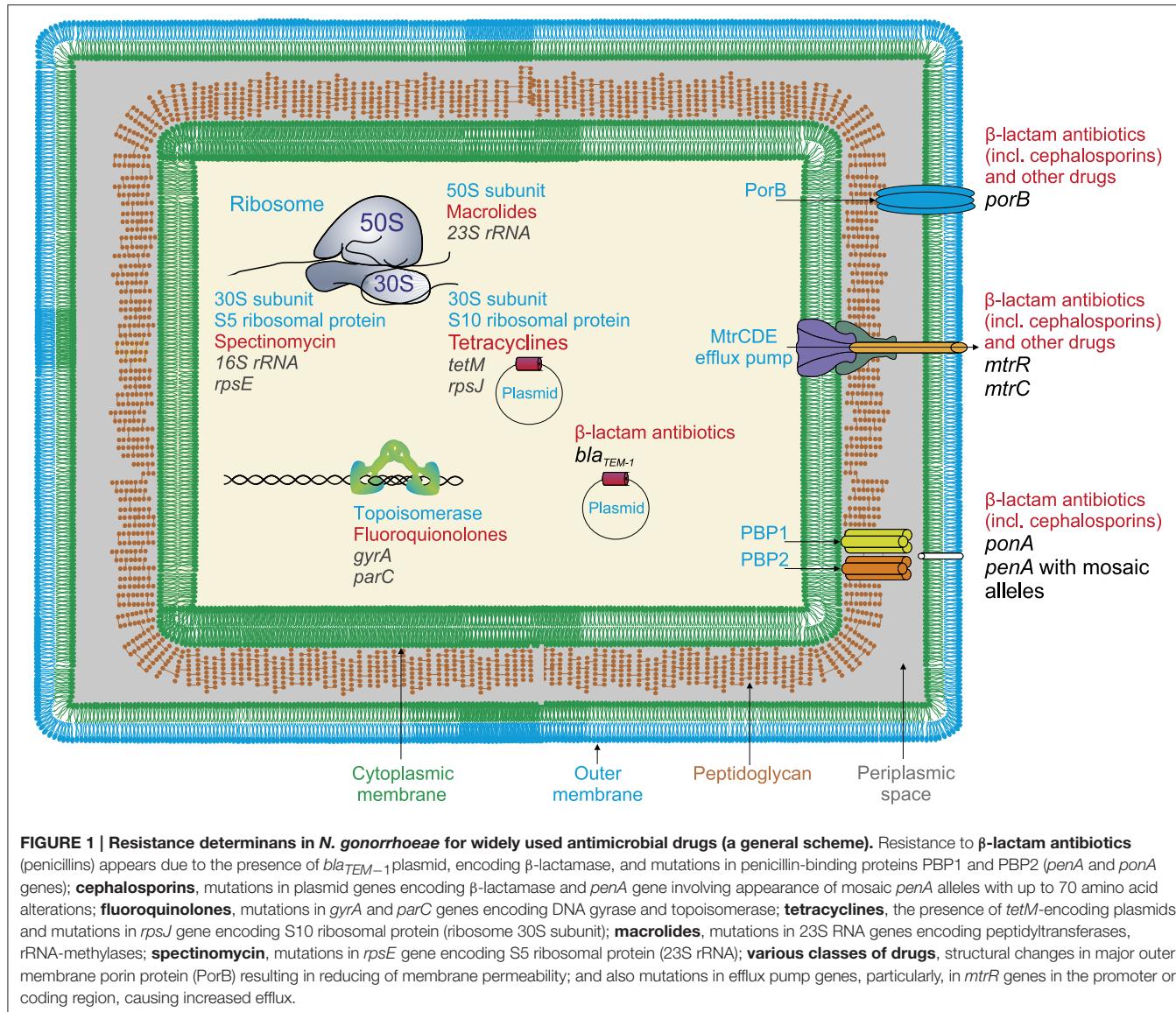
The pathogen *N. gonorrhoeae* is characterized by an extraordinary ability to develop resistance to clinically used

antimicrobial drugs within 10–20 years (Unemo and Nicholas, 2012). *N. gonorrhoeae* is able to quickly accumulate mutations and acquire resistance to drugs, including multiple drug resistance. There are now strains of *N. gonorrhoeae* that are resistant to all major drugs used to treat gonorrhea, including  $\beta$ -lactams, fluoroquinolones, macrolides, tetracyclines, spectinomycin, cephalosporins, and azithromycin. The spread of super-resistant forms of the gonorrhea pathogen is a major concern and requires the strengthening of control on a global scale (WHO, 2014b). Given the rate of increase of *N. gonorrhoeae* resistance to other drugs, some cases of gonorrhea may become incurable by 2021 because no vaccines or new drugs are being developed (Unemo, 2015). Unemo and his coworkers in Sweden and the WHO report have repeatedly stressed that the situation with drug-resistant gonorrhea can spiral out of control; thus, it is extremely urgent to develop rapid genetic analysis methods to monitor the resistance patterns of the pathogen worldwide and to apply these data to the treatment of patients (Unemo et al., 2014; WHO, 2014b).

Gonococci use most of the known mechanisms to acquire resistance: the inactivation of the antibiotic, alteration of the drug binding sites, reduction of membrane permeability, and increased drug efflux (Figure 1). The bacteria acquire these new characteristics via chromosomal mutations, plasmids carrying the determinants of resistance (i.e., TEM-1, TEM-135-encoding plasmids for resistance to penicillins, TetM-encoding plasmids for tetracycline resistance), and horizontal gene transfer from other species, especially from other species of *Neisseria*. Mutations altering the membrane permeability and increasing the activity of the efflux pumps are particularly efficient in the emergence of in *N. gonorrhoeae* because both mechanisms affect a wide variety of drugs.

When the penicillin treatment regimen was initiated in the 1940s, this drug was highly effective against gonococci. However, within the next 10–15 years a gradual decrease in penicillin susceptibility was observed; this decrease was mainly due to the sequential accumulation of chromosomal mutations in the bacteria. As the result, strains with MIC for penicillin as high as 4.0 mg/L were formed. Over the next few years, the same process was repeated with erythromycin, tetracycline, and azithromycin (Unemo and Shafer, 2014).

There are several mechanisms that explain the resistance of *N. gonorrhoeae* to  $\beta$ -lactam antibiotics. First, the bacteria produce TEM  $\beta$ -lactamases using a plasmid gene (*N. gonorrhoeae* does not produce chromosomally-encoded beta-lactamases). Because the gonococcal  $\beta$ -lactamase encoded by the plasmid gene *bla<sub>TEM-1</sub>* is not able to destroy cephalosporins, these antibiotics have taken a leading role in the treatment of gonorrhea (Klausner and Kerndt, 2013). Mutations that decrease the affinity of the corresponding penicillin-binding proteins PBP1 and PBP2 to penicillins have been identified in the chromosomal genes *ponA* and *penA*. The main mutation in the *ponA* gene leading to the development of resistant phenotype is L421P (Lindberg et al., 2007; Endimiani et al., 2014), whereas an insertion in the *penA* gene leads to the appearance of D345 (Lindberg et al., 2007; Jeverica et al., 2014). The insertion of an extra codon for D345 in the wild-type



*penA* gene results in a four-fold reduction in the susceptibility of gonococci to penicillin (Spratt, 1988).

In addition to the various mechanisms of penicillin resistance of *N. gonorrhoeae* described above, a mutation in the *pilQ2* gene (E666K) was found to increase resistance to penicillin *in vitro*. E666K works in combination with mutations in the *penA*, *mtrR*, and *penB* genes (Zhao et al., 2005; Helm et al., 2007). Because mutations in the *pilQ2* gene disrupt the formation of pili, which are required for the pathogenicity of gonococci, it is unlikely that similar genotypes could be found in clinical specimens.

During the past few years, the rate of gonococcal strains resistant to penicillin in the Russian Federation has varied between 9.6 and 13.2% (Kubanova A. et al., 2014). Globally, the proportion of resistant *N. gonorrhoeae* strains has widely varied: 81% in China, 85% in Switzerland, 79% in Hungary, 27% in Poland, and 9.9% in Belarus in 2009 (Unemo, 2015). At present, strains have been isolated that exhibit resistance

to extended-spectrum cephalosporins (including ceftriaxone), which are the last drugs available for gonorrhea monotherapy. The detections of isolates with decreased susceptibility to cephalosporins (especially ceftriaxone) has been reported from all over the world (Unemo and Shafer, 2014). On average, the proportion of strains with reduced susceptibility to cefixime (MIC > 0.125 mg/L) in the European Union is approximately 8% (Unemo, 2015).

The reduced susceptibility mechanism is dependent on the mosaic organization of the *penA* gene, which may result from genetic recombination between the *N. gonorrhoeae*, *N. cinerea*, and *N. perflava* species (Ohnishi et al., 2011). The resistance of *N. gonorrhoeae* (especially broad drug resistance to penicillins and cephalosporins) is associated with the emergence of mosaic alleles of *penA* and non-mosaic alleles carrying the A501 mutation. Mosaic alleles contain up to 70 amino acid substitutions compared to the wild-type protein. The mutations

G545S, I312M, and V316T were found in strains resistant to broad spectrum cephalosporins. Their resistance can be explained by modification of the  $\beta$ -lactam binding site (Unemo et al., 2012; Lewis et al., 2013; Golparian et al., 2014). Notably, the introduction of these three mutations in the wild-type PBP2 has almost no effect on the resistance of the pathogen *N. gonorrhoeae*; these mutations enhance pathogen resistance only in the presence of other mosaic alleles in the *penA* gene. It is possible that this effect is due to epistasis (the suppression of a non-allelic gene) (Tomberg et al., 2010).

Reduced susceptibility of the gonorrhea pathogen to ceftriaxone is also associated with mutations of codon 501 in non-mosaic alleles of *penA* (most often A501V but sometimes A501T). The A501 mutation is assumed to be specific for *N. gonorrhoeae* because it is not found among other *Neisseria*; this mutation possibly appeared spontaneously in gonococci under selective antibiotic pressure and was not acquired from another species (Unemo et al., 2012). A detailed study of mutations in the *penA* gene of *N. gonorrhoeae* leading to its resistance to third generation cephalosporins (cefixime and ceftriaxone) was published by Kubanova et al. (Kubanova et al., 2014). Increased resistance to ceftriaxone was observed in the presence of mutations at positions 346, 505, 511, 517, 543, 567, 575, and 576 of PBP2. The replacement of glycine with serine at position 543 results in a manifold increase in resistance to ceftriaxone.

The first highly resistant strain to ceftriaxone (H041; NG-MAST ST4220) was a sporadic case in Japan. Another strain with a similar level of resistance (F89; NG-MAST ST1407; and MLST ST7363) was first described in France and now has spread throughout the world. These strains cause unsuccessful attempts of patient treatment with cefixime and ceftriaxone (Unemo and Nicholas, 2012; Morita-Ishihara et al., 2014). The *N. gonorrhoeae* H041 and F89 strains are highly resistant to third generation cephalosporins with a minimal inhibitory concentration (MIC) of 2–4 mg/L. An investigation of the *N. gonorrhoeae* strain H041 revealed 13 additional amino acid substitutions in a mosaic allele X *penA* compared to the mosaic *penA* gene from the strain with intermediate-level cephalosporin resistance (specifically, the mutations A311V and T316S located near the  $\beta$ -lactam-binding pocket close to the active-site nucleophile Ser310 and the mutation Thr483 that may interact with the carboxylate of the  $\beta$ -lactam antibiotic) (Tomberg et al., 2013). Two cases of extended-spectrum cephalosporin-resistant *N. gonorrhoeae* were recently isolated from patients in South Africa. These strains were shown to belong to a multidrug-resistant gonococcal clone (MLST ST1901) that was associated with several cefixime treatment failures in Europe and North America (Lewis et al., 2013).

The resistance of *N. gonorrhoeae* to macrolides (azithromycin) is caused by the presence of the *erm* (erythromycin ribosome methylation) and *mef* (associated with the active efflux of antibiotics) genes. The substitution C2611T (Ng et al., 2002) in the 23S rRNA gene (the *rrl* gene) leads to the emergence of strains with moderate resistance, whereas A2059G (Katz et al., 2012; Unemo et al., 2014) leads to the emergence of highly resistant strains. In addition to the nucleotide substitutions themselves, resistance depends on the number of mutant alleles. Unemo et al. (2014) demonstrated that the presence of the C2611T

substitution in all four alleles resulted in a high level of resistance, whereas strains with one mutant allele did not differ from the wild type.

*N. gonorrhoeae* becomes resistant to tetracycline either of the expression of a plasmid-encoded TetM protein or by a combination of three gene mutations: (i) the *mtrR* mutation, which results in overexpression of an nonspecific efflux pump (MtrC-MtrD-MtrE) that promotes the efflux of a range of hydrophobic agents and detergents; (ii) the *penB* determinant, which is a mutated porin IB that decreases the influx of tetracycline into the cell, and (iii) and mutations in the *rpsJ* gene that result in the amino acid substitution V57M in the ribosomal protein S10 (Nguyen et al., 2014). Although the combination of these mutations does not confer a level of tetracycline resistance as high as that observed with tetracycline-specific efflux pumps or the TetM determinant, the *mtrR-penB-rpsJ1* gene triad is highly effective and provides levels of resistance above those clinically achievable at the site of infection. In the same time, the *rpsJ* allele acts independently of other resistance factors and increases the MIC of tetracycline between three- and four-fold (Hu et al., 2005).

There are several determinants of *N. gonorrhoeae* resistance to spectinomycin that binds to the 30S ribosomal subunit and inhibits translation during elongation by blocking the EF-G-catalyzed translocation of peptidyl-tRNA from the A site to the P site. One of these determinants affects the segment formation of the spectinomycin binding site (*rrs* gene) by introducing one mutation in the 16S rRNA (G1064C, G1058C, or C1192U) (Galimand et al., 2000). A high-level spectinomycin-resistant (MIC > 1 mg/L) *N. gonorrhoeae* strain was isolated in Norway. The resistance determinant was a deletion of codon 27 (valine) and a K28E alteration in the ribosomal protein S5 (Unemo et al., 2013). Mutation T24P in the *rpsE* gene leading to spectinomycin resistance has also been described (Ilina et al., 2013). Generally, there are few examples of MIC-verified stains of *N. gonorrhoeae* with high-level resistance to spectinomycin (Kirkcaldy et al., 2013; Unemo, 2015). However, in Russia the proportion of spectinomycin-resistant isolates increased from 0.9 to 11.6% in a short period between 2009 and 2012 (Kubanova A. et al., 2014).

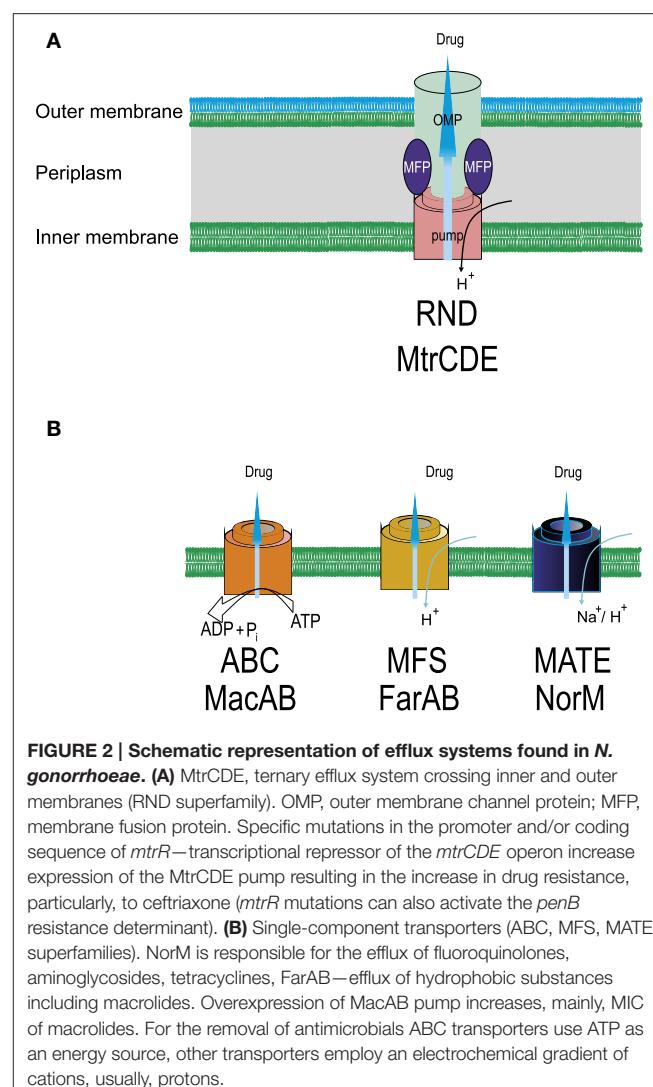
At the end of 80's, since the introduction of quinolones (fluoroquinolones) for the primary treatment of uncomplicated gonorrhea, most of the isolates of gonococci were found to be extremely susceptible to fluoroquinolones. Since the importance of antimicrobial susceptibility studies was understood, data concerning fluoroquinolone-resistant *N. gonorrhoeae* strains became available from all parts of the world (Knapp et al., 1997). In these studies, it was evident that the resistance toward fluoroquinolones, which is chromosomally mediated, develops in an incremental manner. The initial isolates which were less susceptible toward ciprofloxacin were found to have MIC values of 0.06 mg/L, which gradually increased to 1 mg/L (such strains being referred to as intermediate resistant) and later to as high as 16 mg/L (classified as resistant isolates). Strains with MIC > 4 mg/L were considered as high level resistance strains.

The main mechanism of resistance to fluoroquinolones is the decrease in the affinity of the drug for the DNA-enzyme complex. This decrease is caused by mutations in the *gyrA*,

*gyrB*, *parC*, and *parE* genes in the topoisomerase polypeptide chains that form the “quinolone pocket.” The resistance of *N. gonorrhoeae* to fluoroquinolones is associated with mutations S91F and D95N/G in *gyrA* gene and D86N, S88P, and E91K in *parC* gene (Vereshchagin et al., 2004). Mutations in *parC* at positions 87, 104, and 131 have also been described. Isolates with high level resistance may carry several mutations in the *gyrA* and *parC* genes (Vereshchagin et al., 2004; Pottumarthy et al., 2006). The more recent (fourth generation) fluoroquinolones are more active against strains with altered ParC, but are less effective against GyrA mutants, thus, these compounds will in theory, be active against some, but not all, ciprofloxacin-resistant gonococci (Patel et al., 2011).

Resistance of *N. gonorrhoeae* to different antibiotics also arises as the result of modifications in the protein that transports antibiotics across the outer membrane (the porin PorB). In *N. gonorrhoeae*, porins are encoded by the *porB* gene and have a molecular mass ranging from 34 to 38 kDa in different strains (Zeth et al., 2013). Protein molecules form three-dimensional structures that puncture the outer membrane. Analysis of the penicillin- and tetracycline-resistant strains of *N. gonorrhoeae* revealed the G120D, A121D, and G121K mutations in the porin protein. Further studies demonstrated that these mutations were important for the diffusion of antibiotics (Olesky et al., 2006). In the same work, Olesky et al. described the G120K, G120D, and A121D mutations in the gene encoding *N. gonorrhoeae* porins. These mutations in the porin genes are widely spread and have been reported in Russia (Ilina et al., 2008), Switzerland (Endimiani et al., 2014), and Canada (Thakur et al., 2014). Notably, mutations in the *porB* gene do not increase the resistance of *N. gonorrhoeae* to  $\beta$ -lactams in the absence of mutations in the *mtrR* gene (a transcriptional repressor of the *mtrCDE* operon). Thus, the increase in resistance is caused by combined effort of the MtrC-MtrD-MtrE pump and PorB1b, which decreases the concentration of the antibiotic in the periplasm of the microorganism (Veal et al., 2002; Olesky et al., 2006).

Four efflux pump systems (MtrCDE, MacAB, NorM, and FarAB) have been found in gonococci belonging the RND, ABC, MATE, and MFS superfamilies (Alvarez-Ortega et al., 2013; Sun et al., 2014; Li et al., 2015; Sharkey et al., 2016), respectively (Figure 2). The most studied efflux pump from *N. gonorrhoeae* (MtrC-MtrD-MtrE from the RND superfamily) is responsible for the efflux of  $\beta$ -lactam antibiotics, macrolides, rifampin, detergents, fatty acids, steroid hormones, and cationic peptides (Veal et al., 2002). The efflux system involves a linker (MFP), a transporter, and a protein that forms a channel across the outer membrane (OMP protein). Inactivation of the MtrCDE pump in clinical isolates displaying high levels of MDR leads to significant decrease in the resistance to azithromycin, penicillin, and tetracycline (Golparian et al., 2014). Mutations in *mtrR* gene lead to the emergence of penicillin-resistant strains by increasing the expression of the MtrC-MtrD-MtrE efflux pump. Some of these mutations are located in the coding region and cause the amino acid substitutions A39T, R44H, G45D, and L47P (Ilina et al., 2008; Endimiani et al., 2014); others affect the promoter region (e.g., the insertions insTT at position -10 and deletion



**FIGURE 2 | Schematic representation of efflux systems found in *N. gonorrhoeae*.** (A) MtrCDE, ternary efflux system crossing inner and outer membranes (RND superfamily). OMP, outer membrane channel protein; MFP, membrane fusion protein. Specific mutations in the promoter and/or coding sequence of *mtrR*—transcriptional repressor of the *mtrCDE* operon increase expression of the MtrCDE pump resulting in the increase in drug resistance, particularly, to ceftriaxone (*mtrR* mutations can also activate the *penB* resistance determinant). (B) Single-component transporters (ABC, MFS, MATE superfamilies). NorM is responsible for the efflux of fluoroquinolones, aminoglycosides, tetracyclines, FarAB—efflux of hydrophobic substances including macrolides. Overexpression of MacAB pump increases, mainly, MIC of macrolides. For the removal of antimicrobials ABC transporters use ATP as an energy source, other transporters employ an electrochemical gradient of cations, usually, protons.

*delA* at position -35) (Lindberg et al., 2007; Ilina et al., 2008; Endimiani et al., 2014). The most common mutations are the deletion *delA* in the promoter region and the substitution G45D in the coding region of the gene. Other rarer mutations also exist, such as the insertion of 153 bp between the *mtrR/mtrC* promoter and the *mtrC* gene. The resistance of *N. gonorrhoeae* to fluoroquinolones is greatly increased by the G45D or -35 *delA* mutations (Zarantonelli et al., 1999).

The *macA* and *macB* genes for MacAB system are organized as an operon. A SNP in the promoter region increases the transcription of *macAB* and results in an increased resistance to macrolides (Rouquette-Loughlin et al., 2005). The Na/cation antiporter NorM belonging to MATE superfamily in *N. gonorrhoeae* removes fluoroquinolones, aminoglycosides, and cationic dyes. Inactivation of NorM results in an up to eight-fold decrease in the MIC for tetracycline (Golparian et al., 2014). Mutation of the promoter region of the *norM* gene has been shown to reduce susceptibility to the fluoroquinolones ciprofloxacin and norfloxacin (Rouquette-Loughlin et al., 2003).

In addition, the *mef*-encoded efflux pump protein, which is located on a conjugative transposon has been also found in *N. gonorrhoeae* strains. The protein exports macrolides out of the cell, thus, its overexpression results in resistance to macrolides (Luna et al., 2000). There is a synergistic interaction between the change in membrane permeability and the activity of efflux pumps. For example, simultaneous mutations in the porin protein and efflux pump from the RND superfamily in *N. gonorrhoeae* result in a significant increase in pathogen resistance to penicillin and ceftriaxone (Shafer and Folster, 2006).

As mentioned above, a multidrug-resistant strain from South Africa (Lewis et al., 2013) was resistant to cefixime, ciprofloxacin, penicillin, and tetracycline, exhibited intermediate resistance to azithromycin and was susceptible to ceftriaxone and the aminoglycosides, gentamicin and spectinomycin. The pathogen carried determinants of resistance for several antibiotics: mutations in the promoter region of *mtrR* gene (deletion A) resulting in overexpression of the MtrC-MtrD-MtrE efflux pump; G101K and A102N in *porB1b* encoding loop 3 of PorB1b, which decreases the influx of antibacterial drugs into the cell; mutation L421P in the *ponA1* gene that encodes the second penicillin target PBP1, thereby reducing the penicillin acylation of PBP1; and mosaic allele *penA*, type XXXIV, encoding the penicillin-binding protein PBP2.

Thus, *N. gonorrhoeae* has developed resistance to all antimicrobials introduced for first-line therapy during the last 70–80 years, and, probably, for more sustainable future treatment. To overcome this problem it is necessary to focus not only on derivatives of previously developed antimicrobials but also on the development and investigation of new targets, compounds, and approaches for treatment. As an example of such developments, the topoisomerase II inhibitor ETX0914 (also known as AZD0914) belonging to spiropyrimidinetriones was introduced as an antimicrobial compound. The mechanism of its action differs from the mechanisms of all previously and currently used drugs. The ETX0914 pharmacodynamic properties, antimicrobial susceptibility and genetic resistance mechanisms are now extensively studied for the future application as oral monotherapy or in dual combinations with ciprofloxacin, azithromycin, or ceftriaxon (Foerster et al., 2015).

## C. trachomatis

Information concerning the occurrence and clinical importance of *C. trachomatis* resistance to antibiotics is scarce and contradictory (Kohlhoff and Hammerschlag, 2015). Cases of multidrug resistance have been reported in *C. trachomatis* (Shkarupeta et al., 2007; Sandoz and Rockey, 2010). For example, failure of treatment with macrolides and doxycycline was reported in patients with chlamydial infection. Clinically isolated strains resistant to doxycycline, azithromycin, josamycin, spiramycin, and ofloxacin were reported, as were multidrug-resistant strains. Although certain molecular markers associated with resistance to macrolides and fluoroquinolones have been described, no functional relationship between the presence of these markers and the efficacy of antibiotic therapy for Chlamydia has been established. No mutations were found in the *gyrA*, *gyrB*, *parC*, and *parE* genes (resistance to

fluoroquinolones) and in the 23S rRNA V-domain, which contains macrolide resistance-associated mutations in *C. trachomatis* clinical isolates obtained after ineffective therapy of urogenital chlamydiasis with fluoroquinolones and macrolides (Shkarupeta et al., 2007).

It is typical for chlamydia to acquire so-called heterotypic resistance, a form of phenotypic resistance in which a small proportion of an infecting microbial species is capable of expressing resistance at any one time. Strains isolated from patients after the failure of treatment with antimicrobial drugs are fully sensitive to the same drugs *in vitro*, and only a small fraction of the chlamydia population (<1%) survives in the presence of high concentrations of the drugs (Shkarupeta et al., 2007).

## Mycoplasma and Ureaplasma spp.

Because members of the genera *Mycoplasma* and *Ureaplasma* have no cell wall, they are insensitive to all types of  $\beta$ -lactam antibiotics (natural susceptibility). Sulfonamide at physiological concentrations also has no effect on these bacteria due to the absence of the metabolic pathway for the synthesis of folic acid. High rates of resistance to erythromycin and tetracycline in clinical specimens (from 73 to 97%) were reported for *Ureaplasma* species and *M. hominis*. Speciation indicated that *U. parvum* was the predominant *Ureaplasma* spp. that conferred antimicrobial resistance. Specimens resistant to macrolides were found, and cases of clinical and microbiological treatment failure with moxifloxacin were also reported (Redelinghuys et al., 2014). An increase in fluoroquinolone-resistant *M. genitalium* strains in Japan has been recently reported, and the prevalence of macrolide and fluoroquinolone resistance-associated mutations in DNA specimens from men with non-gonococcal urethritis has been determined (Kikuchi et al., 2014).

The resistance of *Mycoplasma* and *Ureaplasma* to antibiotics is primarily associated with mutations in the 23S rRNA (macrolides), *gyrA*, *gyrB*, *parC*, or *parE* gene (fluoroquinolones) (Table 1). The mutations responsible for macrolide, lincosamide, streptogramin, or ketolide group resistance occur in 23S rRNA at positions 2610, 2611, 2057, 2059, and 2062 in *M. hominis* (*Escherichia coli* numbering) and positions 2058 and 2059 in *M. genitalium*. Deletions and insertions in the L4 ribosomal proteins and mutations in the 23S rRNA at positions 2056, 2057, and 2058 are associated with macrolide resistance in *Ureaplasma* spp. (Couldwell et al., 2013; Salado-Rasmussen and Jensen, 2014; Waites and Xiao, 2015). The efflux genes and *erm* gene that contribute to the resistance of *Ureaplasma* to macrolides have been detected in only one work (Lu et al., 2010) and have not been confirmed by other investigators.

Susceptibility of the *M. hominis* and *Ureaplasma* spp. to tetracyclines was studied in South Africa (Redelinghuys et al., 2014). The high-level resistance to tetracyclines in *M. hominis* and *Ureaplasma* spp. is explained by the presence of the *tet(M)* determinant that provides ribosome protection and represents the sole tetracycline resistance mechanism acquired by clinical isolates of human mycoplasmas (Dégrange et al., 2008). For *M. genitalium*, treatment failures with tetracycline were reported but no genes responsible for the resistance were identified (Waites and Xiao, 2015).

**TABLE 1 | Molecular determinants of drug resistance and phenotypic susceptibility in Mycoplasmas and Ureaplasmas.**

Antimicrobial drug	Determinants of drug resistance	Range of minimal inhibitory concentrations for resistant isolates (mg/L)
<b><i>M. genitalium</i></b>		
Macrolides, lincosamides, streptogramins, ketolides	Mutations in the 23S rRNA gene at positions 2058 and 2059* Mutations in L4 ribosomal protein	16–64 for erythromycin
Tetracyclines	No determinants have been detected for isolates obtained in cases of treatment failures	No data available
Fluoroquinolones	Mutations in the <i>gyrA</i> , <i>gyrB</i> , <i>parC</i> , or <i>parE</i> genes	No data available
<b><i>M. hominis</i></b>		
Macrolides, lincosamides, streptogramins, ketolides	Mutations in the 23S rRNA gene at positions 2610, 2611, 2057, 2059, and 2062*	16–64 for clindamycin
Tetracyclines	<i>tet(M)</i> determinant (ribosomal protection)	8–64 for tetracyclines
Fluoroquinolones	Mutations in the <i>gyrA</i> , <i>gyrB</i> , <i>parC</i> , or <i>parE</i> genes	2–32 for levofloxacin 4–8 for ciprofloxacin
<b><i>U. urealyticum</i> and <i>U. parvum</i></b>		
Macrolides, lincosamides, streptogramins, ketolides	Deletions or insertions in L4 ribosomal proteins and/or mutations in the 23S rRNA gene at position 2056, 2057, and 2058 Ribosomal methylation mediated by the <i>ermB</i> gene <i>mrsA/mrsB/mrsD</i> efflux pumps	64–128 for erythromycin
Tetracyclines	<i>tet(M)</i> determinant (ribosomal protection)	2–32 for tetracyclines
Fluoroquinolones	Mutations in the <i>gyrA</i> , <i>gyrB</i> , <i>parC</i> , or <i>parE</i> genes	2–16 for levofloxacin

\*Escherichia coli numbering system.

## DRUG RESISTANCE OF BV ASSOCIATED BACTERIA

### *G. vaginalis*

*G. vaginalis* is treated with metronidazole and clindamycin but only limited data are available with respect to its resistance (Nagaraja, 2008; Tomusiak et al., 2011). The putative mechanism for tetracycline resistance is the presence of the *tetM* gene, which is found in tetracycline-resistant strains of *G. vaginalis* (Harwich et al., 2010). Resistance to metronidazole has been found in some *G. vaginalis* strains. The proposed mechanisms of resistance include (Löfmark et al., 2010):

- A suppressed rate of activation of the drug inside the cell through its reduction;
- Increased activity of DNA repair systems;
- Increased activity of enzymes that consume oxygen (i.e., catalase, peroxidase, and superoxide reductase);
- Accelerated clearance of the drug from the cell by active efflux.

The most well-characterized mechanism of resistance to metronidazole is the inactivation or deletion of genes with nitroreductase activity (Dhand and Snydman, 2009). Some isolates have nitroreductase genes; however, the correlation between these genes and metronidazole resistance has not been studied.

Recent whole-genome sequencing studies have revealed that *G. vaginalis* has a population structure that consists of four clades: clades 1 and 3 are associated with bacterial vaginosis and clades 2 and 4 are not. Metronidazole susceptibility is associated with the population structure, with clade 3 and 4 isolates showing 100% resistance to this drug whereas the resistance of clade 1 and 2 are 35 and 7.1%, respectively (Schuyler et al., 2015b).

### *B. fragilis*

Recently, an increasing resistance to different antimicrobial drugs has been reported for *B. fragilis*. Resistant strains were discovered in both European countries and the United States. (Eitel et al., 2013). Multidrug-resistant *B. fragilis* isolates bearing parallel resistance to imipenem, amoxicillin and metronidazole or clindamycin were also found in Russia (Shilnikova and Dmitrieva, 2015). Antibiotic resistance is spread horizontally among the *B. fragilis* group of clinical isolates due to the antibiotic resistance genes carried on conjugative and mobilizable plasmids, conjugative transposons and integrated genetic elements (Eitel et al., 2013).

The most important mechanism of resistance of *B. fragilis* to β-lactam antibiotics is the production of β-lactamases (Edwards, 1997). The *cepA* gene encodes β-lactamase, which is able to destroy penicillins and most cephalosporins (except cefoxitin). Resistance to cefoxitin-resistant strains was explained by the

presence of the *cfxA* gene located on the MTn4555 transposon. Resistance to carbapenems was explained by the presence of the *cfxA* gene in the bacterial chromosome. Because the *cfxA* gene may be expressed differently or even be silenced, different levels of carbapenem resistance may be detected. As recently shown (Eitel et al., 2013), the *cfxA* gene is not a major factor in determining cefoxitin resistance, *cfxA* was found with a higher prevalence in non-fragilis *Bacteroides* strains than in *B. fragilis*.

Metronidazole remains the drug of choice for the treatment of infections caused by *B. fragilis*, although metronidazole-resistant strains have been described in the literature (Brook, 2004). The resistance is due to the presence of the nitroimidazole resistance genes *nima-nimG* on plasmids or on chromosomes (Carlier et al., 1997).

*B. fragilis* resistance to tetracycline originates from the presence of the *tetQ* gene (ribosome protection mechanism) and the *tetX* and *tetX1* genes encoding FAD-dependent monooxygenases that are able to destroy tetracycline.

*B. fragilis* resistance to clindamycin occurs via two mechanisms. First, the *ermB*, *ermF*, and *ermG* genes encoding various N<sup>6</sup>-methyltransferases modify the 23S RNA. Several transferable plasmids can cause resistance [pBF4, pBFTM10 (pCP1), and pB1136]. The *ermF* resistance gene was found on transposons Tn4351 (pBF4), Tn4400 (pBFTM10), and Tn4551 (pB1136). Among the possible clindamycin resistance genes, *ermF* was the most common and had the largest effect on clindamycin resistance after the *linA* gene. The second mechanism is the action of the efflux pumps encoded by the *msrSA* and *mefA* genes. The *msrSA* gene was first described for *Staphylococcus aureus* and was later also found in *Bacteroides*. The presence of the *ermG-mefA-msrSA* combination was confirmed for clindamycin-resistant *B. fragilis* strains (Eitel et al., 2013).

Members of the *Bacteroides* genus were originally resistant to first and second generation fluoroquinolones. Resistance to the next generation fluoroquinolones appeared in the last few years; however, fluoroquinolones of the third and fourth generations are still effective against *B. fragilis*. The BexA efflux pump encoded by the *bexA* gene may be responsible for the resistance of *B. fragilis* to fluoroquinolones and the elevated moxifloxacin MIC values. The BexA pump belongs to the MATE class. It was first described for *Bacteroides thetaiotaomicron*; disruption of BexA in *B. thetaiotaomicron* made the bacteria more susceptible to norfloxacin, ciprofloxacin, and ethidium bromide. The BexA protein sequence is homologous to the protein sequence of NorM, which is a multidrug efflux transporter of *Vibrio parahaemolyticus* (Miyamae et al., 2001).

## A. vaginalae

Some studies demonstrated that *A. vaginalae* could exhibit a high level of resistance to metronidazole (Geißdörfer et al., 2003). For example, several isolates of *A. vaginalae* described in De Backer et al. (2006) were found to be highly resistant to metronidazole and susceptible to clindamycin, which are the two preferred antibiotics for the treatment of bacterial vaginosis; however, a large amount of variability in the susceptibility to metronidazole was reported (ranging from 2 to more than

256 mg/L). The authors concluded that metronidazole resistance was not an intrinsic feature of *A. vaginalae*. Further research is required to clarify whether this metronidazole resistance might be acquired by the presence and activation of *nim*-genes encoding an alternative reductase that can convert nitroimidazole to a nontoxic derivative, thereby circumventing the toxic effect that causes breakage of the DNA (Löfmark et al., 2010).

A draft genome sequence of a metronidazole-susceptible (MIC 16 mg/L) vaginal isolate of *A. vaginalae* (strain 44061) was recently published (Schuyler et al., 2015a). This information will be useful for comparative studies of the mechanism and the molecular basis of metronidazole resistance in *A. vaginalae*.

## M. mulieris

In different studies, the resistance of *M. mulieris* to metronidazole varied from 50 to 81%. The MIC<sub>90</sub> of metronidazole was 128 mg/L. Reports have suggested that 4% of *Mobiluncus* species are clindamycin-resistant (Spiegel, 1987; Bahar et al., 2005). A high prevalence of metronidazole-resistant *Mobiluncus* species (81%) was found among Turkish women with gynecological infections including bacterial vaginosis (Bahar et al., 2005). Notably, the *tetQ* gene that was associated with resistance to tetracycline was found in *Mobiluncus*.

## CONCLUSION

The emergence of resistance to antimicrobials is a natural consequence of the evolutionary process under increasing pressure from chemotherapy. As a result, resistance of STD pathogens appears to not only natural β-lactam antibiotics, but also to fully synthetic drugs such as fluoroquinolones. In the ongoing war between the disease and its effective treatment, a detailed understanding of the mechanisms of action of antibiotics and the emergence of resistance is necessary to modify existing drugs and to develop new ones directed against new targets. An ever-increasing range of reproductive tract bacterial infections calls for developing rapid, sensitive, and reliable methods that are able to determine resistance to available antimicrobials in individual patients as early as possible. At the same time, the recommended therapeutic regimen should take into account the regional occurrence of resistance to specific drugs obtained by constant monitoring. Moreover, for adequate therapy of diseases of the urogenital tract, in particular, for targeted selection of drugs, it is very important to take into account the possible polymicrobial character of a disease and to be able to simultaneously identify several STD pathogens and BV associated bacteria in individual clinical samples. These strategies will allow the use of existing drugs for a longer time with better efficiency and suppress the occurrence of multidrug-resistant bacterial microflora generated by repetitive failures of treatment with already inefficient antibiotics.

## AUTHOR CONTRIBUTIONS

BS and ED were the primary authors, conceived the study, and drafted the manuscript for publication. AR participated

in writing Chapter Brief Description of the Most Common Bacterial Infection of the Human Reproductive System and the Drugs Used to Treat Them. AL wrote Section *T. pallidum*. DV, XP, and AK participated in writing Section *N. gonorrhoeae* of Chapter Drug Resistance of Std Pathogens. DD participated in writing Sections *C. trachomatis* and Mycoplasma and Ureaplasma spp. of Chapter Drug Resistance of Std Pathogens. DG wrote Chapter Drug Resistance of BV Associated Bacteria, and reviewed the initial and revised versions of the manuscript. All authors read and approved the final manuscript.

## FUNDING

This work was financed by subsidy #14.607.21.0065 (RFMEFI60714X0065) from the Ministry of Education and Science of the Russian Federation.

## ACKNOWLEDGMENTS

We are grateful to Dr. Danila Zimenkov for his useful remarks. We are especially grateful to Dr. Alexander Kolchinsky (Champaign, IL, USA) for his assistance in the preparation of this manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Effect of Tulathromycin on Colonization Resistance, Antimicrobial Resistance, and Virulence of Human Gut Microbiota in Chemostats

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equally to this work.

### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 07 January 2016

Accepted: 22 March 2016

Published: 08 April 2016

### Citation:

Hao H, Zhou S, Cheng G, Dai M, Wang X, Liu Z, Wang Y and Yuan Z (2016) Effect of Tulathromycin on Colonization Resistance, Antimicrobial Resistance, and Virulence of Human Gut Microbiota in Chemostats. *Front. Microbiol.* 7:477.  
doi: 10.3389/fmicb.2016.00477

To evaluate microbiological safety of tulathromycin on human intestinal bacteria, tulathromycin (0, 0.1, 1, 10, and 100 µg/mL) was added into Chemostats. Before and after drug exposure, we monitored (1) population, SCFA products, antimicrobial resistance, and colonization resistance of gut microbiota, and (2) the antimicrobial resistance genes, transferability, virulent genes, pathogenicity of *Enterococcus faecalis*. Results showed that low level of tulathromycin did not exhibit microbiological hazard on resistance selection and colonization resistance. However, high level of tulathromycin (10 and 100 µg/mL) may disturb colonization resistance of human gut microbiota and select antimicrobial resistant *E. faecalis*. Most of the selected resistant *E. faecalis* carried resistant gene of *ermB*, transferable element of Tn1545 and three virulence genes (*esp*, *cylA*, and *ace*). One of them (*E. faecalis* 143) was confirmed to have higher horizontal transfer risk and higher pathogenicity. The calculated no observable adverse effect concentration (NOAEC) and microbiological acceptable daily intake (mADI) in our study was 1 µg/mL and 14.66 µg/kg.bw/day, respectively.

**Keywords:** tulathromycin, gut microbiota, colonization resistance, antimicrobial resistance, chemostat

## INTRODUCTION

Tulathromycin is the first and only member of the triamillide sub-class of macrolide. Tulathromycin is used therapeutically in treatment of respiratory disease in swine and cattle at a single dose of 2.5 mg/kg.bw. It has been registered in more than 30 countries across America, Europe, Oceania, and Asia and played important role in veterinary medicine (FDA, 2004). This agent is characterized by rapid absorption from the injection site, extensive distribution to tissue, and slow elimination. The withdrawal period for cattle and swine is 22 and 5 days, respectively. This drug is excreted primarily unchanged (90%) in feces (2/3) and in urine (1/3), suggesting that it may reach to human colon and remain its antimicrobial activity (Benchaoui et al., 2004; EMEA, 2004; Nowakowski et al., 2004).

The residue of tulathromycin in animal food may have unintended microbiological effects on human gut microbiota. The possible harmful effects may be: (1) shifts in bacterial counts and biochemistry, (2) destruction of colonization resistance, (3) emergence of antimicrobial resistant

bacteria (Cerniglia and Kotarski, 1999; Nutsch et al., 2005). Additionally, antibiotic resistant bacteria may have enhanced fitness or high virulence (Mundy et al., 2000). Human intestinal strains may also serve as reservoirs for antimicrobial resistant determinations and mediates the gene transfer (Salyers et al., 2004).

Based on *in vitro* antibiotic susceptibility tests, microbiological ADIs recommended by Food and Drug Administration (FDA), European Medicine Agency (EMA), and Australian Pesticides and Veterinary Medicines Authority (APVMA) were 50, 10.99, and 5 µg/kg.bw/d, respectively. The large difference in these mADI may due to the disadvantage and limitation of short-term *in vitro* antibiotic susceptibility tests in evaluation of long-term effects of tulathromycin residues posed on human intestinal bacteria and in analyze of impact of tulathromycin on colonization resistance and emergence of antimicrobial resistance of human colonic microbiota (Cerniglia and Kotarski, 2005). Therefore, microbiological safety assessment measures of tulathromycin residues is largely inadequate. It is largely unknown that what is the effect of long-term exposure to tulathromycin on colonization resistance of human intestinal microbiota and on antimicrobial resistance development, virulence change, and gene transfer in some specific intestinal bacteria.

In the present article, the chemostat model of human colonic gut, which is an approach recommended by VICH (VICH GL-36) and some previous studies (Carman and Woodburn, 2001; Carman et al., 2004, 2005), is used to evaluate microbiological safety of tulathromycin on human intestinal microbiota.

## MATERIALS AND METHODS

### Chemicals

The tulathromycin (TUL), erythromycin (ERY), and lincomycin (LIN) were dissolved in methanol and then diluted with deionized water. The ciprofloxacin (CIP), tetracycline (TET), and rifampicin (RIF) were dissolved in deionized water. Tulathromycin was purchased from Liu He animal Pharmaceutical Co., Ltd. (Qingdao, China). The erythromycin, lincomycin, ciprofloxacin, tetracycline, and rifampicin were purchased from SIGMA.

### Bacteria Strains

The *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC29212, and *Micrococcus luteus* ATCC 9341 were purchased from American Type Culture Collection (ATCC). The *E. coli* ATCC 25922 and *E. faecalis* ATCC29212 were used as quality controls for species identification and minimum inhibitory concentration (MIC) determination. The *M. luteus* ATCC 9341 were used as an indicator for determination of the concentration of tulathromycin. *E. faecalis* JH2-2 (Rifampicin MIC > 50 µg/mL) and *E. coli* NK5449 (Rifampicin MIC > 100 µg/mL), purchased from Belgian coordinated collections of microorganisms and Institute of microbiology in Chinese academy of sciences, respectively, were used as receptors for conjugation test. The *Salmonella* Typhimurium SI3 was a ciprofloxacin resistant strain which was selected in our

previous study (Sun et al., 2011). This strain was used as the challenge strain for evaluating the colonization resistance of chemostat.

### Preparation of Fresh Fecal Samples and Strain Isolation

Fresh fecal samples from six adult volunteers who had no history of antibiotic usage and gastrointestinal disturbance within the preceding 3 months were prepared following previous method (Carman and Woodburn, 2001; Hao et al., 2013). The use of human fecal samples was approved by the ethical Committee of Huazhong Agricultural University (approval number hzauch 2013-002).

### Strain Isolation and MIC Determination from Fresh Fecal Samples

The four predominant intestinal bacteria (*E. coli*, *Enterococcus*, *Bifidobacterium*, and *Bacteroides fragilis*) were isolated from the fresh fecal samples of six adult volunteers by selective agars (Eosin methylene blue agar, bile esculin azide agar, BBL media, and *bacteroides* bile esculin agar). Ten isolates of each species were obtained from fresh fecal sample of each volunteer. After species identification by classic biochemical tests and ABI 3130 system (Applied Biosystem, USA), 32 isolated strains of each species were subjected to MIC determination by agar dilution method provide by CLSI (document M7-A5 for aerobic bacterial and document M11-A5 for anaerobic bacterial). The *E. coli* ATCC25922 and *E. faecalis* ATCC 29212 were used as quality control for MIC determination. The minimum concentration to inhibit 50% of isolates (MIC<sub>50</sub>) and the minimum concentration to inhibit 90% of isolates (MIC<sub>90</sub>) was calculated based on the MIC distribution and estimated by probability analysis using SPSS statistical package (Kays and Graff, 2002; Xu et al., 2013).

### Designation of Tulathromycin Dosage in Preliminary Test

The preliminary test for selection of tulathromycin concentration was carried out following the method in previous study (Hao et al., 2013). Briefly, different concentrations of tulathromycin (0, 0.5, 10, 20, 40, 60, 80, 100 µg/mL) were mixed with 50 mL prepared fecal samples at the final concentration of 20% (w/v). The activity of tulathromycin on populations of four different bacteria (*E. coli*, *Enterococcus*, *Bifidobacterium*, and *B. fragilis*) were determined by bacterial colony count using selective agars. The highest concentration of tulathromycin was selected for further experiments as it was able to significantly change the population of four predominant intestinal bacteria.

The low to intermediate concentration of tulathromycin (0.1, 1, and 10 µg/mL) were designed based on the acceptable daily intake (ADI) recommended by FDA (50 µg/kg.bw/d), EMA (10.99 µg/kg.bw/d), and APVMA (5 µg/kg.bw/d). In those designed concentration, 1 µg/mL tulathromycin was the mean MIC<sub>50</sub>-value to the most sensitive *Bifidobacterium* strains. One group was drug free methanol control (0 µg/mL tulathromycin).

## Establishment of Chemostats and the Time Schedule

The establishment of chemostat models and the time schedule were carried out following the method in previous studies (Carman and Woodburn, 2001; Hao et al., 2013). Briefly, 50 mL prepared fecal suspension at the final concentration of 20% (w/v) was inoculated into each culture vessel. After 7 h incubation, culture medium was pumped into and out of the culture vessel at a uniform rate of 35 mL/h to maintain 500 mL of culture medium.

After 7 days running, the chemostats reached to a steady state. From 7<sup>th</sup> to 13<sup>th</sup> day, the chemostats were kept on running on steady state without administration of tulathromycin (Chen et al., 2011, 2014). From 14<sup>th</sup> to 20<sup>th</sup> day, five designed concentrations of tulathromycin (0, 0.1, 1, 10, and 100 µg/mL) were, respectively, infused into the culture medium. During 20<sup>th</sup>–22<sup>nd</sup> day, the tulathromycin was withdrawn from chemostat models and 1 mL of  $6 \times 10^8$  CFU/mL (2X McFarland) *Salmonella Typhimurium* SI3 were daily inoculated into each chemostat. Then the chemostats continued to run for another 7 days (to 29<sup>th</sup> day) without challenge of *Salmonella Typhimurium* (Moffatt, 2007).

## Monitoring Bacterial Counts in the Chemostats

During day 7–20, samples were daily taken from each chemostat. The changes of four predominant bacteria (*E. coli*, *Enterococcus*, *B. fragilis*, and *Bifidobacterium*) were measured by viable cell counting (Hao et al., 2013, 2015).

## Monitoring Short Chain Fatty Acids (SCFAs) in the Chemostats

During day 7–20, samples were daily taken from each chemostat. The concentration of three primary SCFAs (acetic acid, butyric acid, and propionic acid) were monitored using a developed gas chromatographic (GC) method (Hao et al., 2013, 2015).

## Evaluation of Colonization Resistance in the Chemostats

From day 20<sup>th</sup> to 29<sup>th</sup>, samples were taken from each chemostat to monitor the population of *Salmonella Typhimurium* SI3 using bismuth sulfite (BS) agar containing 4 µg/mL ciprofloxacin (Sun et al., 2011; Hao et al., 2013, 2015). The colonization resistance was disrupted if *Salmonella Typhimurium* SI3 was successfully colonized into chemostat models.

## Monitoring Resistance Rate in the Chemostats

During day 8–20, samples were daily taken from each chemostat. The change of resistance rate was also monitored following the method in previous study (Hao et al., 2013, 2015). The resistance rates of *E. coli* and *Enterococcus* were equal to the number of resistant colonies on the selective agars containing 4-fold MIC<sub>90</sub> of tulathromycin divided by the number of total colonies on the selective agars without tulathromycin. The 4-fold MIC<sub>90</sub> was used for selection of tulathromycin resistance on basis of

VICH GL-36 document and some previous reports of resistance selection (Allen and Bierman, 2009; Kadlec et al., 2011).

## Determination of Resistance Pheno- and Geno-type in the Selected *E. faecalis*

During day 8–20, a total of 70 *Enterococcus* isolates were random selected from samples in the chemostat containing 100 µg/mL tulathromycin. After species identification by biochemical tests and PCR amplification, 64 isolates were identified as *E. faecalis*. Among these *E. faecalis*, 34 isolates were obtained before the administration of tulathromycin, while 30 isolates were selected after the treatment with tulathromycin. The susceptibility of the 64 *E. faecalis* to macrolide, lincosamides, and tetracycline were determined by agar dilution method.

The genes involved in macrolide-lincosamides-streptogramins (MLS) resistance with a methylation mechanism were determined by PCR amplification of known *erm* genes using specific primers for *ermA*, *ermB*, and *ermC* (see Table 1). The genes involved in antibiotic efflux systems were determined using specific primers for the *mefA/E* gene (see Table 1). The PCR procedure was followed the method in previous published paper (Portillo et al., 2000).

## Detect Transfer Risk of the Selected Macrolide Resistant *E. faecalis*

The macrolide-resistant isolates containing *ermB* gene were selected to determine the presence of transposons Tn1545 and

**TABLE 1 |** PCR primers and products for detection of macrolides resistance gene and transposons and virulence determinants in *E. faecalis*.

Primers	5'-3'	Target fragment (bp)	GeneBank no.
<i>ermA</i>	F: GTTCAAGAACATAATACAGAG R: GGATCAGGAAAAGGACATTTCAC	421	FN668375
<i>ermB</i>	F: GAAAAGGTACTCAACCAAATA R: AGTAACGGTACTAAATTGTTAC	639	NC_014498
<i>ermC</i>	F: AATCGGCTCAGGAAAAGG R: ATCGTCAATTCCCTGCATG	534	NC_014498
<i>mef (A/E)</i>	F: AGTATCATTAATCACTAGTGC R: TTCTTCTGGTACTAAAAGTGG	346	NC_018641
Tn 1545	F: CTTAGAACAACTTAAGAGTGTGT R: GGTTGAGTA CCTTTCATCGTTAA	382	NC_013644
Tn 917	F: ATCTGACGGTGACATCTCTC R: GGTTGAGTACCTTTCATCGTTAA	652	NC_017312
<i>esp</i>	F: TTGCTAATGCTAGTCCACGACC R: GCGTCAACACTGCGATTGCCGA	932	AF034779
<i>gelE</i>	F: ACCCCGATCATTGGTTT R: ACGCATTGCTTTCCATC	405	M37185
<i>cylA</i>	F: GACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTTAC	688	AD1CLYL
<i>ace</i>	F: GGAATGACCGAGAACGATGGC R: GCTTGATGTTGGCCTGCTCCG	616	AF159247
<i>asal</i>	F: CCAGCCAACATGGCGGAATC R: CCTGTCGCAAGATCGACTGTA	529	SFPASA1

Tn917 using the primers (see **Table 1**) and method established in previous study (Okitsu et al., 2005). Filter mating method was used to investigate the conjugative transfer of the selected macrolide resistant *E. faecalis*. A representative macrolide-resistant isolate containing *ermB* and composite transposon was selected as donor strain. The recipients were two rifampicin-resistant strains, *E. faecalis* JH2-2 (Rif MIC > 50 µg/mL) and *E. coli* NK5449 (Rif MIC > 100 µg/mL). The donor and recipient strains were mixed into the conjugation system at the ratio of 1:9 and incubated on the serum agar for 48 h at 37°C. The number of donor, recipient and transconjugants were counted by agar plate containing rifampicin and/or erythromycin.

### Determination of Virulence in the Selected *E. faecalis*

The presence of virulence factors, including the surface protein (*esp*), haemolysin activator (*cylA*), gelatinase (*gelE*), collagen binding protein (*ace*), and aggregation substances (*asal*), were determined by PCR in the 64 *E. faecalis* isolates obtained in the pre- and post-treatment of tulathromycin in the chemostat. PCR primers used in this study were listed in **Table 1**. The annealing temperatures of five virulence factors were 65°C for *esp*, 51°C for *gelE*, 61°C for *CylA*, 61°C for *ace* and 63°C for *asal* gene, respectively.

In order to compare the changes of pathogenicity of *E. faecalis*, two representative *E. faecalis* isolates (*E. faecalis* 143 and *E. faecalis* 174) were subjected to determine the median lethal dose (LD<sub>50</sub>). Briefly, the 72 SPF Kunming mice with body weight of 25–28 g were randomly divided into nine groups with half males and half females. The groups were, respectively, challenged with *E. faecalis* isolate by intraperitoneal injection of 0.5 mL diluted concentration of bacteria suspension (10<sup>7</sup>–10<sup>10</sup> CFU/mouse). The dead mice were carefully dissected in a bacteria-free operating environment. The LD<sub>50</sub> was calculated by improved Karber method. All experimental procedures were performed according to the guidelines of the committee on the use and care of the laboratory animals in Hubei province China. The study was approved by the Animal Care Center, Hubei Science and Technology Agency in China. All the animals were monitored throughout the study for any sign of adverse effect.

### Statistical Analysis

All experiments were performed in triplicate. The statistical analysis was performed following the previous study (Hao et al., 2013, 2015). Briefly, a “mean pre-treatment” level and 95% prediction interval (PI95) for each parameter was set up based on the data obtained from each culture vessel during steady state (day 7–13) and used as reference boundaries for assessing the effect of tulathromycin on each parameter in the drug exposure period (day 14–20).

### NOAEC and mADI Calculation

The NOAEC was the no observable adverse effect concentration in this study. The microbiological acceptable daily intake (mADI)

for tulathromycin was calculated following the guideline of VICH GL-36. The formula used for mADI calculation was that mADI = NOAEC × daily fecal bolus/(fraction of oral dose available for micro-organisms × weight of human).

## RESULTS

### **MIC<sub>90</sub> of the Strains Isolated from Fresh Fecal Samples**

The 32 strains of each species (*E. coli*, *Enterococcus*, *Bifidobacterium*, and *B. fragilis*) were isolated from fresh fecal samples of six volunteers. The MIC for 90% of isolates (MIC<sub>90</sub>) of tulathromycin for *E. coli*, *Enterococcus*, *Bifidobacterium*, and *B. fragilis* were 5.53, 7.87, 1.35, and 2.30 µg/mL, respectively. After dilution, the methanol used for dissolving tulathromycin did not exert some antimicrobial effect.

### **The Designed Four Tulathromycin Concentration**

From the preliminary test, 100 µg/mL was the highest concentration of tulathromycin which could significantly change the population of four predominant intestinal bacteria. Based on the ADI-values recommended by FDA, EMA, and APVMA, the lower concentration of tulathromycin were designed as 0.1, 1, and 10 µg/mL.

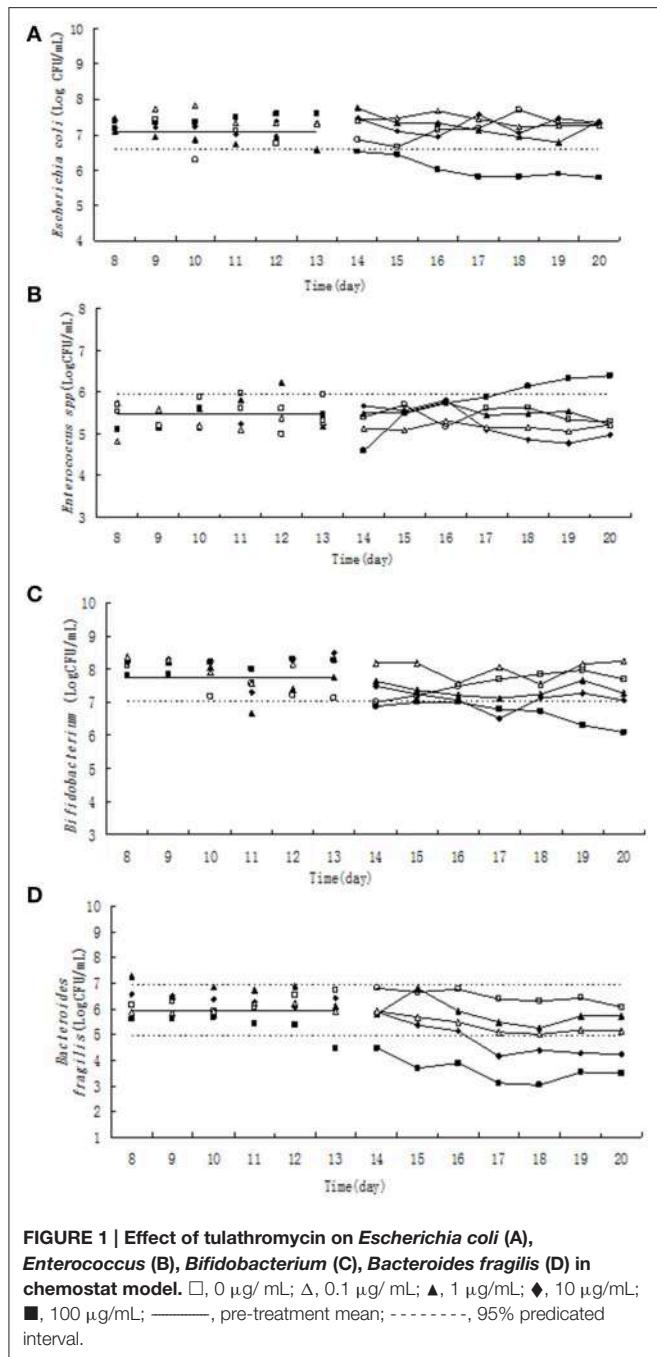
### **Effect of Tulathromycin on Bacterial Counts**

The population of *E. coli* was kept between 3.667 × 10<sup>6</sup> and 5.482 × 10<sup>6</sup> CFU/mL in steady state based on statistical data among five chemostats (**Figure 1A**). Three days after administration of 100 µg/mL tulathromycin, the population of *E. coli* was decreased to 6.556 × 10<sup>5</sup> CFU/mL which was ~6-fold lower than 95% lower confidence limit. However, the three low concentration of tulathromycin (0.1, 1, and 10 µg/mL) did not significantly change the number of *E. coli*.

Pre-treatment of tulathromycin (day 8–13), the *Enterococcus* counts was in the range of 9.526 × 10<sup>4</sup>–8.674 × 10<sup>5</sup> CFU/mL (**Figure 1B**). Under exposure to 10 µg/mL tulathromycin, *Enterococcus* count was decreased ~1.6-fold in the 5<sup>th</sup> day of post-treatment. On the contrary, the total number of *Enterococcus* was increased almost 3-fold from 18<sup>th</sup> day after administration of 100 µg/mL tulathromycin.

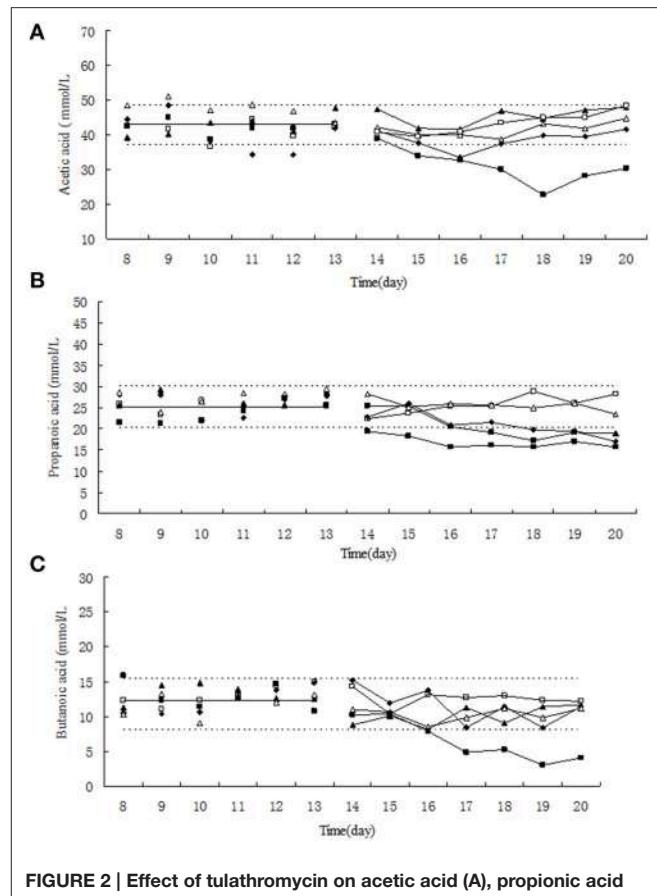
The number of *Bifidobacterium* varied from 1.105 × 10<sup>7</sup> to 2.70 × 10<sup>8</sup> CFU/mL (**Figure 1C**) during day 8–13. The administration of 100 µg/mL tulathromycin significantly decreased the number of *Bifidobacterium* in the chemostat. It was shown that *Bifidobacterium* count was decreased ~10-fold lower than 95% lower confidence limit.

Significant and dose-dependent effects of tulathromycin on *B. fragilis* counts were observed in all tested concentration of tulathromycin. After treatment by 10 and 100 µg/mL tulathromycin, *B. fragilis* count was decreased 6- and 90-fold lower than 95% lower confidence limit, respectively (**Figure 1D**).



## Effect of Tulathromycin on Short Chain Fatty Acid (SCFA)

The relative low concentration of tulathromycin (0, 0.1, and 1  $\mu\text{g}/\text{mL}$ ) did not significantly change the molar concentration of acetic acid, propionic acid and butyric acid (Figure 2). Under exposure to 10  $\mu\text{g}/\text{mL}$  tulathromycin, the concentration of acetic acid and propionic acid was slightly decreased. Upon the administration of 100  $\mu\text{g}/\text{mL}$  tulathromycin, the concentration of acetic acid, propionic acid, and butyric acid were decreased  $\sim$ 1.3, 1.3, and 2.68-fold lower than 95% lower



confidence limit, respectively. Among the three dominant SCFAs, the concentration of propionic acid was changed remarkably under exposure of tulathromycin and present significant dose-dependent effect of tulathromycin.

## Effect of Tulathromycin on Colonization Resistance

In the single growth control group, the growth of *Salmonella* Typhimurium was stable at the level of  $3.75 \times 10^7$ – $1.54 \times 10^8$  CFU/mL, indicating that *Salmonella* Typhimurium grew well in the chemostat model containing no drug and gut microbiome (Figure 3). However, the colony number of *Salmonella* Typhimurium tapered to  $10^2$  CFU/mL on day 31 in the chemostat containing stable gut microbiome, suggesting that the gut microbiome may establish colonization resistance to exogenous pathogens. After treatment of lower concentration of tulathromycin (10, 1, and 0.1  $\mu\text{g}/\text{mL}$ ), the number of *Salmonella* Typhimurium was also decreased to the level of  $10^2$  CFU/mL. In contrast, the colonization number of *Salmonella* Typhimurium was in the range of  $2.08 \times 10^5$ – $7.15 \times 10^6$  CFU/mL in the chemostat administrated by highest concentration of tulathromycin (100  $\mu\text{g}/\text{mL}$ ), suggesting that the high

concentration of drug may disturb the colonization resistance of gut microbiome and induce the proliferation of pathogen (Figure 3).

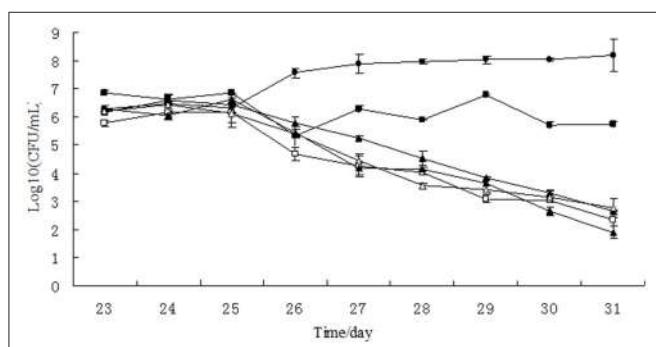
### Effect of Tulathromycin on Resistant Rate

Before the treatment of tulathromycin, 10–30% of tulathromycin resistance was observed in *Escherichia coli* and in *Enterococcus* strains (Figures 4A,B). Under exposure to 10  $\mu\text{g}/\text{mL}$  tulathromycin, the percentage of resistant *Enterococcus* was slight increased (Figure 4B). After treatment with 100  $\mu\text{g}/\text{mL}$  tulathromycin, the resistant rates in *E. coli* and *Enterococcus* were obviously higher than control group, especially in *Enterococcus*, it reach up to 80% (Figures 4A,B). After administration of different concentration of tulathromycin, regular changes were not observed in *Bifidobacterium* and *Bacteroides fragilis* (Figures 4C,D).

### Resistant Phenotype and Genotype of the Selected *E. faecalis*

After species confirmation, 34 and 30 *E. faecalis* isolates were selected from chemostats pre- and post-treatment, respectively. The MIC<sub>50</sub> and MIC<sub>90</sub> of these 64 *E. faecalis* strains to erythromycin, tulathromycin, lincomycin, and tetracycline were showed in Table 2. The 34 *E. faecalis* strains isolated from chemostats before administration of tulathromycin exhibited high susceptibility to erythromycin, tulathromycin and tetracycline (MIC  $\leq 4 \mu\text{g}/\text{mL}$ ), and low resistance to lincomycin (MIC = 16, 32  $\mu\text{g}/\text{mL}$ ). However, after treatment with tulathromycin, 30 *E. faecalis* isolates (except for the strains of 141, 142, 144, and 164) showed high-level resistance to the four tested antibiotics (MIC  $\geq 4 \mu\text{g}/\text{mL}$ ). The MIC<sub>50</sub> of erythromycin, tulathromycin, lincomycin, and tetracycline to *E. faecalis* were considerably increased for 88.9, 49.8, 5.1, and 45.4-fold, respectively.

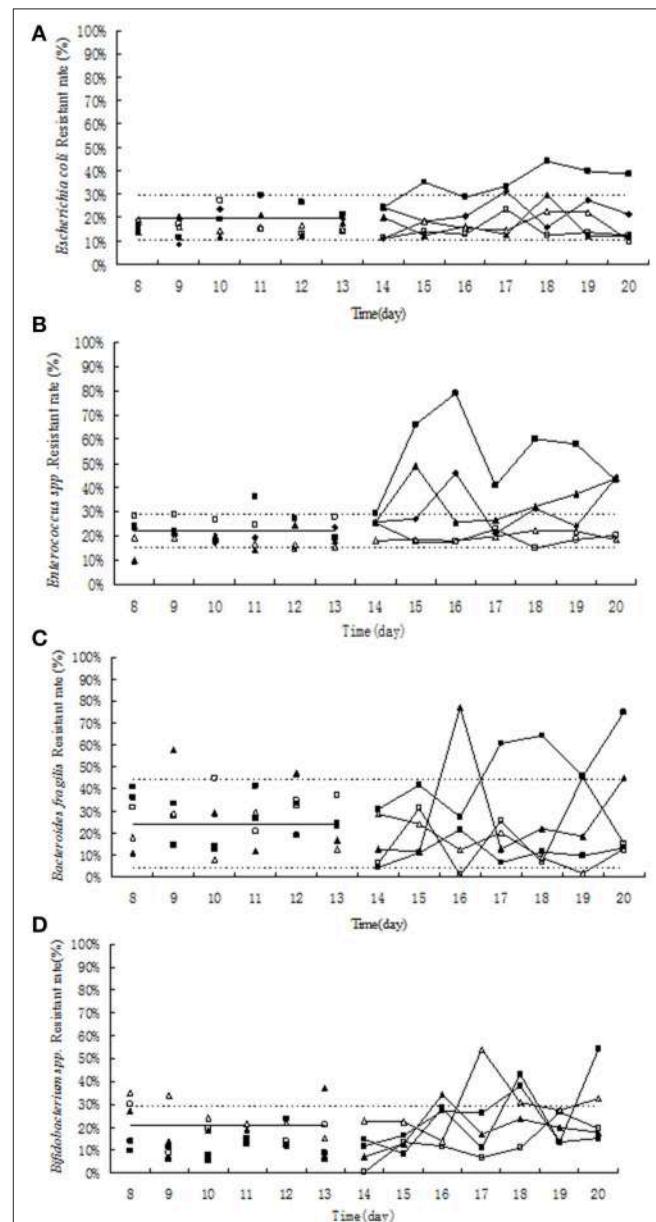
About 88.46% of the tulathromycin resistant *E. faecalis* isolated from chemostats harbored macrolide resistance associated gene of *ermB* (Table 3). However, the tested macrolide resistance associated genes (*ermABC* and *mefA/E*) were free in the tulathromycin resistant *E. faecalis* strains (Table 3).



**FIGURE 3 |** Growth curves of *Salmonella Typhimurium* in chemostat models. □, 0  $\mu\text{g}/\text{mL}$ ; Δ, 0.1  $\mu\text{g}/\text{mL}$ ; ▲, 1  $\mu\text{g}/\text{mL}$ ; ◆, 10  $\mu\text{g}/\text{mL}$ ; ■, 100  $\mu\text{g}/\text{mL}$ ; ●, growth control.

### Transfer Risk of Macrolide Resistant *E. faecalis*

All of *ermB* containing *E. faecalis* strains also harbored composite transposon of Tn1545 (Table 3). When the *E. faecalis* 143 strain containing both *ermB* and Tn1545 was selected as donor strain and subjected to conjugation transfer test, the result showed that macrolide resistant determinants could transfer to *E. faecalis* JH2-2 and *E. coli* NK5449 at the frequencies of 10<sup>-6</sup>–10<sup>-7</sup> (Table 4).



**FIGURE 4 |** Effect of tulathromycin on resistance in *Escherichia coli* (A), *Enterococcus* (B), *Bifidobacterium* (C), *Bacteroides fragilis* (D) in chemostat model. □, 0  $\mu\text{g}/\text{mL}$ ; Δ, 0.1  $\mu\text{g}/\text{mL}$ ; ▲, 1  $\mu\text{g}/\text{mL}$ ; ◆, 10  $\mu\text{g}/\text{mL}$ ; ■, 100  $\mu\text{g}/\text{mL}$ ; —, pre-treatment mean; - - - -, 95% predicated interval.

**TABLE 2 | Comparison of MICs of separated *E. faecalis* strains, before and after treated with four drugs.**

Drug	Pre- or post-treatment	Strains (N)	MICs ( $\mu\text{g/mL}$ )										$\text{MIC}_{50}$ ( $\mu\text{g/mL}$ )	$\text{MIC}_{90}$ ( $\mu\text{g/mL}$ )
			256	128	64	32	16	8	4	2	1	0.5		
ERY	Pre-	34						1	2	26	4	1	2.610	5.211
ERY	Post-	30	17	9					3	1			231.698	—
TLU	Pre-	34						31	2			1	4.904	10.995
TLU	Post-	30	21	4				3	2				244.149	—
LIN	Pre-	34				27	6				1		41.706	221.77
LIN	Post-	30	21	1	2	6							213.721	669.13
TET	Pre-	34						33			1		2.552	4.437
TET	Post-	30		22	2			5		1			115.681	—

$\text{MIC}_{50}$  = minimum concentration to inhibit 50% of isolates;  $\text{MIC}_{90}$  = minimum concentration to inhibit 90% of isolates.  $\text{MIC}_{90}$ - and  $\text{MIC}_{50}$ -values are estimated values by probability analysis by SPSS (Kays and Graff, 2002; Xu et al., 2013); “—” data not given; white areas was pre-treatment group; gray areas was post-treatment group; ERY, erythromycin; TLU, tulathromycin; LIN, lincomycin tetracycline.

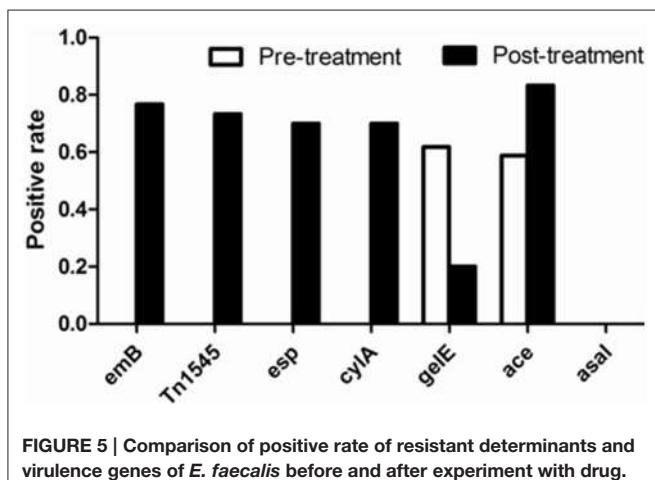
**TABLE 3 | The relationship of MIC, resistant gene and virulence gene in *E. faecalis* isolated during drug administration.**

Isolates no.	MICs				Resistant gene		Virulence determinants				
	ERY	TLU	LIN	TET	<i>ermB</i>	Tn1545	<i>esp</i>	<i>cylA</i>	<i>gelE</i>	<i>ace</i>	
141	2	4	32	2	—	—	—	—	+	+	
142	2	4	32	2	—	—	—	—	+	+	
143	128	256	256	128	+	+	+	+	—	+	
144	2	4	32	2	—	—	—	—	+	+	
145	256	256	32	2	—	+	—	—	+	+	
151	128	256	256	128	+	—	+	+	—	+	
152	128	128	256	128	+	+	+	+	—	+	
154	1	2	32	2	—	—	—	—	+	+	
155	128	128	32	0.5	—	—	—	—	+	+	
161	256	256	256	128	+	+	+	+	—	+	
162	256	256	256	128	+	+	+	+	—	+	
164	256	256	256	128	+	+	+	+	—	+	
165	256	256	256	128	+	+	+	+	—	+	
171	256	256	256	128	+	+	+	+	—	+	
172	128	256	64	64	+	—	—	—	—	—	
174	256	256	64	64	—	+	—	—	—	—	
181	128	256	256	128	+	+	+	+	—	+	
182	256	256	256	128	+	+	+	+	—	+	
183	256	256	256	128	+	+	+	+	—	+	
185	256	256	256	128	+	+	—	—	—	—	
191	128	256	256	128	+	+	+	+	—	+	
192	128	128	256	128	+	+	+	+	—	+	
193	256	256	256	128	+	+	+	+	—	+	
194	256	256	256	128	+	+	+	+	—	+	
195	256	256	256	128	+	+	+	+	—	+	
201	128	128	128	128	+	+	+	+	—	+	
202	256	256	256	128	+	+	+	+	—	+	
203	256	256	256	128	+	+	+	+	—	—	
204	256	256	256	128	+	+	+	+	—	+	
205	256	256	256	128	+	+	+	+	—	+	

The isolate was numbered by isolated day and strain number. For example, isolate 143 was the third isolate obtained in 14<sup>th</sup> day. Gray shades used to distinct the positive and negative of special virulence factor.

**TABLE 4 |** The frequencies of conjugation transfer between donor and host strains.

Receptor strains	Times	Donor strain (CFU/mL)	Host strain (CFU/mL)	Transconjugant (CFU/mL)	Transfer frequency
<i>E. faecalis</i> JH2-2	1	$1.21 \times 10^9$	$2.62 \times 10^8$	$2.04 \times 10^3$	$7.79 \times 10^{-6}$
	2	$4.30 \times 10^9$	$6.75 \times 10^8$	$9.40 \times 10^2$	$1.39 \times 10^{-6}$
	3	$3.72 \times 10^9$	$4.35 \times 10^8$	$2.60 \times 10^3$	$5.98 \times 10^{-6}$
<i>E. coli</i> NK5449	1	$8.42 \times 10^8$	$6.82 \times 10^8$	$1.90 \times 10^2$	$2.79 \times 10^{-7}$
	2	$1.25 \times 10^9$	$3.71 \times 10^8$	$2.20 \times 10^2$	$5.93 \times 10^{-7}$
	3	$1.13 \times 10^9$	$1.65 \times 10^8$	$6.60 \times 10^2$	$4.00 \times 10^{-6}$

**FIGURE 5 |** Comparison of positive rate of resistant determinants and virulence genes of *E. faecalis* before and after experiment with drug.

## Virulence of the Selected *E. faecalis*

In the 34 *E. faecalis* stains selected in the pre-treatment, positive ratios of *esp*, *gelE*, *cylA*, *ace*, and *asal* were 0, 61.76, 0, 58.8% and 0, respectively. However, these changed into 70, 20, 70, 83.3% and 0, respectively, in the 30 *E. faecalis* stains selected in the post-treatment (Figure 5). The positive rates of surface protein (*esp*) and hemolysin activator (*cylA*) were significantly increased (0–70%). To the contrary, the carriage ratios of gelatinase (*gelE*) had a downside (61.76–20%). Aggregation substances (*asal*) were not found in all of the 64 strains *E. faecalis*. As shown in Table 3, most of the antimicrobial resistance in *E. faecalis* selected after treating with tulathromycin carried resistance gene of *ermB*, transferable element of Tn1545 and three virulence gens (*esp*, *cylA*, and *ace*).

After infection with *E. faecalis* 143 (harboring *ermB*, Tn1545, *esp*, *cylA*, and *ace*), mice died within 8 h which was earlier than the death of mice infected with *E. faecalis* 174 (free of *ermB*, *esp*, *cylA*, and *ace*). The LD<sub>50</sub> of the two *E. faecalis* strains was calculated by improved Karber method. The detailed results were shown in Table 5. The LD<sub>50</sub> *E. faecalis* 143 ( $5.970 \times 10^9$  µg/mL) was three-times higher than that of *E. faecalis* 174 ( $1.603 \times 10^9$  µg/mL), suggesting that *E. faecalis* 143 strain harboring virulence factors (*esp*, *cylA*, and *ace*) was more virulent than the strain without these two virulence genes.

## NOAEC and mADI Calculation

Based on our results, the NOAEC was set up as 1 µg/mL. The daily fecal bolus was known as 220 mL. The fraction of oral dose available for microorganisms in the intestinal tract was 0.25 which can be split into two factors of 0.5 each. One factor of 0.5 was based on results for reduced availability of the substance due to interaction with fecal matter (EMEA, 2004) and the other factor of 0.5 was based on the impact of acidic colonic pH on tulathromycin availability for gut bacteria (USFDA, 2004). The weight of human was 60 kg/person. Therefore, our established mADI was 14.66 µg/kg.bw/d.

## DISCUSSION

This study is the first of its kind to assess the effect of tulathromycin on human microbiota using the chemostat model. In addition, this study concentrated on the antimicrobial resistance development and virulence change of *E. faecalis* isolates in chemostats under long term exposure to a range of tulathromycin concentrations.

The present study showed that the intermediate concentration (10 µg/mL) slightly change the population of *Enterococci* and *Bacteroides fragilis*, while the highest concentration (100 µg/mL) inhibited the growth of three type of bacteria except for *Enterococcus*. This result was similar with our previous results that the numbers of some intestinal bacteria (e.g., *B. fragilis*) underwent significant changes during exposure to tilmicosin (Hao et al., 2015). As one of the most predominant bacteria in the intestinal microbiota, *B. fragilis* may be a most sensitive indicator of gut microbiome under exposure to macrolide drugs including erythromycin, tilmicosin, and tulathromycin (Carman et al., 2005; Merck sharp and D. Corp., 2011; Hao et al., 2015).

The present study and our previous investigation suggested that in the chemostat model high concentration of macrolide drugs (tilmicosin and tulathromycin) could significantly reduce the population of *B. fragilis* and subsequently resulted into the decline of the three main SCFAs especially propionic acid (Hao et al., 2015). The related change of propionic acid with *B. fragilis* population that we found was also observed in human flora associated (HFA) mice model (MacNeil, 2005).

Similar to previous studies, the colonization resistance of human intestinal microbiota in the chemostat was disrupted

**TABLE 5 | Comparing the LD<sub>50</sub> of *E. faecalis* 143 and 174 in the mice experiment.**

<i>E. faecalis</i> no.	Inoculated dose (CFU/mouse)	Total mice number	Dead mice number during experiment				IgLD <sub>50</sub>	LD <sub>50</sub> (CFU/mL)
			0–8 h	8–24 h	24–48 h	>48 h		
14-3	1.60 × 10 <sup>10</sup>	8	7	0	0	0	9.205	1.603 × 10 <sup>9</sup>
	1.60 × 10 <sup>9</sup>	8	3	1	1	0		
	1.60 × 10 <sup>8</sup>	8	0	0	0	0		
	1.60 × 10 <sup>7</sup>	8	0	0	0	0		
	0	8	0	0	0	0		
17-4	1.06 × 10 <sup>10</sup>	8	2	1	1	0	9.776	5.970 × 10 <sup>9</sup>
	1.06 × 10 <sup>9</sup>	8	0	0	1	0		
	1.06 × 10 <sup>8</sup>	8	0	0	0	0		
	1.06 × 10 <sup>7</sup>	8	0	0	0	0		
	0	8	0	0	0	0		

under 7 days exposure to high concentration of antibiotics (Carman et al., 2004; Hao et al., 2013). Coincidentally, in our chemostat model, there were significant alterations in the populations of *E. coli*, *B. fragilis*, *Enterococcus*, and *Bifidobacterium* which appeared to be indicator of intestinal microbiota and contributed a lot to the colonization resistance (Corpet, 1993; Nuding et al., 2013). As previously reported, 10<sup>5</sup> CFU/mL of *E. coli* had a complete inhibitory effect on the growth of *Salmonella* (Carman et al., 2004). However, in our previous result, the treatment of tilmicosin did change the colonization resistance (Hao et al., 2015). This difference suggested that disruption of colonization resistance was largely depended on antimicrobial agents and their concentrations (Carman et al., 2004; Ferreira et al., 2011).

Similar to previous results, resistance rate in *E. coli* did not change in the chemostat and HFA rodent models treated by tilmicosin (EMEA, 1997; Cerniglia and Kotarski, 2005; Hao et al., 2015). This may be due to the inherent resistance to macrolide drugs in *E. coli* (Phuc Nguyen et al., 2009). However, the resistance rate of *Enterococcus*, *Bifidobacterium*, and *B. fragilis* was significantly increased under the selective pressure of 100 and 10 µg/mL tulathromycin in our study. Consistently, the subtherapeutic and therapeutic administration of tulathromycin also significantly increased the proportion of erythromycin resistant enterococci in beef cattle (Zaheer et al., 2013).

Our study showed that most of the isolated tulathromycin resistant enterococci contained *ermB*. The *ermB* gene in different gram-positive bacteria has been well-documented (Schmitz et al., 2000; Perreten et al., 2005; Littauer et al., 2006; Tremblay et al., 2011; Zmantar et al., 2011). The occurrence of macrolide resistance mediated by *ermB* was also found in enterococci originating from swine and cattle due to the subtherapeutic use of tylosin and tulathromycin (Jackson et al., 2004; Chen et al., 2008; Zaheer et al., 2013).

Tn1545 was found in almost of the high-level macrolides resistant *Enterococcus* harboring *ermB* gene. Our conjugation test also showed that the *Enterococcus* isolate containing Tn1545

and *ermB* can transfer its resistance to *E. faecalis* JH2-2 and *E. coli* NK5449. The transferability of *ermB* located in transposon may play an important role on the increase of resistance rate in *Enterococcus*, *Bifidobacterium*, and *B. fragilis* (Okitsu et al., 2005; Ciric et al., 2013).

Both the previous study and our study found that *gelE* gene was one of the predominant virulence genes in *E. faecalis* (Di Rosa et al., 2006). A positive correlation between macrolide resistance and *gelE* virulence gene was observed in an epidemiological investigation (Zou et al., 2011; Lins et al., 2013). High level expression of *gelE* has also been observed in multi-drug resistant Enterococci and in macrolide resistant *E. faecalis* containing *ermB* gene (Arciola et al., 2008; Hao et al., 2015). However, the *E. faecalis* containing both *ermB* gene and gelatinase (*gelE*) did not occur in our study.

Most of *E. faecalis* harboring both virulence factors (*esp* and *cylA*) and resistance gene of *ermB* were selected after treatment with tulathromycin, however, no *E. faecalis* isolates containing these two virulence genes was found before tulathromycin treatment. Coincidentally, recent studies also found the existence of a large pool of potentially virulent and multidrug resistant *E. faecalis* in diseased farm animals (Seputiene et al., 2012). The presence and expression of some virulence determinants (e.g., *esp* and *cylA*) may enhance the colonization and invasion of *E. faecalis* to the epithelial cell of host (Trieu-Cuot et al., 1990; Littauer et al., 2006; Heikens et al., 2009; Johanson et al., 2012; Cafini et al., 2015; Kafil and Mobarez, 2015). The acute toxicity test with SPF mice indicated that *E. faecalis* harboring the macrolide resistant gene of *ermB* and two virulence genes (*esp* and *cylA*) had higher toxicity and pathogenicity.

Our established mADI (14.6 µg/kg.bw/d) was slightly higher than the mADI (10.97 µg/kg.bw/d) recommended by EMA-CVMP and the mADI (5 µg/kg.bw/d) recommended APVMA. Recently, EMA-CVMP revised the mADI of tulathromycin to 55 µg/kg.bw/d (EMA/CVMP, 2015). As a new approved drug, more work of safety assessment needs to be done to establish the final mADI of tulathromycin.

## EXECUTIVE SUMMARY

### Dose dependent effect on colonization resistance

10 µg/mL tulathromycin would significantly decrease the population but increase the resistance rate of *Enterococcus* and *Bacteroides fragilis*. 100 µg/mL tulathromycin significantly decreased population of *Escherichia coli*, *Bifidobacterium* and *B. fragilis* and SCFAs. The colonization resistance was disturbed by higher concentration of tulathromycin.

### Antimicrobial resistance inducement and risk of resistant factors

10 µg/mL tulathromycin would significantly increase the resistance rate of *Enterococcus* and *Bacteroides fragilis*. 100 µg/mL tulathromycin largely increased resistant rate of *Enterococcus* and selected multidrug resistant *E. faecalis*. The selected resistant *E. faecalis* always carried *ermB* gene. It was located in transposons Tn1545 and had ability of horizontal gene transfer (HGT).

### The virulence change under drug exposure

The *E. faecalis* harboring resistance gene of *ermB* gene and virulence gene of *esp* and *cylA* was only selected after administration of high concentration of tulathromycin. The *E. faecalis* harboring *esp* and *cylA* had higher pathogenicity.

### Microbiological acceptable daily intake (mADI) of tulathromycin

The mADI was calculated as 14.66 µg/kg bw/day based on our study.

## CONCLUSIONS

In conclusion, the established NOAEC and mADI in our study was 1 µg/mL and 14.66 µg/kg.bw/day, respectively.

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The low concentration of tulathromycin had no significant microbiological hazard with regard to colonization resistance and antimicrobial resistance. However, the long-term exposure to high concentration of tulathromycin (100 µg/mL) may damage the colonization resistance of human gut microbiota and induce the development of antimicrobial resistance in *Enterococcus*. The *E. faecalis* containing both transferable resistance determinant (*ermB* in Tn1545) and virulence genes (*esp* and *cylA*) were selected after administration of high level tulathromycin. More work need to be done to systematically assessment the safety of tulathromycin on human public health.

## AUTHOR CONTRIBUTIONS

Experiment designation: HH, SZ, YW, and ZY; Experiment implement: SZ, HH, and ZL; Data analysis: SZ, HH, XW, YW, and ZY; Manuscript writing: SZ and HH; Manuscript modification: HH, GC, MD, YW, and ZY.

## FUNDING

This work was supported by Grants from National Basic Research program of China (2013CB127200), project supported by the morning program of Wuhan in China (2015070404010191), Fundamental Research Funds for the Central Universities (2662015PY035), National Natural Science Foundation of China (31101856 and 31272614), and National Program for Risk Assessment of Quality and Safety of Livestock and Poultry Products (GJFP2016008).

## ACKNOWLEDGMENTS

We give our sincere appreciation to Carl E. Cerniglia and Sangeeta Khare from National Center of Toxicological Research, USFDA due to their help on modification of this manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Fecal Carriage of *Staphylococcus aureus* in the Hospital and Community Setting: A Systematic Review

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
Frontiers in Microbiology

Received: 08 February 2016

Accepted: 18 March 2016

Published: 10 May 2016

### Citation:

Claassen-Weitz S, Shittu AO, Ngwarai MR, Thabane L, Nicol MP and Kaba M (2016) Fecal Carriage of *Staphylococcus aureus* in the Hospital and Community Setting: A Systematic Review. *Front. Microbiol.* 7:449.  
doi: 10.3389/fmicb.2016.00449

**Background and rationale:** *Staphylococcus aureus* fecal carriage has been identified as a potential source for nosocomial transmission and a risk factor for disease development. This systematic review determined the overall *S. aureus* [including methicillin susceptible and resistant *S. aureus* (MSSA and MRSA)] fecal carriage rates within the community and healthcare settings.

**Methodology:** Peer-reviewed articles indexed in Medline, Scopus, Academic Search Premier, Africa-Wide Information, CINAHL, and Web of Science were identified using applicable and controlled vocabulary through to 11 November 2015. Eligible studies were ascertained by three independent reviewers. Random-effects meta-analyses of proportions were performed to determine *S. aureus*, MSSA and MRSA fecal carriage rates reported by eligible studies.

**Results:** Twenty six studies were included in this review. The pooled estimates for *S. aureus*, MSSA and MRSA fecal carriage were 26% (95% confidence interval (CI): 16.8–36.3%), 86% (95% confidence interval (CI): 65.9–97.9%) and 10% (95% CI: 0.7–27.0%), respectively. Fecal *S. aureus* carriage rates increased on average from 10 to 65% during the first 8 weeks of life, followed by an average carriage rate of 64% at 6 months and 46% at 1 year of life. Genotyping techniques were employed mainly in studies conducted in developed countries and comprised largely of gel-based techniques. Six studies reported on the role of *S. aureus* fecal strains in diarrhea ( $n = 2$ ) and the risk for acquiring infections ( $n = 4$ ). Eight of the 26 studies included in this review performed antibiotic susceptibility testing of *S. aureus* fecal isolates.

**Conclusion:** This study provides evidence that screening for *S. aureus* fecal carriage, at least in populations at high risk, could be an effective measure for the prevention of *S. aureus* transmission and infection in the healthcare and community setting. More well-structured studies need to be conducted and sequence-based genotyping techniques should be employed for the comparison of isolates on a global scale in both developing and developed countries.

**Keywords:** carriage, community, fecal, *Staphylococcus aureus*, systematic review

## INTRODUCTION

*Staphylococcus aureus* is a commensal Gram-positive bacterium, which under certain circumstances may be responsible for pyogenic or toxigenic infections, such as skin and soft tissue infections, toxic shock syndrome and pneumonia (Tong et al., 2015). Its carriage is considered as an important risk factor for subsequent development of hospital and community-acquired infections (Ellis et al., 2004; Wertheim et al., 2004; Maier et al., 2005; Dukic et al., 2013; Levy et al., 2013). The anterior nares is recognized as the primary site for *S. aureus* colonization (Kluytmans et al., 1997; van Belkum et al., 2009; Sollid et al., 2014). Other anatomical niches for *S. aureus* include the skin (Popov et al., 2014), oropharynx (Mertz et al., 2007; Petersen et al., 2013), intestinal tract (Acton et al., 2009), and the vagina (Bourgeois-Nicolaos et al., 2010).

The importance of fecal carriage of *S. aureus* has been recognized more than five decades ago in a study which demonstrated that rectal *S. aureus* carriage preceded those from the nose and throat in new-borns (Hurst, 1960). Thereafter, several studies have provided evidence on the clinical importance of fecal carriage of *S. aureus* [in particular methicillin-resistant *S. aureus* (MRSA)] in the hospital setting (Acton et al., 2009). For example, it has been shown that hospitalized patients with both *S. aureus* fecal and nasal colonization are significantly more likely to have positive skin cultures compared to patients with nasal carriage only (Bhalla et al., 2007). In addition, *S. aureus* fecal carriage may serve as an important source for environmental contamination, which can potentially facilitate nosocomial transmission within the healthcare setting (Bhalla et al., 2007). Furthermore, antibiotic-associated diarrhea attributed to MRSA has also been reported (Lo and Borchardt, 2009; Sizemore et al., 2012; Avery et al., 2015); and patients with MRSA colonized diarrheal stools impact significantly on environmental contamination (Boyce et al., 2007).

Despite the potential role and significance of the sole fecal carriage of *S. aureus* (Lee et al., 1997; Squier et al., 2002; Bhalla et al., 2007) and the transmission dynamics of *S. aureus* in infection, a limited number of studies have focused on fecal *S. aureus* carriage in the hospital and community setting (Acton et al., 2009). This systematic literature review is therefore aimed to determine the overall rate of *S. aureus* [including methicillin susceptible and resistant *S. aureus* (MSSA and MRSA)] fecal carriage amongst individuals in the community and healthcare settings.

## METHODOLOGY

This review followed the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines (Moher et al., 2009). The PRISMA check-list for this review is provided in a Supplementary Table (Table S1).

### Literature Search Strategy

Peer-reviewed articles (written in English and French) published through to 11 November 2015 on *S. aureus* fecal carriage within the community and healthcare settings were evaluated using four electronic databases and a combination of keywords (Table 1). We also explored for additional articles by checking the references cited in the primary eligible studies included in this systematic review.

### Study Selection and Data Extraction

Potentially relevant articles (selected based on their titles and abstracts) were assessed for eligibility (Table 2) by three independent authors. All potentially eligible articles were screened for “predatory journals” using “Beall’s list” (Beall, 2015; Shen and Björk, 2015; Siebert et al., 2015). The corresponding authors of potentially relevant articles were contacted to determine the healthcare exposure status of participants so as to assess their eligibility for inclusion in this systematic review (Table 2). Data extraction was performed independently by two authors using a standardized data extraction form. Disagreements and inconsistencies were resolved by consensus. The following information was extracted from each eligible study: study population, number of participants screened for fecal carriage, participant characteristics (age, health status, exposure to health care settings), sample collection details (sample type, age at which samples were collected, collection site), laboratory techniques (*S. aureus* and MRSA screening methods, genotyping techniques, virulence profile assessment), as well as *S. aureus* and MRSA detection rates.

### Operational Definitions of Terms Used in this Systematic Review

#### Community Setting

#### Healthy participants

- Participants reported to be healthy at the time of screening for *S. aureus* or MRSA fecal carriage without any exposure to healthcare settings during the year preceding screening (McKinnell et al., 2013);

**TABLE 1 |** Search strategy performed in four databases.

Database	Search mode	Keywords
Medline via Pubmed	All fields	(“staphylococcus aureus”) AND (“gut” OR “gastrointestinal” OR “anal” OR “anus” OR “intestinal” OR “rectum” OR “rectal” OR “stool” OR “feces” OR “faeces” OR “fecal” OR “faecal”) AND (“epidemiology” OR “incidence” OR “prevalence” OR “carriage” OR “carriage rate” OR carrier*) AND (“humans” OR “human”)
Scopus via SciVerse	Article title, abstract, keywords	
Academic Search Premier, Africa-Wide Information and CINAHL via EBSCOHost	Boolean/Phrase	
Web of Science via Web of Knowledge	Topic	

**TABLE 2 | Eligibility criteria.**

Inclusion criteria for systematic review	Exclusion criteria for systematic review
<ul style="list-style-type: none"> <li>Studies published from 1920 to 11 November 2015 were included in the search.</li> <li>Studies reporting on <i>S. aureus</i> or MRSA carriage from fecal/rectal/anal specimens from humans.</li> <li>Studies providing information on the prevalence of <i>S. aureus</i> or MRSA fecal carriage.</li> <li>Healthcare exposure data should include information on whether or not participants were:           <ol style="list-style-type: none"> <li>Hospitalized in the 12 months prior to screening nursing home residents, health care workers, or patients transferred from other hospitals or wards (McKinnell et al., 2013).</li> <li>Screened for <i>S. aureus</i> or MRSA fecal carriage within &gt; or <math>\leq</math> 48 hours of healthcare contact (Folden et al., 2005; Millar et al., 2007; Otter and French, 2011).</li> </ol> </li> <li>Studies published in either English or French.</li> </ul>	<ul style="list-style-type: none"> <li>Studies screening for <i>S. aureus</i> or MRSA from samples other than feces/rectal swabs/anal swabs.</li> <li>Fecal samples studied for parasites or bacteria other than <i>S. aureus</i>.</li> <li>Articles reporting on the number of <i>S. aureus</i> or MRSA isolates detected from fecal specimens or on the number of fecal specimens positive for <i>S. aureus</i> or MRSA, but not providing information on the number of participants testing positive for <i>S. aureus</i> or MRSA fecal carriage.</li> <li>Studies not providing the necessary healthcare exposure data for participants (via the published article or via correspondence with the authors), in order to categorize participants into Healthy participants, Out-patients, In-patients and Healthcare personnel.</li> <li>Articles published in predatory journals (Beall, 2015).</li> <li>Articles not obtainable from the electronic databases, the University of Cape Town (UCT) library or the UCT inter-library loans.</li> </ul>
Inclusion criteria for meta-analysis of proportions	Exclusion criteria for meta-analysis of proportions
<ul style="list-style-type: none"> <li>Overall fecal carriage prevalence for <i>S. aureus</i> and/or MRSA must be available.</li> </ul>	<ul style="list-style-type: none"> <li>Studies providing fecal carriage rates for participants for which fecal carriage rates have previously been reported.</li> <li>Studies not providing information on the age at which participants were screened.</li> <li>Studies screening a pre-selected group of participants based on microbiological assessments.</li> <li>Studies for which MRSA was not confirmed using molecular methods.</li> </ul>

- Pregnant women visiting obstetric clinics;
- New-borns and mothers at maternity wards during the time of delivery;
- Mothers and infants reported as healthy at the time of screening for *S. aureus* or MRSA fecal carriage, but exposed to the delivery unit or maternity ward during the year preceding screening.

#### Out-patients

Patients screened for *S. aureus* or MRSA fecal carriage with  $\leq$  48 h of healthcare contact (Folden et al., 2005; Millar et al., 2007; Otter and French, 2011). Patients should not have had contact with healthcare settings in the year preceding the study.

#### Healthcare Setting

##### In-patients

Patients screened for *S. aureus* or MRSA fecal carriage with >48 h of healthcare contact. Patients screened within  $\leq$  48 h after admission should be those transferred from another hospital/ward which will allow for >48 h of hospital contact.

##### Healthcare personnel

Participants screened for *S. aureus* or MRSA fecal carriage working at a healthcare setting with or without any illness.

#### Developed and Developing Countries

Countries were categorized as developed or developing countries based on data from the International Monetary Fund (<http://www.imf.org/external/pubs/ft/weo/2015/01/weodata/groups.htm>).

#### Antibiotic Susceptibility Results

The percentage of isolates (obtained from participants with *S. aureus* or MRSA fecal carriage) resistant to each of the antibiotics assayed was calculated from studies that provided adequate data on antibiotic susceptibility test results. Our review noted susceptibility tests results whether or not the respective studies incorporated published guidelines [such as Clinical Laboratory Standards Institute (CLSI), National Committee on Clinical Laboratory Standards (NCCLS), European Committee on Antimicrobial Susceptibility Testing (EUCAST), Antibiogram Committee of the French Society of Microbiology (CA-SFM), or the Swedish Reference Group for Antibiotics (SRGA) guidelines] in assessing the antibiotic resistance profiles.

#### Statistical Analysis and Data Visualization

The *S. aureus*, MRSA and MSSA fecal carriage rates for studies included in this systematic review were calculated as follows:

$$\text{S. aureus fecal carriage rate (\%)} =$$

$$\frac{\text{Participants positive for } S. \text{ aureus fecal carriage}}{\text{Participants screened for } S. \text{ aureus fecal carriage}}$$

$$\text{MRSA fecal carriage rate (\%)} =$$

$$\frac{\text{Participants positive for MRSA fecal carriage}}{\text{Participants screened for } S. \text{ aureus or MRSA fecal carriage}}$$

$$\text{MSSA fecal carriage rate (\%)} =$$

$$\frac{\text{Participants positive for } S. \text{ aureus fecal carriage} - \text{Participants positive for MRSA fecal carriage}}{\text{Participants screened for } S. \text{ aureus fecal carriage}}$$

$$\frac{\text{Participants screened for } S. \text{ aureus fecal carriage}}$$

Individual reports assessing the same participants for *S. aureus*, MSSA or MRSA fecal carriage were considered as a single report. Calculated fecal *S. aureus* carriage rates were used to derive longitudinal data of individual studies, as well as the average carriage rate amongst these studies, at each time-point. Meta-analyses of proportions were performed to determine the overall *S. aureus*, MSSA and MRSA fecal carriage rates (pooled estimates) among individuals in the community and healthcare settings. Meta-analyses of proportions for MRSA and MSSA did not include studies for which MRSA was not confirmed using molecular methods. For all meta-analyses of proportions, studies screening for MRSA amongst pre-selected vancomycin resistant enterococci (VRE) fecal carriers were excluded. Similarly, meta-analyses of proportions did not include studies that screened for MRSA fecal carriage solely from pre-selected MRSA carriers (MRSA identified from other body sites). Meta-analyses were performed using StatsDirect statistical software version 3.0.165 [England: StatsDirect Ltd. 2016] for studies adhering to the inclusion criteria summarized in **Table 2**. The StatsDirect statistical software version 3.0.165 [England: StatsDirect Ltd. 2016] was also applied to assess the heterogeneity between the studies included in the meta-analyses (Cochran Q-test) (Cochran, 1954) and to determine the inconsistency across the studies included ( $I^2$  statistic) (Higgins et al., 2003). The criterion for statistical significance for the test for heterogeneity was set at alpha = 0.05. The risk of publication bias was assessed and visualized by a Funnel plot (Egger et al., 1997; Sterne et al., 2011).

## RESULTS

### Study Selection and Characteristics

#### *S. aureus* Study Selection

**Figure 1** outlines the study selection process and the broad reasons for exclusion. The search strategy identified 2522 records. An additional record was identified from the reference list of one of the eligible articles included in the review. A total of 124 potentially eligible reports were identified, of which 69 fulfilled the primary inclusion criteria (**Figure 1**). The vast majority (80%; 55/69) of these potentially eligible articles did not provide information on healthcare exposure during the year preceding screening and/or did not indicate the duration for which patients were admitted prior to the time of screening. Following correspondence with authors, seven articles were excluded as these reports did not fulfill our inclusion criteria. Moreover, 36 articles were excluded due to lack of required information from corresponding authors or as a result of unavailable author contact information. Consequently, only 26 (11 and 15 reports based on their full texts and information obtained from the authors, respectively) of the 69 studies could be included in our systematic review. The main findings reported by each of the 26 eligible studies are summarized in detail in **Tables 3, 4**. Select studies that screened for *S. aureus* fecal carriage from both community and healthcare settings are also reported accordingly in **Tables 3, 4**.

### Characteristics of Reports from Community and Healthcare Settings

#### *Reports on S. aureus* fecal carriage

A total of 19 reports investigated fecal *S. aureus* carriage within the community setting, of which five and 14 studies reported on fecal carriage rates from outpatients and healthy participants, respectively (**Table 3**). Moreover, the majority (64%; 9/14) of reports on fecal *S. aureus* carriage rates from healthy participants were of longitudinal design and investigated infants up until one year of age (**Table 3**). Of the five reports on fecal carriage rates from outpatients, a single study performed a longitudinal analysis of *S. aureus* fecal carriage (Efuntoye and Adetosoye, 2003) and another investigated infants during the first year of life (Shehabi et al., 2013). Study sizes for the community setting ranged between 21 and 1761 participants (**Table 3**).

Fecal *S. aureus* carriage within the healthcare setting was noted in 12 reports (**Table 4**). Of these, 10 were from inpatients and two from healthcare personnel. All reports on inpatients were of cross-sectional design and the majority (60%; 6/10) did not provide information on the age of the participants. In addition, the two studies on healthcare personnel were cross-sectional in design and carried out in the United States of America (USA) (Carmeli et al., 1998; Andrews et al., 2009). Study sizes for healthcare-based reports ranged between 37 and 2727 participants (**Table 4**).

#### *Reports on methicillin susceptible and resistant S. aureus* fecal carriage

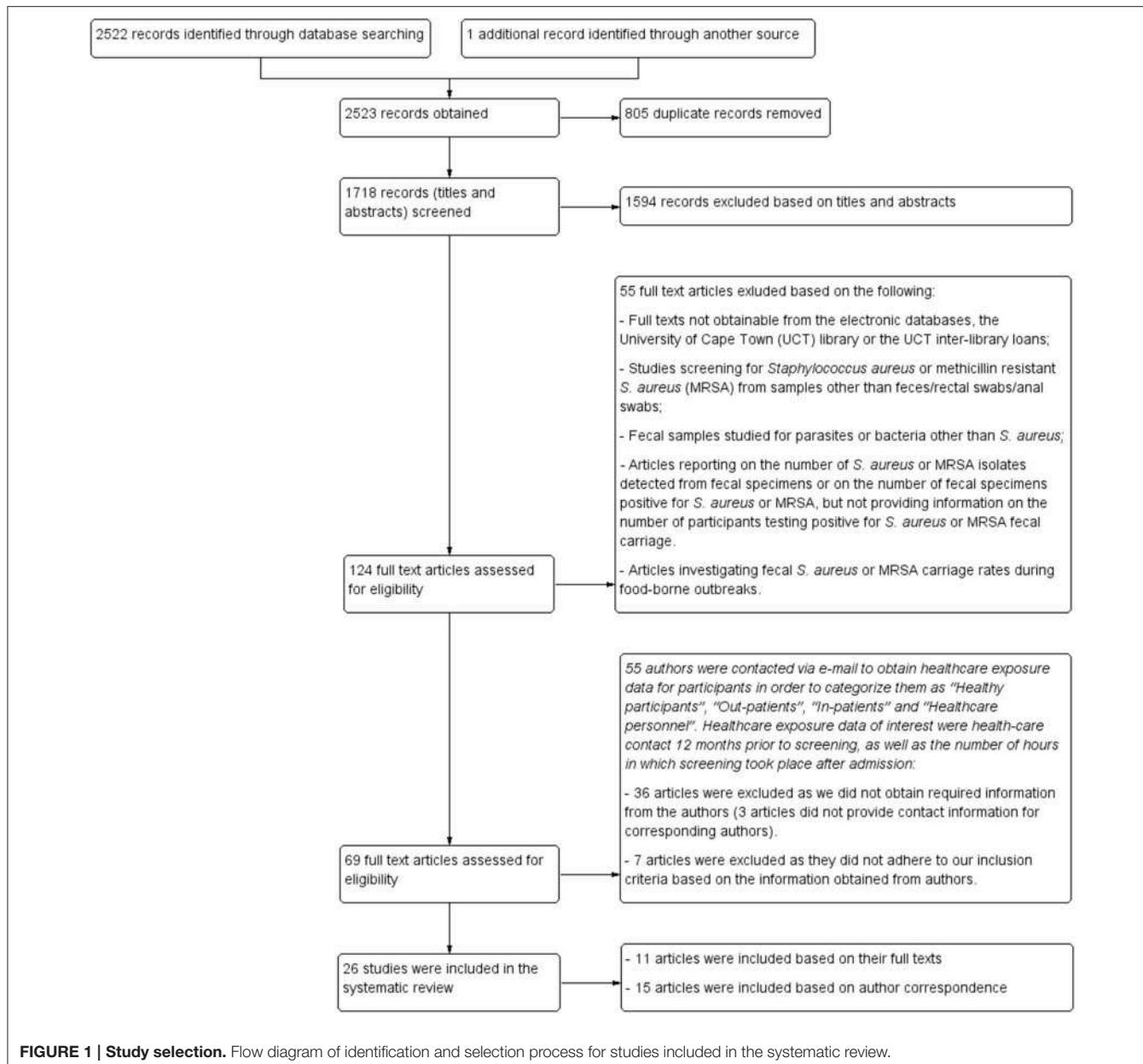
Six of the 19 reports on *S. aureus* fecal carriage from the community setting provided MRSA fecal carriage rates confirmed by molecular methods (**Table 3**). Five of these studies (conducted in developed countries) reported both *S. aureus* and MRSA fecal carriage rates which allowed for the calculation of MSSA fecal carriage rates. Only one study within the healthcare setting (conducted in the USA) confirmed fecal MRSA carriage by screening specimens using a molecular approach (Andrews et al., 2009).

### Pooled Estimates of *S. aureus* Fecal Carriage Rates Assessed by Meta-Analyses

Studies included in all of the proportional meta-analyses were heterogeneous, as determined by the Cochrane Q test and  $I^2$  statistic (**Figures 2–4**). We could not determine pooled MSSA or MRSA fecal carriage rates within the healthcare setting as only a single study was considered eligible for this analysis.

The pooled random-effects estimate for *S. aureus* fecal carriage within the community and healthcare settings was 26% (95% CI = 16.8–36.3; **Figure 5**). Sub-analyses of *S. aureus* fecal carriage within the community and healthcare settings resulted in pooled random-effects estimates of 31% (95% CI = 17.8–46.3) and 5% (95% CI = 1.7–8.9), respectively.

MSSA fecal carriage was estimated at 86% (95% CI = 65.9–97.9) using the random-effects model (**Figure 6**). Within the community setting, the random effects estimate for MSSA fecal



**FIGURE 1 | Study selection.** Flow diagram of identification and selection process for studies included in the systematic review.

carriage was 86% (95% CI = 62.3–98.5). The pooled random-effects estimates for MRSA fecal carriage were 10% (95% CI = 0.7–27.0; **Figure 7**); and 10% (95% CI = 0.4–28.9) within the community setting.

### ***S. aureus* Fecal Carriage Rates According to the Age of Participants**

The report on this section is not based on meta-analysis. *S. aureus* fecal carriage rates within the community setting were higher during the first year of life (**Figure 8**). On average, reports from longitudinal studies revealed an increase in *S. aureus* fecal carriage rates from approximately 10–65% during the first 8 weeks of life (**Figure 8**). At 6 months of age, the average fecal

carriage rate was 64%, thereafter it decreased to approximately 46% at 1 year of life. A longitudinal investigation of fecal MRSA carriage rates from healthy participants from the USA showed an increase in fecal MRSA carriage from 0 to 9% during the first 2 weeks of life (Gries et al., 2009). The highest MRSA fecal carriage rate (23%) reported was from Spanish infants screened at  $\leq 1$  year of life (Benito et al., 2015).

### **Assessment of Antibiotic Susceptibility of Fecal *S. aureus* Isolates**

Eight of the 26 eligible studies (31%) included in this review assayed for antibiotic susceptibility of fecal *S. aureus* or MRSA isolates (**Table 5**). Overall, *S. aureus* or MRSA isolates were

**TABLE 3 |** Characteristics of eligible studies analysing fecal carriage of *Staphylococcus aureus* or MRSA from the community.

Study population setting	Participants screened for fecal carriage (n)	Participant characteristics			Sample collection			Laboratory technique(s)			S. aureus detection & (n/N)	MRSA detection & (n/N)	References
		Age range	Health status	Exposure to healthcare setting 12 months prior to screening†	Sample type	Age at which samples were collected	Site at which S. aureus samples were collected	MRSA detection	Genotyping	Virulence profile analysis			
<b>CATEGORY: HEALTHY PARTICIPANTS</b>													
Italy	100	Birth to 12 months	Healthy	At time of delivery	Rectal swabs	3 days	Delivery unit	Phenotypic NP	RAPD	SE TSST	5 (5/100)¶	NA	Lindberg et al., 2010
				Feces		1 week	Home				15 (15/100)¶		
						2 weeks					24 (24/100)¶		
						4 weeks					34 (34/100)¶		
						8 weeks					45 (45/100)¶		
						6 months					52 (52/100)¶		
						1 year					31 (27/86)¶		
						Overall prevalence					66 (66/100)¶		
Mozambique	121	≤14 days to 1 year	Apparently healthy	NR	Feces	≤14 days to 1 year	Home	Molecular	NP	NP	77 (92/120)	NA	González et al., 2013
Nigeria	120	15–35 years	Healthy	No	Feces	15 to 35 years	Provided by participants	Phenotypic NP	NP	NP	32 (38/120)	34 (13/38)¶	Onanuga and Temedie, 2011
Spain	21	7–35 days	Healthy	At time of delivery¶	Feces	1 week 2 weeks 5 weeks	Home	Phenotypic and Molecular	spa typing agr typing MLST	SE PVL ET PFGE	10 (2/21) 14 (3/21) 48 (10/21)	14 (3/21) 48 (10/21)	Benito et al., 2015
											57 (12/21)	42 (5/12)	
											BAP		
											CNA		

(Continued)

TABLE 3 | Continued

(Continued)

TABLE 3 | Continued

Study population setting	Participants screened for fecal <sup>ψ</sup> carriage (n)		Participant characteristics			Sample collection		Laboratory technique(s)		S. aureus detection	MRSA detection	References	
	Age range	Health status	Exposure to healthcare setting 12 months prior to screening <sup>†</sup>	Sample type	Age at which samples were collected	Site at which S. aureus samples were collected	MRSA detection	Genotyping	Virulence profile analysis	% (n/N)	& (n/N)		
Sweden	50	Birth to 12 months	Healthy	At time of delivery	Rectal swabs	3 days 1 week 2 weeks	Delivery unit Home	Phenotypic NP	RAPD PFGE	ET TSST	20 (10/50) 40 (20/50) 52 (26/50)	NA	Lindberg et al., 2004a
						4 weeks 8 weeks					60 (30/50) 64 (32/50) 68 (34/50)		
						Overall prevalence					24 (9/37)	NA	
37	Apparently healthy <sup>ψ</sup> and non-allergic mothers		Allergic	At time of delivery	Feces	1 week after delivery or at a later stage	Home						
Sweden	81	Birth to 12 months	Healthy	At time of delivery	Rectal swabs	3 days 1 week 2 weeks 4 weeks 8 weeks 6 months 1 year	Delivery unit at 3 days and home at 1 week to 1 year	Phenotypic	Phenotypic RAPD	ET TSST	NR	0 (0/81) <sup>ψ</sup>	Lindberg et al., 2004b
Sweden	49	Birth to 12 months	Healthy	At time of delivery	Rectal swabs Feces	3 days 1 week 2 weeks 4 weeks 8 weeks 6 months 1 year	Delivery unit Home	Phenotypic NP	RAPD	ET TSST	16 (8/49) 57 (28/49)	NA	Lindberg et al., 2000

(Continued)

**TABLE 3 | Continued**

Study population setting	Participants screened for fecal <sup>w</sup> carriage (n)		Participant characteristics		Sample collection		Laboratory technique(s)		<i>S. aureus</i> detection		MRSA detection & (n/N)	References	
	Age range	Health status	Exposure to healthcare setting 12 months prior to screening <sup>†</sup>	Sample type	Age at which samples were collected	Site at which <i>S. aureus</i> samples were collected	MRSA detection	Genotyping	Virulence profile analysis				
United Kingdom	30	2–7 months	Healthy	NR	Feces	2 weeks 10 weeks 7 months Overall prevalence	Home	Phenotypic NP	NP	SE TSST	37 (11/30) 40 (12/30) 40 (12/30)	NA	Harrison et al., 2009
United States of America	147	>18 years	Healthy pregnant women at 35–37 weeks of pregnancy	No <sup>q</sup>	Rectal swabs	>18 years	Obstetric clinics	Phenotypic and Molecular PFGE	SCCmec typing	PVL	4 (6/147) <sup>q</sup>	0 (0/6) <sup>q</sup>	Andrews et al., 2009
United States of America	38	1 day to 2 weeks	Healthy	At time of delivery	Feces	1–2 days 2 weeks Overall prevalence	New-born unit	Phenotypic and Molecular PFGE	SCCmec typing	PVL	0 (0/38) 26 (6/23) 26 (6/23)	0 (0/38) 33 (2/6) 33 (2/6)	Gries et al., 2009
<b>CATEGORY: OUTPATIENTS</b>													
India	100	16–88 years	Patients at admission to other hospitals	No long hospital stay or admission to other hospitals	Feces	16–88 years	Hospital	Phenotypic NP	NP	NP	0 (0/100)	NA	Deepa et al., 2014
Jordan	216	≤28 days to 1 year	NR	No	Feces	≤28 days to 1 year	Clinic	Phenotypic SCCmec typing	ET PVL SE	17 (37/216)	59 (22/37)	Shehab et al., 2013	

(Continued)

TABLE 3 | Continued

Study population setting	Participants screened for fecal <sup>ψ</sup> carriage (n)	Participant characteristics			Sample collection			Laboratory technique(s)			<i>S. aureus</i> detection	MRSA detection	References
		Age range	Health status	Exposure to healthcare setting 12 months prior to screening <sup>†</sup>	Sample type	Age at which samples were collected	Site at which <i>S. aureus</i> samples were collected	MRSA detection	Genotyping	Virulence profile analysis			
Nigeria	1761	≤5 years	Diarrhoeic children	No <sup>ψ</sup>	Feces	<1 year	Hospital	Phenotypic	Phenotypic NP	ET	3 (11/416) 4 (13/323)	NR	Efuntoye and Adetosoye, 2003
						1.1–2.0 years					4 (12/309)	NR	
						2.1–3.0 years					5 (15/292)	NR	
						3.1–4.0 years					5 (21/421)	NR	
						4.1–5.0 years					4 (72/1761)	NR	
						Overall prevalence							
Saudi Arabia	58	NR	Patients at admission (<48 h) <sup>ψ</sup> with diarrhea or abdominal pain	No <sup>ψ</sup>	Feces	NR	Hospital	Phenotypic	Phenotypic NP	NP	NA	9 (5/58)	Babay and Somily, 2009
United States of America	150	Birth to 18 years	Children requiring abscess drainage (n = 60) Children requiring general surgery (n=90)	No <sup>ψ</sup>	Rectal swabs	Birth to 18 years	Hospital	Phenotypic and Molecular	Molecular MLVA SCCmec typing	PVL	47 (28/60)	NR	Faden et al., 2010
											1 (1/90)	NR	

<sup>ψ</sup>Fecal samples, rectal swabs, anal swabs, peri-rectal or peri-anal swabs.<sup>†</sup>Hospital, long-term care facility, nursing homes, maternity wards.<sup>‡</sup>Resistant to cefotaxin.<sup>§</sup>Information obtained from the author.

<sup>¶</sup>Phenotypic identification: culture characteristics on mannitol salt agar, Baird-Parker agar, Tripicase soy agar, Chapman agar, *Staphylococcus* medium 110, positive results for Gram stain, catalase, coagulase and DNase Tests. agr, Accessory gene regulator; AUR, Aureolysin; BAP, biofilm-associated protein; CAN, collagen-binding protein; ET, Exfoliative toxins; MLVA, Multiple-locus variable-number tandem repeat analysis; MRSA, methicillin resistant *Staphylococcus aureus*; NR, Not reported; NP, Not performed; NA, Not performed; PFGE, pulsed-field gel electrophoresis; PVL, Pantón-Valentine Leukocidin; RAPD, random amplified polymorphic DNA; SCCmec, *Staphylococcus aureus* protein A; TSST, Toxic shock syndrome toxin; SE, *Staphylococcal enterotoxins*; spa, *Staphylococcus aureus* protein A; TSS, Toxic shock syndrome toxin.

**TABLE 4 |** Characteristics of eligible studies analysing fecal carriage of *Staphylococcus aureus* or MRSA from the healthcare setting.

Study population setting	Participants screened for fecal <sup>w</sup> carriage (n)	Participant characteristics		Sample collection		Laboratory techniques		S. aureus detection	MRSA detection	Reference
		Age range	Health status	Exposure to healthcare setting 12 months prior to screening <sup>j</sup>	Sample type samples were collected	Age at which S. aureus samples were collected	MRSA detection			
<b>CATEGORY: IN-PATIENTS</b>										
France	748	Mean age: 55 years $\pm$ 12	Liver cirrhosis	Hospitalized for minimum of 2 weeks	Feces	Mean age: 55 years $\pm$ 12	Hospital	Phenotypic	NP	NR (93/748) 2001
France	327	NR	Chronic liver disease, post-surgical patients, patients with alcohol withdrawal and digestive tract diseases	Patients transferred from other hospitals	Feces	NR	Hospital	Phenotypic and Molecular	PFGE	NR (36/327) 2002
Germany	2727	NR	Nosocomial diarrhea	$\geq$ 72 h at the time of study	Feces	NR	Hospital	Phenotypic	NP	NR (198/2727) 2007
Germany	131	NR	NR	Inpatients positive for MRSA	Rectal swabs	NR	Hospital	Phenotypic and Molecular	PFGE and Molecular	NR (61/131) 2005
Jordan	214	$\leq$ 28 days to 1 year	NR	NR	Feces	$\leq$ 28 days to 1 year	NICU	Phenotypic	SCCmec typing	2 (5/214) 2013
Saudi Arabia	122	NR	NR	$\geq$ 72 h at the time of study	Feces	NR	Hospital	Phenotypic	NP	NA 7 (9/122) Babay and Somly, 2009
United States of America	810 (2000-01) NR	Cancer	Inpatients <sup>g</sup>	Rectal swabs	NR	Hospital	Phenotypic and Molecular	spa typing	PVL	NR 0.6 (5/810) <sup>g</sup> et al., 2010
	925 (2006-07)							MLST		2.9 (27/925) <sup>g</sup>

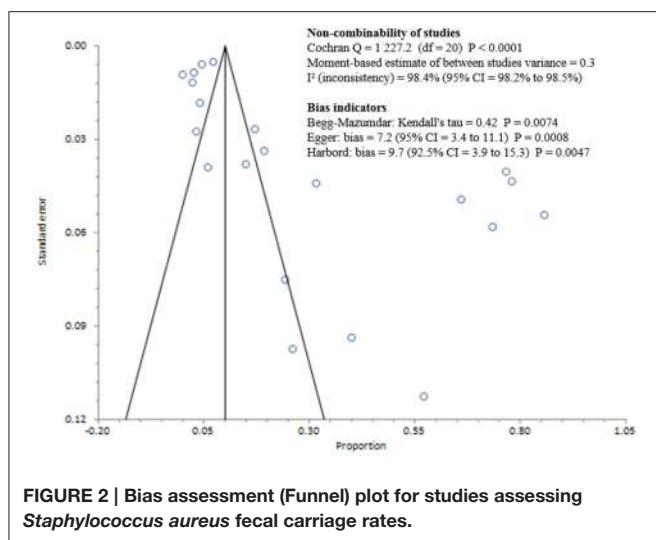
(Continued)

**TABLE 4 | Continued**

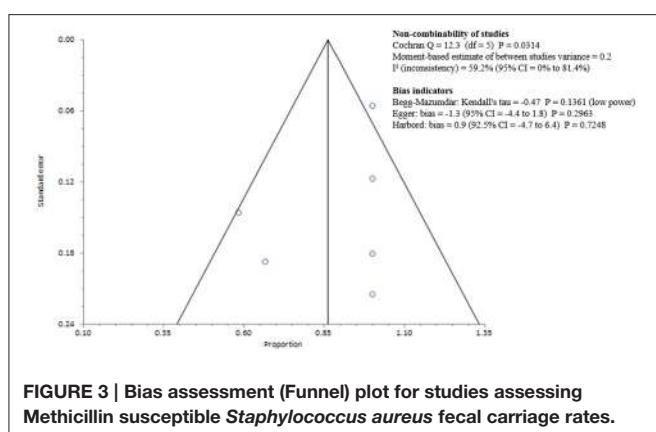
Study population setting	Participants screened for fecal <sup>w</sup> carriage (n)	Participant characteristics			Sample collection			Laboratory technique(s)			S. aureus detection	MRSA detection	Reference
		Age range	Health status	Exposure to healthcare setting 12 months prior to screening <sup>†</sup>	Sample type	Age at which samples were collected	S. aureus detection	MRSA detection	Genotyping profile analysis	% (n/N) & (n/N)			
United States of America	161	57–103 years	Fecal and urinary in-continenence, pressure ulcers, diabetes, COPD, heart failure	Long-term care facility residents	Rectal swabs	57–103 years	Long-term care wards	Phenotypic	PFGE	NP	NA	3 (4/161)	O'Fallon et al., 2009
United States of America	37	48–91 years	Chronic renal failure, Inpatients positive for VRE diabetes, chronic dermatologic infections	Feces	48 to 91 years	Hospital	Phenotypic	PFGE	NP	62 (23/37)	87 (20/23)	Ray et al., 2003	
<b>CATEGORY: HEALTHCARE PERSONNEL</b>													
United States of America	114	NR	NR	Skilled-care patients admitted for long-term care	Rectal swabs	Hospital	Phenotypic	PFGE	NP	NR	5 (6/14)	Trick et al., 2001	
United States of America	62	>18 years	Healthy pregnant women at 35–37 weeks of pregnancy	No <sup>e</sup>	Rectal swabs	> 18 years	Obstetric clinics	Phenotypic and Molecular typing PFGE	SCCmec PFGE	3 (2/62) <sup>w</sup>	0 (0/2) <sup>w</sup>	Andrews et al., 2009	
United States of America	55	36 years±11	NR	Nurses (n = 29), Physicians (n = 15), Others (n = 9), Unknown (n = 2) (mean patient contact years: 13 years±9)	Fecal	36 years±11	NR	NP	Phenotypic NP	NP	NA	0 (0/55)	Carmeli et al., 1998

<sup>w</sup>Fecal samples, rectal swabs, anal swabs, peri-rectal or peri-anal swabs.<sup>†</sup>Hospital, long-term care facility, nursing homes, maternity wards.<sup>e</sup>Information obtained from the author.

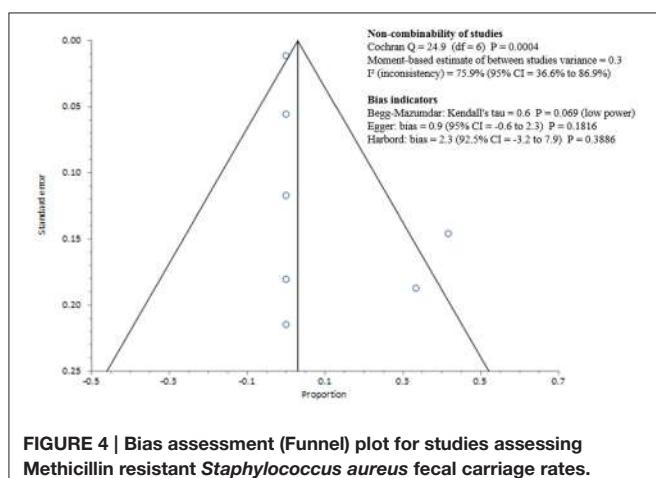
Phenotypic identification: culture characteristics on mannitol salt agar; Chapman agar; Triplicase soy agar; Baird-Parker agar; *Staphylococcus* medium 110, positive results for Gram stain, catalase, coagulase and DNase Tests. agr: Accessory gene regulator; AUF, Aureolysin; BA/P, bifilm-associated protein; CNA, collagen-binding protein; COPD, chronic obstructive pulmonary disorder; ET, Exfoliative toxin; MLVA, Multiple-locus variable-number tandem repeat analysis; MRSA, methicillin resistant *Staphylococcus aureus*; NICU, Neonatal intensive care unit; NR, Not reported; NP, Not performed; NA, Not applicable; PFGE: pulsed-field gel electrophoresis; PVL, Panton-Valentine Leukocidin; SCCmec, staphylococcal cassette chromosome mec; SE, *Staphylococcus aureus* protein A; TSST, Toxic shock syndrome toxin; VRE, Vancomycin-resistance enterococci.



**FIGURE 2 | Bias assessment (Funnel) plot for studies assessing *Staphylococcus aureus* fecal carriage rates.**



**FIGURE 3 | Bias assessment (Funnel) plot for studies assessing Methicillin susceptible *Staphylococcus aureus* fecal carriage rates.**



**FIGURE 4 | Bias assessment (Funnel) plot for studies assessing Methicillin resistant *Staphylococcus aureus* fecal carriage rates.**

screened with 32 different antibiotics across the respective studies using disk diffusion, agar dilution, or the Vitek Legacy System. The use of published guidelines for susceptibility testing were reported by six of the eight studies (Table 5). Susceptibility testing to erythromycin was performed most frequently (88%;

7/8), followed by chloramphenicol, clindamycin, ciprofloxacin, gentamicin, penicillin and vancomycin (75%; 6/8) (Table 5). Vancomycin intermediate or resistant *S. aureus* (VISA/VRSA) were not identified in five of the six studies that screened for vancomycin resistance (Table 5). Only the study by Onanuga and Temedie (2011) reported fecal VRSA carriage of 37% (14/38).

## Genotyping of *S. aureus* Isolated from Fecal Specimens

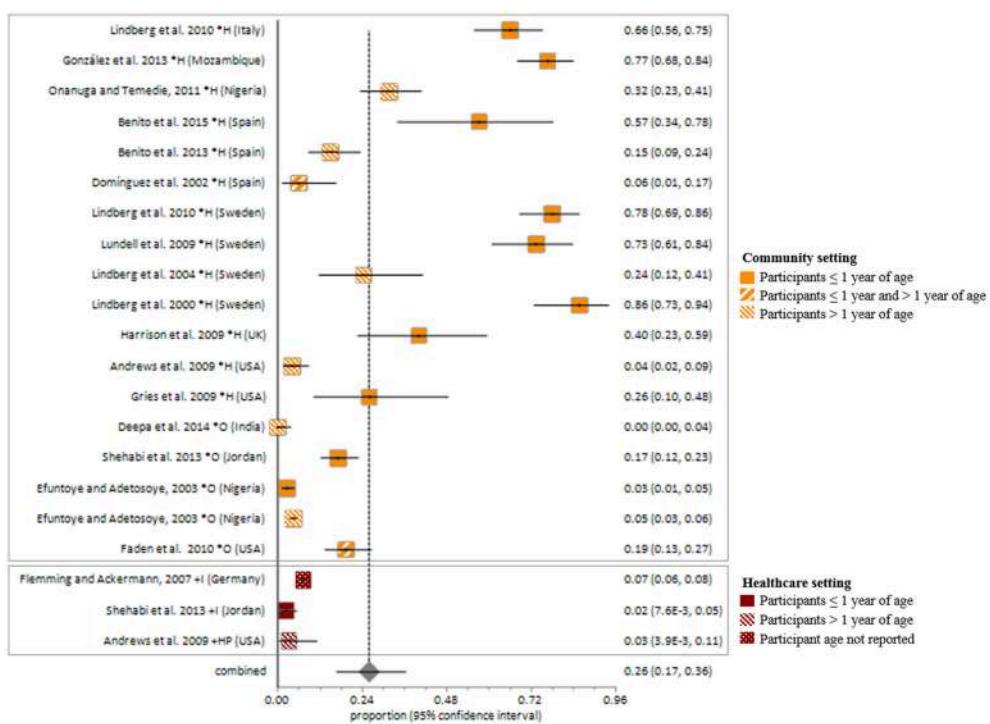
Techniques used to genotype *S. aureus* isolated from fecal specimens included multiple-locus variable-number tandem repeat analysis (MLVA), pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, staphylococcal cassette chromosome *mec* (SCCmec), accessory gene regulator (*agr*) and *Staphylococcus aureus* protein A (*spa*) typing (Tables 3, 4). Genotyping was performed in slightly more reports from the healthcare setting (67%; 8/12) compared to the community (58%; 11/19). Gel-based methods (PFGE, RAPD and MLVA) were employed in 58% (7/12) and 42% (8/19) of studies in the healthcare and community settings, respectively. In addition, similar rates (26% vs. 25%) in the use of sequence-based methods (*spa* typing, SCCmec typing and MLST) for genotyping of *S. aureus* strains were reported from community and healthcare settings. Only a single study conducted in a developing country (Jordan) performed genotyping of the *S. aureus* strains (Shehabi et al., 2013).

## Assessment of the Detection of *S. aureus* Virulence Genes

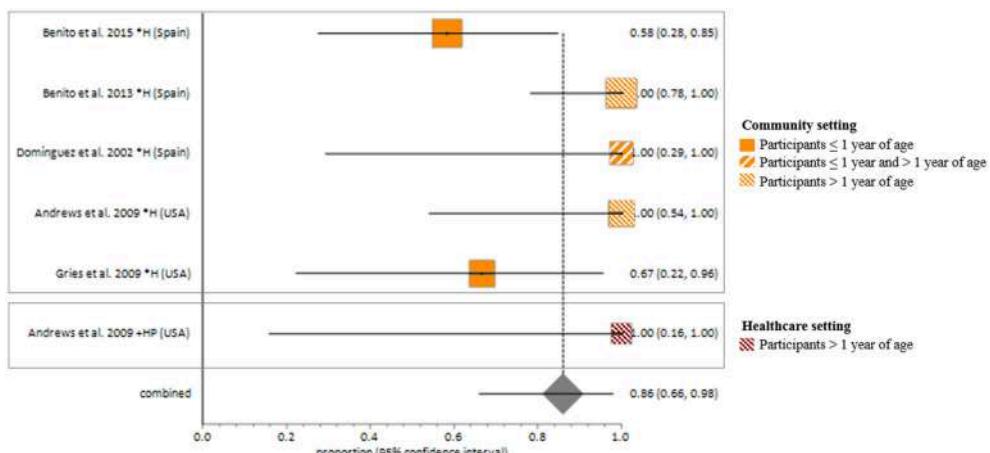
Virulence genes screened included the aureolysin enzyme, biofilm-associated protein, collagen-binding protein, exfoliative toxins (ETs), staphylococcal enterotoxins (SEs), toxic shock syndrome toxin (TSST), and Panton-Valentine leukocidin (PVL) (Tables 3, 4). More community-based investigations screened for *S. aureus* virulence genes compared to reports from the healthcare setting. Thus, 53% (10/19), 37% (7/19), and 37% (7/19) of studies conducted in the community setting reported on TSSTs, SEs, ETs, respectively, using PCR, reverse passive latex agglutination tests or enzyme-linked immunosorbent assays. Approximately one third of the studies conducted in the community setting (6/19) reported on PCR detection of the PVL genes. In studies conducted in the healthcare setting; 8% (1/12), 25% (3/12), 8% (1/12), and 25% (3/12) reported on TSSTs, SEs, ETs, and PVL, respectively.

## *S. aureus* and MRSA Fecal Carriage as Risk Factors for Disease Development

Two studies included in this review identified enterotoxin producing *S. aureus* strains from fecal specimens of patients with diarrhea (Efuntoye and Adetosoye, 2003; Flemming and Ackermann, 2007). Another study reported that all patients colonized with MRSA in both the nares and rectum (8/8) developed an infection (Srinivasan et al., 2010). In addition, two of the nine patients, colonized with MRSA in the rectum only, were concurrently or subsequently infected. *Spa* typing on a



**FIGURE 5 | Meta-analysis of proportions on *S. aureus* fecal carriage rates.** \*, Community setting; +, Healthcare setting; H, Healthy participants; O, Outpatients; I, Inpatients; HP, Healthcare personnel.

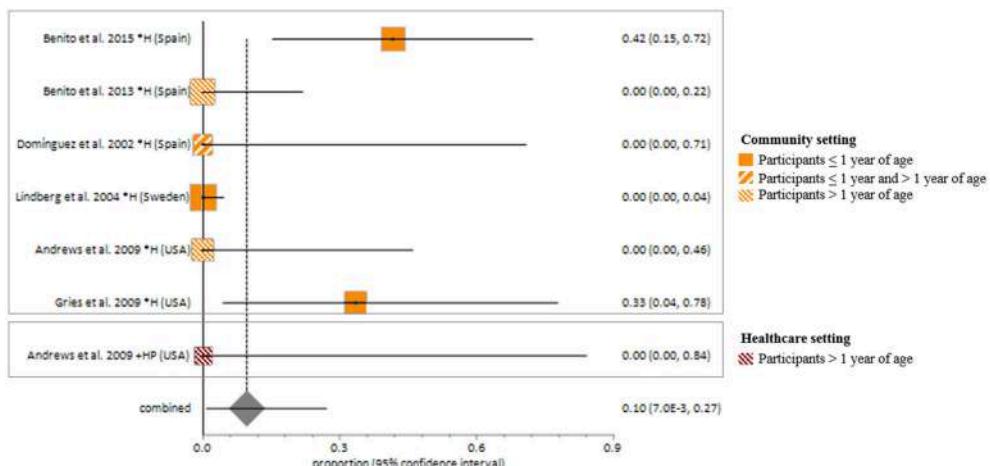


**FIGURE 6 | Meta-analyses of proportions on Methicillin susceptible *Staphylococcus aureus* fecal carriage rates.** Pooled random-effects estimate of MSSA fecal carriage within the community and healthcare setting. \*, Community setting; +, Healthcare setting; H, Healthy participants; HP, Healthcare personnel.

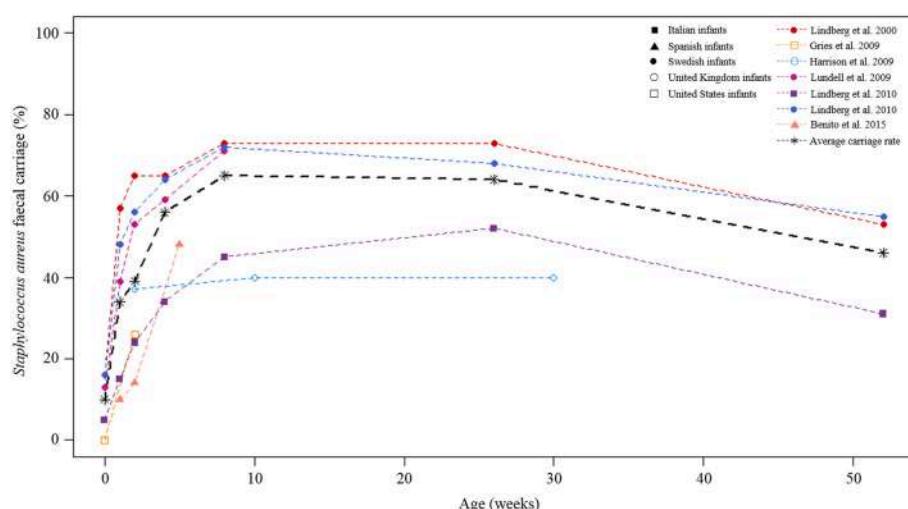
subset of colonizing isolates from the nares and rectum noted that the majority (69%; 9/13) were clonally related to infecting isolates (Srinivasan et al., 2010). In support of the potential of fecal carriage for infection, it has also been shown that *S. aureus* detection occurs more frequently from rectal specimens of children with skin and soft tissue abscesses (47%; 28/60) compared with the control group (1%; 1/90) ( $P = 0.0001$ ) (Faden et al., 2010).

## DISCUSSION

Our results clearly showed that fecal *S. aureus* carriage from healthy infants is high during the first year of life. Specifically, *S. aureus* fecal carriage rates increased during the first 8 weeks of life followed by a gradual decrease towards 1 year of life. The reasons for this abrupt increase in fecal carriage very early in life (especially from healthy infants) is not yet clear,



**FIGURE 7 | Meta-analyses of proportions on Methicillin resistant *Staphylococcus aureus* fecal carriage rates.** Pooled random effects estimate of MRSA fecal carriage within community and healthcare settings. \*, Community setting; +, Healthcare setting; H, Healthy participants; HP, Healthcare personnel.



**FIGURE 8 | Longitudinal *S. aureus* fecal carriage rates during the first year of life.** The lines in color indicated fecal carriage rates (%) at each of the time-points measured by the respective studies. The different cohorts are shown by different symbols at each of the time-points studied. The black broken line is the average fecal *S. aureus* carriage rate calculated from all longitudinal studies at the respective time-points under study.

however a potential explanation may be early life care-giving practices, particularly breastfeeding. For example, colostrum contains the highest levels of human milk oligosaccharides (HMOs) (Bode, 2012), which have been suggested to stimulate *S. aureus* growth (Hunt et al., 2012). Moreover, *S. aureus* strains may be transmitted from parents via skin contact (Lindberg et al., 2004a) or from the mother via breastfeeding (Kawada et al., 2003; Lindberg et al., 2004a; Benito et al., 2015). Furthermore, staphylococci from the maternal GIT or skin surrounding the areola may be transferred to breast milk during lactation (Thum et al., 2012; Fernández et al., 2013). Higher *S. aureus* fecal carriage rates have also been noted from breast-fed in comparison to formula-fed or mixed-fed infants (González et al., 2013; Salminen

et al., 2015). The observed change in the dynamics of *S. aureus* fecal carriage after 8 weeks of life may be explained by the increase in anaerobic bacteria from around 1 week of life (Bezirtzoglou, 1997; Adlerberth et al., 2006; Adlerberth and Wold, 2009; Jost et al., 2012), as well as the introduction of formula feeding (González et al., 2013) and solid foods (Bergström et al., 2014; Voreades et al., 2014). Infant fecal bacterial profiles have also been shown to change during the course of the lactation period (Cabrera-Rubio et al., 2012; González et al., 2013).

This systematic review does not only provide insight into the dynamics of fecal *S. aureus* carriage rates during the first year of life; but also highlights that *S. aureus* and MRSA fecal carriage is a potential risk factor for subsequent infections. Vancomycin is

**TABLE 5 |** Antibiotic resistance profiles across participants screened for fecal *S. aureus* or MRSA.

Study	Guide-lines applied	Techniques applied	Antibiotic resistance profiles of fecal <i>S. aureus</i> or MRSA isolates (%)																							
			Total number of <i>S. aureus</i> or MRSA isolates screened for resistance			Amikoglycosides			Fluorogquinolones			Glycopeptides			Lipopeptides			Macrolides			Oxazolidinones			Penicillins		
Dominguez et al., 2002	NR	Agar dilution method	3	0	0	0	0	0	0	0	0	67	100	0	0	0	0	0	0	0	0	0	0	0	0	0
Efuntoye and Adetosoye, 2003	NCCLS	Disk diffusion method	72	22	0	0	0	0	0	0	0	79	7	100	0	0	0	0	0	0	0	0	0	0	0	0
Lindberg et al., 2004b	SRGA	Disk diffusion method	116	0	0	0	0	0	0	0	0	78	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Flemming and Ackermann, 2007	NR	Disk diffusion method	198	0	0	0	0	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Srinivasan et al., 2010	CLSI	Vitek Legacy System	31*	3	100	0	0	0	0	0	0	81	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Onanuga and Temedie, 2011	CLSI	Disk diffusion and agar dilution method	38	0	5	18	34	24	8	0	0	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Benito et al., 2013	CLSI CA-SFM	Disk diffusion method	15	0	0	0	0	7	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Benito et al., 2015	CLSI EUCAST	Disk diffusion method	25	36	0	16	36	40	4	0	0	36	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(CA-SFM), Antibogram Committee of the French Society of Microbiology; (CLSI), Clinical Laboratory Standards Institute; (EUCAST), European Committee on Antimicrobial Susceptibility Testing; (NCCLS), National Committee on Clinical Laboratory Standards; (SRGA), Swedish Reference Group for Antibiotics; NR, Not reported.

\*Number of MRSA isolates (detected using oxacillin screening agar) screened for antibiotic resistance  
Antibiotic resistance rates (%): █ > 40–50; █ > 30–40; █ > 20–30; █ > 10–20; █ > 0–10; █ 0; █ > 50–60; █ > 60–70; █ > 70–80; █ > 80–90; █ > 90–100.

regarded as one of the drugs of choice for MRSA infections (Tarai et al., 2013); however the emergence of vancomycin resistant *S. aureus* (VRSA) poses yet another threat to infection control (Hiramatsu, 1998; Spagnolo et al., 2014). The intestinal tract, in particular, may be a key potential reservoir for the emergence and transmission of VRSA isolates due to the intestinal coexistence (Ray et al., 2003), and potential transfer of the *vanA* gene from VRE to MRSA (Courvalin, 2006). Although, 23% of the studies included in this review screened for fecal carriage of VRSA within community and healthcare settings (Domínguez et al., 2002; Lindberg et al., 2004b; Srinivasan et al., 2010; Onanuga and Temedie, 2011; Benito et al., 2013, 2015); only a single study, performed in Nigeria, reported VRSA fecal carriage (Onanuga and Temedie, 2011). It is noteworthy, however, that this finding should be interpreted with caution as the disk diffusion method was used to screen for vancomycin resistance at 30 µg/ml, which is not recommended by the CLSI guidelines (Clinical Laboratory Standards Institute, 2012).

Healthcare associated fecal screening for *S. aureus* and MRSA is of key importance in infection control (Campillo et al., 2001; Ray et al., 2003; Bhalla et al., 2007). For example, it has been shown that select staphylococcal enterotoxins (SEs) may contribute to the colonizing success of *S. aureus* strains in the GIT (Nowrouzian et al., 2011), which could potentially facilitate in its transmission. Moreover, *S. aureus* and MRSA fecal carriage may complicate de-colonization, with a potential to contribute to infections within the healthcare setting (Campillo et al., 2001; Dupeyron et al., 2002; Ray et al., 2003; Srinivasan et al., 2010). To prevent nosocomial transmission and infection, two recent studies (Roth et al., 2016; Senn et al., 2016) have also highlighted the importance of screening for *S. aureus* fecal carriage on admission in the following risk groups: patients admitted to surgery or intensive care units with a history of MRSA colonization or infection; hospitalization during the past year; or direct transfer from another healthcare facility. Only a single study was considered eligible for inclusion in our meta-analyses of the proportions on MSSA and MRSA fecal carriage within the healthcare setting. Therefore we could not determine the fecal carriage rate for MSSA or MRSA within this setting.

A major limitation in this systematic review is the poor study design and limited data available from studies assessing the fecal carriage rates of *S. aureus* and MRSA. For example, a large proportion of potentially eligible articles were excluded due to the lack of information regarding participants' contact with healthcare facilities as well as the duration of hospital admission prior to *S. aureus* and MRSA screening. This information is essential in comparing fecal carriage rates from community and healthcare settings. Furthermore, a number of studies could not be included in calculating the pooled estimates for MSSA and MRSA fecal carriage (from both community and healthcare settings) due to the lack of molecular techniques incorporated to confirm MRSA carriage. On the other hand, the extent in which our observations could have changed if unavailable articles were included is unclear. However, based on the rigorous appraisal of various studies in this systematic review, we conclude that the excluded articles are not likely to impact significantly on observations presented in the manuscript. In addition, more

studies from both developed and developing countries are needed in order to determine *S. aureus* and MRSA fecal carriage and transmission within and between the community and healthcare settings. In support of this, rural areas and low socioeconomic status have been shown to contribute to higher fecal transmission rates of *S. aureus* and MRSA (Vale and Vitor, 2010). Finally, there is the need for more sequence-based genotyping data on *S. aureus* and MRSA fecal carriage as the majority of studies from developed countries made use of gel-based methods which are not ideal when comparing isolates on a global level.

## CONCLUSION

*S. aureus*, MSSA and MRSA fecal carriage rates within both the community and healthcare setting are not negligible and estimated at 26, 86, and 10%, respectively. Therefore, preventative strategies which include fecal *S. aureus* screening of high risk patients are necessary for infection control within these settings. More studies are needed to determine the role of fecal *S. aureus* carriage as a risk factor for disease development; as well as fecal carriage rates of MSSA, MRSA, and VRSA from both community and healthcare settings. Furthermore, well-structured research should be conducted and sequence-based genotyping techniques should be employed. The latter will allow for comparison of isolates on a global level in both developing and developed countries.

## AUTHOR CONTRIBUTIONS

MK and SC initiated the project. SC, MRN, and MK searched the databases for potentially eligible articles based on their titles and abstracts. SC extracted the data and contacted authors of potentially eligible publications to obtain healthcare information on participants when this information was unclear or not provided by the articles. SC, MK, and AS reviewed the articles. SC, LT, and MK performed the statistical analysis and interpreted the results. SC, LT, AS, MPN, and MK wrote the manuscript. All the authors reviewed the final version of the manuscript prior to submission for publication.

## FUNDING

This work was supported by the Bill and Melinda Gates Foundation Global Health Grant (OPP1017641), the National Research Foundation (South Africa), the Carnegie Corporation of New York (United States of America), the US National Institutes of Health (1U01AI110466-01A1), and the Wellcome Trust, United Kingdom (102429/Z/13/Z).

## ROLE OF FUNDING SOURCE

Any opinions, findings and conclusions, or recommendations expressed in this review are those of the authors, and therefore do not represent the official position of the funders. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The first and the corresponding author had full access to the study data.

All authors had final responsibility for the decision to submit the article for publication.

## ACKNOWLEDGMENTS

SC is supported by the National Research Foundation and the Drakenstein Child Health Study, University of Cape Town (South Africa), a birth cohort study funded by Bill and Melinda Gates Foundation (OPP1017641). MK was a recipient

of Carnegie Corporation of New York (USA) fellowship, and he is currently supported by Wellcome Trust, United Kingdom (102429/Z/13/Z).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00449>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Genomic Microbial Epidemiology Is Needed to Comprehend the Global Problem of Antibiotic Resistance and to Improve Pathogen Diagnosis

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 16 February 2016

Accepted: 22 May 2016

Published: 15 June 2016

### Citation:

Wyrsh ER, Roy Chowdhury P, Chapman TA, Charles IG, Hammond JM and Djordjevic SP (2016) Genomic Microbial Epidemiology Is Needed to Comprehend the Global Problem of Antibiotic Resistance and to Improve Pathogen Diagnosis. *Front. Microbiol.* 7:843. doi: 10.3389/fmicb.2016.00843

Contamination of waste effluent from hospitals and intensive food animal production with antimicrobial residues is an immense global problem. Antimicrobial residues exert selection pressures that influence the acquisition of antimicrobial resistance and virulence genes in diverse microbial populations. Despite these concerns there is only a limited understanding of how antimicrobial residues contribute to the global problem of antimicrobial resistance. Furthermore, rapid detection of emerging bacterial pathogens and strains with resistance to more than one antibiotic class remains a challenge. A comprehensive, sequence-based genomic epidemiological surveillance model that captures essential microbial metadata is needed, both to improve surveillance for antimicrobial resistance and to monitor pathogen evolution. *Escherichia coli* is an important pathogen causing both intestinal [intestinal pathogenic *E. coli* (IPEC)] and extraintestinal [extraintestinal pathogenic *E. coli* (ExPEC)] disease in humans and food animals. ExPEC are the most frequently isolated Gram negative pathogen affecting human health, linked to food production practices and are often resistant to multiple antibiotics. Cattle are a known reservoir of IPEC but they are not recognized as a source of ExPEC that impact human or animal health. In contrast, poultry are a recognized source of multiple antibiotic resistant ExPEC, while swine have received comparatively less attention in this regard. Here, we review what is known about ExPEC in swine and how pig production contributes to the problem of antibiotic resistance.

**Keywords:** multiple antibiotic resistance, animal production, environmental pollutants, genomic epidemiology, agriculture, *Escherichia coli*

## ANTIMICROBIAL RESISTANCE IN AGRICULTURAL AND CLINICAL SETTINGS

Meat production farming practices have increased in scale over the past 30 years (Landers et al., 2012) and are predicted to continue to increase in line with rising incomes in low- and middle-income countries (Van Boekel et al., 2015). This rationalization of farming has led to intensification of animal production and greater reliance on antimicrobials to control infectious disease, improve feed conversion efficiency, and promote animal growth (Levy, 1992; Sarmah et al., 2006; FAO, 2009; Krishnasamy et al., 2015; Van Boekel et al., 2015). Modeling studies estimate that

global antimicrobial consumption will increase by 67% from an estimated 63,151 tons in 2010 to 105,596 tons in 2030 (Van Boeckel et al., 2015). Much of this rise in consumption is expected to come from China, India, Russia, South Africa, and Brazil; countries where meat consumption is growing due to rising incomes (Van Boeckel et al., 2015). Large scale animal production facilities generate huge volumes of animal waste that is contaminated with veterinary antibiotics. While estimates vary, it is thought that in the USA, 11–14 million kilograms of antibiotics are used annually in the production of animals for food (i.e., livestock; Institute of Medicine; National Research Council; Panel on Animal Health, 1999; Landers et al., 2012). Almost half of these are used for non-therapeutic purposes, which is significantly more than the estimated 1.4 million kilograms of antibiotics used in human medicine (Institute of Medicine; National Research Council; Panel on Animal Health, 1999). Many classes of antibiotics are used in animal production including  $\beta$ -lactams, sulfonamides, tetracyclines, streptogramins, macrolides, lincosamides, polyethers, quinoxalines, elfamycins, glycolipids, arsenicals, and polypeptides (Sarmah et al., 2006). How these compounds are modified within the animal, how they persist in soils and waterways after they are excreted, and their fate when animal slurries are used to fertilize pastures are complex and poorly understood (Sarmah et al., 2006; Zhu et al., 2013). Antimicrobial residues that contaminate groundwater tables, aquatic environments, and land used for crop production exert selection pressure on microbial populations that may promote the lateral transfer of resistance and virulence genes between populations, and contributes to the emergence of novel pathogenic profiles (Venturini et al., 2010, 2013; Baquero and Tobe, 2013; Baquero et al., 2013; Roy Chowdhury et al., 2015). It is perhaps unsurprising that commensal gastrointestinal bacterial populations are commonly resistant to more than one antimicrobial (Bettelheim et al., 2003; Diarrassouba et al., 2007; Bailey et al., 2010) and consequently the frequency of community-acquired infections with resistance to multiple antimicrobials are also increasing (Onken et al., 2015; Paniagua-Contreras et al., 2015; Yahiaoui et al., 2015). Furthermore, surveys have shown the presence of antibiotic resistance genes in the gastrointestinal tracts of humans and animals that have never received antibiotic interventions, specifically in: human feces from remote communities (Bartoloni et al., 2009; Ruppe et al., 2009; Eisenberg et al., 2012), migratory bird populations (Foti et al., 2011; Carroll et al., 2015; Jamborova et al., 2015; Stedt et al., 2015) and various wildlife species (Allen et al., 2010; Baez et al., 2015; Jobbins and Alexander, 2015; Katakweba et al., 2015). This demonstrates that resistance genes in host bacteria can readily spread to naïve microbial populations via waterways, air currents, road incursions into remote areas, animal and bird migration, and land clearance (Tsubokura et al., 1995; Middleton and Ambrose, 2005; Ewers et al., 2009). The application of manure onto pasture from ruminants that have not received recent antibiotic therapy is known to stimulate the expansion of resident flora encoding resistance to  $\beta$ -lactam antibiotics (Udikovic-Kolic et al., 2014). These observations demonstrate our limited understanding of the impact of the application animal waste to agriculture. The mechanisms by which mobile elements

flux through microbial populations are not fully understood, but it is clear that efforts must be made to reduce environmental contamination sourced from human and animal effluent.

Antimicrobial resistance is largely an ecological problem (Summers, 2002, 2006; Stokes and Gillings, 2011; Landers et al., 2012; Berendonk et al., 2015; Gillings et al., 2015). In general, studies have focused on the emergence and carriage of resistance genes in certain key microbial populations, without an appreciation for the wider complexity of the problem, or its origins. Hence, there are numerous under-reported microbial sources of resistance genes. Antibiotic resistance genes often traffic in association with transposons that carry mercury resistance operons; these play an important role in transforming mercury to less biologically toxic forms, thereby regulating the availability of toxic mercury compounds in the environment. Mercury-resistance gene operons have evolved as key components of Tn3-family transposons and these transposons are globally disseminated. Mercury has been released into the environment via geological processes over millions of years and, although anthropogenic activity over the past two centuries has contributed significantly to the release of mercury into waterways and the atmosphere, it is likely that mercury levels were higher prior to the industrial revolution than they are now as a result of extensive volcanic activity (Osborn et al., 1997; Barkay et al., 2003). Despite this, large scale surveillance studies of antimicrobial resistance fail to screen microbial populations for the presence of mercury-resistance genes (Djordjevic et al., 2013). Resistance genes can also accumulate in microbial populations in soils that have been fertilized with animal waste and treated effluent (Heuer and Smalla, 2007; Chee-Sanford et al., 2009; Heuer et al., 2011a,b; Kristiansson et al., 2011; Baquero et al., 2013; Berendonk et al., 2015). Meat, seafood, and vegetables are also important and under-recognized sources of antimicrobial resistance genes in bacteria (Johnson et al., 2005a; Agerso et al., 2014; Kim and Woo, 2014; Ahmed and Shimamoto, 2015a,b; Ben Said et al., 2015; Le et al., 2015; van Hoek et al., 2015; Zhang et al., 2015; Zurfluh et al., 2015).

Anthropogenic industrial processes generate heavy metals including cadmium, lead, arsenic, copper, silver, and a wide range of synthetic organic compounds that pollute natural ecosystems. As such, heavy metal contamination represents yet another selective pressure for the development of antimicrobial resistance (Baker-Austin et al., 2006; Castillo et al., 2008; Li et al., 2010; Zhu et al., 2013). Genes coding for resistance to silver, copper, arsenic and antimony are associated with complex resistance gene loci in bacteria resistant to more than one antimicrobial and have great significance to human, livestock, and plant health (Berg et al., 2005; Seiler and Berendonk, 2012; Hobman and Crossman, 2015; Bondarczuk et al., 2016). The widespread occurrence of genes coding for resistance to heavy metals has, in part, been demonstrated in studies using information mined from publicly available genome sequences highlighting the value of genomic surveillance studies (Hobman and Crossman, 2015).

Genome-wide studies of genes coding for resistance to antibiotics, biocides and heavy metals have shed light on how these genes co-assemble on mobile genetic elements. In an analysis of 2522 fully sequenced bacterial genomes and 4582

plasmid sequences, Pal et al. (2015) were able to identify examples where antimicrobial resistance genes co-occurred. The authors found that the most likely co-selection scenario occurred in bacterial strains that carried plasmid-borne antibiotic resistance genes and biocide/heavy metal resistance genes on the same chromosome. A plasmid localized gene cluster coding for a cadmium/zinc resistance gene (*cadd*) with aminoglycoside and macrolide resistance genes was also identified (Pal et al., 2015). Resistance to these combinations of antimicrobials would benefit bacterial populations found in intensive animal production systems where cadmium and zinc are both used to promote growth and aminoglycosides and macrolides are frequently administered for the treatment of Gram-negative and Gram-positive infections, respectively (Castillo et al., 2008; Li et al., 2010). It is clear that plasmids can carry combinations of genes coding for resistance to more than one microbial agent alongside genes coding for resistance to biocides and heavy metals. Invariably these plasmids are large, conjugative and commonly found in pathogens from hospital and intensive farming environments (Chen et al., 2007; Woodford et al., 2009; Venturini et al., 2010, 2013; Pal et al., 2015). Sub-lethal levels of antibacterial biocides and heavy metals found in mildly polluted aquatic and land-based ecosystems may be sufficient to maintain these mobile elements carrying multiple antibiotic, biocide and heavy metal resistance genes (Stepanauskas et al., 2006; Gullberg et al., 2014).

Although plasmids can vary in size and coding ability, their size is generally positively correlated with the size of the host chromosome (Smillie et al., 2010). Plasmids conferring multiple-drug resistance are usually large, as they code for a suite of resistance genes, alongside associated integrons, transposition factors, and genes for heavy metal resistance, which can be in excess of 20,000 bp or more in size. Small plasmids that acquire resistance to more than one antimicrobial are, however, not without precedent. Labar et al. (2012) described the sequence of a small plasmid (pASL01A; 27,072 bp) that codes for resistance to ampicillin, streptomycin, sulfonamides, trimethoprim, and mercury (Labar et al., 2012). Most of the plasmid (21,904 bp) is comprised of a Tn21 derivate mercury resistance transposon that carries a class 1 integron with a trimethoprim resistance *dfrA7* gene cassette and Tn6029C (Roy Chowdhury et al., 2015). Tn6029C comprises a *bla<sub>TEM-1</sub>*-IS26-*repA/C-sul2-strA-strB* gene cluster flanked by direct copies of IS26 (Labar et al., 2012; Roy Chowdhury et al., 2015). The plasmid backbone (5,168 bp) and related plasmids are widespread in enterobacterial populations in Africa (Labar et al., 2012).

Antimicrobials, including heavy metals, are used widely in animal production to treat clinical disease, to prevent disease outbreaks during critical or vulnerable periods, and to promote growth (McEwen and Fedorka-Cray, 2002; Landers et al., 2012). The use of antibiotics to promote growth is a phenomenon whereby animals receiving very low doses of antimicrobials appear healthier and grow larger (Roy Chowdhury et al., 2014). Although, the mechanism for this growth promoting effect is still poorly understood, the majority of pigs produced in the USA are exposed to tetracyclines and tylosin to prevent disease and promote growth (Apley et al., 2012). While these approaches

have proved effective in controlling disease and aiding farms globally, the uninhibited use of antimicrobials also provides the selection pressure that drives antimicrobial resistance (Pulcini and Gyssens, 2013). As such, concerted efforts to reduce the use of antimicrobials in food production should be a priority.

Given that the persistence and spread of resistance is intrinsically linked with the presence and activity of antimicrobials, it is significant that 30–90% of ingested antibiotics are excreted in an un-metabolized or only partially metabolized form (Sarmah et al., 2006) into waste treatment plants and animal waste-holding facilities. Antibiotic residues are known to persist in secondary effluent despite the treatment process (Sarmah et al., 2006; Watkinson et al., 2007; Martinez, 2008; Le-Minh et al., 2010; Bondarczuk et al., 2016). Furthermore, the pH, temperature, nutrient concentration, and bacterial loads in waste treatment plants are conducive to the evolution and spread of antimicrobial resistance (and virulence) genes (Andersen, 1993; Dolejska et al., 2011). This is in part evidenced by an increase in the presence of the class 1 integrase gene, a reliable proxy for resistance to more than one antimicrobial in bacterial populations sampled in wastewater treatment plants (LaPara et al., 2011; Ma et al., 2013; Gillings et al., 2015), and an increase in the number of resistance genes in wastewater treatment plants and rural domestic wastewater treatment systems (Gao et al., 2012; Chen and Zhang, 2013; Ju et al., 2015; Mao et al., 2015). This indicates that controlling the use of antimicrobials is a prerequisite for slowing the development and spread of pathogens with multiple antimicrobial resistance mechanisms.

## ANTIMICROBIAL RESISTANCE IN *Escherichia coli*

*Escherichia coli* is a widespread and abundant organism capable of causing a wide range of gastrointestinal and extra-intestinal diseases. It can survive and proliferate in a diversity of terrestrial and aquatic environments (van Elsas et al., 2011). As such, its genetic profile is shaped profoundly by horizontal gene transfer, and it is a useful marker species for understanding how antimicrobial resistance and virulence genes accumulate over evolutionary time. Horizontal gene transfer events are known to generate novel combinations of putative and established virulence factors, and generate novel pathotypes of *E. coli* (Wu et al., 2007; Bielaszewska et al., 2014). Although, *E. coli* is a commensal inhabitant of the gastrointestinal tract of warm-blooded animals, pathotypes have evolved that cause intestinal [intestinal pathogenic *E. coli* (IPEC)] and extra-intestinal [extraintestinal pathogenic *E. coli* (ExPEC)] diseases. Different pathotypes of *E. coli* cause diarrheal disease, hemolytic uremic syndrome (HUS), urinary tract infection (UTI), pyelonephritis, septicemia, meningitis, and respiratory disease (pneumonia) in humans and animals. Known IPEC pathotypes include the attaching and effacing *E. coli* (AEEC), Shiga-toxigenic *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse adhering *E. coli* (DAEC) and the recently described enteroaggregative hemorrhagic *E. coli* (EAHEC; Kaper et al., 2004; Brzuszkiewicz

et al., 2011). EHEC are a subgroup of STEC, which colonizes the terminal regions of the gastrointestinal tract of ruminants. It is notable that different serotypes of *E. coli* preferentially colonize different ruminant hosts (Djordjevic et al., 2001, 2004; Hornitzky et al., 2002) and carry different Shiga toxin gene variants (Ramachandran et al., 2001; Brett et al., 2003a,b). These studies underscore the importance of understanding the ecology of disease-causing organisms and how niche adaptation influences lateral gene transfer. Notably, however, ruminants are not a recognized source of ExPEC (Manges and Johnson, 2015).

Many recent molecular surveillance studies on *E. coli* report the frequency of genes that code for extended-spectrum beta lactamase enzymes (ESBLs), which are indicative of resistance to penicillin, cephalosporin, and other clinically important antibiotics, but fail to screen for resistance to the older, first generation antibiotics (Bean et al., 2009; Ewers et al., 2012; Glasner et al., 2013; Roschanski et al., 2015). Genes that code for resistance to first generation antibiotics are often carried in bacteria containing class 1 integrons (Johnson et al., 2005b; Jakobsen et al., 2011; Hansen et al., 2014). From a diagnostic perspective, recent genomic studies on an outbreak of EAHEC and EAEC strain O104:H4, highlighted this issue (Ahmed et al., 2012). EAHEC is a recently emerged *E. coli* pathotype and EAEC strain O104:H4 has acquired the Shiga toxin gene *stx2a*, a diagnostic marker of EHEC. Outbreak strains were found to be resistant to ampicillin, streptomycin, sulfamethoxazole, trimethoprim, and tetracycline but little effort was given to determining how the genes that coded for these antibiotic resistance phenotypes were assembled (Ahmed et al., 2012). We were subsequently able to show that the genes coding for resistance to these antibiotics were assembled in *Tn6029D*, a transposon that abuts a class 1 integron in a *Tn21/Tn1721* hybrid backbone (Roy Chowdhury et al., 2015). The resistance region resided in genomic island 3 which also carried the virulence gene *ag43* encoding a self-associating serine protease auto-transporter, important for biofilm formation. This is an example of how antimicrobial resistance genes and virulence genes co-localize on the same mobile genetic element (Roy Chowdhury et al., 2015). Chromosomal islands are increasingly associated with carriage of class 1 integrons encoding resistance to multiple antibiotics of clinical significance (Roy Chowdhury et al., 2016).

*Salmonella* genomic island 1 (Boyd et al., 2001; Levings et al., 2005), *Salmonella* genomic island 2 (Levings et al., 2008) and several large plasmids in EHEC strain O26:H (Venturini et al., 2010), and an atypical EPEC, strain O111 (Venturini et al., 2013) are also examples of virulence and resistance genes being carried on the same mobile element. Strains of *E. coli* that carry genes that are markers for more than one *E. coli* pathotype pose a dilemma for diagnostic laboratories. There are numerous reports describing novel *E. coli* strains with combinations of virulence genes that are diagnostic of more than one *E. coli* pathotype; this suggests that newly emerging strains are increasingly likely to have complex resistance loci facilitated by the clustering of mobile element-associated resistance and virulence genes. In a study of 265 cases of *E. coli* causing UTI, 28 carried virulence genes typical of IPEC (Bielaszewska et al., 2014). Several other examples of hybrid *E. coli* strains have been described and their presence

demonstrates the need to have genomic surveillance strategies in place to determine how antibiotic resistance and virulence genes assemble in emerging pathogens (Wallace-Gadsden et al., 2007; Abe et al., 2008; Bielaszewska et al., 2014; Mariani-Kurdjian et al., 2014).

Extraintestinal pathogenic *E. coli* is a phylogenetically diverse group comprised of a broad range of *E. coli* sequence types (STs). ExPEC are fecal *E. coli* that rarely cause intestinal disease but have acquired a wide variety of virulence gene cargo that facilitates their ability to cause disease at extraintestinal sites, particularly in the urinary tract. ExPEC are the most frequently isolated Gram negative bacterial pathogen infecting humans incurring significant mortality and morbidity. Notably, ExPEC are a leading cause of sepsis and are increasingly resistant to multiple antibiotics posing a serious health concern (Manges and Johnson, 2015; Poolman and Wacker, 2016). ExPEC have a fecal origin but rarely cause gastrointestinal disease. The acquisition of a diverse range of virulence factors by lateral gene transfer has armed ExPEC with an ability to cause life-threatening blood-borne, urinary tract, respiratory, skin and soft tissue infections, and meningitis in humans of all ages (Russo and Johnson, 2003; Poolman and Wacker, 2016). ExPEC vary widely in the combinations of putative virulence genes that they carry. Many genes encode for adhesins, iron acquisition systems and other putative virulence attributes and this genetic redundancy complicates efforts to develop broadly applicable molecular diagnostic tests to detect ExPEC (Johnson et al., 2001; Spurbeck et al., 2012). Importantly, livestock are important reservoirs for ExPEC (Ewers et al., 2007, 2009; Manges et al., 2007; Vincent et al., 2010; Nordstrom et al., 2013; Manges and Johnson, 2015). Virulence genes are often shared among *E. coli* isolates from samples collected from humans, and the meat and feces of intensively reared livestock, particularly poultry (Rodriguez-Siek et al., 2005; Vandekerchove et al., 2005; Jakobsen et al., 2010a,b). Consistent with this view, ExPEC carry genes coding for resistance to antimicrobials frequently used in veterinary medicine. Hospital diagnostics laboratories do not screen for most of the antibiotics used in veterinary medicine and so resistance to these is under-reported. In a Chinese study of 315 ExPEC isolates from various pig tissues, more than 63% exhibited resistance to 10 antibiotics including ampicillin, trimethoprim, sulfadimidine, tetracycline, neomycin, streptomycin, and kanamycin (Tan et al., 2012). The genes *blaTEM-1* (ampicillin resistance); *strAB* (streptomycin resistance); *sul1* and, *sul2* (sulfonamide resistance); *aphA1* (kanamycin and neomycin resistance); *tetA*, *tetB*, and *tetG* (tetracycline resistance); as well as various *dfrA* genes (trimethoprim resistance) coded for resistance to these antibiotics.

The class 1 integron is widespread in clinical settings. Bacteria that are resistant to more than one antimicrobial often carry class 1 integrons and their presence is a reliable indicator of multiple antimicrobial resistance (Leverstein-van Hall et al., 2003). The class 1 integron structures identified in most clinical samples are thought to be derived from the capture of a chromosomally located class 1 integron, prevalent within environmentally abundant bacterial species belonging

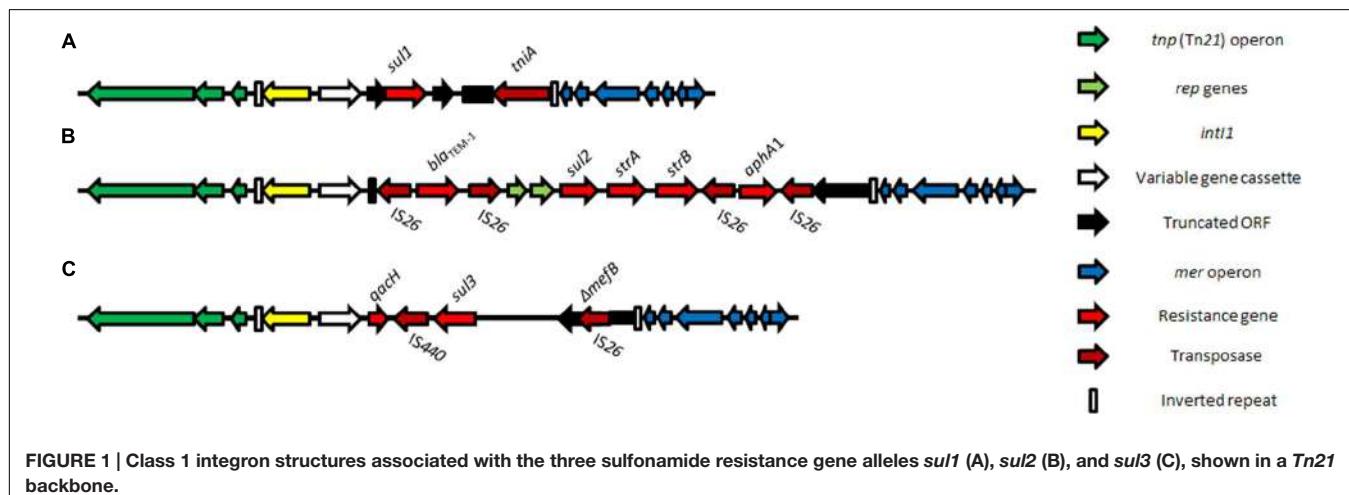
to the broad  $\beta$ -proteobacterium group, by a Tn402 family transposon (Gillings et al., 2008; Gillings, 2014). Global spread of the class 1 integron is in part a result of the propensity for the Tn402 family of transposons to target *res* sites found on a wide variety of conjugative and mobile plasmids, including the widespread mercury-resistance transposon, Tn21, and the broader Tn3 family (Liebert et al., 1999; Gillings et al., 2008, 2015). For this and a variety of other reasons described below, resistance genes cluster on mobile elements. Clinical class 1 integrons have subsequently evolved, typically by the loss of most of the Tn402 *tniB* transposition gene, and partial fusion of the *qacE* gene (coding for an efflux pump for disinfectants, Hall et al., 1994; Partridge et al., 2001) with the sulfonamide resistance gene *sul1*. The most commonly observed structure observed in clinical isolates (Figure 1A) is comprised of a 5' conserved segment (5'-CS), a 3'-conserved segment (3'CS) and a variable region inserted in between them. The 5'-CS contains the *intI1* gene encoding the class 1 integrase; the Tn402 inverted repeat, the two promoters, one required for the transcription of the integrase and the other for the gene cassettes and the *attI1* recombination site. The variable region contains a diverse array of resistance gene cassettes (Partridge et al., 2009) and the associated *attC* sites. The 3' conserved segment (3'-CS) most frequently contains *qacEΔ1* and *sul1* genes, remnants of the transposition module and a terminal Tn402 inverted repeat. Most gene cassettes found in clinical class 1 integrons from nosocomial and livestock settings code for resistance to trimethoprim (*dfrA*) and aminoglycosides (*aadA*); despite this over 130 different gene cassettes have been described (Partridge et al., 2009) and, to date, 410 annotated variants are recognized (Tsafnat et al., 2011).

Further variation in the structure of the 3'-CS has also been described (Pan et al., 2006; Cain et al., 2010; Dawes et al., 2010; Saenz et al., 2010; Venturini et al., 2010, 2013; Mattiello et al., 2015). In one instance, the 3'-CS was almost completely lost and had been replaced with the compound transposon, Tn6026 (Venturini et al., 2013). The Tn6026 structure contains the beta-lactamase *bla<sub>TEM-1</sub>* gene; the sulfonamide resistance gene, *sul2*; the streptomycin resistance gene *strAB*; the neomycin/kanamycin resistance gene, *aphA1* and it is flanked by direct copies of IS26

(Figure 1B). Tn6026 is located between *attI*, the insertion point of the gene cassette, and the defective *tni* module of a clinical class 1 integron, replacing all but the first 24 bp of the 3'-CS (Cain et al., 2010; Dawes et al., 2010; Venturini et al., 2010, 2013). A *sul3* variant structure associated with IS26 has also emerged over the last decade from human- and animal-sourced isolates (Figure 1C). The first report of *sul3* was from Swiss pigs and humans in Grape et al. (2003) and Perreten and Boerlin (2003). Since then, *sul3* has been reported in samples from a number of countries (Pan et al., 2006; Saenz et al., 2010; Tang et al., 2011; Mattiello et al., 2015; Wyrsch et al., 2015; Moran et al., 2016). These atypical class 1 integrons (Figure 1) are often components of larger complex resistance regions, hosted on plasmids that also carry virulence genes (Venturini et al., 2010, 2013).

Small mobile elements, including insertion sequences (IS1, IS26, ISEcp1c, and IS6100 are of note), Insertion Sequence Common Regions (ISCRs) and composite transposons formed from two or more IS elements, play an important role in reshaping the 3'-CS of clinical class 1 integrons (Daly et al., 2005; Dionisi et al., 2009; Venturini et al., 2010). The genes *blaSHV-11*, and *blaSHV-12* (coding for  $\beta$ -lactam resistance; Ford and Avison, 2004; Dionisi et al., 2009); *qnrB19* (coding for quinolone resistance; Dionisi et al., 2009); and *aphA1* (coding for kanamycin/neomycin resistance; Cain et al., 2010) have been shown to be flanked by direct copies of IS26. The mechanism of action of IS26 has been the subject of several recent studies (Harmer et al., 2014; Harmer and Hall, 2015; He et al., 2015). These studies show that resistance loci containing IS26 can be hotspots for the capture of further resistance genes and other genetic cargo flanked by IS26 and demonstrates the important role of IS26 and other mobile elements in the assembly and re-assembly of novel complex resistance loci.

Currently, genomic epidemiological surveillance relies heavily on whole genome and metagenomic sequence information generated using short-read (<400 bp) sequencing platforms. A fall in the cost of high throughput, next generation sequencing with platforms that generate short-read lengths means they have become the leading technology for characterizing microbial evolution and uncultured microbial consortia, and to catalog



**FIGURE 1 |** Class 1 integron structures associated with the three sulfonamide resistance gene alleles *sul1* (A), *sul2* (B), and *sul3* (C), shown in a *Tn21* backbone.

antimicrobial resistance and virulence gene carriage. However, short-read sequencing technologies are unable to assemble complex resistance gene loci, or generate complete genome sequences, without which it is difficult to characterize mobile genetic elements rapidly and reliably. These limitations severely restrict efforts to comprehend the role of lateral gene transfer in the evolution of microbial pathogens and antimicrobial resistance, particularly in regards to the microevolutionary events that lead to the generation of novel complex resistance loci. Long-read sequencing technologies have improved significantly over the past 5 years and are beginning to address this shortfall, but are currently either cost prohibitive for large-scale genomic epidemiological surveillance or require further improvements in base call accuracy (Loman and Pallen, 2015).

Genes that code for resistance to older-generation antibiotics are often incorporated within complex resistance regions. In part, this provides a plausible explanation for why these genes persist despite enforced restrictions on prescribing of older-generation antibiotics. In the UK, strict limitations on the prescription of sulfonamide drugs were enforced in Anonymous (1995). However, functional *sul* genes persist in clinically important Gram negative bacteria and their frequency has not declined since the restriction was imposed (Enne et al., 2001; Bean et al., 2005, 2009). A similar scenario unfolded in Finland when trimethoprim was withdrawn from use for the treatment of UTI (Heikkila et al., 1990). In the Enterobacteriaceae, resistance to sulfonamides, trimethoprim, ampicillin, tetracycline, and fluoroquinolones remains high (Enne et al., 2001; Nozarian and Abdollahi, 2015; Paniagua-Contreras et al., 2015; Uzun et al., 2015; Yahiaoui et al., 2015). Carriage rates for *sul1*, *sul2*, or *sul3* genes in *E. coli* isolated from retail meat, livestock, healthy humans, and humans with clinical disease also remains a problem (Enne et al., 2001; Blahna et al., 2006; Frank et al., 2007; Altalhi et al., 2010; Bailey et al., 2010; Saenz et al., 2010; Vogt et al., 2014; Yahiaoui et al., 2015). Carriage of *sul* genes is linked with the presence of both typical and atypical class 1 integrons, including genes that code for resistance to ampicillin, streptomycin, and trimethoprim (Bettelheim et al., 2003; Bean et al., 2009; Bailey et al., 2010; Soufi et al., 2011; Ewers et al., 2014; Yahiaoui et al., 2015). Combinations of genes coding for resistance to first generation antibiotics contribute significantly to the multiple antimicrobial resistance phenotypes observed in commensal *E. coli* (Bettelheim et al., 2003; Vinue et al., 2008, 2010; Bailey et al., 2010). Genetic studies that have examined resistance to a broad spectrum of antibiotics frequently report the presence of *blatem-1*, *sul1*, *sul2*, *strAB*, *tetA(A)*, and a range of *aadA* and *dfrA* genes (Soufi et al., 2011; Labar et al., 2012; Ewers et al., 2014; Yamamoto et al., 2014; Yahiaoui et al., 2015). The streptomycin resistance gene, *strAB*, and the *sul2* sulfonamide resistance gene are also spread via small plasmids such as RSF1010 (Yau et al., 2010), and IncQ plasmids that are related to RSF1010 (Tietze et al., 1989). The *strAB* gene and the adjacent inverted repeat IR<sub>R</sub>, as seen in RSF1010, probably had their origins in Tn5393c, while *sul2* was recruited from a CR2-containing element (Yau et al., 2010). These molecular events describing the evolution of the *sul2-strAB* configuration observed in RSF1010 have been described previously (Yau et al., 2010). The

*sul2-strAB* gene cluster and flanking sequences from RSF1010 have been captured onto Tn21 derivative transposons, on large and small plasmid backbones, and in chromosomal islands; they are often in association with IS26 and are distributed widely (Daly et al., 2005; Cain et al., 2010; Venturini et al., 2010, 2013; Labar et al., 2012; Reid et al., 2015; Roy Chowdhury et al., 2015).

## ***E. coli* IN INTENSIVE PRODUCTION ANIMALS**

### **Porcine Enterotoxigenic *Escherichia coli* (ETEC)**

Enterotoxigenic *Escherichia coli* (ETEC) are the causative agents of three distinct diseases in young pigs: neonatal, pre-weaning, and post-weaning diarrhea (Fairbrother et al., 1988; Noamani et al., 2003). These diseases vary in severity and are categorized by the age at which the pig develops symptoms. Diarrhea caused by ETEC strains, generally known as enteric colibacillosis, is a problem in all countries involved in pig production, and various methods have been employed to protect pigs from ETEC infection and any subsequent diarrheal disease. These methods include, but are not limited to, treatment with antibiotics, vaccination, and feed modulation (Fairbrother et al., 2005; Virdi et al., 2013). While these protective steps have helped reduce levels of ETEC infection, particularly in neonatal and pre-weaning colibacillosis, antimicrobial resistance has become a global challenge that is severely limiting antimicrobials as a treatment option.

Pathogenic *E. coli* express a wide variety of functional molecules including toxins, adhesins, autotransporters, and invasins (Nataro and Kaper, 1998; Kaper et al., 2004), which enable the *E. coli* cells to damage host tissues. In porcine ETEC, these molecules, referred to as virulence factors, include heat-stable (ST) and heat-labile (LT) enterotoxins, both of which can cause diarrhea via different molecular pathways within the pig's gastrointestinal tract (Nataro and Kaper, 1998). Various adhesins have evolved in ETEC and are expressed on the bacterial cell surface, allowing adhesion to the host's gastrointestinal tract. In pigs, the K88 (F4), K99 (F5), 987P (F6), F18 and F41 fimbrial adhesins all provide host intestinal cell binding, with K88, K99 and F18 fimbriae being particularly prevalent (Nataro and Kaper, 1998; Nagy and Fekete, 1999; Koh et al., 2008). Interestingly, the K88 and F18 fimbrial adhesins are often associated with pre- and post-weaning diarrhea, whilst the K99 fimbria has been associated with neonatal diarrhea in pigs (DebRoy and Maddox, 2001). These binding effects are thought to be supplemented by other common adhesins coded by genes such as *fimH* (Bouckaert et al., 2006) and *eaeH* (Sheikh et al., 2014), which are known to provide additional post-attachment binding. Chapman et al. (2006) employed multiplex PCR assays targeting 58 virulence genes to screen 52 clinical *E. coli* isolates from episodes of porcine neonatal and post-weaning diarrhea, and 23 commensal *E. coli* isolates from healthy pigs. Of the 58 genes, 17 were useful in distinguishing commensal from clinical *E. coli*, nine of which (*iha*, *hlyA*, *aidA*, *east1*, *aah*, *fimH*, *iroN<sub>E.coli</sub>*, *traT*, and *saa*) were

identified for the first time in clinical porcine isolates in Australia (Chapman et al., 2006).

In an attempt to combat *E. coli* expressing these and other virulence genes, antimicrobials have been employed as both curative and prophylactic medicines on farms worldwide. This has placed a selection pressure on both targeted and surrounding microbial populations, and as such resistance to these antimicrobials has become extremely prevalent. For pathogenic *E. coli* in general, the definition and interpretation of virulence has been tempered based on results from large-scale microbial genome sequencing projects (Falkow, 1988; Pallen and Wren, 2007). ExPEC reside in the gut of their host, often in a commensal state, but have the genetic repertoire of virulence genes that enables them to cause life-threatening extraintestinal infections. Due to the degree of redundancy inherent in many ExPEC virulence genes, it remains a challenge to define virulence in ExPEC. These ExPEC-associated virulence genes have also been found in isolates sourced from humans, poultry and swine but rarely in cattle and other ruminants (Johnson et al., 2001; Tan et al., 2012; Manges and Johnson, 2015). Poultry are a well-recognized reservoir of ExPEC that have the potential to cause human disease but the role of swine in this regard has not been investigated sufficiently.

## Current Status of Global Antimicrobial Use and Its Influence on the Porcine *E. coli* Resistome

Rationalization of farming practices has had profound effects on the spread of resistance to antimicrobials, and consequently both human and animal health – and the problem continues to grow (FAO, 2009). An estimated 210 million kilograms of antibiotics are produced annually in China, making it the largest producer and consumer of antibiotics globally. Approximately half of the antibiotics produced are used in livestock production (Hvistendahl, 2012; Zhu et al., 2013). China is also the largest producer and consumer of pork; 690 million pigs were produced in 2011 and 38.1 kg of pork are consumed per person per year (Krishnasamy et al., 2015), now reportedly almost double the pork consumption of the USA population. China is said to contain half the world's pig population, with five times the production rate of the USA (the former largest production center) and a higher per capita meat consumption than any developing nation other than Brazil and Latin America (FAO, 2009). Trends are similar for poultry production in China (Krishnasamy et al., 2015). An estimated 34 million kilograms of antibiotics, particularly tetracyclines, sulfonamides, macrolides, and penicillins were used in Chinese pig production during 2012 (Krishnasamy et al., 2015). Combination therapies of chlortetracycline with sulfathiazole and penicillin, and chlortetracycline with sulfamethazine and penicillin, are used widely. Other individual antibiotics that are used less extensively include tylosin, chlortetracycline, oxytetracycline, bacitracin, and bambemycin amongst others (Krishnasamy et al., 2015). Furthermore, pig production in China is responsible for the production of approximately 618 billion kilograms of manure per annum (Wang et al., 2006), much of which is contaminated with

antibiotic residues and heavy metals, particularly tetracyclines, sulfonamides and copper; some residues are present at levels in the order of 100s of milligrams per kilogram (Pan et al., 2011; Qiao et al., 2012; Zhu et al., 2013). Elevated levels of zinc and arsenic have also been detected in these studies. Of 149 unique antimicrobial resistance genes 63 were detected significantly more frequently in microbes from manure at different stages of processing on three large pig production farms in China, compared with microbes in manure from control animals that received no antimicrobials; this included genes coding for resistance to antibiotics used to treat humans (Zhu et al., 2013). Samples of pig manure from China were also rich in microbes carrying transposase genes, notably those belonging to the IS6-family, predominantly IS26 transposase (Zhu et al., 2013).

In the USA, estimates suggest that the usage of tetracyclines is as high as in China, both in the combinations described above, and as stand-alone treatments. Additionally, bacitracin, carbadox, lincomycin, neomycin, penicillin, tiamulin, tilmicosin, tylosin, and virginiamycin have all been used in the USA pig production industry (Apley et al., 2012). The USA and China combined produce the majority of the world's pigs through intensive farming practices (FAO, 2009), with China producing up to five times more than the USA (Krishnasamy et al., 2015). However, other countries also have pig production systems that rely heavily on antibiotic usage. In Alberta, Canada, the same suite of antibiotics is used as in the USA, with the addition of dimetridazole (Rajic et al., 2006). A similar pattern of use has also emerged in an Australian survey of pig production, with the use of apramycin and neomycin, penicillins, macrolides, sulfonamides, tetracyclines, lincomycin and spectinomycin, tiamulin, olaquindox, ceftiofur, and dimetridazole reported (Jordan et al., 2009). A number of these antibiotics, including lincomycin, spectinomycin, and dimetridazole are also used in the treatment of infectious human diseases. In an Australia-wide study of 114 porcine-derived *E. coli*, resistance to numerous antibiotics was common: tetracycline (88.6%), ampicillin (71.05%), trimethoprim/sulfamethoxazole (67.5%), streptomycin (69.3%), chloramphenicol (44.74%), neomycin (35.96%), apramycin (34.21%), gentamicin (28.95%), florphenicol (26.32%), cefalotin (24.56%), and spectinomycin (21.93%). Resistance to imipenem and amikacin was not detected (Abraham et al., 2015). Of the 114 isolates evaluated 79% were classified as resistant to antibiotics from three or more different classes. Resistance to extended-spectrum cephalosporins (3%) and fluoroquinolones (1%) was detected in isolates belonging to some *E. coli* lineages (ST117, ST744, ST10, and ST1) albeit infrequently (Abraham et al., 2015).

Colistin is a polypeptide antimicrobial used to treat extensively drug-resistant Gram negative infections. It was first used in the 1950s but its use in humans has declined due to concerns about nephrotoxicity and neurotoxicity (Nation and Li, 2009). China, India, and Europe still use colistin extensively for agricultural purposes. In Europe, colistin is used to treat enterobacterial infections in pigs (neonatal diarrhea), poultry (colibacillosis), cattle (neonatal diarrhea in veal calves), sheep, and goats (Timmerman et al., 2006; Pardon et al., 2012); in 2010 it was the fifth most commonly sold antimicrobial after tetracycline,

**TABLE 1 |** Molecular characterisation of published antimicrobial resistant, porcine-derived *Escherichia coli* isolates.

Country	Resistance genes detected		Phenotypic resistances tested?	Molecular typing	Prominent Achtman MLSTs	Plasmid typing	Virulence gene detection?	Reference
	Integron associated	Resistance	Beta-lactamases					
Argentina	intI2			Yes	Serogrouping			Moredo et al., 2007, 2015; Pantozzi et al., 2010
Australia	intI1, intI2, aadA, dfrA, cmrA, cat1, cat2, ereA, aacC4	sul1, sul2, su3, strA, strB, aphiA1, tetA, tetB, tefC	blaTEM, blaCTX-M, blaCMY	Yes	RAPD Serogrouping Phylotyping	ST100 ST29 ST90 ST746	Replicon	Yes Chapman et al., 2006; Wu et al., 2007; Smith et al., 2010; Abraham et al., 2012, 2014, 2015
Austria			blaCTX-M, blaTEM, blaSHV	Yes				Mayrhofer et al., 2004, 2006; Pettfennel et al., 2014
Brazil				Yes	ERIC-PCR Serogrouping			Carvalho et al., 1991; da Costa et al., 2008; Borges et al., 2012; Moraes et al., 2012
Canada	aadA, dfrA, aadB, aacC4, cmrA	sul1, sul2, su3, strA, strB, catA1, aphaA, flor, tetA, tetB, tetC	blaTEM, blaOXA, blaCMY	Yes	ERIC-PCR BOX-PCR Serogrouping			Maynard et al., 2003; Lu et al., 2005; Travis et al., 2006; Duriez and Topp, 2007; Kozak et al., 2009a,b; Rosengren et al., 2009; Deckert et al., 2010; Sheikh et al., 2012
China	intI1, aadA, dfrA	sul1, sul2, oqxAB, aac(6')-Ib-cr, qnrA, qnrB, qnrS, qepA, fosA, norF	blaCTX-M, blaTEM, blaSHV, blaCMY	Yes	Serogrouping XbaI PFGE ERIC-PCR Phylogrouping	ST10 ST206	Replicon	Yes Yang et al., 2004, 2014; Yue et al., 2008; Ho et al., 2009, 2013; Ma et al., 2009; Huang et al., 2012; Tian et al., 2012; Liu et al., 2013; Meng et al., 2014; Xu et al., 2014; Gao et al., 2015; Liao et al., 2015
Denmark	intI1, aacC4	sul1, sul2, su3	blaCTX-M, blaTEM, blaCMY, blaSHV	Yes	XbaI PFGE Phylogrouping Serogrouping	ST10 ST23	Replicon	Yes Olesen et al., 2004; Hammerum et al., 2006, 2014; Jensen et al., 2006; Cavaco et al., 2008; Trobos et al., 2009; Jakobsen et al., 2010a,b; Wu et al., 2010; Agero et al., 2012; Agero and Aarestrup, 2013; Hansen et al., 2013; Herrero-Fresno et al., 2015
Germany	intI1, intI2, aadA, dfrA, aadB	sul1, sul2, su3, strA, strB, tetA, tetB, tetM	blaTEM, blaCTX-M, blaOXA	Yes	XbaI PFGE Phylogrouping Serotyping	ST10 ST58 ST167 ST410	Replicon pMLST	Yes Kadlec and Schwarz, 2008; Schwälger et al., 2010; Bednorz et al., 2013; Schierack et al., 2013; Fischer et al., 2014; Dahms et al., 2015; Garcia-Cobos et al., 2015

(Continued)

TABLE 1 | Continued

Country	Resistance genes detected		Phenotypic resistances tested?	Molecular typing	Prominent Achtman MLSTs	Plasmid typing	Virulence gene detection?	Reference	
	Integron associated	Resistance							
India		<i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>CTX-M</sub>	Yes	Serogrouping			Yes	Murugan et al., 2012; Rajkhowa and Sarma, 2014; Samanta et al., 2015	
Italy		<i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>CTX-M</sub>	Yes					Stefani et al., 2014; Luppi et al., 2015	
Japan	<i>intI1</i> , <i>aadA</i> , <i>dfrA</i> , <i>cmlA</i> , <i>aacA4</i>	<i>cat1</i> , <i>tetA</i> , <i>tetB</i> , <i>tetC</i>	Yes	PFGE Phylogrouping			Yes	Kojima-Tanaka et al., 2003, 2005; Asai et al., 2005; Harada et al., 2005, 2006; Kumai et al., 2005; Katsunuma et al., 2008; Kojima et al., 2009; Makita et al., 2016	
Korea	<i>intI1</i> , <i>aadA</i> , <i>dfrA</i> , <i>aadB</i> , <i>cmlA</i>	<i>aphA</i> , <i>qnrB</i> , <i>qnrS</i> , <i>aac(6')-Ib-cr</i> , <i>tetA</i>	Yes	PFGE HFERP Phylogrouping Serogrouping			Yes	Choi et al., 2002; Han et al., 2002; Kang et al., 2005; Lim et al., 2007, 2009; Rayamajhi et al., 2008; Uno et al., 2010, 2011; Tamang et al., 2012; Lee et al., 2014	
Lithuania	<i>intI1</i> , <i>intI2</i> , <i>aadA</i> , <i>dfrA</i> , <i>estX</i> , <i>sat2</i>		Yes					Pavilonis et al., 2010; Seputiene et al., 2010	
Nigeria		<i>qnrS</i>	<i>bla</i> <sub>TEM</sub>	Yes	ERIC-PCR PFGE Serogrouping	ST10 ST168	pMLST	Yes	Ojo et al., 2010; Fortini et al., 2011; Adenipekun et al., 2015
Norway	<i>intI1</i> , <i>intI2</i> , <i>aadA</i> , <i>dfrA</i> , <i>catB</i> , <i>sat1</i>	<i>sul1</i> , <i>sul2</i> , <i>strA</i> , <i>strB</i> , <i>tetA</i> , <i>tetB</i> , <i>tetC</i>	Yes	PFGE				Sunde et al., 1998; Sunde and Sørum, 1999; Brun et al., 2002; Sunde, 2005; Sunde and Norstrom, 2006; Trobos et al., 2009	
Portugal	<i>intI1</i> , <i>intI2</i> , <i>dfrA</i> , <i>aadA</i> , <i>estX</i> , <i>sat2</i> , <i>catB</i>	<i>strA</i> , <i>strB</i> , <i>qnrB</i> , <i>tetA</i>	Yes	XbaI PFGE	ST10		Replicon pMLST RFLP	Machado et al., 2008; Gonçalves et al., 2010; Jones-Dias et al., 2013; Ramos et al., 2013; Rodrigues et al., 2013	
Spain	<i>intI1</i> , <i>intI2</i> , <i>aadA</i> , <i>dfrA</i> , <i>cmlA</i> , <i>sat2</i> , <i>linF</i>	<i>sul1</i> , <i>sul3</i> , <i>pmrA</i> , <i>pmrB</i>		<i>bla</i> <sub>TEM</sub> , <i>bla</i> <sub>SHV</sub> , <i>bla</i> <sub>CTX-M</sub>	Yes		Phylogrouping REP-PCR ERIC-PCR	Brinas et al., 2002; Sabate et al., 2008; Escudero et al., 2010; Marchant and Moreno, 2013; Marchant et al., 2013; Ojer-Usoz et al., 2013; Quesada et al., 2015	

(Continued)

TABLE 1 | Continued

Country	Resistance genes detected		Phenotypic resistances tested?	Molecular typing	Prominent Achtman MLSTs	Plasmid typing	Virulence gene detection?	Reference
	Integron associated	Resistance						
Switzerland	<i>intI1, aadA</i>	<i>sul1, sul2, sul3, strA, strB, tetA, tetB, tetC</i>	<i>blaCTX-M</i>	Yes	PFGE Serogrouping	ST3 ST529	Yes	Stephan and Schumacher, 2001; Lamz et al., 2003; Perretin and Boerlin, 2003; Zweifel et al., 2006; Stannarius et al., 2009; Endimiani et al., 2012
Taiwan	<i>intI1, aadA, dfrA</i>	<i>sul1, fosA</i>	<i>blaTEM, blaCTX-M, blaCMY</i>	Yes	XbaI PFGE	ST744 ST2310	Yes	Hanson et al., 2002; Phongpaichit et al., 2007; Mathew et al., 2009; Lay et al., 2012; Changkaoe et al., 2015
Thailand	<i>intI1, aadA, dfrA, linF, sat1</i>	<i>sul1</i>	<i>blaCTX-M, blaTEM</i>	Yes	Phylogrouping			Jackson, 1981; Enne et al., 2008; Taylor et al., 2008; Freire Martin et al., 2014; Randall et al., 2014; Cheney et al., 2015
UK	<i>aadA</i>	<i>sul2, floR</i>	<i>blaCTX-M, blaSHV</i>	Yes		pMLST		
USA	<i>intI1, aadA, dfrA, cmvA, sat1</i>		<i>cat2, flo, tetA, tetB, tetC, tetM</i>	Yes	Serogrouping XbaI PFGE Phylogrouping	Replicon	Yes	Bischoff et al., 2002; Schroeder et al., 2002; Bryan et al., 2004; Singh et al., 2005; Lindsey et al., 2011; Xia et al., 2011; Ju et al., 2012; Tadesse et al., 2012; Roug et al., 2013

penicillins, sulfonamides, and macrolides (European Medicines Agency [EMA], 2013). Colistin is also used in aquaculture (Xu et al., 2012). The recent emergence of colistin resistance in commensal porcine *E. coli* isolates in China via the acquisition of plasmid-mediated *mcr-1* gene has serious implications for the treatment of pan-drug-resistant Gram-negative bacteria, particularly isolates of the extremely drug resistant *Acinetobacter baumannii* and carbapenemase-resistant *Klebsiella pneumoniae* (Liu et al., 2015). The seriousness of this finding is clear due to: (i) high *in vitro* transfer rates among *E. coli* by conjugation and the ability of the *mcr-1* plasmid to transfer and be maintained in globally dispersed pathogenic clones such as *E. coli* ST131, *K. pneumoniae* ST11, and *Pseudomonas aeruginosa*; (ii) the high frequency of carriage of *mcr-1* in *E. coli* from livestock and in retail meats sourced from southern China; (iii) detection of *mcr-1*-like genes in Malaysia (Liu et al., 2016). The apparent high rates of carriage of *mcr-1* in isolates of *E. coli* from animals and the apparent low incidence in human-derived *E. coli* populations has led to speculation that the heavy use of colistin in agriculture in China has been the main driver for the emergence of plasmid-mediated colistin resistance (European Medicines Agency [EMA], 2013; Liu et al., 2016).

Genes conferring antimicrobial resistance have been identified in *E. coli* from diverse pig-related sources (Table 1). The genes *blaTEM*, *sul1/2/3*, *aadA*, *dfrA*, and *tetA/B/C* feature

prominently. In order, these genes confer resistance to penicillin, sulfonamides (such as sulphamethoxazole), streptomycin and spectinomycin, trimethoprim, and tetracyclines; all of these are used in the treatment of animals. Additional clinically important cases of resistance are also identified sporadically in the literature, specifically to chloramphenicol (*cmlA*, *cat*), streptothricin (*estX/sat*), florfenicol (*flo*), quinolones (*qnr*), streptomycin (*strAB*), and fosfomycin (*fosA*). There are also a number of known resistance mutations in the *gyr* and *pmr* genes that confer elevated resistance to quinolones and colistin respectively. Already the presence of these genes together in the same isolate and from disparate countries indicates they are both genetically linked in antimicrobial resistance-encoding loci, and globally disseminated. Detection of the genes *bla<sub>OXA</sub>*, *bla<sub>SHV</sub>*, and *bla<sub>CTX-M</sub>* coding for the extended-spectrum beta-lactamases, which are relevant to human medicine, is also of concern (Randall et al., 2014). Of note, these data were collected from a variety of publications for each country, and show some selection bias concerning the genes detected. Whole genome sequencing has become a tool to rectify this bias, and will continue to feature in the detection and characterisation of antimicrobial resistance, its evolution and dissemination (Salipante et al., 2015).

Much needs to be done to reduce the reliance on antimicrobials in food production. There is a growing body

**TABLE 2 | Published complete porcine *E. coli* genome and plasmid sequences.**

Name	Country	Type	Size (bp)	Achtman MLST/Plasmid Inc.	Disease association/source	GenBank accession	Reference
pND11_107	USA	Plasmid	107,138	I	Neonatal diarrhea	HQ114281	Johnson et al., 2011
pPWD4_103	USA	Plasmid	103,297	I	Post-weaning diarrhea	HQ114284	
pUMNF18_69	USA	Plasmid	69,065	I		CP002891	
pND12_96	USA	Plasmid	92,290	I	Neonatal diarrhea	HQ114282	
UMNK88	USA	Chromosome	5,186,416	ST165 cplx	Post-weaning diarrhea	CP002729	Shepard et al., 2012
pUMNK88_Hly	USA	Plasmid	65,549			CP002733	
pUMNK88_Ent	USA	Plasmid	81,475			CP002732	
pUMNK88_K88	USA	Plasmid	81,883			CP002730	
pUMNK88_91	USA	Plasmid	90,868	I		CP002731	
pUMNK88	USA	Plasmid	160,573	A/C		HQ023862	
pTC1	Hungary	Plasmid	91,019		Weaning diarrhea	CP000913	Fekete et al., 2012
pHK23a	China	Plasmid	73,607	FII	Multi-drug resistant isolate	JQ432559	Ho et al., 2013
pSSEC2	China	Plasmid	135,615	A/C	Maxillary lymph node	KF152885	Zhang et al., 2014
pIFM3804	UK	Plasmid	104,399	I		KF787110	Freire Martin et al., 2014
PCN033	China	Chromosome	4,987,957	ST5147	Extraintestinal infection (brain)	CP006632	Liu et al., 2015
PCN033p1	China	Plasmid	3,319			CP006633	
PCN033p2	China	Plasmid	4,086			CP006634	
PCN033p3	China	Plasmid	161,511			CP006635	
PCN061	China	Chromosome	4,603,777	ST46	Extraintestinal infection (lung)	CP006636	
PCN061p1	China	Plasmid	2,014			CP006637	
PCN061p2	China	Plasmid	5,754			CP006638	
PCN061p3	China	Plasmid	6,222	Q1/FIB/FIC/FII		CP006639	
PCN061p4	China	Plasmid	34,692			CP006640	
PCN061p5	China	Plasmid	103,644	I		CP006641	
PCN061p6	China	Plasmid	145,722	N/FIB/X1		CP006642	
pFSEC-01	China	Plasmid	33,885			KR779901	Zhang et al., 2015
pSD11	China	Plasmid	37,672	X4		KM212169	Sun et al., 2015

of evidence suggesting that food production may be a key driver in the evolution of multiple drug resistance in a number of bacterial species of clinical significance to human health. Livestock represent a reservoir of *E. coli* pathotypes with resistance to more than one antimicrobial. While pigs and poultry are significant sources of ExPEC, cattle are the major reservoirs for Shiga toxin producing *E. coli* (EHEC; Manges and Johnson, 2015) and atypical EPEC (Hornitzky et al., 2005; Dawes et al., 2010). The consequences for the environment of large-scale intensive animal production and a growing human population, estimated to be 9 billion by 2050 (United Nations, 2015) are not fully understood. Much remains to be done to determine how antimicrobial resistance genes are assembled on mobile genetic elements and to identify their unique genetic features. Diagnostics tests that target unique features will be useful in tracking the movement of mobile elements that are carrying complex resistance loci and to identify hotspots where they reside. Metagenomic approaches will have a major role to play in understanding the scale of the antimicrobial resistance problem, detecting and tracking emerging pathogens, and the role of gut flora in human and animal health. Studies on microbial populations from the gastrointestinal tracts of intensively reared livestock are needed to identify and characterize novel pathotypes that will undoubtedly emerge in response to the selection pressures that are commonplace in large-scale animal production facilities. Genomic surveillance studies will have an important role to play in identifying complex resistance loci, particularly how they move through different production systems and natural ecosystems (aquatic and terrestrial), and the impact they have on the carriage of multiple antimicrobial resistance genes in bacteria

that colonize and infect humans and animals. Currently, there are just three complete porcine-derived *E. coli* chromosomal sequences published, as well as a variety of complete plasmid sequences, the majority of which have been derived from the study of isolates with closed genome sequences (Table 2). This is at odds with the significant role that intensive pig production plays in the release of antibiotic residues into the environment, and we expect more focus to be placed on the sequencing of pig-associated *E. coli* isolates in the near future.

## AUTHOR CONTRIBUTIONS

EW drafted an early version of the manuscript and produced the figure and tables. PR and IC contributed to several sections in the manuscript. TC and JH edited drafts of the manuscript. SD wrote most the manuscript.

## FUNDING

The research in this article is part of the Ausgem partnership and supported by an Australian Research Council Linkage Project grant LP150100912.

## ACKNOWLEDGMENT

We wish to acknowledge J. K. Pell Consulting for copyediting the manuscript.

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# High genetic similarity of ciprofloxacin-resistant *Campylobacter jejuni* in central Europe

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 30 June 2015

Accepted: 09 October 2015

Published: 23 October 2015

### Citation:

Kovač J, Čadež N, Stessl B, Stingl K, Gruntar I, Ocepek M, Trkov M, Wagner M and Smole Možina S (2015) High genetic similarity of ciprofloxacin-resistant *Campylobacter jejuni* in central Europe. *Front. Microbiol.* 6:1169.  
doi: 10.3389/fmicb.2015.01169

Campylobacteriosis is the leading zoonosis in the European Union with the majority of cases attributed to *Campylobacter jejuni*. Although the disease is usually self-limiting, some severe cases need to be treated with antibiotics, primarily macrolides and quinolones. However, the resistance to the latter is reaching alarming levels in most of the EU countries. To shed light on the expansion of antibiotic resistance in central Europe, we have investigated genetic similarity across 178 ciprofloxacin-resistant *C. jejuni* mostly isolated in Slovenia, Austria and Germany. We performed comparative genetic similarity analyses using allelic types of seven multilocus sequence typing housekeeping genes, and single nucleotide polymorphisms of a quinolone resistance determining region located within the DNA gyrase subunit A gene. This analysis revealed high genetic similarity of isolates from clonal complex ST-21 that carry *gyrA* allelic type 1 in all three of these central-European countries, suggesting these ciprofloxacin resistant isolates arose from a recent common ancestor and are spread clonally.

**Keywords:** *Campylobacter jejuni*, ciprofloxacin resistance, multilocus sequence typing, *gyrA*, QRDR, resistance expansion

## INTRODUCTION

Campylobacteriosis is the leading zoonosis in the European Union (EU), with the majority of cases attributed to *Campylobacter jejuni* (EFSA, 2015). Severe or prolonged cases of campylobacteriosis are conventionally treated with antibiotics from the macrolide (erythromycin) and quinolone (ciprofloxacin) classes. While macrolide resistance is successfully retained at low levels (human isolates EU average, 1.5%), some EU Member States report up to 91.5% prevalence of quinolone resistance (EFSA, 2015). The resistance prevalence among *C. jejuni* from humans was 64.1 and 63.0% in Slovenia and Austria in 2013, respectively (EFSA, 2015). The main mechanism that confers high-level resistance to ciprofloxacin in *Campylobacter* is the occurrence of the Thre86Ile point mutation in the quinolone-resistance-determining region (QRDR) of the gene that encodes DNA gyrase subunit A (*gyrA*), although other mutations within *gyrA*, as well as enhanced

efflux activity can contribute to development of ciprofloxacin resistance (Wieczorek and Osek, 2013). The Thre86Ile resistant mutants have also been demonstrated to have increased chicken colonization fitness (Luo et al., 2005) in certain genetic backgrounds, giving them an advantage over susceptible strains. In other genetic backgrounds, the advantage was not observed (Zeitouni and Kempf, 2011). The resistance to quinolones is especially problematic due to their therapeutic use in human and veterinary medicine, including in food production animals, which allows for transmission of antibiotic resistance along the food-production chain to humans (EMA/ESVAC, 2011).

Several studies have reported the resistance and genotype data that demonstrated increased prevalence of resistance in certain genotypes in different geographical regions (Kinana et al., 2006; D'Lima et al., 2007; Gu et al., 2009; Habib et al., 2009; Wirz et al., 2010; Stone et al., 2013; Wu et al., 2014; Jonas et al., 2015). Furthermore, associations of particular multilocus sequence typing (MLST) clonal complexes with resistance were suggestive of clonal expansion of ciprofloxacin resistance in Slovenia, the UK and Switzerland (Cody et al., 2012; Kittl et al., 2013; Wimalarathna et al., 2013; Kovač et al., 2014).

To provide better insight into the epidemiology of *C. jejuni* antibiotic resistance in central Europe, we performed a comparative genetic similarity analysis of 178 ciprofloxacin-resistant *C. jejuni* strains isolated in Slovenia, Austria and Germany. We used standard MLST coupled with single nucleotide polymorphism (SNP) analysis of the QRDR of *gyrA* to investigate the genetic relatedness of ciprofloxacin resistant *C. jejuni* isolates and shed light on the nature of their expansion in these three central-European countries.

## MATERIALS AND METHODS

### Bacterial Strains

The 178 ciprofloxacin resistant *C. jejuni* strains included in this study were isolated from poultry, cattle, chicken meat, cow milk, surface water, dog, strawberry, a zoo animal, and human stools in central Europe (Slovenia, Austria, Germany); two isolates were obtained from the southern Balkan region (Serbia, Bosnia, and Herzegovina) (**Supplementary Table S1**). The strains were isolated between 2001 and 2013 according to ISO10272, as part of national monitoring of retail meat and production animals, human clinical cases, and from other national and EU research projects.

### Bacterial Growth Conditions and DNA Extraction

Stocks of *C. jejuni* isolates were stored at  $-80^{\circ}\text{C}$  and grown on selective Karmali agar (Oxoid, Hampshire, UK) for 24 h at  $42^{\circ}\text{C}$  under microaerobic conditions (5%  $\text{O}_2$ , 10%  $\text{CO}_2$ , in  $\text{N}_2$ ). DNA used for molecular species confirmation and MLST typing was extracted with PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA), according to the manufacturer protocol. DNA was used for *C. jejuni* species confirmation according to Wang et al. (2002), and amplification

of MLST housekeeping genes and *gyrA* QRDR. All primers used in this study are listed in **Supplementary Table S2**.

### Antibiotic Resistance

Resistances to ciprofloxacin (0.06–4 mg/l), chloramphenicol (2–32 mg/l), erythromycin (0.5–32 mg/l), gentamicin (0.12–16 mg/l), streptomycin (1–16 mg/l) and tetracycline (0.25–16 mg/l) were determined by broth microdilution using Eucamp microtitre plates (Sensititre, Thermo Fischer Scientific) by following manufacturers' instructions. The reference strain ATCC 33560, which is susceptible to all seven tested antibiotics was used as a quality control. The minimum inhibitory concentration (MIC) cut-offs as recommended by EFSA (2015) were used to identify the resistant phenotypes. Briefly, isolates with MICs of ciprofloxacin >0.5 mg/l, chloramphenicol >16 mg/l, erythromycin >4 mg/l, gentamicin >2 mg/l, streptomycin >4 mg/l, and tetracycline >1 mg/l were identified as resistant. The MICs and resistance breakpoints are available in **Supplementary Table S1**.

### Multilocus Sequence Typing

Multilocus sequence typing was carried out according to the established *C. jejuni* MLST scheme, with primers listed in **Supplementary Table S2** (Dingle et al., 2001). Sequences (Sanger sequencing; Macrogen, South Korea) were used for the identification of MLST profiles using Bionumerics 7.1 with the MLST plugin (Applied Maths NV, Sint-Martens-Latem, Belgium). The MLST profiles identified in this study have been deposited in the PubMLST database <http://pubmlst.org/campylobacter> (Jolley and Maiden, 2010). The PubMLST IDs are available in **Supplementary Table S1**. The minimum-spanning tree (**Figure 1**) demonstrating the genetic similarity among strains was built using MLST allele variants in Bionumerics version 7.1 (Applied Maths).

### Quinolone-Resistance-Determining Region

The QRDR of *gyrA* of 178 ciprofloxacin-resistant *C. jejuni* isolates was amplified using primers GZgyrA5 and GZgyrA6 in a single PCR, and Sanger sequenced using primers GZgyrA7 and GZgyrA8 (Zirnstein et al., 1999; **Supplementary Table S2**). The 389-bp-long QRDR sequences spanning from nucleotide positions 92 to 481 within *gyrA* were aligned in MEGA V6.0 (Tamura et al., 2013) to identify the unique variants of *gyrA*. Every sequence with new SNP (synonymous or non-synonymous) observed was designated a new *gyrA* allelic type number. The conserved region of *gyrA* outside of QRDR was previously used by others for epidemiological investigation of *C. jejuni* and *C. coli* host specificity (Ragimbeau et al., 2014). Here we have investigated the QRDR region, as its sequence diversity is directly associated with ciprofloxacin resistance.

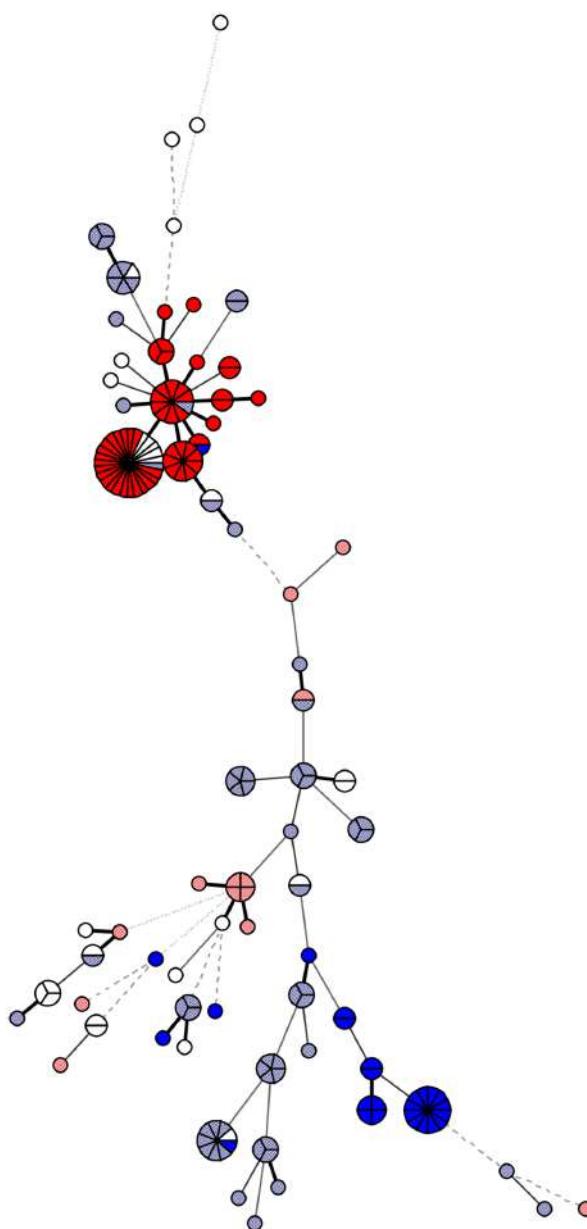
### Statistical Analysis

Statistical associations between *gyrA* types and MLST clonal complexes, and between *gyrA*+MLST types and multidrug

## RESULTS

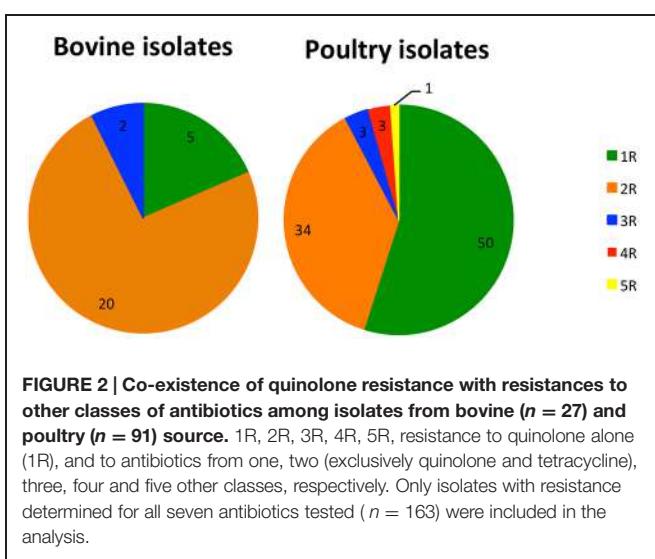
Analyses of antibiotic co-resistance (multidrug resistance) were carried out on the isolates, with the MICs determined for all seven of the antibiotics tested ( $n = 163$ ). The MICs of ciprofloxacin-resistant isolates ranged from 2 to over 4 mg/l, confirming a high-level resistance in all of them (resistance MIC cut-off is  $>0.5$  mg/l). Among them, 76 (46.6%) isolates were either resistant to ciprofloxacin only ( $n = 4$ ) or were cross-resistant to nalidixic acid ( $n = 72$ ). The MICs of nalidixic acid ranged from 32 to over 46 mg/ml. Seventy-six isolates (46.6%) were co-resistant to ciprofloxacin and another antibiotic from a different class, which was exclusively tetracycline. MICs of tetracycline were ranging from 2 to over 16 mg/l, however most of the isolates ( $n = 61$ ; 80.3%) were resistant to concentrations 16 mg/l or higher. Seven of the ciprofloxacin-resistant isolates (4.3%) were additionally resistant to either streptomycin and erythromycin, or streptomycin and tetracycline. Three isolates (1.8%) were resistant to antibiotics from four different classes, and a single isolate (0.6%) was resistant to antibiotics from five different classes (Supplementary Table S1). As demonstrated in Figure 2, higher proportion of resistance only to quinolone (1R) antibiotics was observed among isolates from poultry source ( $n = 50$ ; 34.9%,  $p < 0.001$ ), compared to isolates of bovine origin, where resistance to two classes of antibiotics (quinolones and tetracyclines – 2R) was prevailing ( $n = 20$ ; 74.0%;  $p = 0.003$ ). However, these differences arise mostly due to the high proportion of 1R among Slovenian poultry isolates ( $n = 33$ ; 55%), compared to Austrian ( $n = 8$ ; 17.4%) and German ( $n = 9$ ; 33.3%), suggesting divergent antibiotic treatment practices in Slovenia, Austria, and Germany.

We identified 67 different MLST sequence types (STs) that were distributed among 21 clonal complexes (Table 1). Twenty isolates could not be assigned to any of the existing clonal complexes. Novel variants of alleles *aspA* (*aspA* 387), *glyA*



**FIGURE 1 | Genetic similarity of the 178 analyzed *Campylobacter jejuni* isolates.** The minimum spanning tree was constructed based on the multilocus sequence typing (MLST) allelic profiles using Bionumerics, version 7.1. The genotype specific for clonal complex ST-21 + *gyrA* 1 is presented in dark red and for *gyrA* 1 in combination with a clonal complex other than ST-21 is in light red; the genotype for clonal complex ST-353 + *gyrA* 13 is in dark blue; and for *gyrA* 13 in combination with a clonal complex other than ST-353 is in light blue. The figure demonstrates higher similarity of isolates carrying *gyrA* allelic type 1, which cluster within clonal complex ST-21 (dark red), compared to isolates carrying *gyrA* 13, which are found within clonal complex ST-353 (dark blue), but are also dispersed among other clonal complexes (light blue).

resistance were carried out using Fischer's exact tests in R, version 3.1.2, with the statistical relevance cut-off set at  $p < 0.01$  (R Core Team, 2014).



**FIGURE 2 | Co-existence of quinolone resistance with resistances to other classes of antibiotics among isolates from bovine ( $n = 27$ ) and poultry ( $n = 91$ ) source.** 1R, 2R, 3R, 4R, 5R, resistance to quinolone alone (1R), and to antibiotics from one, two (exclusively quinolone and tetracycline), three, four and five other classes, respectively. Only isolates with resistance determined for all seven antibiotics tested ( $n = 163$ ) were included in the analysis.

**TABLE 1 |** The distribution of ciprofloxacin-resistant *Campylobacter jejuni* isolates, originating from different countries among MLST clonal complexes.

	Austria	Germany	Slovenia	Serbia	Bosnia and Herzegovina	SUM
ST-21 complex	22	15	24			61
ST-353 complex	7	3	15		1	26
UA	10	1	9			20
ST-354 complex	2		8			10
ST-206 complex	5	1	3			9
ST-464 complex	6	1	1			8
ST-257 complex	4	1	1			6
ST-45 complex		4	2			6
ST-443 complex	4		1			5
ST-48 complex	2	1	2			5
ST-52 complex	1		4			5
ST-574 complex	4					4
ST-22 complex	1		1			2
ST-607 complex	1			1		2
ST-658 complex			2			2
ST-1034 complex		1				1
ST-283 complex			1			1
ST-362 complex			1			1
ST-42 complex		1				1
ST-49 complex	1					1
ST-692 complex		1				1
ST-828 complex			1			1
SUM	70	30	76	1	1	178

(599), *tkt* (578), and *uncA* (458) were identified, along with the definition of their novel corresponding STs: ST 7514, ST 7517, ST 7516, and ST 7515, respectively. One new ST, ST 7506, was identified as a novel combination of previously reported allele types, according to the information in the PubMLST database.

Sequence type 50 (clonal complex ST-21) was the most prevalent ST that was common to all three countries, Austria ( $n = 12$ ), Slovenia ( $n = 12$ ) and Germany ( $n = 3$ ). The second-most frequent ST among the Slovenian isolates was ST 5205 (clonal complex ST-353) ( $n = 11$ ), which was identified for the first time in our previous study (Kovač et al., 2014) and has been so far reported only for Slovenian ( $n = 11$ ) and Austrian ( $n = 1$ ) isolates, according to the PubMLST database (accessed on 20 June 2015). The most-prevalent clonal complex in all three of these countries was ST-21, with 24 representatives in Slovenia, 22 in Austria, and 15 in Germany, followed by ST-353, with 15 and seven representatives in Slovenia and Austria, respectively, and ST-45, with four representatives in Germany (Supplementary Table S1). Both, ST-21 and ST-353 were highly represented by chicken and human isolates, but no bovine isolate was assigned to ST-353. The 64.5% ( $n = 20$ ;  $p < 0.001$ ) of bovine isolates carried *gyrA* type 1, while 59.8% ( $n = 49$ ,  $p < 0.001$ ) of chicken isolates carried *gyrA* type 13.

The comparative analysis of the 389-bp-long QRDR sequences revealed 15 different *gyrA* allelic types with variations on 25 nucleotide sites (Supplementary Table S1; GenBank KP794628-KP794805). The sequences were checked for presence

of previously reported resistance-conferring mutations on nucleotide positions 210, 255, 258, 270, and 312 (according to full-length *gyrA*), which corresponded to amino acids Ala70, Asp85, Thr86, Asp90, and Pro104 (Wieczorek and Osek, 2013). A total of 171 isolates carried the main resistance-conferring mutation Thr86Ile. This mutation was not detected in seven isolates, which still exhibited high level resistance to ciprofloxacin (MICs 2 to over 4 mg/l), possibly due to enhanced efflux activity. The *gyrA* types identified in isolates lacking the Thr86Ile mutation were *gyrA* 7, 8, 9, and 10. None of the isolates analyzed carried the alternative resistance-conferring mutations at amino-acid positions 70, 85, or 90, however five of the isolates had the Pro104Thr mutation. Pro104Thr is a newly identified mutation, as only Pro104Ser has been reported so far (Wieczorek and Osek, 2013). Pro104Thr was found in four strains that were isolated in Austria (08/000367, MRC-09/00280, MRC-10/00374, MRC-11/00224), and in a single strain isolated in Germany (Bfr-CA-10467) (Supplementary Table S1). All of the isolates with Pro104Thr mutation possessed the *gyrA* type 6.

The most prevalent *gyrA* STs were *gyrA* type 13 ( $n = 86$ ) and *gyrA* type 1 ( $n = 65$ ), with the remaining *gyrA* STs represented by only up to five isolates. The isolates with *gyrA* type 13 were distributed among 14 clonal complexes, and 26.7% of these isolates were assigned to ST-353 (ST-353 + *gyrA* 13;  $p < 0.001$ ). On the other hand, isolates with *gyrA* type 1 were distributed among eight different clonal complexes, with 80.0% of these isolates clustering in ST-21 (ST-21 + *gyrA* 1;  $p < 0.001$ ). Sequence analysis revealed closer relationships

between isolates carrying *gyrA* type 1, which mostly belonged to clonal complex 21 (ST-21 + *gyrA* type 1), compared to isolates carrying *gyrA* type 13 (**Figure 1**). Both of these *gyrA* types were found in isolates from Slovenia, Austria, and Germany.

Isolates with genotype ST-353 + *gyrA* 13 were in most cases ( $n = 13$ ; 61.9%;  $p < 0.001$ ) resistant only to ciprofloxacin or to ciprofloxacin and nalidixic acid. Similar numbers, but lower proportions, of solely ciprofloxacin resistant isolates ( $n = 18$ ; 39.1%;  $p = 0.2244$ ) were observed also for genotype ST-21 + *gyrA* 1. A higher proportion of isolates with genotype ST-21 + *gyrA* 1 were resistant to two classes of antibiotics ( $n = 25$ ; 54.3%;  $p = 0.222$ ), than of isolates with genotype ST-353 + *gyrA* 13 ( $n = 5$ ; 23.8%,  $p = 0.035$ ). The proportions of isolates resistant to three antibiotics from different classes were low, both for ST-353 + *gyrA* 13 ( $n = 3$ ; 14.3%) and for ST-21 + *gyrA* 1 ( $n = 1$ ; 2.2%). Isolates that showed resistance to four and five antibiotics were represented only by genotype ST-21 + *gyrA* 1.

## DISCUSSION

The current antibiotic-resistance problem among zoonotic agents demonstrates the need for improvements to resistance management policy. The EU ban on antibiotic use for growth promotion in animal husbandry was a step forward (European Commission, 2011). However, the extensive amounts of clinically important antibiotics that are applied for treatment and control of pathogens in food-production animals still support the spread and persistence of resistant bacterial populations in the food production chain, and therefore compromise efficient treatment of human infections (EMA/ESVAC, 2014).

Although the ban on the use of antibiotics in food animals may not produce the immediate targeted effects, studies from several countries that have established such policies have provided evidence for its sustainability, in terms of decreasing the prevalence of antibiotic resistance (Norström et al., 2006; Nelson et al., 2007; Skjøt-Rasmussen et al., 2009). Nevertheless, the implications of such policies and risk analyses for the expansion of antibiotic resistance through the international trade in chicken meat have not been evaluated to date, although such investigations are urgently needed.

Here, we have investigated the genetic similarity of ciprofloxacin-resistant strains isolated from the environment, animals, food and human in three central-European countries over the last 12 years to gain knowledge about their distribution among different genotypes that could potentially explain the mechanisms driving the increase in ciprofloxacin resistance prevalence in *C. jejuni*. We have found that resistance to ciprofloxacin alone (1R) is more frequently observed among poultry isolates, compared to bovine isolates where resistance to ciprofloxacin and tetracycline (2R) is more common. Although the associations were statistically significant according to

Fischer's exact test, the observations are biased, as substantially higher proportion of 1R isolates originates from Slovenia, compared to Austria and Germany. According to an opinion of Slovenian veterinary expert familiar with the field use of antibiotics in poultry, this can be explained with preferential use of enrofloxacin, rather than tetracyclines for poultry treatment in Slovenia. Main reason for that is the shorter withdrawal period and less problems with antibiotic precipitation in water.

Ciprofloxacin-resistant isolates clustered in two groups of high genetic similarity, as inferred based on MLST and SNP analyses of the QRDR. These two groups comprised isolates from MLST clonal complex ST-353 with *gyrA* type 13 and isolates from clonal complex ST-21 with *gyrA* type 1. While isolates from both of these MLST clonal complexes were significantly associated with their corresponding *gyrA* types, the sequence similarity analysis revealed closer relationships between isolates carrying *gyrA* type 1. Isolates with *gyrA* type 13 were widely dispersed also in clonal complexes other than ST-353, while isolates with *gyrA* type 1 belonged almost exclusively to MLST clonal complex 21, indicating they have expanded from a recent common ancestor. Furthermore, strains isolated from all three countries over several different years can be found within the group.

We have found 17 STs appearing between animal, as well as human isolates. Five of those (STs 50, 21, 104, 5205, and 572) were found more than once among human isolates. Of those, ST 50, 21, and 104 belong to clonal complex ST-21, ST 5205 to clonal complex ST-353, while 572 was unassigned. These findings suggest epidemiological linkage between animal and human isolates from clonal complexes ST-21 and ST-353, which also had the highest accumulation of ciprofloxacin resistant isolates.

The ST-21, and in particular ST 50 were highly represented among our set of ciprofloxacin resistant isolates, nevertheless previous studies in other geographical regions had not found significant associations between this genotype and quinolone resistance or susceptibility (Cody et al., 2012; Kittl et al., 2013). On the other hand, they have found ST 464 and ST 45 to be significantly associated with quinolone resistance, and susceptibility, respectively (Cody et al., 2012; Kittl et al., 2013). These two STs, however, were not identified frequently enough among our ciprofloxacin resistant isolates, to draw statistically relevant conclusions.

It is becoming increasingly evident that the global escalation of quinolone resistance in *Campylobacter* is associated with spreading of certain resistant genotypes (Cody et al., 2012; Kittl et al., 2013; Wimalaratna et al., 2013; Kovač et al., 2014), which may be driven by globalization in the poultry trade (AVEC, 2014) and/or of travel. Our findings suggest that the ciprofloxacin resistance is expanding with widespread *C. jejuni* clonal complex ST-21 in central Europe. Further

research focused on identifying the genetic traits other than antibiotic resistance determinants that may facilitate this process is needed, in order to better understand the antibiotic resistance epidemiology, and implement successful resistance control measures.

## ACKNOWLEDGMENTS

This study was funded by project PROMISE (FP7-265877) and the Slovenian Research Agency (CRP V4-1110). The authors thank to Dr. Sandra Jelovcan from AGES and Dr. Marija Lušicky from NLZOH for providing *C. jejuni* strains,

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- and Eva Sobočan Zajc and Diletta DiMarco for technical assistance.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmich.2015.01169>

**TABLE S1 | Isolates used in this study.** The source of isolates, their antibiotic resistance profiles, MLST types and *gyrA* QRDR types.

**TABLE S2 | Primers used in this study.**

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# IncF plasmid diversity in multi-drug resistant *Escherichia coli* strains from animals in China

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equally to this work.

### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 18 April 2015

Accepted: 31 August 2015

Published: 22 September 2015

### Citation:

Yang Q-E, Sun J, Li L, Deng H, Liu  
B-T, Fang L-X, Liao X-P and Liu Y-H  
(2015) IncF plasmid diversity in  
multi-drug resistant *Escherichia coli*  
strains from animals in China.  
*Front. Microbiol.* 6:964.  
doi: 10.3389/fmicb.2015.00964

The purpose of this study was to characterize a collection of 103 multidrug resistance IncF plasmids recovered from *Escherichia coli* of food producing and companion animals between 2003 and 2012. A total of 103 incF plasmids were characterized using an established PCR-based IncF replicon sequence typing (RST) system to identify FII, FIA, and FIB (FAB) groups. Plasmids were also analyzed using-restriction fragment length polymorphism (RFLP). Antibiotic Resistance determinants *bla*<sub>CTX-M</sub>, plasmid-mediated quinolone resistance (PMQR) genes and *rmtB* and plasmid addiction systems (PAS) were identified by PCR screening. A total of 20 different RSTs from 103 IncF plasmids were identified. The groups F2 and F33 with the RST formulae A-: B- were the most frequently encountered types (63.1%). The antibiotic resistance genes (ARGs) *bla*<sub>CTX-M</sub>, *rmtB*, and *oqxB* were carried by 82, 37, and 34 IncF plasmids, respectively. Most of these plasmids carried more than one resistance gene (59.2%, 61/103). The IncF plasmids also had a high frequency of addiction systems (mean 2.54) and two antisense RNA-regulated systems (*hok-sok* and *srnBC*) and a protein antitoxin-regulated system (*pemKl*) were the most prevalent. Not surprisingly, RFLP profiles among the IncF plasmids were diverse even though some shared identical IncF-RSTs. This is the first extensive study of IncF plasmid-positive *E. coli* isolates from animals in China. Our results demonstrate that IncF is the most prevalent plasmid family in *E. coli* plasmids and they commonly carry multiple resistance determinants that render them resistant to different antibiotic classes simultaneously. IncF plasmids also harbor addiction systems, promoting their stability and maintenance in the bacterial host, under changing environmental conditions.

**Keywords:** *Escherichia coli*, IncF plasmid, multi-resistance, addiction systems, RFLP

## Introduction

Increasing antibiotic resistance in bacteria has potentially disastrous consequences for human and animal health around the world. Recent studies have demonstrated that plasmids act as efficient vehicles for the spread of antibiotic resistance genes (ARGs) more frequently than previously believed (Taylor et al., 2004; García-Fernández et al., 2009; Dolejska et al., 2011; Accogli et al., 2013; Carattoli, 2013; Dahmen et al., 2013b). Thus far, 27 different plasmid incompatibility (Inc.) groups are recognized in the Enterobacteriaceae

(Couturier et al., 1988; Carattoli et al., 2005; Villa et al., 2010). A smaller number of particular plasmid families play a major role in the diffusion of specific resistance genes among Enterobacteriaceae and are the most frequently encountered. The IncF plasmids represent one of the most prevalent incompatibility types, and have been identified worldwide in Enterobacteriaceae from different origins and sources (Carattoli, 2009; Mathers et al., 2015). Currently, a total of 924 plasmids have been deposited at the plasmid multilocus sequence typing (pMLST) database (<http://pubmlst.org/plasmid/>, 30 January, 2015). Of these, 214 plasmids belong to IncF group and 158 of them (73.8%) recovered from *Escherichiacoli* could be classified in 90 different subgroups on the basis of their FII, FIA and FIB (FAB) alleles.

IncF plasmids have systems which guarantee their autonomous replication but also encode addiction systems frequently based on toxin-antitoxin factors. These systems contribute to control their copy number and ensure the instable inheritance during cell division (Cohen, 1976; Kado, 1998; Hayes, 2003). In our previous studies, we found that IncF plasmids could integrate a wide range of genes conferring resistance to all major classes of antimicrobials including  $\beta$ -lactams, aminoglycosides, tetracyclines, chloramphenicol, and quinolones (Liao et al., 2013; Liu et al., 2013). The extended-spectrum  $\beta$ -lactamases (ESBLs), especially those of the CTX-M type, are often associated with plasmid-mediated quinolone resistance (PMQR) and aminoglycoside resistance genes. Plasmids belonging to the IncF group have been the primary hosts for these traits (Boyd et al., 2004; Yao et al., 2011; Matsumura et al., 2013; Ogbolu et al., 2013).

The spread of such multidrug resistance plasmids among Enterobacteriaceae strains could also affect clinical treatments. For instance, IncF-*bla*<sub>CTX-M</sub> association was observed from both human and animal *E. coli* isolates such as the IncF-like plasmid R100 involved in the dissemination of *bla*<sub>CTX-M-14</sub> in Hong Kong, United Kingdom and France (Woodford et al., 2009; Ho et al., 2012; Dahmen et al., 2013a). The genes *rmtB*, *qepA*, *qnr*, *fosA3*, and *oqxAB* have been recently identified on IncF plasmids in *E. coli* from China, Korea and Spain (Tamang et al., 2008; Li et al., 2012; Ruiz et al., 2012; Ho et al., 2013). All together, the IncF-related family has the potential to be a major contributor worldwide to the diffusion of different resistance genes.

In general, the IncF plasmids are not a homogeneous group and vary in size (50–200 kb) and replicon type (Garcillán-Barcia et al., 2009; de Been et al., 2014; Lanza et al., 2014). Most importantly, plasmids encoding virulence-associated traits fall almost exclusively within IncF group (Johnson and Nolan, 2009). Therefore, knowledge of the prevalence and IncF plasmid subtypes in resistant populations could be a useful aid in exploring novel plasmid-targeting strategies to treat resistant bacteria (Osborn et al., 2000; DeNap and Hergenrother, 2005; Baquero et al., 2011). We investigated variations in IncF plasmids and the phylogenetic relationship among IncF plasmids from different sources using RST and RFLP analyses. This study characterized IncF plasmids according to resistance genes, genetic relatedness and addiction systems in 103 *E. coli* isolates from pigs, poultry and companion animals.

## Materials and Methods

### Transconjugants and Antibiotic Susceptibility Testing

A total of non-duplicate 795 *E. coli* were isolated from animals between 2002 and 2012, according to the data published in our previous study (Liao et al., 2013; Liu et al., 2013, 2014). Among them, 696 *E. coli* were isolated from liver, heart or faces samples of diseased food-producing animals including 318 from avian (177 from ducks, 110 from chicken and 31 from geese) and 378 from pigs. The food-producing animals originated from more than 80 farms all over Guangdong province. The remaining 99 isolates were from rectal swab samples of pets including 71 dogs and 28 cats in five animal hospitals in Guangdong province. Further information about these animals, the underlying disease and possible antimicrobial pretreatment were unfortunately not available. One suspected colony with typical *E. coli* morphology and size was selected from all agar plates of each sample and was identified by Matrix Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF VITEK MS RUO System) using the Saramis™ database (bioMérieux, France). The genetic relatedness among partial *E. coli* isolates randomly selected were determined by pulsed-field gel electrophoresis (PFGE) with *Xba*I according to a protocol described previously (Gautom, 1997). Isolates encoding *bla*<sub>CTX-M</sub>, *rmtB* and/or PMQR gene (535/795), were selected for conjugation experiments by the broth-mating method using streptomycin-resistance *E. coli* C600 as the recipient. Transconjugants were selected on MacConkey agar plates containing streptomycin (1000 mg/L) and cefotaxime (2 mg/L)/amikacin (32 mg/L)/olaquindox (64 mg/L), respectively. The transconjugants harboring *bla*<sub>CTX-M</sub>, *rmtB*, and/or PMQR gene mentioned above were confirmed by PCR as previously.

One hundred and three transconjugants, each carrying one single IncF plasmid, were selected from a collection of clonally unrelated *E. coli* strains (15 pets, 43 avian, and 45 pigs) in our study. Table S1 contains detailed information for this population. Within this group 28 IncF plasmids had been previously characterized (Liao et al., 2013; Liu et al., 2013, 2014). Antimicrobial susceptibility tests were determined by agar dilution method on Mueller-Hinton agar plates. The antimicrobial drugs studied included quinolones (nalidixic acid), fluoroquinolones (ciprofloxacin, enrofloxacin, and levofloxacin), ampicillin, third-generation cephalosporins (ceftiofur, cefotaxime and ceftazidime), aminoglycoside (streptomycin, amikacin, kanamycin, and gentamycin) and other antimicrobials (olaquindox, chloramphenicol, florfenicol, and tetracyclines). The susceptibility tests were carried out and evaluated according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The breakpoints for each antimicrobial were those recommended by the CLSI (M100-S19), CLSI (Vet01-A4/Vet01-S2), and DANMAP 98 (olaquindox) (Clinical and Laboratory Standards Institute, 2008, 2013). *E. coli* ATCC 25922 was used for quality control purposes.

**TABLE 1 |**The distribution of addiction systems and resistance genes according to FAB type identified in the 103 multi-resistance plasmids.

FAB	No. (size: kb)	Addiction systems (No.)						Mean						*Resistance genes (No.)							
		<i>hok-sok</i>	<i>pemK1</i>	<i>snpBC</i>	<i>vagCD</i>	<i>ccdB</i>	<i>pndAC</i>	Total	<b>M55</b>	<b>M14</b>	<b>M27</b>	<b>M65</b>	<b>M15</b>	<b>M3</b>	<b>M24</b>	<b>rmtB</b>	<b>qnrB</b>	<b>qnrS</b>	<b>qepA</b>		
F2:A:B-	35(70~194)	28	18	0	3	14	3	66	1.9	5	8	14	2	0	1	1	16	11	3	5	6
F2:A1:B-	3(120~145)	3	0	3	3	0	0	9	3	1	2	0	0	0	0	0	0	0	0	1	0
F2:A1:B1	2(145~194)	2	1	2	2	2	0	9	4.5	0	0	0	0	0	0	0	2	1	0	1	0
F2:A:B10	1(~120)	1	0	1	1	0	0	3	3	0	1	0	0	0	0	0	0	0	0	0	0
F14:A-B-	1(ND)	0	1	0	1	0	0	2	2	0	1	0	0	0	0	0	0	0	1	0	0
F16:A-B-	3(~97)	3	1	1	0	0	0	5	1.7	0	0	0	0	0	0	0	0	0	3	0	2
F16:A-B1	1(~60)	1	1	0	0	1	0	3	3	1	0	0	0	0	0	0	0	0	0	0	0
F18:A-B-	3(135~145)	3	3	0	0	0	0	9	3	1	1	0	1	0	0	0	0	0	1	0	0
F18:A-B1	1(145~194)	8	8	9	1	2	1	29	2.9	5	0	0	0	0	0	0	0	0	1	6	0
F18:A-B8	1(~194)	1	0	1	0	1	0	3	3	0	0	0	0	0	0	0	0	0	0	0	0
F31:A4:B1	3(~194)	1	3	3	2	3	0	12	4	0	0	0	0	0	0	0	0	0	0	0	0
F33:A-B-	30(60~194)	26	25	29	1	1	2	84	2.8	16	0	0	9	0	0	0	0	0	17	8	0
F33:A1:B-	1(ND)	1	0	0	1	1	0	3	3	0	1	0	0	0	0	0	0	0	0	0	0
F33:A-B1	1(ND)	1	1	0	0	0	0	3	3	0	0	0	0	0	0	0	0	0	1	1	0
F35:A-B-	3(~97)	3	0	3	2	1	0	9	3	0	3	0	0	0	0	0	0	0	0	0	0
F36:A1:B1	1(~160)	1	1	0	1	0	0	4	4	0	1	0	0	0	0	0	0	0	0	0	0
F43:A-B-	1(~110)	1	0	0	1	0	0	2	2	0	1	0	0	0	0	0	0	0	0	0	0
F43:A3:B-	1(~145)	1	1	1	1	0	0	5	5	1	0	0	0	0	0	0	0	0	0	1	0
F46:A-B24	1(~130)	1	0	1	0	0	0	2	2	0	0	0	0	0	0	0	0	0	1	0	1
F68:A-B-	1(~145)	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0
Total No.	103	86	64	59	18	29	6	262	2.54	30	20	14	13	3	1	1	37	34	3	11	6

\*M55/14/27/65/15/3/24 is resistance genes abbreviations for bla<sub>CTX-M-55</sub>/bla<sub>14</sub>/bla<sub>27</sub>/bla<sub>65</sub>/bla<sub>15</sub>/bla<sub>3</sub>/bla<sub>24</sub>, respectively.

ND, not determined.

## Detection of Antimicrobial Resistance Genes

The total DNA of transconjugants harvested from LB broth were extracted by TIANPrep Plasmid Mini Kit (TIANGEN Biotech, Beijing, China), according to the manufacturer's instructions. The *bla<sub>CTX-M</sub>*, *rmtB*, and PMQR genes were screened by PCR using primers as described previously (Cano et al., 2009; Wang et al., 2012). The *bla<sub>CTX-M</sub>*, *rmtB*, and *oqxB*-positive transconjugants were also evaluated for the presence of other PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qepA*). PCR products were sequenced and compared with the reported sequences from GenBank ([www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)).

## IncF Plasmid Analysis

Plasmid DNA was extracted by QIAGENPrep Plasmid Midi Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. PCR-base replicon typing (PBRT) was performed on all transconjugants carrying a single plasmid, as previously described (Carattoli et al., 2005). To better characterize IncF, replicon sequencing typing (RST) was performed according to protocols described previously (Villa et al., 2010), and alleles were assigned by comparing the amplicon sequence to the plasmid MLST database (<http://pubmlst.org/plasmid/>).

Plasmid sizes in the transconjugants were determined by pulsed-field gel electrophoresis using nuclease S1 (TaKaRa Biotechnology, Dalian, China) digestion of plasmid DNA prepared in agarose blocks (S1-PFGE). DNA fragments were separated by PFGE on a CHEF-DR III apparatus (Bio-Rad, Hercules, CA, USA) for 15 h at 6 V/cm at 14°C with an initial pulse time of 1 s and a final pulse time of 12 s. Southern hybridization was performed following S1-PFGE using *bla<sub>CTX-M</sub>*, *rmtB*, and *oqxB*-specific digoxigenin (DIG)-labeled probes. Hybridization was detected using the DIG Nucleic Acid Detection Kit (Roche Diagnostics). All plasmids were also analyzed by restriction fragment length polymorphism (RFLP) as follows: Plasmid DNA was digested with *EcoRI* and electrophoresed in 1.0% agarose at 5 V/cm for 2 h. DNA was visualized by EtBr staining. Cluster analysis of digestion patterns generated dendograms and plasmid similarity was measured using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice Similarity Index (DSI) (tolerance 1.5% and optimization 1.5%). Plasmids with a DSI ≥80% were assigned to the same cluster (designated with numbers 1, 2, 3, and 4). Letters were used to discriminate RFLP patterns assigned to the same cluster that differed by one or two restriction bands (i.e., 1a, 1b, 1c etc.).

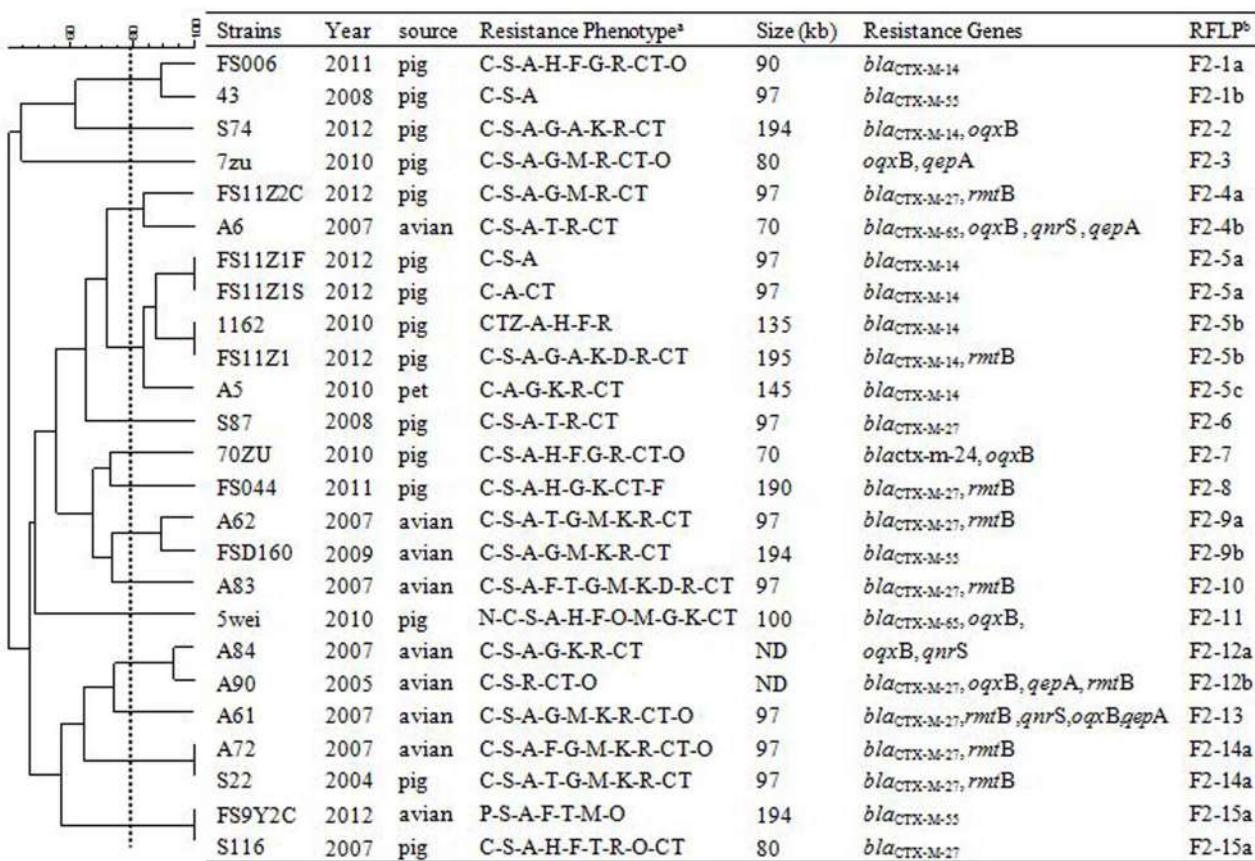


FIGURE 1 | RFLP patterns and characterization of multi-resistance 25 F2: A- B- plasmids.

## Identification of Plasmid Addiction Systems

Plasmid-mediated addiction systems were identified using primers and amplification conditions previously described (Mnif et al., 2010). We screened for 8 major addiction systems: *ccdB*-*ccdB* (involved in cell division), *pemK*-*pemI* (for plasmid emergency maintenance), *relB*-*relE* (relaxed control of stable RNA), *parD*-*parE* (DNA replication), *vagC*-*vagD* (virulence-associated protein), *hok*-*hok* (host-killing), *snrB*-*snrC* (RNA stability), and *pndA*-*pndC* (promotion of nucleic acid).

## Statistical Analysis

Statistical significance for the comparison of prevalence data and proportions was determined by the  $\chi^2$ -test. *p*-values less than 0.05 were deemed to be statistically significant.

## Results

### Antibiotic Resistance Phenotypes

A total of 103 transconjugants were examined in this study and 96% exhibited multi-drug resistance phenotypes with to ampicillin and streptomycin. More than half of the isolates also showed resistance to ceftazidime, ceftriaxone, gentamicin, kanamycin and ceftiofur (Table S1). The antimicrobial resistance rates to other antibiotics tested as follows: amikacin (43.7%), olaquindox (42.7%), tetracycline (40.8%), chloramphenicol (34.0%), and florfenicol (26.2%). Most of these isolates were still susceptible to levofloxacin, enrofloxacin and ciprofloxacin.

### Detection of Resistance Genes

Results of screening for resistance genes among 103 transconjugants are shown in Table S1. The predominant resistance genes identified were *bla*<sub>CTX-M</sub>, *rmtB*, and *oqxB* found in 82, 37, and 34 IncF-type plasmids, respectively. Interestingly, there was a range of *bla*<sub>CTX-M</sub> genotypes including

M55 (*n* = 30), M14 (*n* = 20), M27 (*n* = 14), M65 (*n* = 13), M15 (*n* = 3) and M24 and M3 (1 each). A smaller subset also harbored *qnrS*, *qepA*, and *qnrB* genes (11, 6, and 3, respectively). Sixty-one plasmids carried more than one resistance gene, and the most prevalent combinations were *bla*<sub>CTX-M</sub>-*rmtB* (*n* = 28) and *bla*<sub>CTX-M</sub>-*oqxAB* (*n* = 15). The *qnrA*, *qnrC*, and *qnrD* loci were not found in any of the plasmids tested.

### Characterization of IncF Plasmids

S1-PFGE and Southern blot hybridization analysis showed that all the 103 multi-drug resistant strains contained plasmids ranging in size from 60 to 194 kb (Table 1). This group contained very diverse IncF-RST patterns. A total of 20 IncF-RSTs were found that had replicon FII alone or in combination with FIA or FIB. Various alleles were observed for each of FII (F2, F14, F16, F18, F31, F33, F35, F36, F43, F46, and F58), FIA (A-, A1, A3, and A4) and FIB (B-, B1, B8, B10, and B24) replicons. Meanwhile, we found identical combinations were detected in a number of different plasmids; F2: A-: B- (*n* = 35) and F33: A-: B- (*n* = 30) were the most common. Curiously, more than half of F2: A-: B- positive *E. coli* strains (54.8%, 19/35) were recovered from pigs, while F33: A-: B- (46.7%, 14/30) and F18: A-: B- (50.0%, 5/10) were mainly found in *E. coli* strains recovered from avians.

Further analysis of IncF plasmids bearing multi-resistance genes was performed to see whether a particular resistance gene was associated with a specific plasmid backbone defined by replicon type and addiction system content. Notably, there was higher prevalence of resistance genes located on F2: A-: B- and F33: A-: B- plasmids than on the remaining replicon types (Table 1). For instance, *bla*<sub>CTX-M-27</sub>, *qepA*, and *qnrS* were preferentially associated with the F2: A-: B-. The *bla*<sub>CTX-M-55</sub>, which was the most common identified  $\beta$ -lactamase in our study, combined with F33: A-: B-; while *rmtB* and *oqxB* were mainly found in F2: A-: B- and F33: A-: B-.

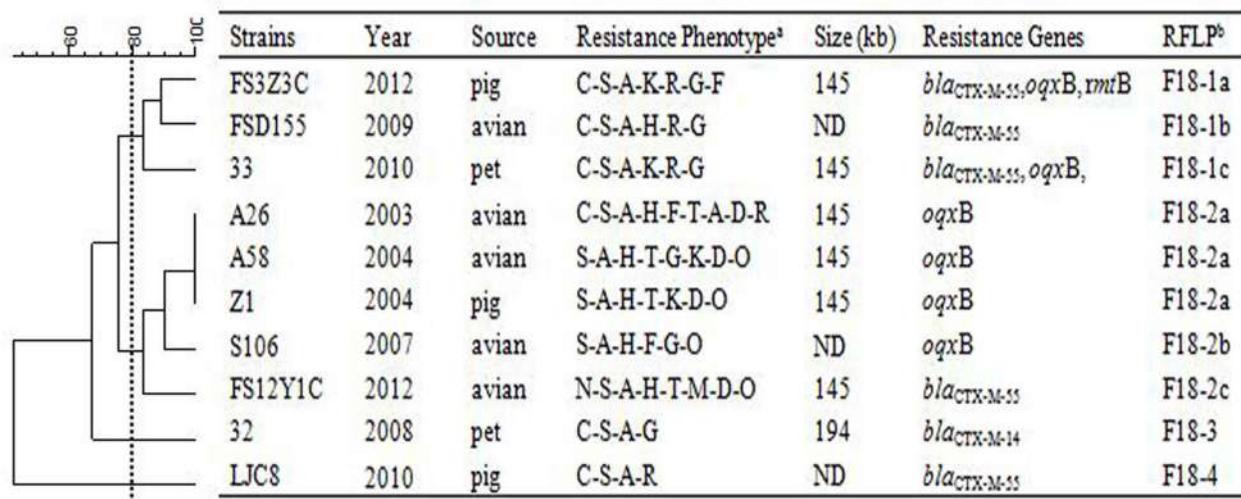


FIGURE 2 | RFLP patterns and characterization of multi-resistance 10 F18: A-: B1 plasmids.

In order to assess relationships among the different replicon types of 103 IncF plasmids encoding variable resistance determinants, RFLP analysis was conducted which allowed visualization of variable clustering. However, our RFLP results showed diverse plasmid profiles even among identical IncF-RST groups. For instance, among 27/30 F33: A-: B- and 25/35 F2: A-: B- plasmids, 12 and 15 RFLP patterns were identified, respectively. In addition, antimicrobial susceptibility, plasmid size and antimicrobial resistance genes varied (Figures 1–3).

## Addiction Systems

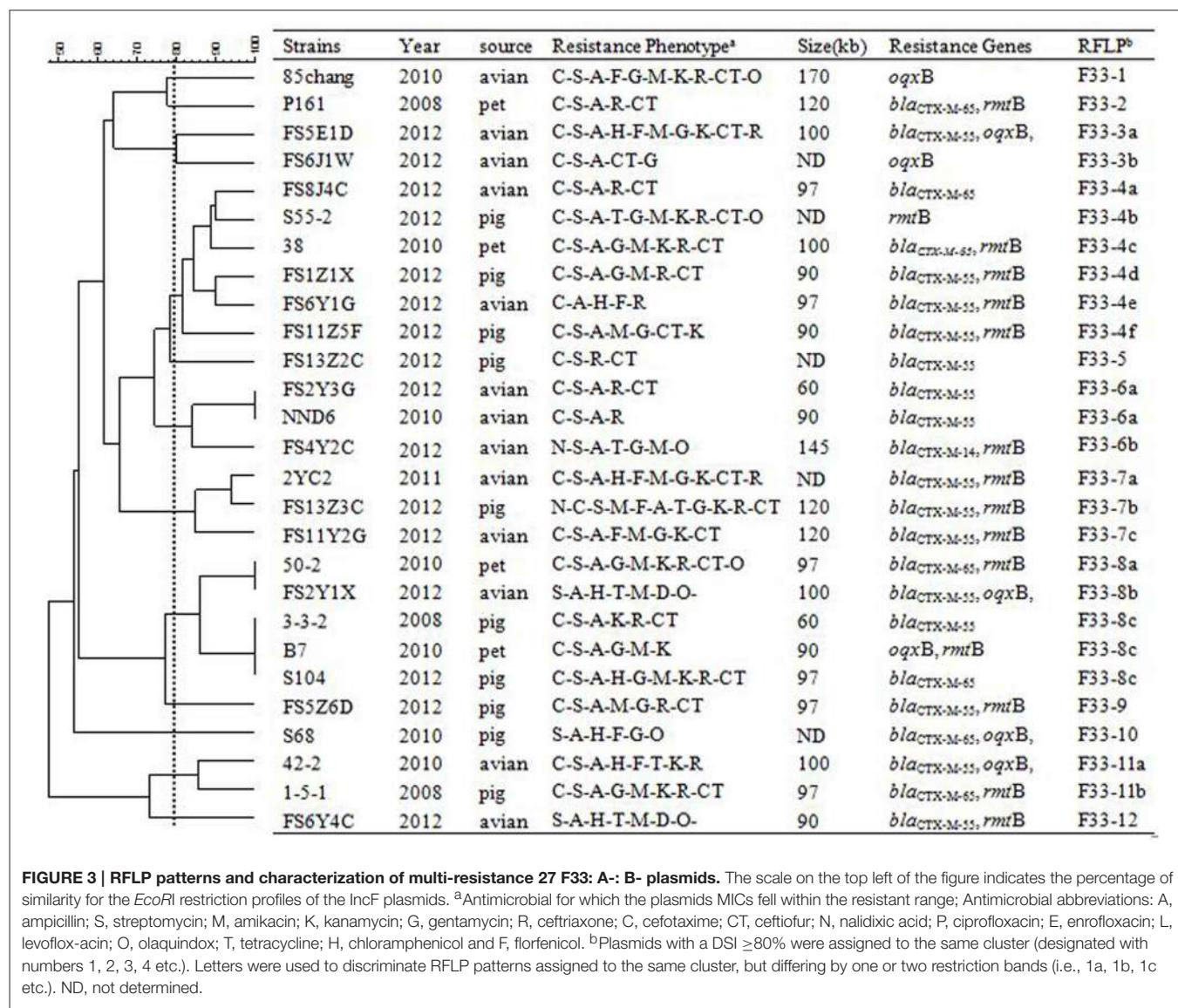
Of the eight addiction systems, the most frequently represented systems were *hok-sok* ( $n = 86$ ), followed by *pemKI* ( $n = 64$ ), *snrBC* ( $n = 59$ ), *ccdB* ( $n = 29$ ), *vagCD* ( $n = 18$ ), and *pndAC* ( $n = 6$ ). None of the IncF-type plasmids harbored *parDE* and *relBE*. The average mean of addiction system was 2.54 with a range of 0–5 per plasmid. Interestingly, when the replicon

FII was associated with FIA and FIB, the isolate also carried more addiction systems than in the absence of this association (Table 1). For instance, between the groups F2: A1: B1 and F2: A-: B-, there was a mean of 4.5 vs. 1.9, and the combination of F16: A-: B1 and F16: A-: B- with the mean of 3.0 vs. 1.7.

The number of different types of addiction systems also varied between the most prevalence replicon types in this study, F2: A-: B- and F33: A-: B-. The number of *snrBC* loci on F33: A-: B- was significantly higher than on F2: A-: B- ( $P < 0.01$ ). In contrast, this number was reversed in the case of the *ccdB* system (Table 1).

## Discussion

In the present study, the prevalence of the resistance determinants *bla<sub>CTX-M</sub>*, PMQR genes and *rmtB* in IncF plasmids from farm animals and pets in China was investigated and the



**FIGURE 3 | RFLP patterns and characterization of multi-resistance 27 F33: A-: B- plasmids.** The scale on the top left of the figure indicates the percentage of similarity for the EcoRI restriction profiles of the IncF plasmids. <sup>a</sup>Antimicrobial for which the plasmids MICs fell within the resistant range; Antimicrobial abbreviations: A, ampicillin; S, streptomycin; M, amikacin; K, kanamycin; G, gentamycin; R, ceftazidime; C, cefotaxime; CT, ceftiofur; N, nalidixic acid; P, ciprofloxacin; E, enrofloxacin; L, levofloxacin; O, olaquindox; T, tetracycline; H, chloramphenicol and F, florfenicol. <sup>b</sup>Plasmids with a DSI  $\geq 80\%$  were assigned to the same cluster (designated with numbers 1, 2, 3, 4 etc.). Letters were used to discriminate RFLP patterns assigned to the same cluster, but differing by one or two restriction bands (i.e., 1a, 1b, 1c etc.). ND, not determined.

characteristics of IncF plasmids were elucidated. One-hundred and three IncF plasmids were recovered, the majority from pigs and avians (85.4%, 88/103), and the remaining obtained from pets (14.6%, 15/103), demonstrating that IncF plasmids are prevalent among multi-drug resistant *E. coli* strains from animals in China. The IncF plasmids identified in our study were associated with multidrug resistance profiles that conferred resistance to different classes of antimicrobials simultaneously. Most of IncF plasmids harbored *bla*<sub>CTX-M</sub> and, frequently carried other resistance genes including *qnr*, *qepA*, *oqxB*, and *rmtB*. These results are in agreement with previous findings that the *rmtB* and PMQR genes are often linked with *bla*<sub>CTX-M</sub>-located on the same plasmid (Dhanji et al., 2011; Li et al., 2012; Matsumura et al., 2013; Chen et al., 2014). Together, these data suggest that the spread of multiple ARGs on the same plasmid has been an important dissemination mechanism of multidrug resistance (Liebert et al., 1999).

The most impressive finding in this study was the diversity of IncF plasmids. The result is similar to previous reports that showed IncF plasmids are not a homogeneous group (Osborn et al., 2000; Coque et al., 2008; Villa et al., 2010; Shin et al., 2012). Moreover, heterogeneous RFLP patterns among identical IncF-RST groups indicate that the cargos such as resistance genes are also likely associated with the diversity of IncF plasmids. Interestingly, we identified some particular resistance genes were associated with the specific plasmid backbone defined by replicon type. For instance, *bla*<sub>CTX-M-3</sub> was located on F2: A-: B-, whereas it has been previously on detected IncL/M plasmids (Golebiewski et al., 2007). As for *bla*<sub>CTX-M-14</sub>, which was primarily found on F2: A-: B- in our study, but in Spain and France, this gene was presented on the IncK plasmids (Diestra et al., 2009; Valverde et al., 2009). Although the movements of these resistance genes were attributed to transposable elements, it is certain that the epidemic plasmids may accelerate their widespread dissemination.

Interestingly, although RFLP patterns were diverse among the plasmids, some with the same IncF-RST grouping did show similar RFLP patterns with minor variations. These data agree with previous studies reporting that IncF plasmids form a very heterogeneous group (Dahmen et al., 2013b). Thus, the extensive diversity of RFLP patterns among IncF plasmids from the collection of *E. coli* used in this study may be due to frequent

recombination and acquisition resistance genes between the plasmids (Partridge et al., 2011; Wiedenbeck and Cohan, 2011; Cain and Hall, 2012).

In contrast to RFLP patterns, the content of addiction systems seemed to differ by replicon type. For instance, the *snrBC* system was widely represented in F33: A-: B- but absent in F2: A-: B-, while *ccdB* was associated with F2: A-: B-. This finding indicates a linkage between addiction systems and specific plasmid backbones, but this must be investigated further. As previously reported, plasmid addiction systems (PAS) contribute to the stability and maintenance of plasmids and they have been shown to facilitate plasmid dissemination even in the absence of antibiotic selection (Hayes, 2003; Moritz and Hergenrother, 2007). Together, these data suggest a hypothesis that the diversity of IncF plasmids can be driven by a combination of acquired multiple resistance genes and addiction modules. In this way, host maintenance would be facilitated by their spread in the *E. coli* population.

In conclusion, we identified extensive diversity of RSTs, plasmid sizes, addiction systems and RFLP patterns among IncF plasmids, carrying *bla*<sub>CTX-M</sub> or PMQR or aminoglycoside resistance genes. Considering the fact that the IncF plasmids have been common reservoirs for diffusion of resistance genes, its wide dissemination among Enterobacteriaceae especially among *E. coli* could pose a serious threat to public health.

## Acknowledgments

This work was supported by the Program for Changjiang Scholars and Innovative Research Team in University of Ministry of Education of China (Grant No. IRT13063), the National Natural Science Foundation and Natural Science Foundation of Guangdong Province, China (Grant No. U1201214), the Natural Science Foundation of Guangdong Province (Grant No. S2012030006590) and the National Natural Science Funds of China (Grant No. 31402247).

## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.00964>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Mechanism and *In Vivo* Evaluation: Photodynamic Antibacterial Chemotherapy of Lysine-Porphyrin Conjugate

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### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 22 October 2015

Accepted: 15 February 2016

Published: 02 March 2016

### Citation:

Xu Z, Gao Y, Meng S, Yang B, Pang L, Wang C and Liu T (2016) Mechanism and *In Vivo* Evaluation: Photodynamic Antibacterial Chemotherapy of Lysine-Porphyrin Conjugate. *Front. Microbiol.* 7:242. doi: 10.3389/fmicb.2016.00242

Lysine-porphyrin conjugate 4i has potent photosensitive antibacterial effect on clinical isolated bacterial strains such as Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and *Pseudomonas aeruginosa*. The mechanism of photodynamic antibacterial chemotherapy of 4i (4i-PACT) *in vitro* and the treatment effect *in vivo* was investigated in this paper. Atomic force microscopy (AFM) revealed that 4i-PACT can effectively destroy membrane and wall of bacteria, resulting in leakage of its content. This was confirmed by dual fluorescent staining with acridine orange/ethidium bromide and measuring materials absorption at 260 nm. Agarose gel electrophoresis measurement showed that 4i-PACT can damage genomic DNA. Healing of wound in rat infected by mixed bacteria showed that the efficiency of 4i-PACT is dependent on the dose of light. These results showed that 4i-PACT has promising bactericidal effect both *in vitro* and *in vivo*.

**Keywords:** lysine-porphyrin conjugate, photodynamic antimicrobial chemotherapy, antibiotic resistance, photosensitizer, mixed bacterial-infected wounds

## INTRODUCTION

The antibiotics for treating common infections have become less effective in recent years. Nearly 1000 of resistance-related  $\beta$ -lactamases, which inactivate the antibiotics, have been identified, a 10 times increase since 1990 (Davies and Davies, 2010; Laxminarayan et al., 2013). This situation made us at the dawn of post antibiotic era (Ryono, 2014). So, it is urgent to find alternative approaches against antibiotics-resistant bacteria. Generally, there are two solutions to this problem. One is to develop novel antibacterial agents, the other is to develop novel physical sterilization. The latter includes radiation sterilization (Nusbaum and Rose, 1979), high pressure electric field sterilization (Sasagawa et al., 2006), microwave sterilization (Dovigo et al., 2009), magnetic sterilization (Haile et al., 2008), and pulse light sterilization (Krishnamurthy et al., 2004). Chemical agents and physical approaches are combined in Photodynamic antimicrobial chemotherapy (PACT) (Wainwright, 1998), which consists of a non-toxic photosensitizer (PS), illumination light of an appropriate wavelength, and ambient molecular oxygen. However, not all of the PS can be used in PACT treatment of bacterial infection. Only special designed PS can match the PACT demand (Meng et al., 2015). So from this point of view, PACT falls between novel antibacterial agents and novel

physical sterilization approaches, namely, a physicochemical treatment. With the advantage of circumventing the bacterial multidrug resistance in the treatment of infection, PACT has received great attention in recent years.

The PS is the crucial element in PACT. In the past few decades, several types of PSs have been reported, such as porphyrin, heme, phthalocyanine, phenothiazinium dyes, BODIPY, methylene blue, conjugated polyelectrolytes, cationic functionalized fullerene, as well as nanoparticals (Wainwright and Crossley, 2004; Wainwright, 2010; Fu et al., 2013; Sperandio et al., 2013). As an ideal PS candidate for photodynamic therapies (Stojiljkovic et al., 2001), Porphyrin can efficiently kill Gram-positive bacteria. Meanwhile, in combination with agents that permeabilize the highly organized outer membrane of Gram-negative bacteria, only cationic PSs, or noncationic PSs are able to kill Gram-negative species (Mbakidi et al., 2013; Prasanth et al., 2014).

In a previous paper, we have reported the synthesis, characterization, and *in vitro* photodynamic antimicrobial activity of basic amino acid-porphyrin conjugates (Meng et al., 2015), a new class of PSs. Among them, compared with other PSs, 4i exhibits a broader spectrum of photoactivation *in vitro*, improved biocompatibility and stability (Bertolini et al., 1990; Nitzan et al., 1992; Tomé et al., 2004; Liu et al., 2012; Gomes et al., 2013; Mbakidi et al., 2013; Prasanth et al., 2014). This paper reported the mechanism of bacterial inactivation by 4i-PACT and its effectiveness *in vivo* on experimental mixed bacterial infected rat wounds. The results showed that 4i-PACT had potent bactericidal effect both *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Bacterial Culture

In this experiment, three clinical bacterial strains, MRSA, *Escherichia coli*, and *Pseudomonas aeruginosa* were isolated at the Tianjin Armed Police Hospital. These bacterial strains were cultured in Luria Bertani (LB) medium. A single colony was used to inoculate 10 mL of liquid medium. The bacteria was grown under aerobic condition at 37°C in a shaking incubator (200 rpm) for 18 h. The bacteria was then collected by centrifugation and resuspended in an equal volume of PBS (Zhao et al., 2014).

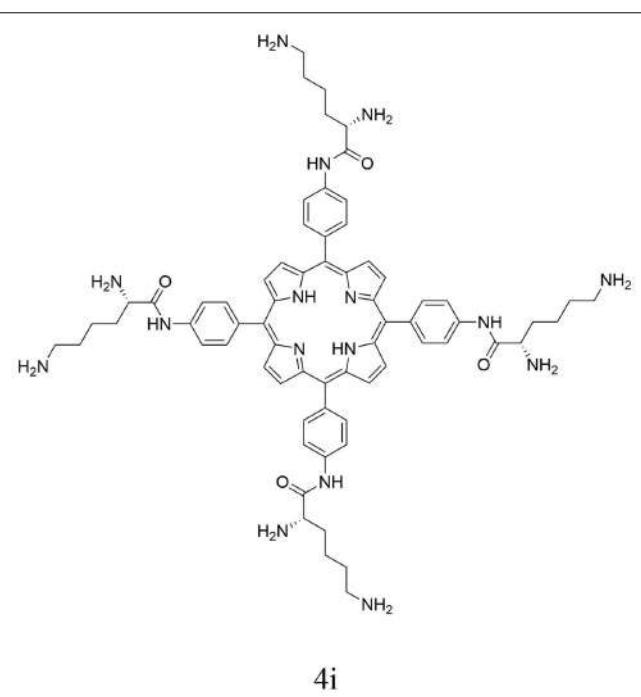
### Chemicals and Instruments

Porphyrin derivative 4i was synthesized and characterized with the procedure reported by Meng et al. (2015). For clarity, synthetic procedure and characterization detail are omitted. The chemical structure of porphyrin derivative 4i is shown in Figure 1.

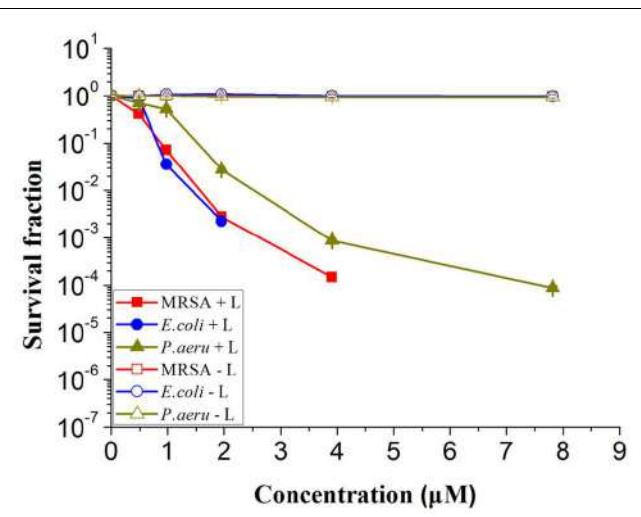
Light from a semiconductor laser (7404, Intense, USA) with a wavelength of 650 nm was delivered on the sample via an optic fiber. The energy density of light spot was measured by a light power meter (LM1; Carl Zeiss).

### MIC and MBC Determinations

Three bacterial strains were treated with the same procedure. As an example of all of the strains, the treatment of MRSA



**FIGURE 1 |** Structure of 5,10,15,20-trakis(4-((S)-2,6-diaminohexanamido)-phenyl) porphyrin (4i).



**FIGURE 2 |** Survival fraction of three bacterial strains treated by different concentration of 4i with and without illumination. Light toxicity marked by MRSA + L, *E. coli* + L, *Pseudomonas aeruginosa* + L and dark toxicity marked by MRSA - L, *E. coli* - L, *P. aeruginosa* - L respectively.

is described in detail. Experiments were performed in 96-well flat bottom plates. Twenty microliter of MRSA suspension and 180 μL of the compounds were added to each well. The final concentration of the bacterial in the mixture was 10<sup>6</sup> colony-forming units/mL (CFU/mL). 4i with different mole concentrations were prepared, e.g., 0.98, 1.95, 3.91, 7.8, 15.6,

31.25, 62.5, 125, 250, and 500  $\mu\text{M}$ , respectively. Plates were kept in the dark for 30 min at 37°C and then exposed to light illumination for 30 min or kept in the dark to provide dark control samples. After light exposure, the samples were incubated in dark at 37°C and CFU was evaluated after 18 h. Three sets of independent experiments were performed (Rodrigues et al., 2013).

## Dose-Dependent Photoactivation Effects

Suspensions of bacteria ( $10^7$  CFU/mL) were incubated at 37°C for 30 min in the dark with 4i (3.91, 7.81, 15.63, 31.25, and 62.50  $\mu\text{M}$ ). The suspensions were then loaded into a 96-well plate and illuminated with light with energy density of 6  $\text{J}/\text{cm}^2$  for the photoreaction. After illumination, 100  $\mu\text{L}$  mixture from each well was taken to determine the CFU. The mixtures were serially diluted to  $10^1$ ,  $10^2$ ,  $10^3$ ,  $10^4$ , and  $10^5$  times of the initial concentrations using PBS. 100  $\mu\text{L}$  of each dilution was plated onto LB agar plates and incubated for 18 h at 37°C in dark. The CFUs were counted, and each experiment was performed in triplicate. The surviving fractions of bacteria were expressed as ratios of CFU produced by bacteria treated with PSs and light (Grinholc et al., 2008).

## Atomic Force Microscopy (AFM) Imaging

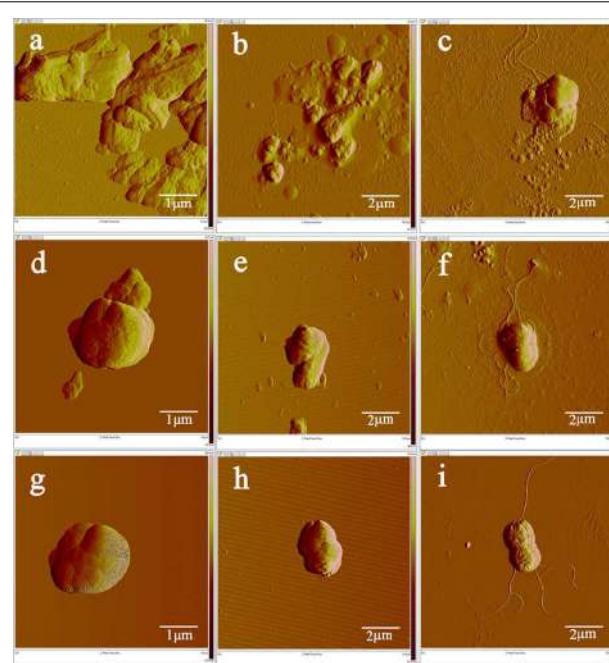
The bacteria were examined with atomic force microscope (Veeco Multi Mode 8/B0021, Veeco German) equipped with Antimony doped silicon of conical shape (Veeco). The force constant is 3 N/m. Bacterial samples in a concentration of  $10^7$  CFU/mL were dropped on to the mica plate surface (about  $0.5 \text{ cm}^2$ ), naturally dried at ambient temperature, then were scanned in intelligent mode (Wu et al., 2014).

## Fluorescence Imaging

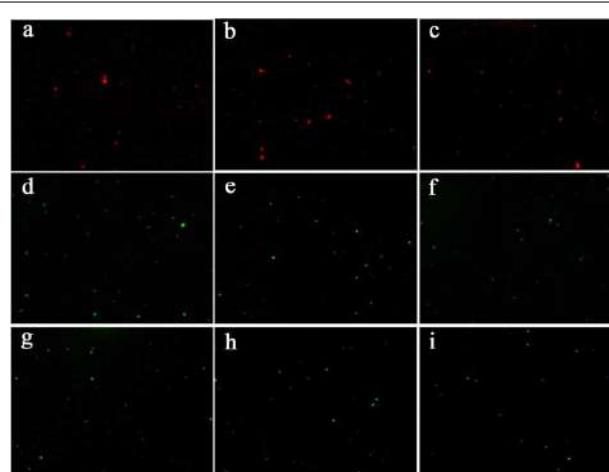
In order to study 4i-PACT effect on the permeability of the bacterial membrane, the samples after 4i-PACT treatment were stained with 4  $\mu\text{L}$  of the acridine orange/ethidium bromide (AO/EB) double fluorescent dyes mixture (100 mg/L of AO and 100 mg/L of EB) for 5 min in dark. Then 5  $\mu\text{L}$  of the stained mixture was dropped on a microscopic glass slide with cover glass and immediately checked by the fluorescent microscope (Nikon Eclipse Ti/B0004, Nikon, Japan) at 200 $\times$  magnification (Sui et al., 2013).

## Genomic DNA Extraction and Electrophoresis

Bacterial suspensions ( $1 \times 10^7$  CFU/mL) were treated with 10  $\mu\text{M}$  4i as mentioned in experiment 2.4 above. After exposing to the laser light, the genomic DNA was extracted from bacteria immediately using a Genomic DNA Purification Kit (Solarbio, Beijing). DNA samples were gently mixed with 6x loading buffer (Solarbio, Beijing). DNA was analyzed by electrophoresis with 0.8% agarose gel in 1x TAE buffer at 70 V for 30 min. DNA green was incorporated into the agarose gel. The 15000 bp DNA Marker (TAKARA) was used as a molecular weight marker with DNA fragments between 250

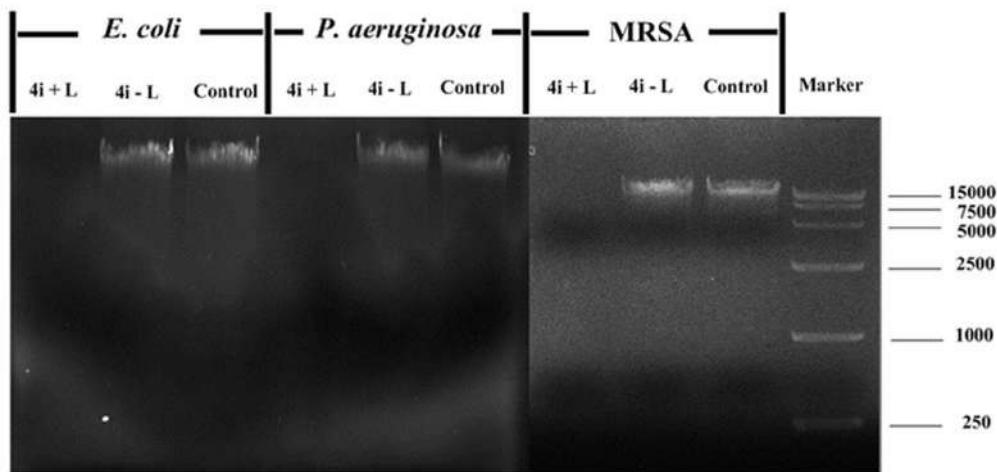


**FIGURE 3 |** Fluorescence microscopy image of three bacterial strains treated by  $10 \mu\text{M}$  4i with and without illumination. **(a)** MRSA treated by 4i-PACT, **(d)** 4i with no light, **(g)** control; **(b)** *P. aeruginosa* treated by 4i-PACT, **(e)** 4i with no light, **(h)** control; **(c)** *E. coli* treated by the 4i-PACT, **(f)** 4i with no light, **(i)** control.



**FIGURE 4 |** AFM image of three bacterial strains treated by  $10 \mu\text{M}$  4i with and without illumination. **(a)** MRSA treated by 4i-PACT, **(d)** 4i with no light, **(g)** control; **(b)** *P. aeruginosa* treated by 4i-PACT, **(e)** 4i with no light, **(h)** control; **(c)** *E. coli* treated by 4i-PACT, **(f)** 4i with no light, **(i)** control.

and 15000 bp. The light dose had been optimized *in vitro* in our experiments. Light dose higher than  $6 \text{ J}/\text{cm}^2$  did not improve the efficacy, while the light with lower dose results in a lower bactericidal ability. Without PS 4i, light exposure alone on bacteria *in vitro* did not have significant bactericidal effect.



**FIGURE 5 |** Agarose gel electrophoresis of three bacterial strains after treated by  $10 \mu\text{M}$  4i with and without illumination. Bacteria treated with 4i-PACT (4i + L), treated with 4i in the dark (4i-L) and no treatment (control).

## Exudation Study for Measuring Materials Absorption at 260 nm

DNA and RNA have absorbance at 260 nm, so exudation study was conducted by measuring the materials absorption at 260 nm for bacteria treated by  $10 \mu\text{M}$  4i-PACT,  $10 \mu\text{M}$  4i alone, and PBS, respectively. After treatment,  $400 \mu\text{L}$  of the bacterial suspensions ( $1 \times 10^8$ ) were filtered to remove the bacteria. OD at 260 nm (OD<sub>260</sub>) of the supernatant was recorded for treated and pre-treatment control groups.

## Excisional Wound Model and Establishment of Infection

The wound healing characteristic of the 4i-PACT was evaluated using a rat model (Dash et al., 2001). **The Animal Care and Use Committee of PUMC approved all experimental protocols involving rats.** Sprague-Dawley rats from Beijing HFK Bioscience CO., LTD, weighing about 220 g, were housed at one rat one cage and maintained in dark except PACT treatment. The rats were first anesthetized via intraperitoneal injection of chloral hydrate (300 mg/kg). Then their back was shaved with an electric razor, followed by a depilatory agent. Four circular wounds (1.5 cm in diameter) with a depth of the skin layers were prepared along both sides of the spine. Immediately after that, suspension ( $50 \mu\text{L}$ ) of mixed bacteria ( $10^9 \text{ CFU/mL}$  MRSA,  $5 \times 10^8 \text{ CFU/mL}$  *E. coli*,  $5 \times 10^8 \text{ CFU/mL}$  *P. aeruginosa*) in sterile PBS was inoculated onto the surface of each wound with a pipet tip and then smeared onto the wound surface with an inoculating loop. Next, a bandage was wrapped around each rat to protect the shaved skin and the wounds from other harm. This state was kept for 1 day. Then these rats were used as the wounds model for mixed bacterial infection.

## 4i-PACT In Vivo

Thirty rats with wound infected by mixed bacteria were divided into five groups: (A) no treatment control; (B) 4i +  $100 \text{ J/cm}^2$

Light; (C) 4i +  $50 \text{ J/cm}^2$  Light; (D) 4i +  $25 \text{ J/cm}^2$  Light; (E) 4i +  $12.5 \text{ J/cm}^2$  Light. Since light illumination doses alone in this study do not have significant effect on the wound healing, we did not provide light illumination doses alone groups.

Twenty four hours after infection,  $50 \mu\text{L}$  of 4i solution ( $40 \mu\text{M}$  in PBS) was injected under eschar of the wound of groups B–E. Then after 30 min for the PS uptake by the bacteria, rats in groups B–E were illuminated with  $650 \text{ nm}$  laser ( $100 \text{ mW/cm}^2$ ), the light dose was kept at  $100, 50, 25, 12.5 \text{ J/cm}^2$  for group B–E, respectively. On the next day, the same light dose was given to increase the antibacterial effect of 4i-PACT. The above treatment was repeated three times.

The day, on which rats were infected, was regarded as day 1, the widths and lengths of the wounds were measured using a vernier caliper on days 1, 2, 3, 4, 5, 6, 7, 8, 10, and 12. The viability of each group of rats after infection was recorded, respectively. Meanwhile bacterial CFUs in the wound were calculated in the following ways. Swabs collected from the rat wounds were cultured in PBS. Viable bacteria in the PBS were counted by making 10-fold serial dilutions and culturing the dilutions on LB agar for 18 h at  $37^\circ\text{C}$ . Then CFUs were counted manually (Kumar et al., 2011; Sudheesh Kumar et al., 2012).

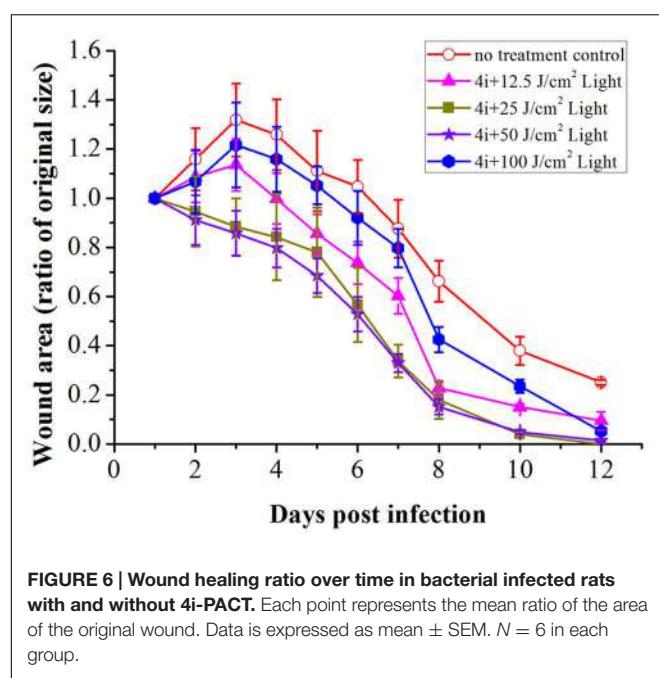
## Statistical Analysis

All data are presented as mean  $\pm$  SEM. The significance of differences between sample means was determined by Student's *t*-test using SPSS 19.0.

## RESULTS AND DISCUSSION

### MIC and MBC Determinations

The minimum inhibitory and bactericidal concentration (MIC and MBC) of 4i toward bacteria was studied. Bacterial suspensions ( $10^6 \text{ CFU/mL}$ ) were incubated with the 4i in dark for 30 min at  $37^\circ\text{C}$  and then exposed to light illumination



**FIGURE 6 |** Wound healing ratio over time in bacterial infected rats with and without 4i-PACT. Each point represents the mean ratio of the area of the original wound. Data is expressed as mean  $\pm$  SEM.  $N = 6$  in each group.

(650 nm, 6  $\text{J}/\text{cm}^2$ ). The concentration of 4i required to make the suspensions ( $10^6 \text{ CFU/mL}$ ) change visibly from turbid to clear was regarded as the minimum inhibitory concentration (MIC), while the concentration at which no more than five colonies were observed on the plates was regarded as the minimum bactericidal concentration (MBC). As shown in Table 1, compound 4i had high bacterial photoinactivation ability with MIC values of  $3.91 \mu\text{M}$  against MRSA,  $1.95 \mu\text{M}$  against *E. coli*, and  $7.81 \mu\text{M}$  against *P. aeruginosa*, respectively; MBC values of  $7.81 \mu\text{M}$  against MRSA,  $3.91 \mu\text{M}$  against *E. coli*, and  $15.60 \mu\text{M}$  against *P. aeruginosa*. Meanwhile the dark toxicity of the 4i is relatively weak, with MIC >  $62.5 \mu\text{M}$ , MBC >  $125 \mu\text{M}$  for

**TABLE 1 |** Minimal inhibitory concentration (MIC,  $\mu\text{M}$ ) and minimal bactericidal concentration (MBC,  $\mu\text{M}$ ) of 4i against MRSA, *Pseudomonas aeruginosa*, *Escherichia coli*.

Clinical bacterial strain	Light toxicity		Dark toxicity	
	MIC	MBC	MIC	MBC
MRSA	3.91	7.81	62.50	125.00
<i>P. aeruginosa</i>	7.81	15.60	250	> 500
<i>E. coli</i>	1.95	3.91	62.50	125.00

**TABLE 2 |** Bacterial content exudation in the control (PBS), 4i alone, and 4i-PACT treatment groups ( $n = 4$ , mean  $\pm$  SD) measured at 260 nm.

Clinical bacterial strain	Increase in OD <sub>260</sub> relative to pre-treatment (%)		
	Control	4i alone	4i-PACT
MRSA	$2.86 \pm 0.34$	$10.98 \pm 0.41$	$247.87 \pm 17.87$
<i>P. aeruginosa</i>	$2.04 \pm 0.29$	$9.54 \pm 0.28$	$188.98 \pm 19.32$
<i>E. coli</i>	$2.98 \pm 0.33$	$13.31 \pm 0.38$	$336.67 \pm 28.08$

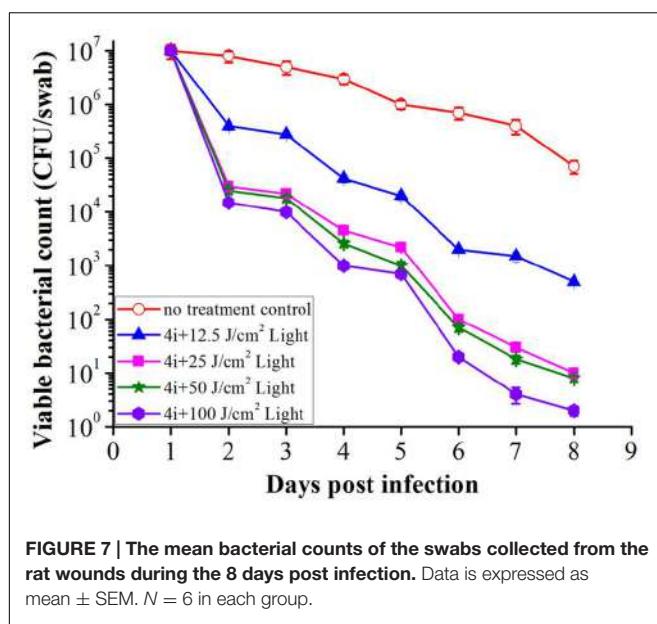
these three strains. From the values of MBC, it can be concluded that 4i-PACT is very effective toward *E. coli*, *P. aeruginosa* is the most resistant strain and MRSA is at the middle of them. So 4i-PACT seemly shows higher bactericidal activity toward Gram-negative strains than that of Gram-positive strains. This trend is different from the PACT of other positive charged PS (Sahu et al., 2009; Kharkwal et al., 2011; Ke et al., 2014), which show higher photoinactivation toward the Gram-positive strains than the Gram-negative counterparts. The latter possess a highly organized outer membrane which imparts a higher permeability barrier for the PSs to penetrate into the bacteria. In this study, 4i-PACT shows higher bactericidal effect toward *E. coli* than MRSA and *P. aeruginosa*. This unexpected result can be interpreted based upon the chemical structure of 4i,  $\pi-\pi$  interaction of large planar porphyrin rings, hydrogen bonding interaction and charge interaction from four lysine moieties. The ensemble interaction and difference of the outside membrane structure of these strains lead to the unexpected bactericidal effect of 4i-PACT.

## Dose-Dependent Photoinactivation Effects

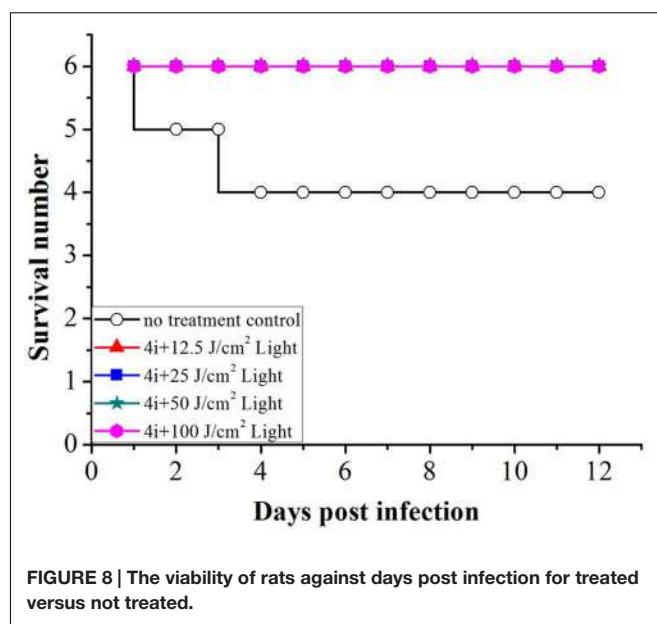
The photoinactivation effect of 4i against MRSA, *E. coli* and *P. aeruginosa* strains was dose-dependent (Figure 2). A sharp decrease in bacterial survival fraction with increase of the concentration was observed. These three strains were incubated with  $3.9 \mu\text{M}$  4i in the dark for 30 min at  $37^\circ\text{C}$ , then illuminated by the 650 nm laser with power of  $6 \text{ J}/\text{cm}^2$ .  $4.35$ ,  $6.96$ , and  $3.56 \log_{10}$  reduction in bacterial survival fraction was observed for the clinical isolated MRSA, *E. coli* and *P. aeruginosa*, respectively. As a control, 4i did not show any bactericidal effect to these strains in dark at less than  $10 \mu\text{M}$ .

## Morphology Changes

Changes of the bacterial structure can be clearly revealed at morphological level by AFM. The representative results are shown in Figure 3. The normal ball shape structure of MRSA (Figure 3g), the rod-like shape of *P. aeruginosa* (Figure 3h) and *E. coli* (Figure 3i) was observed without any treatment. The size and shape of the bacteria agree with that reported in previous study (Eaton et al., 2008). For these three strains treated by 4i in the dark (Figures 3d-f), it can be seen that the bacterial structure is complete. Particles scattered at the surface of bacteria may be the aggregates of 4i. In the groups treated by 4i-PACT (Figures 3a-c), it was found that bacteria is damaged and fragmented. Some irregular aggregation of the dead corpse was detected. Researchers have found that the roughness, size and shape of bacterial strains are slightly altered after PACT treatment with Toluidine Blue O (Sahu et al., 2009; Jin et al., 2010; Lin et al., 2012). The leakage from *Staphylococcus aureus* cell suspension is greatly increased (Sahu et al., 2009), suggesting that cytoplasmic materials are lost through the damaged bacterial membrane. Results in this study showed that bacterial strains are completely broken into pieces. Thus, 4i-PACT treatment appeared to have potent effect on the bacterial envelope, including damaging the bacterial wall and membrane.



**FIGURE 7 |** The mean bacterial counts of the swabs collected from the rat wounds during the 8 days post infection. Data is expressed as mean  $\pm$  SEM.  $N = 6$  in each group.



**FIGURE 8 |** The viability of rats against days post infection for treated versus not treated.

## Membrane Integrity

The fluorescence microscopy was used to investigate the permeability of bacterial membrane before and after 4i-PACT. Fluorescent dyes (AO/EB), as the indicator of cell membrane damage, were used to assess the antibacterial activity of 4i-PACT. Living bacteria was observed as green and dead as red, respectively. Figures 4g–i show fluorescence microscopy images of the control samples, where no 4i was added, the bacteria is dispersed and alive with green color. Figures 4a–c are the fluorescence microscopy images of MRSA, *P. aeruginosa* and *E. coli* treated by 4i-PACT ( $10 \mu\text{M}$ ,  $6\text{J}/\text{cm}^2$ ,  $650\text{ nm}$ ), respectively, where most of the bacteria is dead with red color. On the other hand, fluorescence microscopy images of MRSA (Figure 4d), *P. aeruginosa* (Figure 4e), and *E. coli* (Figure 4f) treated by  $10 \mu\text{M}$  4i without illumination showed little bactericidal effect. The above results mean that 4i-PACT can damage the membrane of bacteria and change the permeability of the bacterial membrane. This again explains why the antimicrobial effect of 4i-PACT is so strong.

## Photodynamic Effect on Genomic DNA

The influence of 4i-PACT on bacteria was further studied from a genomic point of view such as DNA leakage or damage. The electrophoresis graph (Figure 5) shows that the DNA bands, extracted from the processed bacterial cells, disappear after 4i-PACT ( $10 \mu\text{M}$ ,  $6 \text{ J}/\text{cm}^2$ ,  $650 \text{ nm}$ ), while a clear DNA band is detected in the control groups with no 4i-PACT or treated by 4i alone. This result is consistent with the AFM morphological study as well as the fluorescent staining, which reveal that the 4i-PACT breaks the bacterial membrane and wall and causes the leakage of genomic DNA. The absence of DNA bands can be attributed to either the leakage of bacteria membrane or the break of the DNA. So we conducted another independent experiment. The naked DNA extracted from each strains was treated by 4i-PACT. The agarose gel electrophoresis graph still shows no

DNA bands. This result indicated that 4i-PACT can break DNA into pieces. This phenomenon is consistent with the previous results (Menezes et al., 1990; Choi et al., 2010; Lin et al., 2012). Generally, the reason for multidrug resistance in cellular level is due to the drug only blocked or weakened the partial binding site or signal pathway, while the whole cellular bodies were kept, so it is easy to develop multidrug resistance. Double effects of 4i-PACT, destroying bacterial membrane and damaging the DNA, can account for the vanish of DNA band in electrophoresis graph. This is the reason why bacterial strains have no resistant to 4i-PACT.

## Bacterial Content Exudation

Release of intracellular components is a good indicator of membrane integrity. Small ions such as potassium and phosphate tend to exude first, followed by large molecules such as DNA, RNA, and other materials. Since these nucleotides have strong UV absorption at  $260 \text{ nm}$ , measuring the absorbance at  $260 \text{ nm}$  can reveal the information of membrane integrity (Denyer and Hugo, 1991; Chen and Cooper, 2002). As shown in Table 2, after  $10 \mu\text{M}$  4i-PACT, the bacterial content exudation from MRSA, *P. aeruginosa*, and *E. Coli* are increased by 247.87, 188.98, 336.67%, respectively, compared with pre-treatment samples, while changes less than 10% was observed in the control or 4i group. This result is consistent with that of AFM and fluorescent images. These data are larger than that reported by Sahu et al. (2009), which were only 20–60% increase compared with the control groups.

## In Vivo Antibacterial Activity and Wound-Healing Effect

*In vivo* antibiotics experiment, generally 5 or 10 times MIC was chosen as the working concentration.  $40 \mu\text{M}$  of 4i, five times of MIC (Singh et al., 2006; Dai et al., 2009) was chosen as the working dose. The antibacterial effect of 4i-PACT with

different light dose was studied. The wound healing rate is highly light dose dependent (**Figure 6**). Control group A, with no treatment, showed less wound healing at each observation time point. A rapid wound healing was observed in the treatment of groups C (50 J/cm<sup>2</sup>) and D (25 J/cm<sup>2</sup>) during the day 4 to 8 after infection ( $p < 0.05$ ). After day 8, wound healing became slow till completely healed on day 12. However, in the groups B (100 J/cm<sup>2</sup>) and E (12.5 J/cm<sup>2</sup>), rapid wound closure happened from day 7 to 10. On the day 8, mean wound area ratios of B–E were  $0.4252 \pm 0.0512$ ,  $0.1515 \pm 0.0312$ ,  $0.1823 \pm 0.0777$ , and  $0.2273 \pm 0.0239$ , respectively, but in the control group wound area ratio was  $0.6619 \pm 0.0834$ , indicating B–E groups had better wound healing ratio compared with the control group. Almost total wound closure was observed by the day 12 post infection in all treated groups.

The viable bacteria in wound tissue were counted as an index of the bactericidal effect of 4i-PACT. On days 1, 2, 3, 4, 5, 6, 7, 8 post infection, PACT-treated groups exhibited obvious reduction in bacterial viability compared with the no treatment control group (**Figure 7**). At the same time point, the decrease of viable bacterial count is in the order of light illumination dose, B > C > D > E. This suggests that 4i-PACT accelerates wound healing via bactericidal effect against MRSA, *P. aeruginosa*, and *E. coli*. However, the wound healing ratio is in the order of C > D > E > B, instead of paralleling of the bactericidal effect. This indicates that 4i-PACT effect on normal tissue should be considered. A rationalized interpretation can be given based upon the multiple function of 4i-PACT *in vivo*: 4i-PACT not only photoinactivates bacteria, but also causes the normal tissue damaged and repaired. The observed effect is the balance of the ensemble results. For either a weaker 4i-PACT with a lower illumination dose (12.5 J/cm<sup>2</sup> in group E), or a stronger 4i-PACT with a higher illumination dose (100 J/cm<sup>2</sup> in group B), the total results are not good. The best result is from treatment with medium light dose like group C (50 J/cm<sup>2</sup>). So for the practical application of PACT further study is required.

In the no treatment control group, two rats died on day 2 and day 4, rats in other groups all survived the experiment. Therefore, the viability of rats post treatment indicates that 4i-PACT is more efficient in the treatment of mixed bacterial infection (**Figure 8**).

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## CONCLUSION

This paper studied the photoinactivation mechanism of 4i-PACT *in vitro* and its wound healing effect on wound rat model infected by mixed bacteria. 4i-PACT (3.91  $\mu$ M, 6J/cm<sup>2</sup>, 650 nm) had a broad potent antibacterial spectrum: 4.35, 6.96, and 3.56 log<sub>10</sub> reduction in bacterial survival for MRSA, *E. coli*, and *P. aeruginosa*, respectively. AFM revealed that 4i-PACT can destroy bacterial wall and membrane. This is further confirmed by Dual fluorescent staining with AO/EB. Agarose gel electrophoresis indicated that 4i-PACT can damage the genomic DNA of bacteria. Absorbance at 260 nm reveals that 4i-PACT could cause bacterial content leakage. 4i-PACT breaks not only bacterial wall but also the genomic DNA. That is why the bacterial strains have no resistance to 4i-PACT. Wound healing effect on mixed bacterial infected rat model revealed that 4i-PACT *in vivo* is highly potent and light-dose dependent. The ensemble effect is the balanced effect of 4i-PACT both to normal tissue and bacterial strains. The best light dose is 50 J/cm<sup>2</sup>. Overall, 4i-PACT is a highly efficient treatment modality for bacterial infection *in vitro* or *in vivo*.

## AUTHOR CONTRIBUTIONS

TL designed experiments; SM, ZX, LP, and YG carried out experiments; YG and ZX analyzed experimental results, CW analyzed sequencing data. TL, ZX, and YG wrote the manuscript. TL designed experiments; SM, ZX, LP, and YG carried out experiments; YG and ZX analyzed experimental results, CW analyzed sequencing data. TL, ZX, and YG wrote the manuscript.

## ACKNOWLEDGMENTS

This work was supported by the key technologies R & D program of Tianjin (12ZCDZSY11900), Peking Union Medical College Peking Union Youth Fund grant to ZX (NO.3332015030) and the National Natural Science Foundation of China (81202415).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Mycoplasma bovis: Mechanisms of Resistance and Trends in Antimicrobial Susceptibility

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*Mycoplasma bovis* is a cell-wall-less bacterium and belongs to the class *Mollicutes*. It is the most important etiological agent of bovine mycoplasmoses in North America and Europe, causing respiratory disease, mastitis, otitis media, arthritis, and reproductive disease. Clinical disease associated with *M. bovis* is often chronic, debilitating, and poorly responsive to antimicrobial therapy, resulting in significant economic loss, the full extent of which is difficult to estimate. Until *M. bovis* vaccines are universally available, sanitary control measures and antimicrobial treatment are the only approaches that can be used in attempts to control *M. bovis* infections. However, *in vitro* studies show that many of the current *M. bovis* isolates circulating in Europe have high minimum inhibitory concentrations (MIC) for many of the commercially available antimicrobials. In this review we summarize the current MIC trends indicating the development of antimicrobial resistance in *M. bovis* as well as the known molecular mechanisms by which resistance is acquired.

## OPEN ACCESS

### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 14 December 2015

**Accepted:** 11 April 2016

**Published:** 27 April 2016

### Citation:

Lysnyansky I and Ayling RD (2016)  
*Mycoplasma bovis: Mechanisms of Resistance and Trends in Antimicrobial Susceptibility*. *Front. Microbiol.* 7:595.  
doi: 10.3389/fmicb.2016.00595

## INTRODUCTION

*Mycoplasma bovis* is a cell-wall-less bacterium and belongs to the class *Mollicutes* (Razin, 1978). It is the most important etiological agent of bovine mycoplasmosis in North America and Europe, causing respiratory disease, mastitis, otitis media, arthritis, and reproductive disease (Nicholas and Ayling, 2003). Until *M. bovis* vaccines are universally available, sanitary control measures and antimicrobial treatment are the only approaches that can be used in attempts to control *M. bovis* infections. However, *M. bovis* is refractory to  $\beta$ -lactams and to all antimicrobials that target the cell wall. In addition, mycoplasmas are also naturally resistant to polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampin (Taylor-Robinson and Bebear, 1997). Antimicrobials that are mycoplastastatic provide an opportunity for the host's immune response to develop and counteract infection, but *M. bovis* has defense mechanisms that include an ability to vary its surface proteins (Lysnyansky et al., 1996) and form biofilms (McAuliffe et al., 2006).

The potential effectiveness of antimicrobials *in vivo* can be assessed by *in vitro* susceptibility testing to determine the minimum inhibition concentration (MIC). Guidelines for *in vitro* testing of veterinary mycoplasma species have been published (Hannan, 2000). However, the data produced are subject to interpretation as there are currently no *in vitro* testing standards and interpretive breakpoints have not been determined. Standards for testing *Mollicutes* that cause clinical infections in humans *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* have

been published (Waites et al., 2012). However, the growth requirements of these species differ from those of *M. bovis*. In addition, the authors are not aware of specific published *M. bovis* pharmacokinetics (PK)/pharmacodynamics (PD) studies which can influence the interpretation of *in vitro* antimicrobial sensitivity.

Herein, we review *in vitro* MIC data that indicate that *M. bovis* is developing antimicrobial resistance, which is increasingly being supported by molecular evidence of genetic mutations consistent with antimicrobial resistance and by reports of treatment failure.

## ANTIMICROBIAL CLASSES ACTIVE AGAINST *M. BOVIS*

Control of *M. bovis* infection requires early identification and treatment with antimicrobials including the tetracyclines, macrolides and some fluoroquinolones. However, few are specifically approved for treating *M. bovis* in cattle and with the exception of the fluoroquinolones, which are mycoplasmacidal, and the aminoglycosides, which are mycoplasmacidal at high concentrations, the remaining antimicrobials are mycoplasmastatic, and generally work by inhibiting protein synthesis. The typical tetracyclines, chlortetracycline and doxycycline which are polycyclic structures of the perhydronaphthacene carboxamide inhibit protein synthesis in the ribosome by binding to the 30S ribosomal subunit and blocking an attachment of aminoacyl-tRNA to the A site (Bryskier, 2005a). Other plasmid related resistance, or efflux mechanisms present in some *Mycoplasma* species have not been described in *M. bovis*.

The macrolides which are known to be good for treating respiratory infections have been shown to have a preferential distribution *in vivo*, concentrating in diseased lungs, up to three times the concentration detected in healthy lungs (Reeve-Johnson, 1999). Macrolides are hydrophobic molecules that have a central 12-16-membered-ring lactone with few or no double bonds. They inhibit protein synthesis possibly by preventing peptidyltransferase from adding the growing peptide attached to tRNA to the next amino acid as well as inhibiting ribosomal translation. Another potential mechanism is premature dissociation of the peptidyl-tRNA from the ribosome. Macrolides can penetrate phagocytic cells thereby allowing treatment of intracellular infections. Lincosamides consist of an amino acid and a sugar connected by an amide bond. They act on the 50S subunit of the ribosome, preventing transpeptidation by inhibiting peptidyl-transferase.

The fluoroquinolones are synthetic antimicrobials and all have a pyridine- $\beta$ -carboxylic nucleus. Their activity depends on several factors: the aromatic system associated with the pyridine- $\beta$ -carboxylic acid nucleus, the substituents, and their spatial disposition. These substituents produce greater affinity for the target enzymes (DNA gyrase and topoisomerase IV) but also allow penetration of the bacterial outer membrane (Bryskier, 2005b). The activity of these antimicrobials can be affected by pH, magnesium and calcium ions.

The aminoglycosides are amino sugars and fall into two broad groups based on their chemical structures: streptomycin and its derivatives and deoxystreptamines. The ribosome is the prime target of their action, but other actions on membranes and modifications of RNA synthesis have been observed. They are considered mycoplasmacidal, but that is concentration dependent.

The phenicols are derived from dichloroacetic acid with an aromatic nucleus and an alkyl group and an aminopropanediol chain. The fluoro derivative of chloramphenicol, florfenicol, is commonly used in the veterinary field. It inhibits protein synthesis by binding to the 50S ribosomal subunit inhibiting the peptidation reaction and the translation of bacterial mRNA.

Pleuromutilins, such as tiamulin and valnemulin are semisynthetic derivatives that have a tricyclic diterpenoid structure with different side chains. They have been used in veterinary medicine; they bind to the peptidyl transferase component of the 50S ribosomal subunit to inhibit protein synthesis (Long et al., 2006). Tiamulin was the first pleuromutilin compound to be approved for veterinary use in 1979, followed by valnemulin in 1999 (Novak and Shlaes, 2010).

## *M. BOVIS IN VITRO SUSCEPTIBILITY PROFILES AND TRENDS*

*M. bovis* treatment failures are increasingly being associated with antimicrobial resistance. However, different research groups report variations in the proportion of *M. bovis* isolates with decreased susceptibility. This may be related to geographical origin, year of isolation, type of livestock production system, clinical presentation, or site of isolation of the strains tested. However, this may also indicate differences among the countries in regulatory practices for use of antimicrobials. Not all studies included epidemiology or typing of *M. bovis* isolates, but those which did, reported that in some countries the acquisition of resistance to antimicrobials in *M. bovis* was attributed to the emergence and spread of a single clone (Becker et al., 2015) while in other the genetic heterogeneity of *M. bovis* isolates with decreased susceptibility was identified (Lysnyansky et al., 2009).

*M. bovis* susceptibility testing has a role in epidemiological monitoring of acquired resistance and can guide the veterinarian in the selection of the most likely effective antimicrobial treatment, but published MIC data requires some interpretation, as there is no agreed standard method or controls for susceptibility testing of *M. bovis*. Although guidelines were produced by Hannan (2000), variations in the growth and approaches to determining the endpoint by different laboratories are reported and make it hard to compare results from different laboratories. However, these differences do not detract from the trends observed, which are now being supported by genetic evidence of resistance correlated with high MIC values (Lysnyansky et al., 2009; Sato et al., 2013; Lerner et al., 2014; Amram et al., 2015; Khalil et al., 2015). Nevertheless, standardized methods, controls and breakpoint interpretations would be highly beneficial for future epidemiology assessment of antimicrobial sensitivities and treatment guidance. In Table 1 we

**TABLE 1 | Minimal inhibitory concentration ranges ( $\mu\text{g/ml}$ ) and  $\text{MIC}_{50}$  and  $\text{MIC}_{90}$  ranges for various antimicrobials against *M. bovis*<sup>a</sup>.**

Antimicrobials	<b>MIC<sup>b</sup> Range</b>	<b><math>\text{MIC}_{50}</math> Range</b>	<b><math>\text{MIC}_{90}</math> Range</b>
<b>Tetracyclines</b>			
Tetracycline	<0.03–>64	1–8	4–64
Oxytetracycline	0.05–128	2–128	4–128
Chlortetracycline	0.25–256	2–128	2–256
Doxycycline	≤0.03–1	0.5	1
<b>MLSK Group</b>			
Erythromycin	4–>512	>3.2–512	32–512
Tylosin	0.2–>256	1–>128	6.25–>128
Tilmicosin	≤0.5–>512	2–512	4–>512
Gamithromycin	128–>128	128	128
Tildipirosin	128–>128	128	128
Tulathromycin	≤0.063–>128	0.25–128	8–>64
Kitasamycin	1–128	4	64
Josamycin	0.78–12.5	1.56	6.25
Spiramycin	0.39–>100	3.12	25
Lincomycin	0.06–>256	0.78–>64	3.12–>64
Clindamycin	0.03–>256	0.5–8	>32
Pirlimycin	<0.125–0.5	0.25	0.5
<b>Pleuromutilins</b>			
Tiamulin	<0.03–2	0.06–0.2	0.78–1
Valnemulin	<0.03	<0.03	<0.03
<b>Fluoroquinolones</b>			
Enrofloxacin	0.025–>32	0.12–1	0.12–32
Danofloxacin	0.078–32	0.156–0.5	0.312–8
Marbofloxacin	0.25–>32	0.625–1.25	0.625–32
Flumequine	16–128	64	128
Ciprofloxacin	0.125–1	0.5	1
<b>Aminoglycosides</b>			
Gentamycin	2–>64	8	32
Kanamycin	8–>128	32–64	64–>128
<b>Aminocyclitol</b>			
Spectinomycin	0.03–>256	1–>256	2–>256
<b>Chloramphenicols/Phenicols</b>			
Chloramphenicol	0.25–>32	2–8	2–32
Thiamphenicol	3.12–>512	6.25–>512	12.5–>512
Florfenicol	0.06–64	1–8	2–32

<sup>a</sup>The data are compiled from multiple published studies (Ayling et al., 2000; Hirose et al., 2003; Thomas et al., 2003; Rosenbusch et al., 2005; Godinho, 2008; Gerchman et al., 2009; Uemura et al., 2010; Soehnlen et al., 2011; Kroemer et al., 2012; Hendrick et al., 2013; Ayling et al., 2014; Gautier-Bouchardon et al., 2014; Kawai et al., 2014; Sulyok et al., 2014; Kong et al., 2016) in which different methodologies (microbroth and agar dilution, but not E-test) and different antimicrobial concentrations were used (adapted from Bebear and Kempf, 2005; Waites et al., 2014).

<sup>b</sup>Only MIC data obtained for *M. bovis* isolated after 1990 were included.

present the susceptibility profiles (ranges of MICs,  $\text{MIC}_{50}$ , and  $\text{MIC}_{90}$ ), obtained by microdilution and agar dilution methods, of *M. bovis* isolated in different countries since 1990. Both the  $\text{MIC}_{50}$  and  $\text{MIC}_{90}$  may indicate shifts in the susceptibility of bacterial populations to antimicrobials since  $\text{MIC}_{50}$  values show the general potency of an antimicrobial against a specific type of bacteria, while the  $\text{MIC}_{90}$  values indicates the potential

and first stages of specific bacteria developing resistance to an antimicrobial.

## Tetracyclines

*In vitro* susceptibility data from all over the world reveal heterogeneity in the susceptibility pattern to tetracyclines with  $\text{MIC}_{50}$  values for oxytetracycline ranging from 2 to <8  $\mu\text{g/ml}$  for isolates from Belgium, North America and Israel (Thomas et al., 2003; Rosenbusch et al., 2005; Gerchman et al., 2009; Soehnlen et al., 2011; Hendrick et al., 2013); while the  $\text{MIC}_{50}$  of isolates from the UK, the Netherlands, France, Hungry and Japan (Ayling et al., 2000, 2014; Hirose et al., 2003; Uemura et al., 2010; Gautier-Bouchardon et al., 2014; Kawai et al., 2014; Sulyok et al., 2014) was reported to be  $\geq 8 \mu\text{g/ml}$ . Notably, when susceptibility to tetracyclines was tested in two cohorts of respiratory *M. bovis* isolates isolated in the UK in 2004 and 2009, a significant increase in the  $\text{MIC}_{50}$  from 1 to 32  $\mu\text{g/ml}$  was identified (Ayling et al., 2014). In contrast, in France, the group of archived isolates (1978–1979) already demonstrated high  $\text{MIC}_{50}$  to oxytetracycline (32  $\mu\text{g/ml}$ ), which increased to  $\geq 32 \mu\text{g/ml}$  in recent isolates (2010–2012) (Gautier-Bouchardon et al., 2014). The authors suggested that less susceptible *M. bovis* isolates had developed by 1978–1979.

## Macrolides and Lincosamides

Acquired resistance to macrolides in *M. bovis* is a widely known phenomenon and isolates with high MICs are frequently identified (Table 1). For example,  $\text{MIC}_{50}$  values of  $\geq 32 \mu\text{g/ml}$  for tilmicosin alone or for tylosin and tilmicosin were identified in recent British and Hungarian isolates (Ayling et al., 2014; Sulyok et al., 2014) as well as in isolates from western Canada, Japan and the USA (Rosenbusch et al., 2005; Uemura et al., 2010; Hendrick et al., 2013; Kawai et al., 2014). All recent French isolates had MICs of  $\geq 64 \mu\text{g/ml}$  for tylosin and tilmicosin (Gautier-Bouchardon et al., 2014). Gerchman et al. (2009) reported marked differences in susceptibility profiles to tylosin and tilmicosin in isolates from different geographical regions. Israeli isolates were significantly more resistant to macrolides than isolates from calves imported from Hungary (2005–2007). However, recent Hungarian isolates (2010–2013) are more resistant to these antimicrobials ( $\text{MIC}_{50}$  and  $\text{MIC}_{90} \geq 128 \mu\text{g/ml}$ ) (Sulyok et al., 2014). Such data emphasize the requirement for periodic antimicrobial susceptibility testing on a regional basis.

Both archived and recent French isolates possessed high MICs for the new-generation macrolides, gamithromycin and tildipirosin. In addition, all tested French isolates and 27% of British tested isolates had MIC values of  $\geq 128 \mu\text{g/ml}$  to tulathromycin, a relatively new semi-synthetic macrolide (Ayling et al., 2014; Gautier-Bouchardon et al., 2014). Interestingly, results of a susceptibility study comparing MICs for tulathromycin in *M. bovis* respiratory isolates isolated in Europe prior to 2002 and between 2004 and 2006 showed an increase in  $\text{MIC}_{50}$  (from 0.25 to 4  $\mu\text{g/ml}$ ) without a change in the  $\text{MIC}_{90} (>64 \mu\text{g/ml})$  (Godinho, 2008). The authors suggested that a high MIC does not necessarily reflect a lack of clinical efficacy of tulathromycin since an isolate with an MIC  $>64 \mu\text{g}$

/ml was effectively treated in an experimental trial (Godinho et al., 2005; Godinho, 2008).

The MIC<sub>50</sub> for lincomycin ranged from 0.78 to  $\geq 64 \mu\text{g/ml}$  in several studies (Hirose et al., 2003; Thomas et al., 2003; Uemura et al., 2010; Ayling et al., 2014; Sulyok et al., 2014) and MIC<sub>50</sub> of  $0.19 \mu\text{g/ml}$  (but with MIC<sub>90</sub> of  $>256 \mu\text{g/ml}$ ; tested by E-test) for clindamycin were reported from Canada (Francoz et al., 2005). Interestingly, Ayling et al. (2014) reported decreased MIC<sub>50</sub> values for lincomycin (from 8 to  $1 \mu\text{g/ml}$ ) and clindamycin (from  $\geq 32$  to  $0.25 \mu\text{g/ml}$ ) in *M. bovis* isolates isolated in the UK in 2004 and 2009. Low MIC<sub>50</sub> and MIC<sub>90</sub> of 0.25 and  $0.5 \mu\text{g/ml}$ , respectively for pirlimycin (**Table 1**) were reported in one study from Japan (Kawai et al., 2014).

## Fluoroquinolones

In contrast to acquired resistance to macrolides which has been reported for a long time, the resistance to veterinary fluoroquinolones is a relatively new phenomenon. For example, only a single fold increase in MIC<sub>50</sub> and MIC<sub>90</sub> (in both cases from 0.25 to  $0.5 \mu\text{g/ml}$  for enrofloxacin and danofloxacin; and from 0.5 to  $1 \mu\text{g/ml}$  for marbofloxacin) was reported between 1978–1979 and 2010–2012 for French isolates (Gautier-Bouchardon et al., 2014). The same increase in the MIC<sub>50</sub> was also found in UK isolates isolated between 2004–2005 and during 2007–2009. However, their MIC<sub>90</sub> values differed considerably ( $0.5\text{--}1$  vs.  $8\text{--}>32 \mu\text{g/ml}$ ) (Ayling et al., 2014). Relatively low enrofloxacin MIC<sub>50</sub> values of 0.12 to  $0.5 \mu\text{g/ml}$  were reported in North America, Japan and some European countries (Hirose et al., 2003; Thomas et al., 2003; Rosenbusch et al., 2005; Uemura et al., 2010; Soehnlen et al., 2011; Hendrick et al., 2013; Gautier-Bouchardon et al., 2014; Kawai et al., 2014; Sulyok et al., 2014). This may reflect differences in therapeutic use of antimicrobials in different countries.

## Aminocyclitol and Aminoglycosides

Several studies have demonstrated relatively low MIC<sub>50</sub> and MIC<sub>90</sub> values for spectinomycin (up to  $8 \mu\text{g/ml}$ ) (Rosenbusch et al., 2005; Uemura et al., 2010; Hendrick et al., 2013), while others reported that the difference between the MIC<sub>50</sub> ( $1\text{--}8 \mu\text{g/ml}$ ) and MIC<sub>90</sub> ( $>32 \mu\text{g/ml}$ ) was high (Ayling et al., 2000, 2014; Thomas et al., 2003; Soehnlen et al., 2011; Sulyok et al., 2014). In addition high MIC<sub>50</sub> and high MIC<sub>90</sub> ( $\geq 256 \mu\text{g/ml}$ ) values for spectinomycin were also reported (Ayling et al., 2000, 2014; Thomas et al., 2003; Soehnlen et al., 2011; Sulyok et al., 2014). Interestingly, higher spectinomycin MIC values have been reported for isolates from milk. Rosenbusch et al. (2005) reported that 6 out of 14 USA milk isolates had MIC values  $>16 \mu\text{g/ml}$  while Soehnlen et al. (2011) reported a significant difference between MIC<sub>50</sub> of milk ( $>256 \mu\text{g/ml}$ ) and lung ( $8 \mu\text{g/ml}$ ) isolates. MIC values for gentamycin ranged from 2 to  $>64 \mu\text{g/ml}$  (Thomas et al., 2003; Sulyok et al., 2014) and kanamycin from 8 to  $>128 \mu\text{g/ml}$  (Uemura et al., 2010; Kawai et al., 2014).

## Chloramphenicols

The MIC<sub>50</sub> values for florfenicol ranging from 1 to  $8 \mu\text{g/ml}$  were identified in North America, Europe and China (Rosenbusch et al., 2005; Soehnlen et al., 2011; Hendrick et al., 2013; Kong et al., 2016). In addition, *M. bovis* isolates from Japan gave MIC<sub>50</sub>

values of  $6.25\text{--}8 \mu\text{g/ml}$  and  $6.25\text{--}>512 \mu\text{g/ml}$  to chloramphenicol and thiamphenicol, respectively (Hirose et al., 2003; Uemura et al., 2010). Due to the potential toxicity of phenicols and chloramphenicols (not florfenicol), their use is now illegal in food animals in many countries (Papich and Riviere, 2013).

## Pleuromutilins

The few MIC studies on tiamulin and valnemulin showed low MIC values for both antimicrobials (**Table 1**; Thomas et al., 2003; Gautier-Bouchardon et al., 2014). The efficacy of valnemulin against *M. bovis* infection was also demonstrated *in vivo* (Stipkovits et al., 2001, 2005).

## MOLECULAR MECHANISMS OF *IN VIVO* ACQUIRED RESISTANCE BY *M. BOVIS*

In general, until now, the only mechanisms of acquired *in vivo* resistance described in *Mollicutes* are target modification and ribosome protection by the *tet(M)* determinant (Bebear and Kempf, 2005).

## Tetracyclines

The genetic background for decreased tetracycline susceptibility has been elucidated in greater detail in mycoplasmas of human origin than in *M. bovis* (reviewed by Waites et al., 2014). Ribosomal protection by *tet(M)* determinants in *M. hominis* and *Ureaplasma* spp. tetracycline-resistant isolates was identified (Roberts et al., 1985, 1986) while target modification with point mutation(s) in the 16S rRNA genes was described for *in vitro* obtained mutants of *M. pneumoniae* with MICs of  $\leq 2 \mu\text{g/ml}$  (Degrange et al., 2008). Recent correlation between decreased susceptibility to tetracycline hydrochloride in *M. bovis* isolates and mutations identified in the primary binding pocket for tetracycline (Tet-1 site) located in 16S rRNA-encoding genes (*rrs3* and *rrs4* alleles) was shown (Amram et al., 2015). An increase in MICs to tetracycline (MICs  $\geq 2 \mu\text{g/ml}$ ) was correlated with the number of mutated nucleotide positions within the Tet-1 site. Indeed, decreased susceptibilities to tetracycline were associated with mutations present at two (A965 and A967; *E. coli* numbering) or three positions (A965, A967, and G1058) in the two *rrs* alleles. No *tet(M)*, *tet(O)*, or *tet(L)* determinants were identified in any of the 70 *M. bovis* isolates tested. Notably, no *M. bovis* isolates with high MICs ( $\geq 32 \mu\text{g/ml}$ ) to tetracycline were available and tested in this study.

## Macrolides and Lincosamides

Macrolide resistance in mollicutes, which have 1–3 ribosomal operons, is caused by point mutations of the macrolide-binding site located in the 23S rRNA genes or in the genes encoding ribosomal proteins L4 and L22 (reviewed by Waites et al., 2014). Recently, *M. bovis* decreased susceptibility to tylosin and tilmicosin was attributed to any one of the point mutations G748A, C752T, A2058G, A2059G, or A2059C (*E. coli* numbering) identified in one or both alleles of the 23S rRNAs (Lerner et al., 2014). The data revealed that a combination of mutations in two domains (II and V of 23S rRNA) was necessary to achieve higher MICs ( $\geq 128 \mu\text{g/ml}$ ) to tylosin, while a single

mutation in one of the domains could be sufficient to cause a decrease in susceptibility to this antimicrobial. In that study, amino acid (aa) substitution L22-Q90H (*E. coli* numbering) was also identified in 24 of 32 representative *M. bovis* isolates with different MICs, but no clear correlation between this mutation and decreased MICs to tylosin and tilmicosin was found. In addition, multiple aa substitutions were identified in the L4 protein, including at positions 185–186 (positions 64 and 65 in *E. coli*), which are adjacent to the macrolide-binding site (Lerner et al., 2014). However, the actual impact of L4 aa substitutions is hard to define since they were identified in isolates with various MICs, some of which also possessed nt substitution/s within the macrolide binding site of 23S rRNA gene/s. Clarification of the role of mutations at positions 90-L22 and 64/or 64-65-L4 in decreased macrolide susceptibility by *M. bovis* is required.

## Fluoroquinolones

The only mechanism of acquired fluoroquinolone resistance described in *Mollicutes* is alterations within the quinolone resistance-determining regions (QRDRs) of DNA gyrase subunits GyrA and GyrB and/or topoisomerase IV subunits ParC and ParE. However, an active efflux mechanism has also been demonstrated *in vitro* (Raherison et al., 2002). Amino acid substitution in the QRDR of GyrA (Ser83Phe) alone or with concurrent mutation Asn84Asp in ParC was found in *M. bovis* isolates with MICs to enrofloxacin ranging from 0.32–1.25 to 2.5–5 µg/ml, respectively (Lysnyansky et al., 2009). In another study, mutation Ser83Leu in GyrA was identified in 32 isolates with MICs of 0.25–2 µg/ml to enrofloxacin, orbifloxacin and danofloxacin; mutations in Ser83Leu of GyrA and Ser81Pro of ParC were present in three isolates (4–16 µg/ml) while mutations Ser83Phe in GyrA and Ser80Ile in ParC were found in four isolates (8–16 µg/ml) (Sato et al., 2013). The mutations Ser83Phe in GyrA and Ser80Ile in ParC were also identified in a Chinese *M. bovis* isolate with MICs ≥16 µg/ml to ciprofloxacin, levofloxacin, lomefloxacin and norfloxacin (Mustafa et al., 2013). In addition, Sato et al. (2013) showed that laboratory-derived fluoroquinolone-resistant mutants selected from two isolates with Ser83Leu in GyrA possessed an aa substitution Ser80Ile or Ser81Tyr in ParC. Results of both studies (Lysnyansky et al., 2009; Sato et al., 2013) suggest a cumulative effect of the mutations in GyrA and ParC on fluoroquinolone resistance. Previously, *in vivo* acquired resistance by veterinary mycoplasmas to the fluoroquinolones has been attributed to aa changes at positions 80 or 84 in the ParC as well as positions 83 in the GyrA (Hirose et al., 2004; Le Carrou et al., 2006; Vicca et al., 2007).

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## Aminoglycosides

No enzymes responsible for chemical modification of aminoglycosides have been identified in the *M. bovis* genome. However, decreased susceptibility to streptomycin in *M. bovis* was associated with nt T at position 912 (*E. coli* numbering) in the 16S rRNA genes (Konigsson et al., 2002), a position converting to resistance in *E. coli* (Frattali et al., 1990).

In an aminocyclitol antimicrobials, spectinomycin, isolates with high MIC (>512 µg/ml) were found to exhibit a C to A transition at position 1192 (*E. coli* numbering) either in *rrs3* or in both *rrs* alleles (C. Schnee et al., abstract, IOM 2014, p. 60) as has been previously shown for spectinomycin-resistant *E. coli* (Sigmund et al., 1984).

## Chloramphenicols and Pleuromutilins

To the best of our knowledge, no investigations of the phenicols /chloramphenicols and pleuromutilins resistance mechanisms for *M. bovis* have been published.

## SUMMARY

Control of *M. bovis* infections in cattle is inherently difficult. The resulting clinical signs and disease are increasingly recognized as having a significant adverse impact on animal welfare and the economy of cattle farming around the world. Other than sanitary preventative measures, treatment with antimicrobials is the only approach for disease treatment. However, as emphasized herein, the number of potentially effective antimicrobials is limited as *M. bovis* lacks a cell wall. Among the few antimicrobials licensed for treatment of *M. bovis*, there is increasing evidence for resistance. Based on MIC levels and genetic analysis, antimicrobial resistance by *M. bovis* to the tetracyclines, macrolides, lincosamides, aminoglycosides, chloramphenicols, and fluoroquinolones has been reported and appears to be increasing. The mechanisms of *M. bovis* antimicrobial resistance have largely been based on genetic point mutations; few studies have examined efflux mechanisms and no plasmids have so far been detected in *M. bovis*.

This review highlights the necessity to agree a standardized method and controls for animal mycoplasma antimicrobial susceptibility testing. In addition breakpoints should be determined and the use of molecular resistance markers could be used to define them.

## AUTHOR CONTRIBUTIONS

IL, RA designed the study and analyzed and interpreted data. IL, RA drafted the manuscript. All authors read and approved the final version of the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Prevalence of extended-spectrum cephalosporin-resistant *Escherichia coli* in a farrowing farm: ST1121 clone harboring IncHI2 plasmid contributes to the dissemination of *bla*<sub>CMY-2</sub>

## OPEN ACCESS

### Edited by:

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equally to this work.

### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 06 August 2015

**Accepted:** 19 October 2015

**Published:** 03 November 2015

### Citation:

Deng H, Si H-B, Zeng S-Y, Sun J, Fang L-X, Yang R-S, Liu Y-H and Liao X-P (2015) Prevalence of extended-spectrum cephalosporin-resistant *Escherichia coli* in a farrowing farm: ST1121 clone harboring IncHI2 plasmid contributes to the dissemination of *bla*<sub>CMY-2</sub>. *Front. Microbiol.* 6:1210.  
doi: 10.3389/fmicb.2015.01210

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During a regular monitoring of antimicrobial resistance in a farrowing farm in Southern China, 117 *Escherichia coli* isolates were obtained from sows and piglets. Compared with the isolates from piglets, the isolates from sows exhibited higher resistance rates to the tested cephalosporins. Correspondingly, the total detection rate of the *bla*<sub>CMY-2</sub>/*bla*<sub>CTX-M</sub> genes in the sow isolates (34.2%) was also significantly higher than that of the piglet isolates (13.6%; *p* < 0.05). The *bla*<sub>CMY-2</sub> gene had a relatively high prevalence (11.1%) in the *E. coli* isolates. MLST and PFGE analysis revealed the clonal spread of ST1121 *E. coli* in most (7/13) of the *bla*<sub>CMY-2</sub>-positive isolates. An indistinguishable IncHI2 plasmid harboring *bla*<sub>CMY-2</sub> was also identified in each of the seven ST1121 *E. coli* isolates. Complete sequence analysis of this IncHI2 plasmid (pEC5207) revealed that pEC5207 may have originated through recombination of an IncHI2 plasmid with a *bla*<sub>CMY-2</sub>-carrying IncA/C plasmid like pCFSAN007427\_01. In addition to *bla*<sub>CMY-2</sub>, pEC5207 also carried other resistance determinants for aminoglycosides (*aacA7*), sulfonamides (*sul1*), as well as heavy metals ions, such as Cu and Ag. The susceptibility testing showed that the pEC5207 can mediate both antibiotic and heavy metal resistance. This highlights the role of pEC5207 in co-selection of *bla*<sub>CMY-2</sub>-positive isolates under the selective pressure of heavy metals, cephalosporins, and other antimicrobials. In conclusion, clonal spread of an ST1121 type *E. coli* strain harboring an IncHI2 plasmid contributed to the dissemination of *bla*<sub>CMY-2</sub> in a farrowing farm in Southern China. We also have determined the first complete sequence analysis of a *bla*<sub>CMY-2</sub>-carrying IncHI2 plasmid.

**Keywords:** *Escherichia coli*, clonal spread, *bla*<sub>CMY-2</sub>, IncHI2 plasmid, farrowing farm

## INTRODUCTION

Antimicrobial agents are often used as feed and water additives in food animals to treat or prevent disease and to promote general overall health (McEwen and Fedorka-Cray, 2002; Cabello et al., 2013). However, antimicrobial resistance driven by the intensive use of antimicrobial agents in animal husbandry is increasing worldwide (Witte, 1998). In the swine industry, antimicrobial resistance patterns can be traced to particular farms associated with certain management practices (Rasschaert et al., 2012; Brooks et al., 2014). In farrowing farms, antimicrobial resistance in piglets has been shown to be a reflection of antimicrobial use in sows (Mathew et al., 2005; Callens et al., 2015), because sows are an important reservoir of antimicrobial resistant bacteria for their offspring (Callens et al., 2012; Crombe et al., 2013). Additionally, piglet transfer from farrowing to finishing farms increases the likelihood of the transmission of resistant bacteria, thus increasing the risk of antimicrobial resistance transfer between swine farms (Sandvang et al., 2000; van Duijkeren et al., 2008). Therefore, surveillance for antimicrobial resistance in the farrowing farm is important for controlling the dissemination of antimicrobial resistance.

*Escherichia coli* is an important cause of intestinal and extraintestinal diseases in animals and humans worldwide, and  $\beta$ -lactams are widely used in veterinary medicine to treat colibacillosis. However, the use of extended-spectrum cephalosporins (ESCs) in animals has contributed to  $\beta$ -lactam resistance in *E. coli* (Greko et al., 2009). Resistance to ESCs in *E. coli* has been associated with the extended-spectrum  $\beta$ -lactamases (ESBLs) and plasmid-mediated Ambler class C cephamycinases (pAmpC  $\beta$ -lactamases; Seiffert et al., 2013). ESBLs are the major contributors to ESC resistance in *E. coli* and confer resistance to cephalosporins with an oxyimino side chain (cefotaxime, ceftriaxone, and ceftazidime; Bradford, 2001).

Unlike ESBLs, pAmpC  $\beta$ -lactamases exhibit activity against cephamycins, such as cefoxitin and cannot be inactivated by  $\beta$ -lactamase inhibitor clavulanate (Bradford, 2001; Jacoby, 2009). Among them, CMY-2, the most common pAmpC  $\beta$ -lactamase, has been documented worldwide in bacteria of human and animal origin (Li et al., 2007; Jacoby, 2009). The *bla*<sub>CMY-2</sub> gene which likely originated from the chromosomal AmpC locus of *Citrobacter freundii* has been horizontally transmitted through plasmids in *E. coli* from different sources (Martin et al., 2012). Various plasmid types are associated with *bla*<sub>CMY-2</sub>, including IncA/C, IncF, IncI1, IncL/M, IncP, IncK, and IncHI2. Of these, IncA/C and IncI1 plasmid are the most common carriers of *bla*<sub>CMY-2</sub> (Verdet et al., 2009; Folster et al., 2011; Martin et al., 2012; Bortolaia et al., 2014; Guo et al., 2014).

The *bla*<sub>CMY-2</sub> gene is prevalent in ESC-resistant *E. coli* of livestock (Li et al., 2007; Seiffert et al., 2013). In swine, the detection rate of *bla*<sub>CMY-2</sub> in *E. coli* is quite different, ranging from 0 to up to 80% (Seiffert et al., 2013). Although the detection methods of CMY-2 vary between reports, this may still reflect the different prevalence of *bla*<sub>CMY-2</sub> worldwide. In mainland China, the *bla*<sub>CMY-2</sub> gene was detected for the first time in *E. coli* of swine origin in Tian et al. (2012), Zheng et al. (2012), and subsequently occurred in swine *E. coli* isolates carrying

plasmid-mediated quinolone resistance (PMQR) genes (Liu et al., 2013a). Recently, a surveillance study identified *bla*<sub>CMY-2</sub> mainly in *E. coli* of pig origin, highlighting the role of CMY-2 in the ESC resistance of swine *E. coli* (Guo et al., 2014). In the present study, we make an investigation on the prevalence of drug resistance and ESBL/pAmpC genes in *E. coli* isolates from a swine farm in Southern China. The *bla*<sub>CMY-2</sub>-positive isolates were further analyzed to characterize the transmission mechanisms of the *bla*<sub>CMY-2</sub> gene.

## MATERIALS AND METHODS

### Bacterial Isolates and Antimicrobial Susceptibility Testing

In August 2011, a regular monitoring of antimicrobial resistance was conducted in a farrowing farm in Southern China. This farm had been in operation about 8 years and consisted of 2,300 sows with production of about 40,000 piglets for market annually. Rectal swab samples were randomly taken from one pig in every batch pen. The rectal swabs were taken by inserting the sterile swab about 2 cm into the rectum, rotated gently and then immersed in sterile PBS. After collection, the swabs were immediately brought to the laboratory in cool conditions. A total of 137 swab samples were collected from sows (1–5 years-old) and piglets (1–60 days-old). The collected samples were plated on MacConkey agar and then incubated at 37°C for 24 h. One suspected colony with typical *E. coli* morphology was selected from each sample and was identified with API 20E system (BioMerieux, France). Minimal inhibitory concentrations (MICs) of ampicillin (AMP), ceftazidime (CAZ), cefoxitin (FOX), cefotaxime (CTX), ceftiofur (CEF), amikacin (AMK), kanamycin (KAN), florfenicol (FFC), doxycycline (DOX), enrofloxacin (ENR), and trimethoprim-sulfamethoxazole (SXT) were determined by the agar dilution method in accordance with the standard provided by the Clinical and Laboratory Standards Institute (2013a,b). *E. coli* ATCC 25922 was used as the quality control strain.

### Detection of ESBL/pAmpC Genes

Extended-spectrum  $\beta$ -lactamase genes (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-1G</sub>, *bla*<sub>CTX-M-9G</sub>, *bla*<sub>CTX-M-2G</sub>, and *bla*<sub>CTX-M-25G</sub>) among the *E. coli* isolates were analyzed by PCR amplification using previously published primers and protocols (Liu et al., 2013a). Purified PCR products were sequenced and compared using the  $\beta$ -lactamase classification system<sup>1</sup> to confirm the subtypes. Detection of pAmpC genes was performed by a multiplex PCR as previously described (Perez-Perez and Hanson, 2002). For amplification of the entire *bla*<sub>CMY-2</sub> gene, PCR-positive isolates were re-amplified and sequenced with specific primers (Perez-Perez and Hanson, 2002).

### Molecular Typing

All *bla*<sub>CMY-2</sub>-positive isolates were classified according to *Xba*I-pulsed-field gel electrophoresis (PFGE) type (Tenover et al.,

<sup>1</sup><http://www.lahey.org/studies/webt.asp>

1995). Comparison of PFGE patterns was performed by BioNumerics® v6.6 (Applied Maths, Ghent, Belgium) with a cut-off at 90% of the similarity values to indicate identical PFGE types. Multilocus sequence typing (MLST) was performed by using the primers and protocol specified at the *E. coli* MLST web site<sup>2</sup>.

### Transferability of *bla*<sub>CMY-2</sub>

Conjugation experiments were performed as previously described (Chen et al., 2007a), using *E. coli* C600 (streptomycin-resistant; MIC >2000 µg/ml) as a recipient. Putative transconjugants were selected on MacConkey agar plates with streptomycin (2000 µg/ml) and cefoxitin (32 µg/ml), examined for the presence of *bla*<sub>CMY-2</sub> by PCR assay, and finally confirmed by ERIC-PCR (Versalovic et al., 1991). All transconjugants were tested for antimicrobial susceptibility as described above.

The susceptibility of transconjugant EC5207-35T to heavy metals (Cu, Ag) was tested by microdilution in an aerobic atmosphere, as previously described, with some modifications (Mourao et al., 2015). Briefly, the transconjugant was incubated in Mueller-Hinton broth with serial dilutions of CuSO<sub>4</sub> (0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 32, and 36 mM, adjusted to pH 7.2) and AgNO<sub>3</sub> (0.0125, 0.025, 0.06, 0.08, 0.125, 0.16, 0.25, 0.32, 0.5, 1.0, 1.5, and 3 mM, adjusted to pH 7.4). *E. coli* C600 was used as the reference strain.

### Plasmid Analysis

Plasmids of the transconjugants were typed with PCR-based replicon typing (PBRT; Carattoli et al., 2005). The size of *bla*<sub>CMY-2</sub>-carrying plasmid in the transconjugants was determined using S1 nuclease-digested (TaKaRa Biotechnology, Dalian, China) genomic DNA followed by PFGE and Southern blot hybridization with a *bla*<sub>CMY-2</sub>-specific probe (Barton et al., 1995). Plasmid DNA from the transconjugants was extracted using the QIAGEN Plasmid Midi Kit, and was further analyzed by restriction fragment length polymorphism (RFLP) using *Xba*I (TaKaRa Biotechnology, Dalian, China).

### Complete Sequence of *bla*<sub>CMY-2</sub>-carrying IncHI2 Plasmid pEC5207

In order to further characterize the *bla*<sub>CMY-2</sub>-carrying IncHI2 plasmids of the ST1121 clone in this study, the plasmid pEC5207 from transconjugant EC5207-35T was sequenced using SMRT sequencing approach and assembled by HGAP2.2.0 method (Chin et al., 2013). Open reading frames (ORFs) prediction and annotation were performed with the RAST tools (Aziz et al., 2008). Sequence comparison and map generation were performed using BLAST<sup>3</sup> and Easyfig version 2.1 (Sullivan et al., 2011).

### Statistical Methods

Statistical significance for the comparison of prevalence data was determined by the  $\chi^2$  test. Differences were considered statistically significant at  $p < 0.05$ .

### Nucleotide Sequence Accession Number

The complete DNA sequence of plasmid pEC5207 was assigned GenBank accession number KT347600.

## RESULTS

### Antimicrobial Susceptibility

Rectal swab samples were randomly taken from one pig in every batch pen and 117 *E. coli* samples were isolated from 137 sow and piglet rectal swabs. The incidence for each group was approximately 85% (Table 1). Antimicrobial susceptibility tests showed that part of the isolates were resistant to CEF (30.8%), CTX (27.4%), FOX (14.5%), and CAZ (11.1%). Except for CAZ, cephalosporin resistance rates for sow isolates were significantly higher than those for isolates from piglets ( $p < 0.05$ ). However, the resistance rate to CAZ for isolates from sows was still three times of that for piglet isolates (Table 2). In addition to cephalosporins, different levels of resistance to other classes of antimicrobials, including SXT (100%), DOX (98.3%), FFC (96.6%), AMP (94.0%), KAN (83.8%), ENR (64.1%) and AMK (29.9%), were also observed in the tested isolates (Table 2). The vast majority of the isolates (95.7%) exhibited a multidrug resistance (MDR) phenotype, and were resistant to four or more tested antimicrobial agents.

### Detection of ESBL/pAmpC Genes

Among the 117 *E. coli* isolates, CTX-M-type ESBL genes were detected in 18 isolates (15.4%). The most common *bla*<sub>CTX-M</sub>-type were *bla*<sub>CTX-M-122</sub> ( $n = 8$ ), followed by *bla*<sub>CTX-M-65</sub> ( $n = 5$ ), *bla*<sub>CTX-M-55</sub> ( $n = 3$ ) and *bla*<sub>CTX-M-64</sub> ( $n = 2$ ). TEM- and SHV-type ESBL genes were not detected in any of the isolates. Compared with the diverse *bla*<sub>CTX-M</sub> genes detected in this study, *bla*<sub>CMY-2</sub> was the only identified pAmpC gene, and was found in 13 isolates (11.1%). All the *bla*<sub>CMY-2</sub>-positive isolates were resistant to FOX with MICs ranging from 64 to 256 µg/ml, and also exhibited multi-resistance to AMP, CTX, CEF, KAN, FFC, DOX, ENR, and SXT (Figure 1).

The co-existence of ESBL and pAmpC genes was not observed in any of the isolates. The total detection rate of

**TABLE 1 |** Information on the samples and *Escherichia coli* isolates in this study.

Sample source	No. of samples	No. (%) of <i>E. coli</i> isolates
Sow	84	73 (86.9)
Piglet	53	44 (83.0)
Total	137	117 (85.4)

<sup>2</sup><http://mlst.ucc.ie/mlst/dbs/Ecoli>

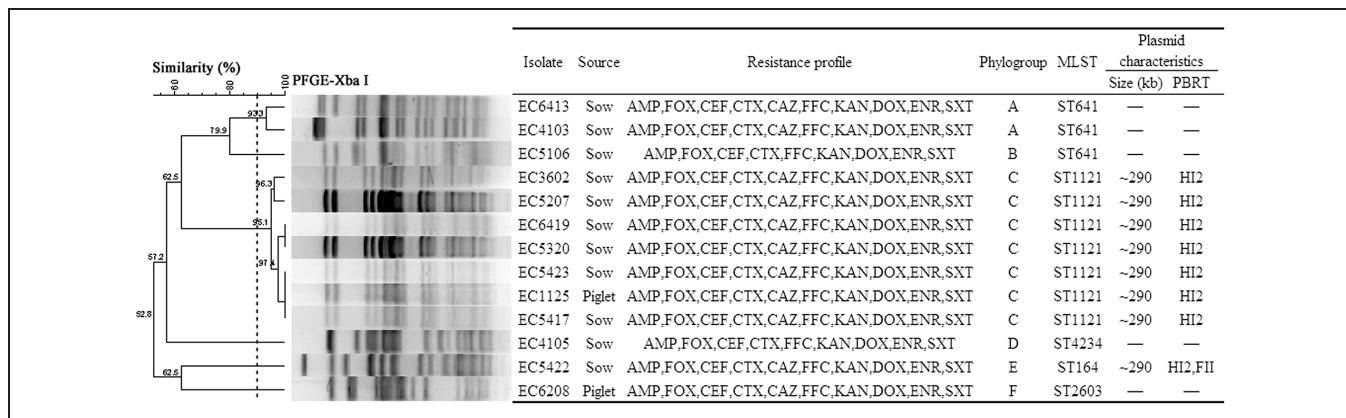
<sup>3</sup><http://blast.ncbi.nlm.nih.gov>

**TABLE 2 | Susceptibility of 117 *E. coli* isolates to 11 antimicrobial agents.**

Antimicrobial agent <sup>a</sup>	MIC range ( $\mu\text{g/ml}$ )	MIC <sub>50</sub> ( $\mu\text{g/ml}$ )	MIC <sub>90</sub> ( $\mu\text{g/ml}$ )	Resistant (%)		
				Sow ( <i>n</i> = 73)	Piglet ( <i>n</i> = 44)	Total ( <i>n</i> = 117)
CAZ	0.06–64	0.25	16	15.1	4.5	11.1
FOX	2–256	4	64	20.5	4.5*	14.5
CEF	<0.125–>256	1	128	38.4	18.2*	30.8
CTX	0.03–>256	0.125	32	35.6	13.6*	27.4
AMP	4–>512	>512	>512	94.5	93.2	94.0
AMK	1–>256	2	>256	17.8	50.0*	29.9
KAN	1–>512	>512	>512	78.1	93.2	83.8
FFC	4–512	256	512	95.9	97.7	96.6
DOX	4–256	64	128	97.3	100	98.3
ENR	0.016–128	4	32	60.3	70.5	64.1
SXT	2/38–>16/304	>16/304	>16/304	100	100	100

<sup>a</sup>CAZ, ceftazidime; FOX, cefoxitin; CEF, ceftiofur; CTX, cefotaxime; AMP, ampicillin; AMK, amikacin; KAN, kanamycin; FFC, florfenicol; DOX, doxycycline; ENR, enrofloxacin; SXT, trimethoprim-sulfamethoxazole.

\**p* < 0.05 for resistance rate of isolates from piglets vs. sows.



**FIGURE 1 | Clonal relationship and plasmid characteristics of the 13 blaCMY-2-positive *Escherichia coli* isolates. - , not detected. AMP, CAZ, FOX, CEF, CTX, KAN, FFC, DOX, ENR, and SXT are represented as in Table 2.**

the bla<sub>CMY-2</sub>/bla<sub>CTX-M</sub> genes in the sow isolates (34.2%) was significantly higher than that in the piglet isolates (13.6%; *p* < 0.05). The distribution of ESBL/pAmpC genes among the isolates are listed in Table 3.

**TABLE 3 | Distribution of ESBL/pAmpC genes among *E. coli* isolates.**

Gene type	No. isolates (%)		Total ( <i>n</i> = 117)
	Sow ( <i>n</i> = 73)	Piglet ( <i>n</i> = 44)	
<b>ESBL</b>	14 (19.2)	4 (9.1)	18 (15.4)
bla <sub>CTX-M-55</sub>	3 (4.1)		3 (2.6)
bla <sub>CTX-M-64</sub>	1 (1.4)	1 (2.3)	2 (1.7)
bla <sub>CTX-M-65</sub>	4 (5.5)	1 (2.3)	5 (4.3)
bla <sub>CTX-M-122</sub>	6 (8.2)	2 (4.5)	8 (6.8)
<b>pAmpC</b>			
bla <sub>CMY-2</sub>	11 (15.1)	2 (4.5)	13 (11.1)
<b>Total</b>	25 (34.2)	6 (13.6)*	31 (26.5)

\**p* < 0.05 for gene detection rate of isolates from piglets vs. sows.

## Molecular Typing

We used cluster analysis of the bla<sub>CMY-2</sub>-positive isolates to generate dendograms from PFGE profiles (Figure 1). Six phylogenetic groups (designated A–F) each with more than 90% similarity were represented in these 13 isolates. Group C contained seven isolates, Group A two, and the other four groups one each.

Multilocus sequence typing analysis of the 13 bla<sub>CMY-2</sub>-positive isolates identified five different STs including a novel one (ST4234). The most prevalent STs were ST1121 (*n* = 7) and ST641 (*n* = 3). The remaining isolates were each of a single ST type (Figure 1). Interestingly, the seven ST1121 isolates from six sows and one piglet were all contained in Group C. The three ST641 isolates from sows were divided between two groups (Figure 1).

## Conjugation Assays and Plasmid Analysis

Eight bla<sub>CMY-2</sub>-positive transconjugants were successfully obtained from the seven ST1121 and one ST164 *E. coli* isolates. S1 nuclease-PFGE analysis identified a single plasmid in each of

the seven transconjugants from ST1121 isolates. Two plasmids were identified in the transconjugant EC5422-25T derived from the ST164 isolate EC5422 (Supplementary Figure S1). Subsequently, Southern blot hybridization identified the *bla<sub>CMY-2</sub>* gene located on a ~290 kb plasmid in each of the transconjugants (Supplementary Figure S1). Replicon typing revealed the presence of the IncHI2 replicon in each of the eight transconjugants, but one of them (EC5422-25T) carried two replicons (IncHI2 and IncFII; **Figure 1**).

The IncHI2 plasmids of the ST1121 isolates shared indistinguishable RFLP profiles that were generated using *XbaI* digestion (data not shown). All of the transconjugants were resistant to FOX, CEF,CTX, AMP, KAN, and AMK. In addition, transfer of resistance to FFC, DOX, and SXT was also observed in the transconjugant EC5422-25T.

The metal susceptibility testing showed that the MICs of CuSO<sub>4</sub> and AgNO<sub>3</sub> for transconjugant EC5207-35T were higher than that of *E. coli* C600 (MIC<sub>CuSO<sub>4</sub></sub> = 12 vs. 8 mM; MIC<sub>AgNO<sub>3</sub></sub> = 1 vs. 0.0125 mM).

### Complete Sequence of Plasmid pEC5207

We determined the DNA sequence of the plasmid derived from the *bla<sub>CMY-2</sub>*-positive transconjugants that were obtained from the seven ST1121 isolates. This plasmid pEC5207 is 272,865 bp in length with a GC content of 46.16% and harbors 253 predicted ORFs. The plasmid backbone is organized similarly to that of pSH111\_227 (GenBank JN983042) from *Salmonella* sp. and encodes typical IncHI2 plasmid replication, partition, maintenance and transfer functions (**Figure 2**). The replication region of pEC5207 included a *repHI2* gene. The *parA* and *parB* genes were involved in the plasmid partition. Two *tra* transfer regions and a *hipAB* toxin-antitoxin gene cluster were associated with the transfer and maintenance function of pEC5207. Interestingly, pEC5207 also contained a large number of genes encoding resistance to heavy metals including tellurium (*terABCDEFWXYZ*), silver (*silABCEPRS*) and copper (*copABCE*; **Figure 2**).

The *bla<sub>CMY-2</sub>* gene in pEC5207 is contained within a 48,888 bp variable region (**Figure 2**). The variable portion is located in a region between *umuD* and *dcm* in pEC5207, where is the *hp161* locus in pSH111\_227. The variable region showed high homology (>99%) with two parts of the *bla<sub>CMY-2</sub>*-carrying IncA/C plasmid pCFSAN007427\_01 (GenBank CP009413) from *Salmonella* and contained an IS26 and a truncated *TnAs1* at the ends. In addition to *bla<sub>CMY-2</sub>*, a new class I integron harboring aminoglycoside resistance gene *aacA7* and sulfonamide resistance gene *sull* was also found in this variable region.

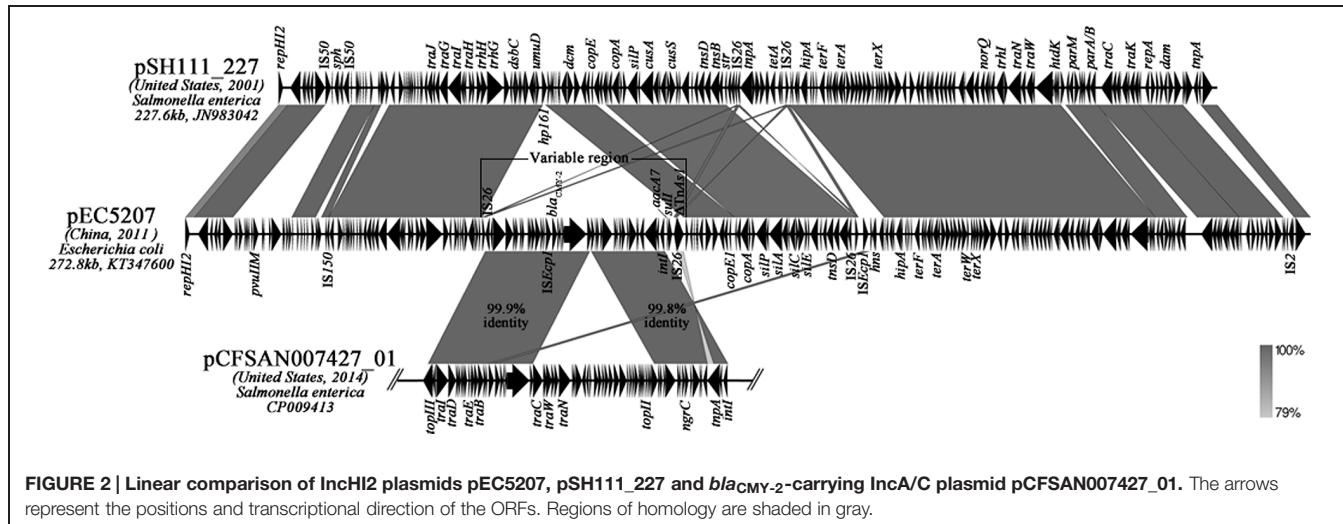
## DISCUSSION

Antimicrobial susceptibility testing showed different resistance levels to cephalosporins and other classes of antimicrobials in *E. coli* isolates from a farrowing farm in Southern China. The antimicrobial usage records for this farm showed that doxycycline and the first generation cephalosporin cefradine were frequently added to drinking water or compound feeds

as prophylactics. Other antimicrobials such as ceftiofur, enrofloxacin, florfenicol, and sulfamethoxazole were also commonly used for treatment during production. This background may be favorable for developing resistance to cephalosporins and other antimicrobials in this farm. The ESC-resistant *E. coli* usually carry additional genes conferring resistance to other veterinary antimicrobial agents like quinolone, aminoglycoside and florfenicol. In our previous study, the ESBL and pAmpC genes were detected in 67.5% of the PMQR-positive *E. coli*, indicating a strong association between ESC and quinolone resistance (Liu et al., 2013a). The 16S rRNA methylase genes, especially *rmtB*, and the florfenicol resistance gene *floR* are also commonly identified in ESBLs and pAmpC-producing *E. coli* isolates (Kang et al., 2009; Wu et al., 2009; Yu et al., 2010; Deng et al., 2011; Liu et al., 2013b; Guo et al., 2014). The use of different antimicrobial agents may increase the potential risk for selection of multidrug resistant isolates, and contribute to the MDR phenotypes of *E. coli* isolates in this farm.

Compared with the isolates from piglets, the isolates from sows showed significantly higher resistance rates to FOX, CEF and CTX, as well as a relatively higher resistance rate to CAZ. This coincided with the higher occurrence of the *bla<sub>CMY-2</sub>* and *bla<sub>CTX-M</sub>* genes in the isolates from sows than in the isolates from piglets. Previous studies have shown that cephalosporin treatment can result in the selection of ESBL and AmpC producing *E. coli* in animals and aggravate the problem of cephalosporin resistance (Tragesser et al., 2006; Cavaco et al., 2008; Kanwar et al., 2013; Barton, 2014). The longer antibiotic exposure times of the sows may promote the persistence of ESBL/AmpC-producing *E. coli* in the gastrointestinal tract, thus resulting in the serious cephalosporin resistance and relatively high prevalence of ESBL/AmpC genes observed in sow isolates.

In this study the prevalence of the *bla<sub>CMY-2</sub>* gene (11.1%) was lower than that of the *bla<sub>CTX-M</sub>* genes (15.4%). However, this level was much higher than those in our previous study (2.9%; Fang et al., 2015) and others' (1.0–3.0%) in China (Liu et al., 2007; Guo et al., 2014; Rao et al., 2014). Previous studies have shown that cefoxitin-ceftiofur-resistant isolates from *E. coli* and *Salmonella* had a high degree of association with the production of CMY enzymes (Barton, 2014). Therefore, the use of ceftiofur in this farm probably contributed to the selection of *bla<sub>CMY-2</sub>*-producing *E. coli* isolates. MLST typing revealed a major ST type, ST1121, in seven of the thirteen *bla<sub>CMY-2</sub>*-positive isolates. Since they shared a high similarity (>95%) in PFGE profiles, this suggested a clonal spread of these ST1121 isolates. Furthermore, plasmid analysis of the transconjugants from the seven ST1121 isolates revealed an indistinguishable IncHI2 plasmid harboring *bla<sub>CMY-2</sub>* in these isolates. Thus, the clonal spread of ST1121 isolates harboring IncHI2 plasmid may play an important role in the dissemination of *bla<sub>CMY-2</sub>* in this farm. Notably, among the 13 *bla<sub>CMY-2</sub>*-positive isolates, one of the ST1121 isolates and the ST2603 isolate were isolated from piglets for market, which would indicate that the *bla<sub>CMY-2</sub>*-positive isolates may have been introduced into other swine farms by piglet trading and therefore would accelerate the spread of *bla<sub>CMY-2</sub>*. Further surveillance is necessary to determine



the prevalence of *bla*<sub>CMY-2</sub>-positive isolates in these swine farms.

Since its first identification on plasmid from *Klebsiella pneumoniae* (Bauernfeind et al., 1990), the *bla*<sub>CMY-2</sub> gene has been associated with various plasmid types. In our previous surveillance study, the *bla*<sub>CMY-2</sub> gene was identified on IncA/C, IncHI2, and IncX plasmids (Fang et al., 2015). In the present study, *bla*<sub>CMY-2</sub>-carrying IncHI2 plasmids were not only detected in the clonal ST1121 isolates but also in a ST164 isolate. Additionally, the strong association of *bla*<sub>CMY-2</sub> with IncHI2 plasmid was also observed in *Salmonella* (Shi, 2015). This highlighted the role of the IncHI2 plasmid in the transfer of *bla*<sub>CMY-2</sub>. Besides *bla*<sub>CMY-2</sub>, IncHI2 plasmid is also associated with another pAmpC gene *bla*<sub>CMY-8</sub> (Chen et al., 2007b). In addition, IncHI2 plasmids have been implicated in the spread of *bla*<sub>CTX-M</sub> genes and are also frequently linked with the other antimicrobial resistance genes such as *bla*<sub>SHV</sub>, *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *arma*, *qnrA1*, *qnrS1*, and *qnrB2* (Garcia Fernandez et al., 2007; Veldman et al., 2010; Coelho et al., 2012). Together, our data indicate that the IncHI2 plasmid played a significant role in the dissemination of antimicrobial resistance. However, to the best of our knowledge, there was no complete sequence analysis of a *bla*<sub>CMY-2</sub>-carrying IncHI2 plasmid to date. Therefore, in order to further characterize the *bla*<sub>CMY-2</sub>-carrying IncHI2 plasmids of ST1121 isolates, plasmid pEC5207 was sequenced in our study.

This plasmid possesses a typical IncHI2 plasmid backbone organized similarly to that of pSH111\_227. A portion of *hp161* in pSH111\_227 was replaced with a *bla*<sub>CMY-2</sub>-harboring variable region in pEC5207. A sequence comparison revealed that this variable region might originate from IncA/C plasmid pCFSAN007427\_01, and is flanked with IS26 and a truncated TnAs1. Considering the potential for genetic rearrangements by insertion sequence and transposon (Hallet and Sherratt, 1997; Bennett, 2004), we speculate that IS26 and TnAs1 transposition into the *hp161* locus of a pSH111\_227-like IncHI2 plasmid was followed by recombination with a pCFSAN007427\_01-like *bla*<sub>CMY-2</sub>-carrying IncA/C plasmid. This could set the stage for the integration of *bla*<sub>CMY-2</sub> into the IncHI2 plasmid.

Apart from the *bla*<sub>CMY-2</sub> gene, pEC5207 also carried other resistance determinants for aminoglycosides (*aacA7*), sulfonamides (*sul1*), as well as heavy metals ions, such as Cu and Ag. And the result of susceptibility tests also confirm the functionality of pEC5207 in the transfer of copper and silver resistance. Copper is often present as trace element feed additives for animal production in China (Wang et al., 2013). Silver is used as disinfectants in water or surfaces in the animal production setting (Mourao et al., 2015). Under the selective pressure of heavy metals, cephalosporins, and other antimicrobials, plasmid pEC5207 could play a critical role in the persistence of host bacteria in the intestine of pigs, thereby increasing the risk for co-selection of isolates carrying *bla*<sub>CMY-2</sub> gene. This may further explain the high prevalence of *bla*<sub>CMY-2</sub> in this farm.

## CONCLUSION

The present study revealed a relatively high prevalence of *bla*<sub>CMY-2</sub> in a farrowing farm in Southern China. The clonal spread of ST1121 type *E. coli* harboring an IncHI2 plasmid mediated the dissemination of *bla*<sub>CMY-2</sub> in this farm. Nucleotide sequence analysis and comparisons indicated that the *bla*<sub>CMY-2</sub>-carrying IncHI2 plasmid pEC5207 may have been generated by recombination with an IncA/C plasmid. pEC5207 may play an important role in the persistence of host bacteria under the selective pressure of heavy metal and antimicrobials. Although limited to a farrowing farm, our study indicates that there is a serious risk of dissemination of the *bla*<sub>CMY-2</sub> gene by clonal spread and piglet training. Broad and longitudinal studies to determine the prevalence of *bla*<sub>CMY-2</sub>-positive *E. coli* in swine farms are required in the future.

## ACKNOWLEDGMENTS

This work was supported by the Program for Changjiang Scholars and Innovative Research Team at University of Ministry of Education of China (Grant No. IRT13063), the Special Fund

for Agro-scientific Research in the Public Interest (Grant No. 201203040), the National Natural Science Foundation (Grant No.31272609), and the Science and Technology Program of Guangzhou, China(Grant No. 2011J2200054).

## ETHICS STATEMENT

This study protocol was reviewed and approved by the South China Agriculture University Animal Ethics Committee.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Prevention and Control of Antimicrobial Resistant Healthcare-Associated Infections: The Microbiology Laboratory Rocks!

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## OPEN ACCESS

### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 22 December 2015

**Accepted:** 23 May 2016

**Published:** 07 June 2016

### Citation:

Simões AS, Couto I, Toscano C, Gonçalves E, Póvoa P, Viveiros M and Lapão LV (2016) Prevention and Control of Antimicrobial Resistant Healthcare-Associated Infections: The Microbiology Laboratory Rocks! *Front. Microbiol.* 7:855.

doi: 10.3389/fmicb.2016.00855

In Europe, each year, more than four million patients acquire a healthcare-associated infection (HAI) and almost 40 thousand die as a direct consequence of it. Regardless of many strategies to prevent and control HAIs, they remain an important cause of morbidity and mortality worldwide with a significant economic impact: a recent estimate places it at the ten billion dollars/year. The control of HAIs requires a prompt and efficient identification of the etiological agent and a rapid communication with the clinician. The Microbiology Laboratory has a significant role in the prevention and control of these infections and is a key element of any Infection Control Program. The work of the Microbiology Laboratory covers microbial isolation and identification, determination of antimicrobial susceptibility patterns, epidemiological surveillance and outbreak detection, education, and report of quality assured results. In this paper we address the role and importance of the Microbiology Laboratory in the prevention and control of HAI and in Antibiotic Stewardship Programs and how it can be leveraged when combined with the use of information systems. Additionally, we critically review some challenges that the Microbiology Laboratory has to deal with, including the selection of analytic methods and the proper use of communication channels with other healthcare services.

**Keywords:** microbiology, healthcare-associated infections, antibiotics, antibiotic stewardship, information systems, prevention, control, communication

## INTRODUCTION

Healthcare-associated infections (HAIs) are a significant cause of morbidity and mortality leading to 37,000 deaths/year in Europe<sup>1</sup> and 75,000 deaths in USA in 2011 (Magill et al., 2014). The HAIs economic impact is also significant: about 9.8 billion dollars/year/USA for the five major infections (Zimlichman et al., 2013). Antibiotic resistance is one of the major

<sup>1</sup>[http://ecdc.europa.eu/en/healthtopics/Healthcare-associated\\_infections](http://ecdc.europa.eu/en/healthtopics/Healthcare-associated_infections)

problems associated with HAIs (Cosgrove, 2006; Neidell et al., 2012): the Centers for Disease Prevention and Control (CDC) estimates that over two million people/year acquire antibiotic resistant infections, and 23,000 die as a result of it (CDC, 2013). In Europe, 25,000 people/year die with drug-resistant infections (ECDC, 2009).

Prevention through education is the most used strategy for HAIs control: the benefits from prevention can be as high as 5.5 billion dollars (Zimlichman et al., 2013). Reinforcing hand washing, staff education, environmental cleaning practices, Antibiotic Stewardship Programs (ASPs) and improved communication systems are measures implemented worldwide to control HAIs. However, to efficiently control HAIs, clinicians need to act quickly, which implies gathering all relevant information about the infection as soon as possible. That is why the Microbiology Laboratory is so important in HAIs prevention and control since it is in the front line for the early identification of infection, characterization of antibiotic resistance patterns and recognition of outbreaks (Diekema and Saubolle, 2011; Davey et al., 2013).

Traditionally, the tasks of the Microbiology Laboratory are to isolate, identify and determine antibiotic susceptibility patterns of pathogens (Wilson and Spencer, 1999). However, its scope covers other areas that are critical for Infection Control Programs as well as to ASPs, which should ideally include a member of the Microbiology Laboratory staff (Kolmos, 1999; Benbachir, 2008; CDC, 2014). The success of these kind of programs depends largely on the active involvement of the Microbiology Laboratory in activities beyond the regular microbiology exams, namely in results report, surveillance, communication, and other daily routine tasks of Infection Control Teams (Kalenić and Budimir, 2009).

In this paper, we describe the multiple chores of the Microbiology Laboratory highlighting its importance in HAIs prevention and control, and in ASPs, especially when combined with properly designed information systems. We also review some of the problems that the Microbiology Laboratory has to deal with when assisting Infection Control Teams, including the selection of the most appropriate analytic methods to provide fast and accurate results.

## TASKS OF THE MICROBIOLOGY LABORATORY

### Microbial Isolation and Identification

The Microbiology Laboratory main task is to isolate and identify the infection etiological agent (Benbachir, 2008), using the most appropriate, rapid and accurate diagnostic method. To ensure this, the Microbiology Laboratory needs to keep up-to-date materials, culture media, reagents, equipment, identification methods and trained personnel (CDC, 2003). The staff needs continuous on-the-job training in microbiological techniques and to be updated on the internationally endorsed methods for isolation and characterization of pathogens (Pfaller and Herwaldt, 1997; Benbachir, 2008). In addition, external and internal quality control and assurance programs must be

implemented to guarantee the quality of the results (Benbachir, 2008).

### Determination of Antimicrobial Susceptibility Patterns

The Microbiology Laboratory should provide frequently updated information on antimicrobial resistance patterns, essential to design appropriate hospital prescription guidelines, help clinicians to choose the most appropriate empiric therapy and to create a culture of patient safety (Pfaller and Herwaldt, 1997; Benbachir, 2008). This data can be analyzed in different perspectives, including infectious agent, specimen, ward, clinical specialty, antibiotics prescribed, or anatomic site of infection, among others (Pfaller and Herwaldt, 1997; Benbachir, 2008).

The availability of periodic reports on local antimicrobial resistance patterns is also relevant in ASPs, since it can be used to evaluate trends of antimicrobial resistance rates, to educate clinicians on optimal antimicrobial use and to assess the impact of prevention measures (Diekema and Saubolle, 2011).

### Report of the Results

The laboratory work is completed only when it is effectively reported (Newton and Novak-Weekley, 2011). All laboratory results (preliminary and final) should be reported as soon as possible to clinicians and Infection Control Teams (Kalenić and Budimir, 2009). Daily reports on significant microbiology results and periodic reports with frequency of isolated pathogens and prevalence of resistant microorganisms provide clinicians and Infection Control Teams with accurate and timely information, essential to follow trends of hospital infections and control urgent situations (Pfaller and Herwaldt, 1997; Benbachir, 2008).

These reports can be delivered through meetings, phone, information systems alerts, paper, or e-mail. However, making results accessible through an information system is an advantage by ensuring that all results are available in an organized, easily accessible, and timely manner, and also permits links to other surveillance data systems (Cantón, 2005). Information systems that incorporate information about the patient, disease, infectious agent and antimicrobial susceptibility are fundamental because they promote timely exchange of information between healthcare workers (Schreckenberger and Binnicker, 2011). For instance, ARTEMIS (Teodoro et al., 2012) and HAItool<sup>2</sup> (Pinto et al., 2016) are good examples of this kind of systems. They analyze heterogeneous data sources and can be used to build antimicrobial resistance surveillance networks participating in the management and prevention of antibiotic resistant HAIs, and by doing so, optimizing human and economic resources<sup>2</sup> (Teodoro et al., 2012). Some of these systems alert the clinician (or the pharmacist) when laboratory results reveal that the antibiotic(s) in use may not be optimal or when de-escalation treatment is indicated (Schreckenberger and Binnicker, 2011; Pinto et al., 2016). The “processed information” generated by these systems are very useful for surveillance purposes and in supporting and leveraging ASPs (Davey et al., 2013).

<sup>2</sup><http://haitool.ihmt.unl.pt/>

## Surveillance and Outbreak Detection

Surveillance enables the identification of infected patients, the origin of HAIs and to understand their paths of spread. Since most of this data comes from microbiological isolates and other laboratory identification tests (Emori and Gaynes, 1993; Peterson and Brossette, 2002), the Microbiology Laboratory has a central role not only on the surveillance and early detection of outbreaks but also on monitoring and reporting unusual laboratory results (e.g., clusters of pathogens, emergence of multidrug-resistant organisms, isolation of unusual pathogens).

To detect outbreaks early enough to mitigate their impact on morbidity and mortality is one of the major challenges of an efficient surveillance program (Diekema and Saubolle, 2011). The Microbiology Laboratory, in association with the Infections Control Team, is the first to detect an outbreak because unusual clusters of pathogens or resistance patterns are easily noticed (Arias, 2010). During an outbreak, these two entities have to work side by side to: (i) provide information on the epidemiology of the etiologic agent; (ii) identify and store the isolates involved for further testing; (iii) define/select appropriate selective isolation media and drug susceptibility testing (if needed/when applicable); (iv) perform the appropriate tests for strain typing (or provide its dispatch to a reference laboratory); (v) and perform supplemental microbiological surveillance of patients, personnel, or environmental sources of infection (Emori and Gaynes, 1993; Pfaller and Herwaldt, 1997; Arias, 2010).

As described above, the use of surveillance systems can enhance surveillance programs by aggregating all the information related with patient, disease, infectious agent and antimicrobial susceptibility, making easier outbreaks detection.

## Education

To maximize the efficacy of Infection Control programs, the Microbiology Laboratory should provide training and information on basic microbiology and biosafety for healthcare workers in areas such as: specimen collection, handling and transport, epidemiologically important pathogens vs. normal flora, colonization vs. infection, interpretation of microbiological results (Kalenić and Budimir, 2009). Written guidelines about sampling, handling and transport should be available in every ward, which can also include details on the tests available for proper isolation, identification, and typing of microorganisms (Grosek, 1999).

## ANTIBIOTIC STEWARDSHIP PROGRAMS

Antibiotic Stewardship Programss should be included in all HAIs prevention and control programs and are essential for gathering information about epidemiological and molecular markers of resistance, and changes in resistance patterns. They not only contribute to the optimization of antimicrobial therapy, by ensuring proper use (indication, dose, route of administration, and duration) and minimizing side effects, but also promote education on it (Davey et al., 2013). The adoption of these programs leads to a reduction in the prevalence of antimicrobial resistance and costs (Malani et al., 2012). These programs have

been implemented throughout the world and there are guidelines and recommendations for their use in the USA (CDC, 2014; Fridkin et al., 2014) and Europe (Pina, 2002; Department of Health Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infection, 2011; Lower et al., 2013; Simões et al., 2015).

Also here, computerized surveillance and decision support systems are a good support for ASPs, since have proven to be effective in the prescription errors reduction, medical care improvement and compliance with recommendations (Evans et al., 1998; Pestotnik, 2005).

A schematic representation of the network of interactions in ASPs is shown in **Figure 1**. The laboratory imparts an important function in ASPs, generating most of the relevant information needed to characterize the biology of the pathogen (and the hosts), namely its identification, antimicrobial susceptibility patterns and epidemiological connections. Therefore, it is recommended that all ASPs include a microbiologist (CDC, 2014). A good example of the role of Microbiology Laboratory in ASPs is described by MacKenzie et al. (2007) who demonstrated that hospitals with routine reports on antimicrobial susceptibility patterns for restricted antibiotics had lower usage (and misusage) of these antibiotics.

## ISSUES ON THE LABORATORY EFFICACY

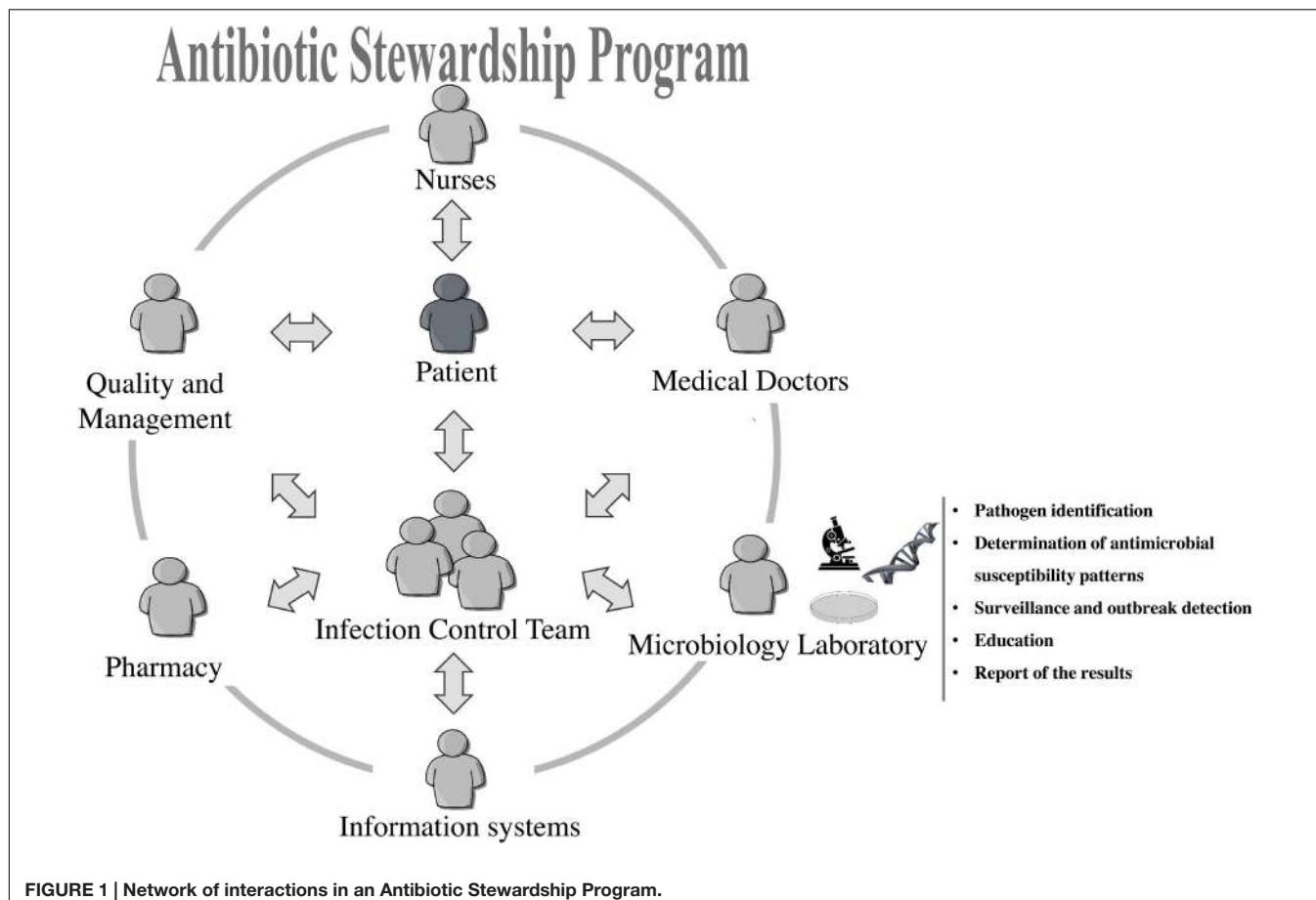
### Sample's Quality and Access to Clinical Data

The quality of the laboratory diagnosis is closely related with the quality of the collected samples (Benbachir, 2008). Samples should be taken on the correct time and from appropriate sites, using proper techniques and in amounts that makes possible to perform all the tests necessary for the isolation and identification (and other testing) of the pathogen (Department of Health Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infection, 2011). Samples that are not properly collected and transported may lead to false results (Benbachir, 2008).

Relevant epidemiological and clinical information data are also important for accurate laboratory diagnosis. Every request for exam should include: name of the patient, name of the clinician in charge, location of the patient, date and time of specimen collection, short anamnesis including suspected diagnosis and underlying patient conditions and comorbidities (Benbachir, 2008; Kalenić and Budimir, 2009). The access to this pre-analytic data facilitates significantly the guidance toward pathogen detection and identification. These kind of information should be easily accessed throughout an information system.

### Isolation in Culture or Molecular Identification Methods, Which One to Choose?

The use of laboratory culture methods for isolation of pathogens followed by identification procedures (biochemical, molecular, serologic, or other) has been the gold standard in Medical



**FIGURE 1 |** Network of interactions in an Antibiotic Stewardship Program.

Microbiology continues to play a vital role in the overall management of infectious diseases. As an example, blood culture is still mandatory for isolation and identification of blood pathogens and guiding therapy (Book et al., 2013). However, isolation in culture has limitations, namely being time consuming (Singh et al., 2006). Results provided 48 to 72 h after the onset of the infection often have limited impact on therapy (Tenover, 2010) and the ability of the laboratory to provide results in a timely manner is essential.

Conversely, the major benefit of direct molecular methods is related to time saving. Using Polymerase Chain Reaction (PCR) based methods, results can be obtained within 6 to 8 h, reducing greatly the time-to-result and implementation of appropriate therapy (Book et al., 2013). Molecular methods have been described as a powerful tool against the spread of microorganisms in hospital environment, in particular during outbreaks (Singh et al., 2006), and many have been used to rapidly identify microorganisms (e.g., multiplex PCR, Real-time PCR, MALDI-TOF MS), to find resistance patterns (e.g., PCR/hybridization screening of resistance determinants) and to estimate epidemiological links between bacteria (e.g., pulsed-field gel electrophoresis, multi-locus sequence typing), enabling rapid and appropriate therapeutic responses (Cantón, 2005; Simões et al., 2011; Sabat et al., 2013; Patel, 2015; Srinivasan et al., 2015). More recently whole-genome sequencing (WGS) promises to

transform Infection Control. WGS provides almost all the genetic information needed for epidemiological studies (Köser et al., 2012) and has been widely used to identify and control outbreaks of antibiotic resistant HAIs (Reuter et al., 2013; Price et al., 2013). WGS can be used to quickly identify pathogenic agents from specimens and unravel many genomic single nucleotide polymorphisms and markers for drug resistance, allowing the implementation of immediate and appropriate control measures (Coll et al., 2014). However, the translation of the large amount of data generated by WGS to the clinical utility is still under development (Burke and Kornigebel, 2015).

Molecular methods have been associated with global cost reduction, due to their high specificity, sensitivity and rapid turnaround (Cantón, 2005; Tenover, 2010; Currie, 2011). Combining molecular typing with surveillance programs was shown to be cost effective and result in significant reduction of HAIs rates (Hacek et al., 1999). More recently, the integration of rapid identifications methods with stewardship interventions has been described as a way to improve time to optimal antibiotic therapy, decrease length of hospital stay and reduce mortality and healthcare costs (Huang et al., 2013; Bauer et al., 2014; Perez et al., 2014).

However, molecular methods do not solve all the problems. Several authors have described drawbacks associated with molecular methods: limited number of detectable pathogens,

possibility of false positives and complex sample preparation procedures (Laxminarayan et al., 2013). In addition, molecular methods are more expensive, require specialized equipment, and training (Morshed et al., 2007) and their value in diagnosis of some infections has not been fully proven. For instance, in a recent study testing the accuracy of SeptiFast multi-pathogens real-time PCR, authors concluded that, despite providing faster results, this method has limited utility in bloodstream infections, when compared with conventional blood culture (Warhurst et al., 2015). Additionally, there are several microorganisms for which isolation by culture methods is the most effective and recommended procedure especially when dealing with drug-resistant inducible geno/phenotypes.

The dispute on isolation in culture followed by identification versus direct identification from specimen by molecular methods seems endless and unnecessary since both methods can be used in concert. Several authors suggested a combination of culture and molecular methods in order to increase the rate, efficacy and accuracy of pathogen detection. (Dolinger and Jacobs, 2011; Brown-Elliott and Wallace, 2012; Huttunen et al., 2013). **Table 1** summarizes some pros and cons of both strategies.

## Antimicrobial Resistance Surveillance

With the emergence and spread of antimicrobial resistant pathogens, antimicrobial resistance surveillance is becoming an important task of the Microbiology Laboratory. Antimicrobial resistance surveillance is an ongoing (and organized) data collection that after being analyzed and reported provides useful information for empirical antimicrobial therapy (Cornaglia et al., 2004).

Nevertheless, a good surveillance program is time consuming and involves dedicated human resources. In addition there are several challenges on data collection, management, analysis, interpretation and reporting. For instance, promoting the use of new and low-cost technologies to improve laboratory work and

to prioritize which bacteria are most important to track are issues that should be addressed (Solomon and Ijaz, 2015). Regarding interpretation, uniformization is needed: currently, different guidelines and breakpoints for evaluation of antimicrobials susceptibility patterns values are adopted in the United States of America and within several European countries (Vernet et al., 2014). Finally, the results should be presented in formats easily understandable by the clinicians (Grundmann et al., 2011).

Information systems are a good way to keep it working smoothly (Lapão, 2007). As stated above, surveillance information systems ensure that antimicrobial resistance related data are available and organized, making it easy to report it retrospectively. In addition, surveillance information systems allow better antibiotic resistance management and help to provide evidence-based results that can be used for the development of control policies (Evans et al., 1998; Pestotnik, 2005).

## Communication

Effective communication is critical for the Microbiology Laboratory procedures. Nevertheless, it can also be one of its major issues. Effective communication is the base of a healthy collaboration between laboratory, healthcare workers and Infection Control Teams (Kalenić and Budimir, 2009). The dialog between healthcare workers and laboratory staff must be easy and effective (Peterson et al., 2001). The existence of a dedicated laboratory staff element (privileged interlocutor) and the participation of microbiologists in regular clinician's meetings is recommended. Efficient communication between clinicians and microbiologists about the presumptive diagnosis, accelerates the diagnosis and avoids problems with inappropriate specimens (Baron et al., 2013).

In order to facilitate communication, it is recommended that the director of the Microbiology Laboratory be a clinician or a laboratory scientist with expertise in infectious diseases and

**TABLE 1 | Comparison of culture and molecular identification methods.**

	Culture methods	Molecular methods
Specificity	Moderate	High
Sensitivity	Low	High
Antimicrobial susceptibility	Isolates can be tested for susceptibility to relevant antibiotics	Allows the detection of some resistance markers without isolation/culturing
Amount needed to detect pathogens	High	Low
Time to obtain results	Long (especially for slow-growers organisms)	Short
Cost	Low	High (variable)
Detection of non-viable bacteria (patient in antibiotic treatment)	No	Yes
Equipment	Requires non-specialized equipment	Requires specialized equipment
Biosafety	Potential biosafety concerns	Minimizes biosafety concerns
Feasibility	Requires basic training	Requires advanced training
Others	No specialized workflow required  Allows visual inspection of colony morphology  Allows biochemical characterization of phenotype  Limited potential for false positives and/or false negatives	Assays may not be commercially available  Allows high resolution analysis  Potential false positives (by cross-reaction with closely related species or contaminated amplicons)  Potential false negatives (by inhibition components or target mutations)

Adapted from (Dolinger and Jacobs, 2011).

microbiology (Thomson et al., 2010) since their background on disease pathologic process facilitates the discussion of clinical cases. Additionally, information systems have an important role in communication within the hospitals by facilitating the exchange of clinical and microbiological relevant data between clinicians and laboratories (Lapão, 2007). However, it is important that information systems are defined and designed together with the healthcare workers in order to really improve communication, data quality, be useful on decision-making and be easy to use (Pinto et al., 2016).

Another reality that can affect the communication is the location of the Microbiology Laboratory. There are an increasing number of off-site laboratories providing services to hospitals and in the cases of in-house laboratories, they usually are in the basement or in an annex outside the hospital main building. It has been described that off-site laboratories delay and decrease communication, could jeopardize infection diagnosis and treatment and weaken infection prevention and antibiotic stewardship infrastructures (Peterson et al., 2001; Dancer et al., 2015).

## CONCLUSION

The Microbiology Laboratory plays a key role in HAIs prevention and control. From ensuring appropriate specimen collection and

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- transport, to the wise selection of isolation and identification methods and finally on the antibiotic therapy guidance plus effective report and communication of the results, the laboratory covers all important aspects of infection control process. The microbiologist is a fundamental and enriching member of ASPs and Infection Control Teams. The new identifications methods (including WGS) combined with the emergence of innovative and centralized information systems that integrate the microbiology results with clinical data will revolutionize HAIs prevention and control strategies, help decision-making and resolve some of the difficulties felt by the microbiologists.

## AUTHOR CONTRIBUTIONS

ASS, IC, CT, EG, PP, MV, and LVL contributed to the draft and revision of the paper and approved the final version to be published.

## FUNDING

This work was supported by project "HAITool – A Toolkit to Prevent, Manage and Control Healthcare-Associated Infections in Portugal" EEA Grants, 000182DT3; and by FCT for funds to GHTM – UID/Multi/04413/2013.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# QnrS1- and Aac(6')-Ib-cr-Producing *Escherichia coli* among Isolates from Animals of Different Sources: Susceptibility and Genomic Characterization

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 29 January 2016

Accepted: 22 April 2016

Published: 23 May 2016

### Citation:

Jones-Dias D, Manageiro V, Graça R, Sampaio DA, Albuquerque T, Themudo P, Vieira L, Ferreira E, Clemente L and Caniça M (2016) QnrS1- and Aac(6')-Ib-cr-Producing *Escherichia coli* among Isolates from Animals of Different Sources: Susceptibility and Genomic Characterization. *Front. Microbiol.* 7:671. doi: 10.3389/fmicb.2016.00671

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*Salmonella enterica* and *Escherichia coli* can inhabit humans and animals from multiple origins. These bacteria are often associated with gastroenteritis in animals, being a frequent cause of resistant zoonotic infections. In fact, bacteria from animals can be transmitted to humans through the food chain and direct contact. In this study, we aimed to assess the antibiotic susceptibility of a collection of *S. enterica* and *E. coli* recovered from animals of different sources, performing a genomic comparison of the plasmid-mediated quinolone resistance (PMQR)-producing isolates detected. Antibiotic susceptibility testing revealed a high number of non-wild-type isolates for fluoroquinolones among *S. enterica* recovered from poultry isolates. In turn, the frequency of non-wild-type *E. coli* to nalidixic acid and ciprofloxacin was higher in food-producing animals than in companion or zoo animals. Globally, we detected two *qnrS1* and two *aac(6')-Ib-cr* in *E. coli* isolates recovered from animals of different origins. The genomic characterization of QnrS1-producing *E. coli* showed high genomic similarity (O86:H12 and ST2297), although they have been recovered from a healthy turtle dove from a Zoo Park, and from a dog showing symptoms of infection. The *qnrS1* gene was encoded in a IncN plasmid, also carrying *blaTEM-1*-containing Tn3. Isolates harboring *aac(6')-Ib-cr* were detected in two captive bottlenose dolphins, within a time span of two years. The additional antibiotic resistance genes of the two *aac(6')-Ib-cr*-positive isolates (*blaOXA-1*, *blaTEM-1*, *blaCTX-M-15*, *catB3*, *aac(3)-Ila*, and *tetA*) were enclosed in IncFIA plasmids that differed in a single transposase and 60 single nucleotide variants. The isolates could be assigned to the same genetic sublineage—ST131 *fimH30-Rx* (O25:H4), confirming clonal spread. PMQR-producing isolates were associated with symptomatic and asymptomatic hosts, which highlight the aptitude of *E. coli* to act as silent vehicles,

allowing the accumulation of antibiotic resistance genes, mobile genetic elements and other relevant pathogenicity determinants. Continuous monitoring of health and sick animals toward the presence of PMQR should be strongly encouraged in order to restrain the clonal spread of these antibiotic resistant strains.

**Keywords:** pathogenicity, *E. coli*, clone, PMQR, multidrug resistance, veterinary

## INTRODUCTION

Antibiotic resistance has been critically increasing over time and now constitutes one of the major health concerns worldwide. The uncontrolled use of antibiotics in human and veterinary practices, animal production and agriculture and the increasingly easiness in global transportation contributed to the dissemination of multidrug resistant pathogens that constitute a risk for humans, animals and the environment (Marshall and Levy, 2011; EFSA, 2015). Nowadays, antibiotic resistant *Salmonella enterica* and *Escherichia coli* are among the most problematic zoonotic bacteria, causing severe gastroenteritis in animals and humans (EFSA, 2015).

Fluoroquinolones constitute a group of broad spectrum antibiotics of critical importance, presenting applications in both human and veterinary medicines (Poirel et al., 2012). Therefore, resistance might easily emerge in animals and get transferred to humans through the food chain and direct or indirect contact. Several examples of such transmission have already been documented (Gomes-Neves et al., 2014; Damborg et al., 2015; Schmithausen et al., 2015). Fluoroquinolone resistance has emerged rapidly due to two main types of mechanisms: mutation of the chromosomal quinolone targets DNA gyrase and topoisomerase IV, and acquisition of the transferable plasmid-mediated quinolone resistance (PMQR) determinants *qnr*, *qepA*, *aac(6')-Ib-cr*, and *oqxAB* (Veldman et al., 2011; Poirel et al., 2012). The alteration of chromosomal quinolone targets can lead to higher levels of resistance than PMQRs that are only able to guarantee low-level quinolone resistance. However, the ability of the latter to be spread by horizontal gene transfer constitutes a serious concern that should be addressed (Poirel et al., 2012). In fact, antibiotic resistance genes are frequently associated to mobile genetic elements such as insertion sequences (ISs), phages, transposons and plasmids, which enhance their ability to efficiently spread among different bacterial species (Stokes and Gillings, 2011). The most worrying mechanisms of resistance, which also show a transboundary spread between animals, humans and the environment, are, in fact, encoded by mobile antibiotic resistance genes. The occurrence of mobile genetic elements harboring multiple antibiotic resistance genes is also frequent, and enables the development of bacterial multidrug resistance, which may be responsible for therapeutic failures in animals or humans (Poirel et al., 2012).

In animals, as well as in humans, several factors can affect the progression and severity of an acute infection. The synchronized presence of antibiotic resistance genes, virulence factors, mobile genetic elements and other pathogenicity determinants, is ideal to the successful spread of these microorganisms in any environment (Cosentino et al., 2013).

In this study, PMQR-producing *E. coli* isolates were gathered from a collection of *S. enterica* and *E. coli* recovered from food-producing, companion and zoo animals, in the scope of their phenotypic and genotypic characterization. To further explore the genetic diversity of these isolates, as well as to understand the molecular features contributing to their spread and ability to cause infection, complete genomic sequencing was performed.

## MATERIALS AND METHODS

### Collection of bacterial Isolates

This study included 89 *S. enterica* isolates recovered from breeders ( $n = 12$ ), broilers ( $n = 33$ ), layers ( $n = 33$ ), swine ( $n = 6$ ), and food products of animal origin ( $n = 5$ ) (Table 1). In poultry farms, samples were collected from feces and environment using sterile boots/sock swabs. Food products included uncooked fresh products such as minced meat, hamburgers, meat cuts, sausages, and table eggs, randomly recovered at a variety of retail stores. Samples from other animal species (pigeons, partridges, ducks, pets, and exotic animals) consisted of blood cultures and organs (lung, liver, spleen, kidneys, and intestine) collected during post-mortem examination. All samples were examined according to ISO norm 6579:2002 applied to *Salmonella* detection in food and animal feeding stuffs. After biochemical confirmation, *Salmonella* spp. isolates were sent to the *Salmonella* National Reference Laboratory (INIAV, Lisbon) in triple sugar iron slopes or SMID plates.

This study also included 91 *E. coli* isolates (Table 1) collected from food-producing animals [(bovine, swine and poultry), ( $n =$

**TABLE 1 | Distribution of the *S. enterica* ( $n = 89$ ) and *E. coli* ( $n = 91$ ) isolates.**

Source	<i>S. enterica</i>		Source	<i>E. coli</i>
	Enteritidis	Other serotypes <sup>a</sup>		
Breeders	12	0	Food	32
Layers	24	9	Companion	36
Broilers	32	1	Zoo	23
Swine	0	6		6
Food of animal origin	3	2		5
Total	71	18	Total	91

<sup>a</sup>Salmonella 4,5:i:- ( $n = 1$ ), Salmonella 6,7,14:-:1,2 ( $n = 1$ ), Salmonella Bradenburg ( $n = 1$ ), Salmonella Gallinarum ( $n = 1$ ), Salmonella Give ( $n = 1$ ), Salmonella Hadar ( $n = 1$ ), Salmonella Heidelberg ( $n = 1$ ), Salmonella Iilla 48:z10:- ( $n = 1$ ), Salmonella Mbandaka ( $n = 1$ ), Salmonella Rissen ( $n = 2$ ), Salmonella Typhimurium ( $n = 3$ ), Salmonella Virchow ( $n = 4$ ).

32)], pets [(dogs, cats, horses, and cage birds), ( $n = 37$ )] and zoo animals [(terrestrial and aquatic mammals, birds and reptiles), ( $n = 22$ )]. Samples consisted of swabs from organic fluids and cavities, fecal samples, urine samples, blood cultures, and organs collected during post-mortem examination and submitted for bacteriological analysis. Suspected *E. coli* colonies obtained in MacConkey agar plates were confirmed by API 20E strips (bioMérieux, Marcy-l'Étoile, France).

### Serotypes of *S. enterica*

*S. enterica* isolates were serotyped by the slide agglutination method, using the method of Kauffmann-White scheme (Grimont and Weill, 2007).

### Antibiotic Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution following standard recommendations, using a panel of 10 antimicrobial compounds: ampicillin, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, streptomycin, chloramphenicol, tetracycline, sulfamethoxazole, and trimethoprim (Table 2). Isolates harboring PMQR determinants were further studied by determination of the MICs to a larger panel of fluoroquinolones, which included moxifloxacin, gatifloxacin, levofloxacin, ofloxacin, enrofloxacin, and norfloxacin. To assess non-wild-type isolates, interpretation of results was performed according to the epidemiological cut-off values suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, <http://mic.eucast.org/Eucast2/>). For *Salmonella* spp., the cut-off value used for sulfamethoxazole was that for sulfonamides from Clinical Standards Laboratory Institute (<http://cls.i.org>). MIC<sub>50</sub> and MIC<sub>90</sub> were calculated as reported elsewhere (Schwarz et al., 2010). *E. coli* ATCC 25922 was used as the quality control strain. Isolates were considered multidrug resistant (MDR) if they presented non-wild-type phenotypes against three or more structurally unrelated antibiotics (Magiorakos et al., 2011).

### Molecular Characterization of Resistance

All isolates were evaluated regarding the presence of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* genes, using primers and conditions previously described (Jones-Dias et al., 2013), and *oqxAB* genes using primers and conditions first described in this study (*oqxA*-F, 5'-AGAGTTCAAGGCCACGCTG-3' and *oqxb*-R, 5'-CTCCTGCATCGCCGTACCA-3'; initial denaturation of 94°C for 5 min; 94°C for 30 s, 64°C for 30 s and 72°C for 1 min, for 30 cycles; final step of extension of 72°C for 5 min). PMQR-producing isolates were also characterized regarding the production of β-lactamase-encoding genes and conventional Multilocus sequence typing (MLST), as described elsewhere (Jones-Dias et al., 2015).

### Genomic Characterization of PMQR-Producing *E. coli*

The genomes of the four PMQR-producing *E. coli* (LV46221, LV46743, LV36464, and LV27950) were characterized. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Aarhus) and quantified using Qubit 1.0 Fluorometer (Invitrogen, Waltham). The Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA) was used to prepare sequencing

libraries from 1 ng of genomic DNA, according to the manufacturer's instructions. Paired-end sequencing of 150 bp reads was performed on a MiSeq (Illumina). Sequence reads were then trimmed and filtered according to quality criteria, and assembled *de novo* using CLC genomics workbench version 8.5.1 (QIAGEN, Aarhus). RAST (Rapid Annotation using Subsystem Technology) was used for subsystem annotation of the genomes (Aziz et al., 2012; Overbeek et al., 2014).

### Identification of Pathogenicity-Related Genes

Pathogenicity-related genes were detected using a variety of online web tools. PathogenFinder 1.1, ResFinder 2.1, VirulenceFinder 1.4, SerotypeFinder 1.1, MLST 1.8, pMLST 1.4, and PHAST were used to estimate the pathogenicity determinants, acquired antibiotic resistance genes, virulence factors, serotypes, MLST, plasmid MLST and phage regions, respectively in the genomes of PMQR-producing *E. coli* (Zhou et al., 2011; Larsen et al., 2012; Zankari et al., 2012; Cosentino et al., 2013; Carattoli et al., 2014; Joensen et al., 2014, 2015). ISSaga was also used to detect and annotate insertion sequences in the draft genomes of the *E. coli* isolates (Varani et al., 2011). Specific analysis of antibiotic resistance genes and respective flanking regions was carried out with CLC genomics workbench version 8.5.1 (Qiagen, Aarhus). Contigs carrying antibiotic resistance genes were manually assembled whenever necessary and blasted against GenBank to identify their genetic location.

### Nucleotide Sequence Genbank Accession Numbers

The draft genomes of isolates LV46221, LV46743, LV36464, and LV27950 have been deposited at DDBJ/EMBL/GenBank under the accessions LRXG00000000, LRXH00000000, LRXI00000000, and LRXJ00000000, respectively. The versions described in this paper are version LRXG01000000, LRXH01000000, LRXI01000000, and LRXJ01000000, respectively.

## RESULTS

### Serotypes of *Salmonella* spp.

*S. enterica* serotype Enteritidis is one of the most common serotype in humans (EFSA, 2015) and it was the most frequently detected among the 89 *S. enterica* isolates (71/89, 79.8%), being present in all food animals except swine. The remaining *Salmonella* serotypes were detected in a less extent and were comprised of *Salmonella* 4,5:i:- ( $n = 1$ ), *Salmonella* 6,7,14:-:1,2 ( $n = 1$ ), *Salmonella* Bradenburg ( $n = 1$ ), *Salmonella* Gallinarum ( $n = 1$ ), *Salmonella* Give ( $n = 1$ ), *Salmonella* Hadar ( $n = 1$ ), *Salmonella* Heidelberg ( $n = 1$ ), *Salmonella* IIIa 48:z10:- ( $n = 1$ ), *Salmonella* Mbandaka ( $n = 1$ ), *Salmonella* Rissen ( $n = 2$ ), *Salmonella* Typhimurium ( $n = 3$ ), and *Salmonella* Virchow ( $n = 4$ ).

### Antimicrobial Susceptibility of *S. enterica* and *E. coli* Isolates

Susceptibility profiles of *S. enterica* and *E. coli* isolates differed with the animal group (Table 2). Although, high rates of non-wild-type *S. enterica* were detected for nalidixic acid (from 82

**TABLE 2 | MIC<sub>50</sub> and MIC<sub>90</sub> for *S. enterica* (n = 89) and *E. coli* (n = 91) isolates.**

Antibiotic	<i>S. enterica</i>				<i>E. coli</i>		
	Food animals				Food animals (n = 32)	Zoo Animals (n = 23)	Companion Animals (n = 36)
	Breeders (n = 12)	Broilers (n = 33)	Layers (n = 33)	Others <sup>a</sup> (n = 11)			
<b>Na</b>							
MIC <sub>50</sub>	128	128	128	128	8	4	4
MIC <sub>90</sub>	128	128	128	128	128	128	128
% Wt	0	12	6	18	59	77	78
%N-Wt	100	88	94	82	41	23	22
<b>Cp</b>							
MIC <sub>50</sub>	0.25	0.25	0.25	0.25	0.03	0.015	0.015
MIC <sub>90</sub>	0.25	0.25	0.25	0.5	8	8	8
% Wt	0	3	18	36	59	77	72
%N-Wt	100	97	82	64	41	23	28
<b>A</b>							
MIC <sub>50</sub>	2	4	0.5	8	8	8	8
MIC <sub>90</sub>	4	4	8	64	64	64	64
% Wt	100	100	94	64	53	50	53
%N-Wt	0	0	6	36	47	50	47
<b>Ct</b>							
MIC <sub>50</sub>	0.125	0.06	0.125	0.125	≤0.06	0.06	≤0.06
MIC <sub>90</sub>	0.125	0.125	0.125	2	0.125	0.125	0.125
% Wt	100	100	94	82	100	95	94
%N-Wt	0	0	6	18	0	5	6
<b>G</b>							
MIC <sub>50</sub>	0.25	0.25	0.25	0.5	0.5	0.5	0.5
MIC <sub>90</sub>	0.5	0.5	0.5	1	2	1	1
% Wt	100	100	100	100	91	91	100
%N-Wt	0	0	0	0	9	9	0
<b>St</b>							
MIC <sub>50</sub>	2	2	4	32	4	4	4
MIC <sub>90</sub>	8	4	128	64	256	256	128
% Wt	100	100	82	36	69	64	86
%N-Wt	0	0	18	64	31	36	14
<b>T</b>							
MIC <sub>50</sub>	2	2	2	4	32	2	2
MIC <sub>90</sub>	4	4	4	64	64	64	64
% Wt	92	100	91	55	47	59	75
%N-Wt	8	0	9	45	53	41	25
<b>C</b>							
MIC <sub>50</sub>	8	4	8	8	4	8	8
MIC <sub>90</sub>	8	8	8	16	64	8	16
% Wt	100	97	100	91	84	100	92
%N-Wt	0	3	0	9	16	0	8
<b>Su</b>							
MIC <sub>50</sub>	128	128	128	128	16	32	16
MIC <sub>90</sub>	128	128	128	>512	>512	>512	>512
% Wt	92	100	100	64	63	59	78
%N-Wt	8	0	0	36	38	41	22
<b>Tp</b>							
MIC <sub>50</sub>	0.5	0.5	0.5	0.25	0.5	0.5	0.5
MIC <sub>90</sub>	0.5	0.5	0.5	32	32	32	32
% Wt	92	100	100	73	81	68	81
%N-Wt	8	0	0	27	19	32	19

<sup>a</sup>Others, pigs (n = 6) and food products of animal origin (n = 5).

Na, nalidixic acid; Cp, ciprofloxacin; A, ampicillin; Ct, cefotaxime; G, gentamicin; St, streptomycin; T, tetracycline; C, chloramphenicol; Su, sulfamethoxazole; Tp, trimethoprim.

to 100%) and ciprofloxacin (from 64 to 100%) in all groups, they were particularly evident in poultry, and predominant in breeders. *S. enterica* isolates recovered from other sources (swine and food products,  $n = 11$ ), showed higher non-wild-type phenotypes for ampicillin (36%), streptomycin (64%), tetracycline (45%), sulfamethoxazole (36%), and trimethoprim (27%) (**Table 2**). The poultry groups of breeders and broilers were mainly susceptible to ampicillin (100%), cefotaxime (100%), gentamicin (100%), and streptomycin (100%).

The frequency of non-wild-type isolates was globally higher for *E. coli* than for *S. enterica* against ampicillin (minimum value of 47 vs. 0%, respectively), tetracycline (minimum value of 25 vs. 0%, respectively), sulfamethoxazole (minimum value of 22 vs. 0%, respectively) and trimethoprim (minimum value of 19 vs. 0%, respectively). Although, no major discrepancies were noticed for *E. coli* in rates of non-wild-type isolates for the different animal groups, isolates recovered from food animals still presented more non-wild-type phenotypes than zoo or companion animals against nalidixic acid (41%), ciprofloxacin (41%), tetracycline (53%), and chloramphenicol (16%).

In *S. enterica* isolates from poultry, similar MIC<sub>50</sub> and MIC<sub>90</sub> values were observed for the majority of the antibiotics tested; major differences ( $\geq 3$  fold dilutions) were observed for the group “others” for ampicillin (8 and 64 mg/L), tetracycline (4 and 64 mg/L), sulfamethoxazole (128 and >512 mg/L) and trimethoprim (0.25 and 32 mg/L). For *E. coli*, the most significant differences in MIC<sub>50</sub> and MIC<sub>90</sub> values were observed for nalidixic acid (4 and 128 mg/L), ampicillin (8 and 64 mg/L), streptomycin (4 and 128 mg/L), tetracycline (2 and 64 mg/L), sulfamethoxazole (16 and >512 mg/L) and trimethoprim (0.5–32 mg/L).

While for *S. enterica* only 9.0% (8/89) MDR isolates were detected, for *E. coli*, MDR was registered in 38.9% (35/90) of the isolates, which were distributed among 16/90 isolates from food-producing animals, 9/90 isolates from companion animals, and 10/90 isolates from zoo animals.

## Molecular Characterization of *S. enterica* and *E. coli* Isolates

Overall, among the 180 isolates studied, we have detected and identified four PMQR determinants in *E. coli* isolates: two *qnrS1* were detected in isolates recovered from a captive turtle dove (LV46221) and a pet dog (LV46743), and two *aac(6')-Ib-cr* were isolated from *E. coli* recovered from captive bottlenose dolphins (LV36464 and LV27950). The detection of  $\beta$ -lactamase-encoding genes showed the presence of *bla<sub>TEM-1</sub>* in isolates LV46221 and LV46743, and *bla<sub>TEM-1</sub>*, *bla<sub>OXA-1</sub>*, and *bla<sub>CTX-M-15</sub>* in LV36464 and LV27950. No other PMQR- or  $\beta$ -lactamase-encoding genes were identified in the collection of *E. coli* and *S. enterica* isolates.

## Genomic Characterization of QnrS1-Producing *E. coli*

The assembly of the genome sequences of the two *qnrS1*-harboring *E. coli*, LV46221 and LV46743, yielded 200 and 199 contigs (each >200 bp long), which together comprised 4,799,985

bp and 4,801,518 bp, respectively. The average coverage of LV46221 was 135.9, while LV46743 displayed 114.1 fold. The maximum contig length obtained for these genomes was 398,205 bp and 333,601 bp, respectively (**Table 3**).

The automated annotation of the draft genomes showed that LV46221 (63%, 2879/4618) and LV46743 (63%, 2873/4609) presented a similar number of sequences attributed to specific subsystems (Figure S1). General annotation of both genomes showed 109 coding sequences associated with virulence, disease and defense, as well as 143 sequences coding for functions related with mobile genetic elements, such as phages, prophages, transposable elements, and plasmids. Globally, the proportion of each subsystem was equally represented in the genomes of the two isolates (Figure S1). According to RAST annotation system, LV46221 and LV46743 isolates carried 77 and 86 RNAs, respectively. The bioinformatics analysis of the genetic relatedness was carried out with regard to serotype and MLST: the serotypes of both isolates were defined as O86:H12, and they also shared the assigned MLST-ST2297 (**Table 4**).

*In silico* analysis of the antibiotic resistance genes (90% identity and 40% minimum length) confirmed the presence of a *qnrS1*, and identified *bla<sub>TEM-1</sub>* gene in both isolates (**Table 4**). *qnrS1* was detected in a contig with an approximate length of 11,000 bp in both cases, showing 99% of homology with a resistance region from *S. enterica* subsp. *enterica* serovar Infantis pINF5 plasmid. By mapping all contigs against this plasmid, we detected *bla<sub>TEM-1</sub>*-containing *Tn3*, and a disrupted IS2-like element upstream of *qnrS1*, as well as IS26 transposase downstream of the gene. Other contigs showed complementary regions, revealing the presence of a fragment encoding conjugation transfer genes upstream of *Tn3* that showed homology with *Salmonella* Virchow plasmid pVQS1 (99%).

LV46221 and LV46743 showed no additional PMQR or other acquired antibiotic resistance genes. Moreover, no mutations were detected in the quinolone resistance determining region (QRDR) of genes *gyrA*, *gyrB*, *parC*, and *parE*, which are known to confer high level resistance to fluoroquinolones (Veldman et al., 2011). The isolates were also characterized with regard to specific mobile genetic elements of different classes. The screening of typable plasmids (>98% homology) enabled the identification of IncN plasmids, which were further typed as ST1

**TABLE 3 | Genome analysis of *E. coli* LV46221, LV46743, LV36464, and LV27950.**

Isolates	LV46221	LV46743	LV36464	LV27950
Genome size (bp)	4,799,985	4,801,518	5,180,399	5,156,819
Number of contigs	200	199	136	209
Average coverage	135.9	114.1	178.7	150.1
N50 (bp) <sup>a</sup>	119,356	119,356	158,975	158,977
Maximum contig (bp)	398,205	333,601	399,998	369,918
Minimum contig (bp)	208	201	486	218
Protein-coding genes	4618	4609	5107	5069
RNAs	77	86	77	76

<sup>a</sup>Minimum contig length of at least 50% of the contigs.

**TABLE 4 |** General features of PMQR-harboring *E. coli* isolates recovered from animals of different sources.

Isolate	Origin	Year	Serotype	PMQR						Other resistance genes	Virulence factors	MLST	Plasmids	pMLST			
				Mx	Cp	Ga	Le	Of	Ef	Na							
LV46221	Dove	2008	O86:H12	0.75	0.38	0.75	0.38	1	1.5	8	0.75	qnrS1	blaTEM-1	gad, iss	ST12297	IncN	ST1
LV46743	Dog	2008	O86:H12	0.5	0.38	0.5	0.5	1.5	2	8	1.5	qnrS1	blaTEM-1	gad	ST12297	IncN	ST1
LV36464	Dolphin	2009	O25:H4	>32	>32	8	>32	>32	>32	>256	>256	aac(6')-lb-cr	aac(3')-Ila, blaCTX-M-15	iss, sat, gad	ST131	IncFA, IncX	F-A1:B-
LV27950	Dolphin	2011	O25:H4	12	>32	8	8	>32	>32	>256	>256	aac(6')-lb-cr	blaTEM-1, blaOXA-1	catB3, tetA	ST131	IncFA	F-A1:B-
												aac(3')-Ila, blaCTX-M-15	blaTEM-1, blaOXA-1	catB3, tetA			

Mx, moxifloxacin; Cp, ciprofloxacin; Ga, gatifloxacin; Le, levofloxacin; Of, ofloxacin; Ef, enrofloxacin; Na, nalidixic acid; Nx, norfloxacin.

PMQR, Plasmid-mediated quinolone resistance.  
MLST, Multilocus sequence typing.

by pMLST (**Table 4**). ISSaga allowed the specialized annotation of insertion sequences and revealed a different distribution of the same elements for LV46221 and LV46743: IS1 (3.23 and 1.96%, respectively), IS200\_IS605 (3.23 and 3.92%, respectively), IS21 (3.23 and 3.92%, respectively), IS3 (24.19 and 21.57%, respectively), IS4 (3.23 and 3.92%, respectively), IS481 (1.61 and 1.96%, respectively), IS5 (1.61 and 1.96%, respectively), IS6 (1.61 and 1.96%, respectively), ISAs1 (27.42 and 27.45%, respectively), ISKra4 (6.45 and 5.88%, respectively), ISL3 (12.9 and 9.8%, respectively), ISNCY (9.68 and 11.76%, respectively), and finally Tn3 (1.61 and 1.96%, respectively). ISAs1 was the most frequent element detected in both isolates, and IS66 was exclusively detected in LV46743 (1.96%). Moreover, in LV46221 we identified 10 prophage regions among which three were questionable but seven were intact. The latter included prophage regions reaching up to 90.6Kb, containing 133 coding sequences (Table S1). In turn, in LV46743, 14 different prophages were detected that included one questionable, four incomplete and nine intact phage regions; intact zones ranged between regions of 10.4 Kb carrying 12 coding sequences, and 70.3 Kb with 88 protein coding DNA fragments. Overall, among the prophages showing higher scores for both genomes were serotype-converting *Shigella flexneri* bacteriophage and Enterobacteria lambda phages (Table S1).

The total number of pathogenicity determinants, which according with PathogenFinder includes, for instance, virulence factors, antibiotic resistance genes and mobile genetic elements, detected a similar number of sequences in the genomes of *E. coli* LV46221 and LV46743: 607 and 611 different pathogenic families showed a 93.5% certainty of the isolates being human pathogens. Finally, the virulence factor glutamate decarboxylase (gad) was detected in both isolates, while the increased serum survival factor iss was exclusively identified in LV46221 (**Table 4**).

## Genomic Characterization of Aac(6')-ib-cr-Producing *E. coli*

The genome sequences of isolates LV36464 and LV27950, which were known to produce Aac(6')-Ib-cr and CTX-M-15, were also compared. Their *de novo* assembly yielded 5,180,399 bp for LV36464 and 5,156,819 bp for LV27950 and displayed a mean coverage of 178.6 and 150.1 fold, respectively. Approximately, 136 and 209 contigs (each >200 bp long) were recovered for LV36464 and LV27950 with a maximum contig length of 399,998 and 369,918 bp, respectively (**Table 3**).

The automated annotation of the genomes showed a total number of coding sequences of 5107 for LV36464 and 5069 for LV27950, excluding 77 and 76 annotated RNA molecules. The distribution of the annotated coding sequences by subsystem showed an identical representation of functions in both isolates (LV36464: 368%, 3062/5107; LV27950: 61%, 3081/5069; Figure S2).

The serotypes of the LV36464 and LV27950 isolates obtained upon the analysis of *fliC*, *wzy*, and *wzx* genes, were defined as O25:H4. The epidemiology and diversity of *E. coli* isolates was also explored, assigning both of them to ST131 and to sublineage *fimH30-Rx*.

Globally, in isolates LV36464 and LV27950 seven different acquired antibiotic resistance genes were detected: *aac(6')Ib-cr*, *bla<sub>OXA-1</sub>*, *bla<sub>TEM-1</sub>*, *bla<sub>CTX-M-15</sub>*, *catB3*, *aac(3)-IIa*, and *tetA*. By mapping, the main difference between the plasmids carried by these isolates was the deletion of a 2400 bp sequence that displayed 99.7% homology with the transposase of Tn5403. Moreover, 60 single nucleotide variants have been detected between them. Both plasmids displayed an IncF plasmid from a ST131 *E. coli* isolate (JJ2434, unpublished) as its best blast hit. The comparative analysis with JJ2434 showed the absence of two regions of 9329 and 1740 bp that corresponded to deletions of genes coding for unknown functions, replication proteins, endonucleases, transcriptional regulators, and conjugation transfer proteins in LV36464 and LV27950 plasmids. The analysis of the QRDR of genes *gyrA* (from 67 to 106 aminoacids), *gyrB* (from 415 to 470 aminoacids), *parC* (from 47 to 133 aminoacids), and *parE* (from 450 to 528 aminoacids) revealed the presence of amino acid substitutions in *gyrA* (S83L and D87N) and *parC* (S80I and E84V) in both isolates.

A high number of mobile genetic elements was detected in the draft genomes of these isolates. Both harbored a plasmid (>98% homology) from incompatibility group IncFIA, which according to PlasmidFinder was classified as an IncFIA type 1. LV36464 accommodated an additional IncX plasmid. The distribution of insertion sequences present in LV36464 and LV27950 genomes was also globally similar: IS1 (5.77 and 5.66%, respectively), IS110 (3.85 and 1.89%, respectively), IS1380 (1.92 and 1.89%, respectively), IS200\_IS605 (5.77 and 3.77%, respectively), IS21 (3.85 and 3.77%, respectively), IS3 (23.08 and 26.42%, respectively), IS30 (1.92 and 3.77%, respectively), IS4 (5.77 and 3.77%, respectively), IS481 (3.85 and 3.77%, respectively), IS6 (1.92 and 1.89%, respectively), IS66 (11.54 and 11.32%, respectively), ISAs1 (1.92 and 1.89%, respectively), ISL3 (19.23 and 18.87%, respectively) and ISNCY (9.62 and 9.43%, respectively). It is worth mentioning that the worldwide disseminated Tn3 was only represented in the genome of LV36464 (3.39%), and IS92 (1.89%) in LV27950. The specialized annotation of phage and prophages revealed that LV36464 harbored 17 regions: 8 intact, 6 incomplete and 4 questionable. These intact prophage regions ranged between 17.4 and 51.5 Kb, showing different numbers of coding sequences that varied between 24 and 88. In turn, LV27950 harbored 13 prophage regions: it displayed 10 intact regions spanning between 20.6 and 86.1 Kb. Globally, regions from five phages were present in the genomes of both and two were exclusive of each isolate (Table S1).

The detection of virulence factors in the genome of LV27950 revealed the presence of an increased serum survival factor provided by an ISS-encoding gene and a secreted autotransporter toxin denominated *sat* (Table 4). LV36464 shared the same virulence factors and, in addition, harbored a glutamate decarboxilase-encoding gene (*gad*). The overall estimation of pathogenicity factors present in the genome of the isolates, using known proteins with recognized involvement in pathogenicity as reference, enabled us to determine that the assembled contigs of LV36464 and LV27950 matched 553 and 544 pathogenic families,

which resulted in the estimation of both isolates being human pathogens (93.1 and 93.3%), confirming their zoonotic potential.

## DISCUSSION

The prevalence of antibiotic resistance genes in isolates from animal origin has been fairly assessed (Szmolka et al., 2011; Tamang et al., 2011; Bardon et al., 2013; Clemente et al., 2015). However, taking in account the current availability of genomic characterization tools, we are now able to proceed with more detailed characterizations of these genes, in a broader context. In this study, we characterized the genome of PMQR-producing *E. coli*. To understand the antibiotic susceptibility background of these specific isolates we have also evaluated the antibiotic susceptibility phenotypes of a collection of *S. enterica* and *E. coli* recovered from animals of different origins, in which the isolates were originally included.

The levels of non-wild type phenotypes revealed to be very distinct among *S. enterica* and *E. coli*. Non wild-type isolates for fluoroquinolones were particularly evident among poultry isolates recovered from *S. enterica*. Regarding *E. coli* isolates, the frequency of non-wild-type phenotypes to nalidixic acid and ciprofloxacin was higher in food-producing animals than in companion and zoo animals, which might be due to the high consumption of veterinary antibiotics in animal industrial units, particularly tetracyclines, sulphonamides, and fluoroquinolones (EFSA, 2015). Portugal still represents a European country with high antibiotic use in animals. This fact raises concerns regarding antibiotic resistance in veterinary settings (EMA, 2014). Different MIC<sub>50</sub> and MIC<sub>90</sub> (3-fold dilutions) were noted for some groups of each species: *E. coli* isolates for nalidixic acid, ciprofloxacin, ampicillin, streptomycin, tetracycline, sulfamethoxazole and trimethoprim, and *S. enterica* for ampicillin, cefotaxime, tetracycline, and trimethoprim.

Although, PMQR determinants are typically responsible by low level resistance, their presence has been increasingly reported in animals, resulting in an additional effect on the nonsusceptibility of fluoroquinolones (Ahmed and Shimamoto, 2013; Donati et al., 2014; Jamborova et al., 2015). The high MIC values of 128 mg/L against nalidixic acid and 8 mg/L against ciprofloxacin observed in some of the isolates of our collection may be associated with amino acid alterations in the quinolone resistance-determining region (QRDR). Indeed, although the fluoroquinolone non-susceptibility is frequently compromised by target modification, the PMQR-encoding genes have the potential to spread and promote co-selection of other antibiotic resistance genes (EMA, 2014). Late reports even suggest that the spread of PMQR may not be triggered by selection pressure, which justifies the low rates of these determinants in animals, despite the high use of fluoroquinolones (Veldman et al., 2011).

Considering the high level MICs, most likely caused by QRDR chromosomal mutations that might mask the presence of PMQR, we decided in this study to retrospectively search for these determinants in all isolates of the collection, regardless of the MIC value. We have detected four PMQR-encoding genes (4/180) (two *qnrS1* and two *aac(6')-Ib-cr*) in *E. coli* LV46221, LV46743, LV36464, and LV27950 recovered from animals of

different origins: a healthy turtle dove from a Zoo Park (2008), a diseased pet dog (2008), a bottlenose dolphin from a Zoo Park showing signs of respiratory infection (2009), and a second but healthy bottlenose dolphin from the same Zoo Park (2011) (**Table 4**).

The comparison of the genomes of QnrS1-producing *E. coli* revealed that isolates LV46221 and LV46743 were very similar in terms of their global pathogenicity potential, although they were recovered from animals of different classes and completely different backgrounds (**Table 4**). The absence of chromosomal mutations in the QRDR of isolates LV46221 and LV46743 corroborated the low fluoroquinolone MIC values obtained, which spanned between 0.38 mg/L for ciprofloxacin and 8 mg/L for nalidixic acid, highlighting the low level resistance conferred by QnrS1 determinants (Cavaco and Aarestrup, 2009). The plasmid region in which the *qnrS1* was enclosed in both isolates, that included the association with Tn3, has already been described in association with *qnrS1* genes in plasmids from *Shigella flexneri* recovered from food products, *Salmonella* Infantis from avian origin, and human clinical *Klebsiella pneumoniae* isolates, respectively (Hata et al., 2005; Chen et al., 2006; Kehrenberg et al., 2006). Moreover, we have previously detected other *qnrS1* from animals in Portugal, associated with a similar genetic environment, exclusively in food-producing animals (Jones-Dias et al., 2013). IncN plasmids harbored by LV46221 and LV46743 were assigned to ST1 by pMLST, which have also been associated with chickens and wild bird water in Czech Republic and the Netherlands, respectively (Ben Sallem et al., 2014).

Few genomic differences were noticed between the two *aac(6')-Ib-cr*- and *bla<sub>CTX-M-15</sub>*-harboring *E. coli*. In fact, the isolates could be assigned to the same genetic sublineage—ST131 *fimH30-Rx*, confirming clonal spread. Although, samples have been recovered within a reasonable time span of 2 years, their origin refers to two bottlenose dolphins of the same species held captive in the same Zoo Park. The presence of four chromosomal alterations in the QRDR region of isolates LV36464 and LV27950 was reflected in the high levels of fluoroquinolone MICs, which ranged between 8 and >256 mg/L. All antibiotic resistance genes detected in LV36464 and LV27950 [*aac(6')Ib-cr*, *bla<sub>OXA-1</sub>*, *bla<sub>TEM-1</sub>*, *bla<sub>CTX-M-15</sub>*, *catB3*, *aac(3)-IIa*, and *tetA*] could be traced back to a single multidrug resistance IncFIA plasmid that showed 99.9% of homology with a plasmid submitted this year to Genbank in U.S.A (JJ2434, unpublished). Although, 60 single nucleotide variants have been detected between the LV36464 and LV27950 plasmids, the main difference consisted of a single deletion that involved part of a transposase-encoding gene. The absence of a set of conjugation transfer proteins (*tra* genes), among other genes, highlighted the preponderance of clonal spread over horizontal gene transfer in ST131 *E. coli* (Nicolas-Chanoine et al., 2014). Although, several isoforms of identical plasmids have been detected worldwide, the simultaneous resistance to β-lactams, fluoroquinolones, aminoglycosides, chloramphenicol and tetracyclines has been a permanent feature, which reinforces the advantage that it confers (Boyd et al., 2004; Zhou et al., 2015). The detection of a ST131 *fimH30-Rx* *E. coli* in two dolphins, which are continuously in

contact with a live audience, constitutes a public health concern. These clinically relevant multidrug resistant *E. coli* isolates have been on the rise for years (Nicolas-Chanoine et al., 2014). Initially restricted to clinical contexts, recent findings suggest that their prevalence in non-clinical settings is maintained by the constant exchange of isolates throughout the time, as verified in this study (Mathers et al., 2015).

Although, *E. coli* is a common inhabitant of the gastrointestinal tract of humans and animals, the detected transposons, plasmids and bacteriophages are essential to the acquisition of pathogenicity factors that enlarge their ability to adapt to new niches, allowing bacteria to increase the capacity to cause a broad spectrum of diseases (Bien et al., 2012). All isolates displayed genomic factors that may be critical to cause a zoonotic infection and that were reflected in high probabilities for the isolates to be human pathogens (>93%). Concerning virulence factors, we detected the presence of glutamate decarboxylase, increased serum survival gene and a secreted autotransporter toxin, irregularly distributed across the four isolates (**Table 3**), which did not denote any relation with the conditions of their respective hosts. These virulence factors confer resistance to extreme acid conditions of the intestines, enable the isolate to survive complement system and cause defined damage to kidney epithelium, being indicative of their ability to cause disease (Johnson et al., 2008; Becker Saidenberg et al., 2012). Indeed, *E. coli* isolates can frequently encode a number of virulence factors, which enable the bacteria to colonize the urinary tract and face highly effective host defenses (Bien et al., 2012).

Although, fluoroquinolones are consistently used in veterinary medicine, results presented in this study indicate that PMQR determinants occurred at a low frequency in these isolates (2.2%), as previously reported (Donati et al., 2014; Jamborova et al., 2015). However, the studied groups of animals should still be considered potential reservoirs for PMQR-producing isolates, especially because there is the inherent potential for transboundary dissemination. These isolates presented a set of genetic features essential to promote their own successful spread: multiple antibiotic resistance genes carried by well-known mobile genetic elements, virulence factors adequate to zoonotic transmission and numerous other pathogenicity factors.

The analysis of many bacterial genomic features showed us great genetic relatedness between the two *qnrS1*- and *aac(6')-Ib-cr*-harboring isolates. The data gathered throughout this study illustrates two scenarios: the presence of the same strain in different hosts inhabiting remote locations and the persistence of a unique strain in a single niche during a long period of time. The strains were each associated with a case of symptomatic infection (LV46743 and LV36464) and with a report of microbiological control of an asymptomatic host (LV46221 and LV27950), which reinforces the ability of *E. coli* isolates to act as silent vehicles, allowing the accumulation of antibiotic resistance determinants, mobile genetic elements and other relevant pathogenicity determinants (Mathers et al., 2015). It is not certain whether these bacteria spread from humans to animals, between different animals or from the environment to animals. However, in the case of companion

animals, but particularly zoo animals, surveillance is essential to prevent continuous dissemination. The contact between animals and owners, zookeepers, visitors, and handlers raises concerns, considering that these bacteria might easily spread to humans and to other animals (Veldman et al., 2011; Ewers et al., 2012).

Permanent surveillance of health and sick animals should be strongly encouraged, regardless of their origin, in order to monitor future trends in the dissemination of resistance to fluoroquinolones and other antibiotics. Furthermore, this genome project contains valuable scientific data which will certainly be helpful for molecular epidemiological surveys of ST131 *E. coli* clonal group.

## AUTHOR CONTRIBUTIONS

DJD designed the study, acquired laboratory data, analyzed the data and wrote the manuscript. VM designed the study, analyzed the data and reviewed the manuscript. RG acquired laboratory data. DAS acquired laboratory data, TA, acquired laboratory or epidemiological data. PT acquired laboratory or epidemiological data. LV acquired laboratory data and reviewed the manuscript. EF acquired laboratory data and reviewed the

manuscript. LC acquired laboratory or epidemiological data and reviewed the manuscript. MC designed the study and reviewed the manuscript. All authors read and approved the final manuscript.

## FUNDING

These work was supported by Fundação para a Ciência e a Tecnologia (grant numbers PTDC/CVT/65713 and PEst-OE/AGR/UI0211/2011-2014). DJD has received research funding from Fundação para a Ciência e a Tecnologia (FCT, grant number SFRH/BD/80001/2011). VM was supported by FCT fellowship (grant SFRH/BPD/77486/2011), financed by the European Social Funds (COMPETE-FEDER) and national funds of the Portuguese Ministry of Education and Science (POPH-QREN).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00671>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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OPEN ACCESS

**Edited by:**

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University of Trás-os-Montes e Alto  
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equally to this work.

**Specialty section:**

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 29 July 2015

**Accepted:** 07 September 2015

**Published:** 23 September 2015

**Citation:**

Li P, Niu W, Li H, Lei H, Liu W, Zhao X,  
Guo L, Zou D, Yuan X, Liu H, Yuan J  
and Bai C (2015) Rapid detection  
of *Acinetobacter baumannii* and molecular epidemiology  
of carbapenem-resistant  
*A. baumannii* in two comprehensive  
hospitals of Beijing, China.  
*Front. Microbiol.* 6:997.  
doi: 10.3389/fmicb.2015.00997

# Rapid detection of *Acinetobacter baumannii* and molecular epidemiology of carbapenem-resistant *A. baumannii* in two comprehensive hospitals of Beijing, China

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*Acinetobacter baumannii* is an important opportunistic pathogen associated with a variety of nosocomial infections. A rapid and sensitive molecular detection in clinical isolates is quite needed for the appropriate therapy and outbreak control of *A. baumannii*. Group 2 carbapenems have been considered the agents of choice for the treatment of multiple drug-resistant *A. baumannii*. But the prevalence of carbapenem-resistant *A. baumannii* (CRAB) has been steadily increasing in recent years. Here, we developed a loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *A. baumannii* in clinical samples by using high-specificity primers of the *bla*<sub>OXA-51</sub> gene. Then we investigated the OXA-carbapenemases molecular epidemiology of *A. baumannii* isolates in two comprehensive hospitals in Beijing. The results showed that the LAMP assay could detect target DNA within 60 min at 65°C. The detection limit was 50 pg/μl, which was about 10-fold greater than that of PCR. Furthermore, this method could distinguish *A. baumannii* from the homologous *A. nosocomialis* and *A. pittii*. A total of 228 positive isolates were identified by this LAMP-based method for *A. baumannii* from 335 intensive care unit patients with clinically suspected multi-resistant infections in two hospitals in Beijing. The rates of CRAB are on the rise and are slowly becoming a routine phenotype for *A. baumannii*. Among the CRABs, 92.3% harbored both the *bla*<sub>OXA-23</sub> and *bla*<sub>OXA-51</sub> genes. Thirty-three pulsotypes were identified by pulsed-field gel electrophoresis, and the majority belonged to clone C. In conclusion, the LAMP method developed for detecting *A. baumannii* was faster and simpler than conventional PCR and has great potential for both point-of-care testing and basic research. We further demonstrated a high distribution of class D carbapenemase-encoding genes, mainly OXA-23, which presents an emerging threat in hospitals in China.

**Keywords:** *Acinetobacter baumannii*, LAMP, rapid diagnosis, carbapenem resistance, molecular epidemiology

## Introduction

*Acinetobacter baumannii* is an important opportunistic pathogen associated with a variety of nosocomial infections, such as ventilator-associated pneumonia, central line-associated bloodstream infections, urinary tract infections, surgical-site infections, and other types of wound infections, especially in intensive care units (ICUs; Peleg et al., 2008). This organism is well adapted to hospital environments, being capable of continually spreading to new patients and making itself a nosocomial pathogen of particular clinical concern and a public health threat (Dijkshoorn et al., 2007).

In recent years, multidrug-resistant *A. baumannii* (MDR-AB) isolates have been increasingly reported worldwide. MDR-AB strains are associated with an enhanced risk of mortality and prolonged durations of hospitalization (Kempf and Rolain, 2012; Antunes et al., 2014; Lemos et al., 2014). Carbapenems, mainly imipenem (IPM) and meropenem (MPM), have been used to treat MDR-AB infections (Perez et al., 2007). However, the incidence of carbapenem resistance in *A. baumannii* is growing steadily in China and many other countries (Poirel and Nordmann, 2006; Tiwari et al., 2012a,b). According to the CHINET 2010 year reports, the resistant to IPM and MPM in *A. baumannii* isolates was 62.3% and 63.8%, respectively (Xi et al., 2012). However, few data are available regarding the molecular epidemiology and antibiotic resistance of *A. baumannii* infections in hospitals in Beijing, China.

The accurate and rapid identification of *A. baumannii* is critical for appropriate infection control in hospital settings. To date, the most common and widespread detection methods include characterization via a phenotypic system and commercial phenotypic methods (e.g., the VITEK 2 system [Biomerieux] and the API 20 NE system) or DNA-based testing such as PCR (e.g., 16S rRNA gene amplification), which have been used to successfully identify most *Acinetobacter* species. However, there are some limitations in these methods (Janda and Abbott, 2007; Karah et al., 2011; Álvarez-Buylla et al., 2012). For example, several days are needed for incubation, and the laboratory diagnosis of *A. baumannii* is actually for that of the *A. calcoaceticus*-*A. baumannii* complex (ABC), which includes *A. calcoaceticus*, *A. baumannii*, *A. pittii* (three species), and *A. nosocomialis* (species 13TU; Peleg et al., 2008). These bacterial species are different in terms of symptomatology, dissemination patterns, mechanisms of antibiotic resistance, and epidemiology (Golanbar et al., 2011; Chiang et al., 2012).

The loop-mediated isothermal amplification (LAMP) method, which is based on autocycling strand displacement DNA synthesis in the presence of Bst DNA polymerase, can be used to amplify target DNA with high specificity (as four or six specific primers that recognize six or eight different sequences on the DNA target) under isothermal conditions in less than 60 min (Notomi et al., 2000). LAMP is highly sensitive and able to detect DNA at as few as six copies in the reaction mixture, and less prone to the presence of irrelevant DNA than PCR (Notomi et al., 2000). This novel method has been developed widely for the detection of numerous pathogens, including influenza

A subtypes H1N1 (Nakauchi et al., 2011), H5N1 (Dinh et al., 2011), H7N9 (Nakauchi et al., 2014), as well as *Mycoplasma pneumoniae* (Gotoh et al., 2013), *Mycobacterium tuberculosis* (Kumar et al., 2014), severe acute respiratory syndrome virus coronavirus (Poon et al., 2004), and human immunodeficiency virus (Zeng et al., 2014).

In present study, we describe a LAMP method for the rapid detection of *A. baumannii* in clinical samples targeting the *blaOXA-51* gene, which was based on visual testing. We also investigated molecular-epidemiology and antibiotic-resistance profiles, and detected several common oxacillinase genes from *A. baumannii* isolates obtained at two comprehensive hospitals in Beijing, China.

## Materials and Methods

### Bacterial Strains, Antimicrobial Susceptibility Testing, and Pulsed-Field Gel Electrophoresis (PFGE)

A total of 34 strains representing 9 *Acinetobacter* species (2 *A. baumannii* strains and 10 other *Acinetobacter* strains) and 22 non-*Acinetobacter* species used in this study to develop the LAMP assays. Three hundred and fifty-five clinical sputum samples and nasopharyngeal swabs were obtained from the ICU hospitalized patients with clinically suspected multi-resistant infections in 307th (Affiliated Hospital of Academy of Military Medical Sciences), and the 309th Hospital of PLA in China. Pertinent information and the source of all strains are listed in Table 1. The species identification were identified by the Vitek 2 system (Biomerieux Vitek, Inc., Hazelwood, MO, USA). *A. baumannii* bacteria were grown overnight at 37°C in Luria-Bertani (LB) broth, while non-*Acinetobacter* species were cultured at 37°C in brain heart infusion (BHI) broth overnight.

Antibiotic susceptibility testing was conducted by disk diffusion in accordance with the guidelines of the Clinical and Laboratory Standards Institute and was confirmed by the Vitek 2 System with the following antibiotics: ampicillin, ampicillin/sulbactam, aztreonam, ciprofloxacin, ceftazidime, ceftriaxone, cefepime, gentamycin, levofloxacin, IPM, MPM, piperacillin/tazobactam, piperacillin, tobramycin, and sulfamethoxazole. *Pseudomonas aeruginosa* ATCC 27853 was used as a control.

Genomic DNA was prepared in agarose plugs, and digested with the restriction enzyme *Apa*I (New England BioLabs, Beverly, MA, USA). The DNA restriction fragments were separated using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA, USA). Gel images were analyzed using BioNumerics software, version 6.01 (Applied-Maths, Belgium). The interpretation of gels was performed by visual inspection using the criteria of Tenover et al. (1995).

### Preparation of Isolated DNA

Genomic DNA from *Acinetobacter* species was prepared using the boiling method described by Olive and Bean (1999), with some modifications. Briefly, colonies of each isolate were picked from LB plates and suspended in 100 µl of ddH<sub>2</sub>O. The bacterial

**TABLE 1 |** Bacteria strains used in this study.

Bacteria strains	Source	Strain type
<b>Acinetobacter species</b>		
<i>A. baumannii</i>	CGMCC	Reference strain
<i>A. baumannii</i> ATCC 22933	CICC	Reference strain
<i>A. calcoaceticus</i>	CGMCC	Reference strain
<i>A. nosocomialis</i>	CGMCC	Reference strain
<i>A. haemolyticus</i>	CGMCC	Reference strain
<i>A. Pittii</i>	CICC	Reference strain
<i>A. johnsonii</i>	CGMCC	Reference strain
<i>A. junii</i>	CGMCC	Reference strain
<i>A. calcoaceticus</i>	The Microorganism Center <sup>a</sup>	Reference strain
<i>A. Iwoffii</i>	The Microorganism Center (three strains)	Reference strain
<b>Non-Acinetobacter species</b>		
<i>Bacillus megatherium</i> 4623	The Microorganism Center	Reference strain
<i>Beta-haemolytic streptococcus</i> group A CMCC32213	The Microorganism Center	Reference strain
<i>Bordetella pertussis</i> ATCC 18530	The Microorganism Center	Reference strain
<i>Brucella suis</i> 3572	The Microorganism Center	Reference strain
<i>Corynebacterium diphtheria</i> CMCC38001	The Microorganism Center	Reference strain
<i>Enteropathogenic Escherichia coli</i> 2348	The Microorganism Center	Reference strain
<i>Enterotoxigenic E. coli</i> 44824	The Microorganism Center	Reference strain
<i>E. coli</i> ATCC25922	The Microorganism Center	Reference strain
<i>Mycobacterium tuberculosis</i> 8362	The Microorganism Center	Reference strain
<i>Neisseria meningitidis</i> group B CMCC29022	The Microorganism Center	Reference strain
<i>Pseudomonas aeruginosa</i> ATCC27853	The Microorganism Center	Reference strain
<i>Salmonella aberdeen</i> 9264	The Microorganism Center	Reference strain
<i>Salmonella enteritidis</i> 50326-1	The Microorganism Center	Reference strain
<i>Stenotrophomonas maltophilia</i> 3859	The Microorganism Center	Reference strain
<i>Shigella flexneri</i> 4536	The Microorganism Center	Reference strain
<i>Shigella sonnei</i> 2531	The Microorganism Center	Reference strain
<i>Staphylococcus aureus</i> 2740	The Microorganism Center	Reference strain
<i>Vibrio carchariae</i> 5732	The Microorganism Center	Reference strain
<i>Vibrio cholera</i> 3802	The Microorganism Center	Reference strain
<i>Vibrio parahaemolyticus</i> 5474	The Microorganism Center	Reference strain
<i>Yersinia enterocolitica</i> 1836	The Microorganism Center	Reference strain
<i>Yersinia pestis</i> 2638	The Microorganism Center	Reference strain

CGMCC, China General Microbiological Culture Collection Center; CICC, China Center of Industrial Culture Collection.

<sup>a</sup>The Microorganism Center of the Institute of Disease Control and Prevention of the Academy of Military Medical Sciences.

suspensions were boiled at 95°C for 15 min, centrifuged at 12,000 rpm for 5 min, and the supernatants were collected and transferred to fresh Eppendorf tubes as templates for use in LAMP and PCR assays. To determine the sensitivity and specificity of the LAMP assays, bacterial genomic DNA was extracted from *A. baumannii* using the Wizard® Genomic DNA Purification Kit A1125 (Promega, Madison, WI, USA). The DNA concentration was measured by using OD260 measurements (ND-1000 spectrophotometer, NanoDrop Technologies, Inc., Wilmington, DE, USA) and prepared by serial 10-fold dilutions to yield concentrations ranging from 500 ng/μl to 0.05 pg/μl.

### Primer Design for LAMP Assay

The sequence of *blaOXA-51* (GenBank No. DQ385606.1) was downloaded from the NCBI GenBank database and used to design *blaOXA-51*-specific LAMP primers. The sequence was further analyzed by Primer Explorer software (version 4;

<http://www.primerexplorer.jp/lamp/>), and five sets of primers were designed, including the outer forward primer (F3), the outer backward primer (B3), the forward inner primer (FIP), the backward inner primer (BIP), and additional loop primers (loops F and B), as shown in Table 2.

### LAMP Reaction and Product Detection

LAMP reactions were performed in a total volume of 25 μl and contained 12.5 μl reaction mixtures (DNA Amplification Kit; Eiken Chemical Co., Ltd., Tochigi, Japan), 2.6 μl primer mixture (40 pmol for FIP and BIP, 20 pmol for LF and LB, and 5 pmol for F3 and B3), and 1 μl of *Bst* polymerase (eight units, New England BioLabs, Ipswich, MA, USA). Finally, 1-μl DNA of genomic template DNA (the concentration is above 50 ng/μl) was added to the reaction tubes. Reaction was performed in a LA-320CE instrument (Eiken Chemical Co., Ltd., Tochigi, Japan) at 65°C for 60 min and stopped by heating to 80°C for 5 min, according

**TABLE 2 | Sequence of primers used for specific amplification of *bla<sub>OXA-51-like</sub>* and the OXA carbapenemases PCR detection.**

Primer	Primer type	Sequence (5'-3')
OXA-4F3	Forward outer	CTTATATAGTGACTGCTAACCAA
OXA-4B3	Backward outer	ATTAAGCATTGAGGTGCA
OXA-4FIP	Forward inner	ACCCGTAGTGTGACTTCGTTAA
		ATTTTACAGCGCTTAAATCTGA
OXA-4BIP	Backward inner	TTAGTTATCCAACAAGGCCAAACTT
		TTAGCAGGTACATACTCGGTC
OXA-4LB	Loop forward	AAAGCTATGGTAATGATCTTGCTCG
<i>bla<sub>OXA-23</sub>-F</i>	Forward	ATGAATAAATTTACTTG
<i>bla<sub>OXA-23</sub>-R</i>	Reverse	TTAAATAATATTCAAGCTGTT
<i>bla<sub>OXA-24</sub>-F</i>	Forward	ATGAAAAAATTATACTTCTCT
		ATATTCAAGC
<i>bla<sub>OXA-24</sub>-R</i>	Reverse	TTAAATGATTCCAAGATTTCTAGC
<i>bla<sub>OXA-51-like</sub>-F</i>	Forward	TAATGCTTGTGATCGGCCTTG
<i>bla<sub>OXA-51-like</sub>-R</i>	Reverse	CTATAAAATACCTAATTCTCTAA
<i>bla<sub>OXA-58</sub>-F</i>	Forward	ATGAAATTATTAAGGATATTGAGT
<i>bla<sub>OXA-58</sub>-R</i>	Reverse	ATAAATAATGAAAACACCCAA

to the manufacturer's instructions. The LA-320CE instrument can monitor the turbidity of real-time LAMP products through spectrophotometric analysis by recording the optical density at 650 nm every 6 s with the help of a Loopamp real-time turbidimeter. In addition, a visual color-change detection method was employed. Briefly, 1  $\mu$ l of fluorescence-detection reagent (Eiken Chemical Co., Ltd., Tochigi, Japan) was added to each 25  $\mu$ l LAMP-reaction mixture prior to initiating the reactions. Positive reactions were identified by a green color change, while negative reactions remained orange in color. The color change could be observed by the naked eye under natural light, or with the aid of ultraviolet light excitation at 365 nm.

To compare the sensitivity and specificity of LAMP with PCR, normal PCR reactions were performed using the *bla<sub>OXA-51-like</sub>-F* and *bla<sub>OXA-51-like</sub>-R* primers (Table 2).

The primers were synthesized commercially by Sangon Biotech Co., Ltd. (Beijing, China). PCR was performed as described previously (Kuo et al., 2012).

## PCR Detection of Carbapenemase Genes

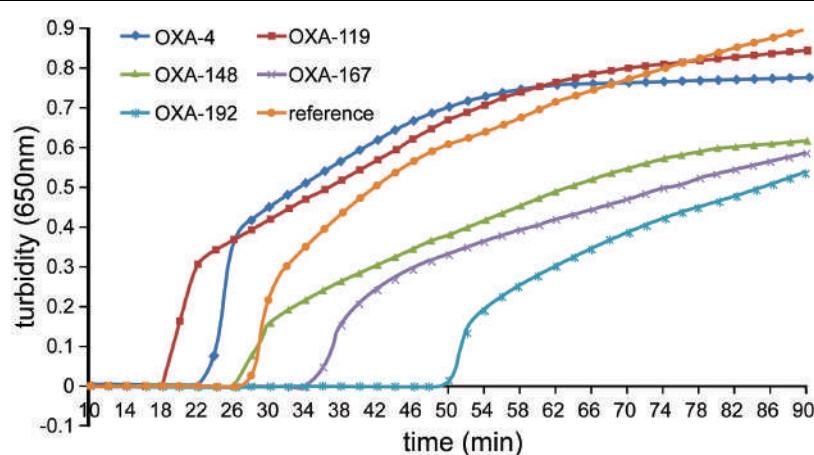
The class D OXA carbapenemases of *Acinetobacter* sp. are represented by 4 main phylogenetic subgroups: OXA-23-like, OXA-24-like, OXA-51-like, and OXA-58. The primers used for detecting these genes by PCR are listed in Table 2. PCR was performed in a 25- $\mu$ l reaction mixtures containing 12.5  $\mu$ l PCR Master Mix Reagent (Tiangen Biotech Co., Ltd., Beijing, China); 1  $\mu$ l of forward primer (10 pmol), 1  $\mu$ l of reverse primer (10 pmol), and 1  $\mu$ l of DNA template. Reaction mixtures were initially heated to 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The final extension step was performed at 72°C for 7 min. The PCR-amplified products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. Images were acquired using a Bio-Rad Gel Doc EQ imaging system.

## Results

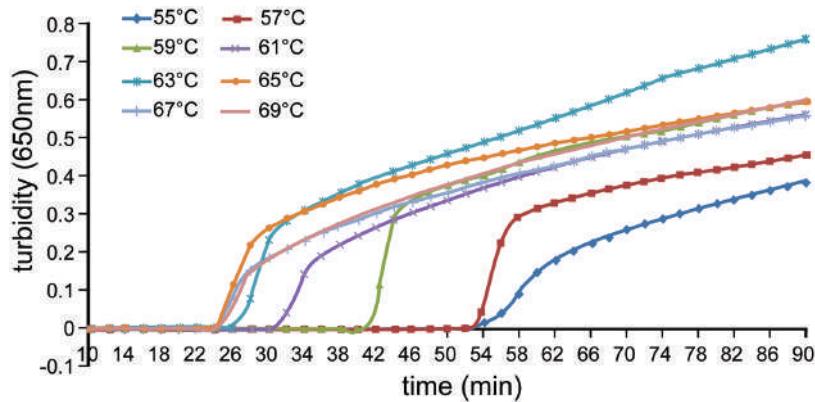
### Optimization of the LAMP Assay Targeting *bla<sub>OXA-51-like</sub>*

Five sets of different primers were initially tested for detection of the *bla<sub>OXA-51-like</sub>* gene, and the primers for *pgaD* gene of *A. baumannii* were added as a control (Wang et al., 2013). Four of the five primer sets enabled successful amplification (Figure 1). The OXA-4 primer set amplified the target sequence within the shortest time and was, therefore, chosen as the optimal primer set (Table 2).

Reaction temperatures ranging from 55 to 69°C at a 2°C intervals were compared to determine optimal amplification conditions. The amplification efficiency was highest at 65°C (Figure 2) and was, therefore, used in subsequent experiments.



**FIGURE 1 | Five sets of primers were used to amplify the indicated target genes of *Acinetobacter baumannii* under the same conditions.** Turbidity was monitored using a Loopamp real-time turbidimeter by measuring the absorbance at 650 nm every 6 s. The OXA-4 primer set was chosen as the most appropriate primers for the rapid detection of *A. baumannii*.



**FIGURE 2 | Effect of differing temperatures on the efficiency of detection of *A. baumannii* by loop-mediated isothermal amplification (LAMP).** Turbidity was monitored using a Loopamp real-time turbidimeter by measuring the absorbance at 650 nm every 6 s.

### Specificity and Sensitivity of the LAMP Assay

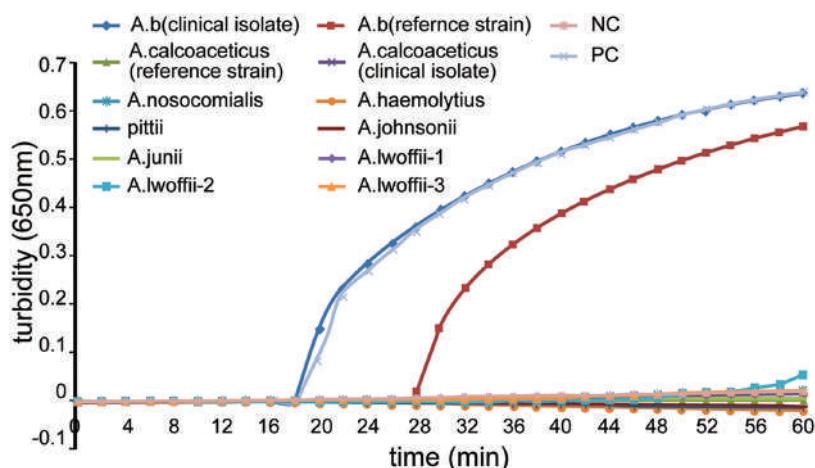
To evaluate the specificity of LAMP detection for *A. baumannii*, genomic DNA was extracted from 2 *A. baumannii* strains, as well as other 7 *Acinetobacter* species (10 strains) and 22 non-*A. baumannii* reference strains, and tested using real-time turbidity or visual detection of color changes as readouts. Genomic *A. baumannii* DNA (ATCC 22933) and distilled water were used as positive and negative controls, respectively. As shown in Figure 3, both methods of analysis positively identified the *A. baumannii* isolates. All other strains (as well as the blank control) tested negatively, indicating that the LAMP assay was specific for *A. baumannii*. Interestingly, the LAMP assay could differentiate *A. baumannii* from other *Acinetobacter* species.

To compare the detection limit of traditional PCR with that of LAMP using either real-time turbidity or color-change measurements, 10-fold serial dilutions (50 ng/ $\mu$ l–5 pg/ $\mu$ l) were tested using genomic DNA extracted from *A. baumannii* ATCC 22933. As shown in Figure 4, the detection limits of real-time

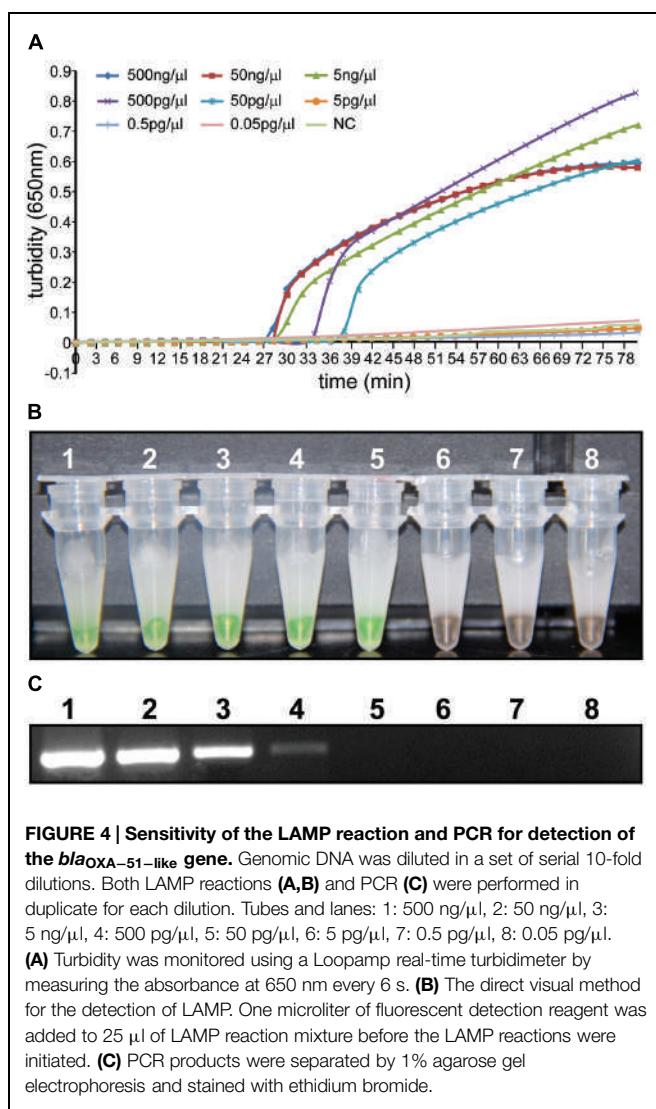
turbidity and visual detection were both 50 pg/ $\mu$ l, which was 10-fold more sensitive than traditional PCR assay.

### Detection of *A. baumannii* in Clinical Samples

A total of 355 clinical sputum samples and nasopharyngeal swabs were collected for LAMP-based surveillance of *bla*<sub>OXA-51-like</sub> from ICU patients suspected of having multidrug-resistant infections in two hospitals in Beijing, China. Ten pairs of sputum samples and nasopharyngeal swabs from healthy people were collected as controls. All clinical samples were simultaneously analyzed by LAMP and PCR. Of the 355 clinical samples, the LAMP assay detected 228 positive samples and 127 negative samples, while the PCR assay detected 221 positive samples, and 134 negative samples. Then, *A. baumannii* was successfully cultured from all 228 samples that were positively identified by LAMP. Samples from the healthy control subjects all tested negatively by LAMP and PCR. The LAMP assay showed 100% specificity compared to 91.89% by PCR assay. Thus, the results



**FIGURE 3 | Specificity of the LAMP reactions in detecting the *bla*<sub>OXA-51-like</sub> gene.** Turbidity was monitored using a Loopamp real-time turbidimeter by measuring the absorbance at 650 nm every 6 s. Amplification was performed at 65°C for 60 min.



showed the LAMP assays were more sensitive and specific than PCR for the diagnosis of *A. baumannii* in clinical samples.

### Antibiotic Susceptibility and Oxacillinase Distribution

A total of 228 clinical *A. baumannii* isolates were characterized by antibiotic susceptibility testing using both the VITEK®2 system and disk diffusion. Resistance rates were very high, with nearly 90% isolates being resistant to ampicillin, 68.2% being MDR-AB, 66.5% being resistant to IPM, and 63.6% being resistant to MPM.

Among all 145 isolates resistant to both IPM and MPM, 100% harbored the *bla<sub>OXA-51-like</sub>* gene, 92.3% (134 strains) co-occurred with the *bla<sub>OXA-23</sub>* gene, 1 isolate carrying both the *bla<sub>OXA-51-like</sub>* gene and *bla<sub>OXA-58</sub>* gene, and no isolate carried the *bla<sub>OXA-24</sub>* gene. Ten isolates were negative for the tested plasmid-mediated OXA-carbapenemase genes.

We also investigated the presence of the *bla<sub>OXA-51-like</sub>* gene in *A. baumannii* isolates susceptible to carbapenem treatment. All of these isolates harbored the naturally occurring *bla<sub>OXA-51-like</sub>*

gene, making it an excellent candidate for the identification of *Acinetobacter* species.

### Molecular Epidemiology of Carbapenem-Resistant *A. baumannii*

Forty-one out of 145 carbapenem-resistant *A. baumannii* isolates were analyzed by PFGE. Genomic DNA digested with *Apa*I showing similar patterns were designated as the same type, and those with 1–3 different bands compared with this type were designated as subtypes. Digested DNA with >3 different bands compared with one type were designated as other types. Bionumerics software V6.01 was used to perform cluster analysis of these data.

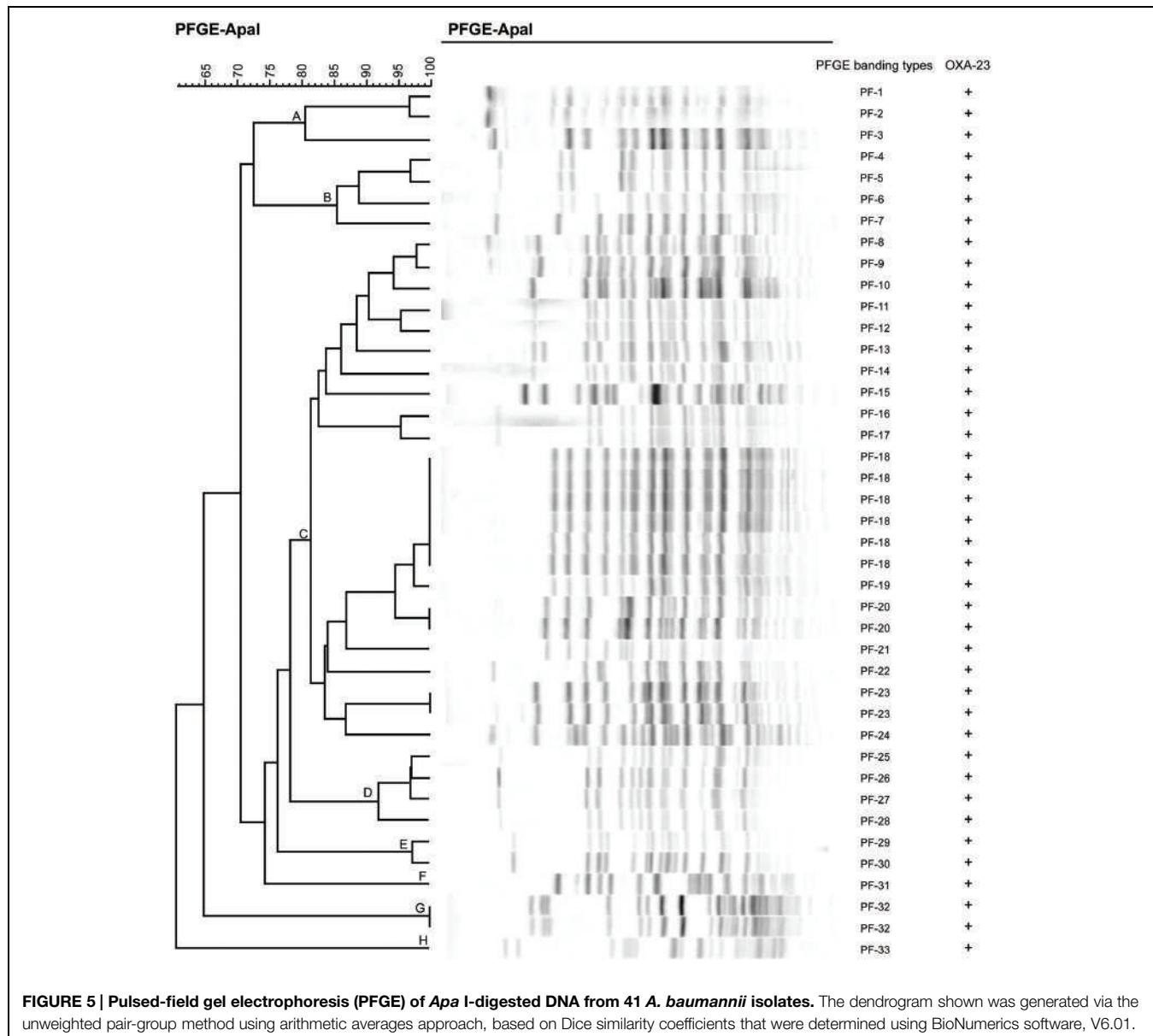
A total of 33 PFGE-banding types were identified, suggesting a diverse population of *A. baumannii* rather than the spread of a specific clone. According to the dice similarity index (80%), these types could be clustered into eight distinct PFGE patterns (clones A–H), and clone C was the dominant clone with 24 isolates (Figure 5). Clinical isolates with PFGE-banding type 18 (PF-18) represented the dominant epidemic strain in the ICU during the testing period.

### Discussion

Carbapenems including IPM and MPM are used as a last resort for treating MDR-AB. The incidence of carbapenem resistance in *A. baumannii* has increased steadily over the past decade and now represents a global problem. The Meropenem Yearly Susceptibility Test Information Collection program revealed a considerable worldwide increase in IPM and MPM resistance rates, which increased from 10 and 35% in 1999 to 47.9 and 57.4% in 2008, respectively (Rhomburg and Jones, 2009). Similarly, the SENTRY program documented an overall increase in IPM resistance from 34.5% in 2006 to 59.8% in 2009 (Gales et al., 2011). In the USA and Europe, carbapenem resistance accounted for 65% of *A. baumannii*-related pneumonia in 2012 (Farrell et al., 2014). In Asia, more than 60% of *A. baumannii* isolates causing hospital-acquired pneumonia were pan-resistant bacteria and resistant to carbapenem (Zarrilli et al., 2013; Tiwari and Moganty, 2014).

In this study, we observed that 66.5% (152 strains) or 63.6% (145 strains) of test isolates were resistant to IPM or MPM, respectively. The 228 clinical *A. baumannii* isolates were mainly collected from the ICUs of two hospitals; thus, the incidence of carbapenem resistance in *A. baumannii* was relatively high. Prolonged hospitalization, invasive medical procedures, and the prior use of broad-spectrum antibiotics (third-generation cephalosporins and fluoroquinolones) are risk factors for the acquisition of MDR-AB (Karageorgopoulos and Falagas, 2008). These findings indicated that treatment with carbapenem may potentially induce carbapenem resistance.

During PFGE analysis, 33 PFGE-banding patterns were identified and classified into 8 distinct clones, based on Tenover's criteria (Tenover et al., 1995). Clone C was the dominant clone, making it possibly responsible for the *A. baumannii* epidemic in the ICUs of the two comprehensive hospitals



**FIGURE 5 | Pulsed-field gel electrophoresis (PFGE) of *Apa I*-digested DNA from 41 *A. baumannii* isolates.** The dendrogram shown was generated via the unweighted pair-group method using arithmetic averages approach, based on Dice similarity coefficients that were determined using BioNumerics software, V6.01.

from which we obtained the isolates. Clinical isolates of subtype PF-18 represented the main epidemic strain during the testing period. Previous results have demonstrated that carbapenem-resistant *A. baumannii* isolates showing the same PFGE patterns possess the same carbapenemase-associated genes (Yan et al., 2010; Park et al., 2012); however, our results were not consistent with these observations. Three isolates carrying only the *bla<sub>OXA-51</sub>*-like gene, but not the *bla<sub>OXA-23</sub>* gene, were grouped into different clones. However, isolates carrying both the *bla<sub>OXA-51</sub>*-like gene and the *bla<sub>OXA-23</sub>* gene were clustered into distinct clones, either. This may be due to the complicated resistance mechanism that underlies carbapenem resistance in *A. baumannii*.

Carbapenem susceptibility in *A. baumannii* is compromised by a variety of mechanisms. By far the most common mechanism

are carbapenemases (Pogue et al., 2013). Carbapenem-hydrolyzing class D  $\beta$ -lactamases (CHDLs, Ambler class D), also known as oxacillinas, are the most common mediator of carbapenem resistance in *A. baumannii* (Poirel and Nordmann, 2006), although they are relatively inefficient compared with other types of carbapenemases, such as class B metallo- $\beta$ -lactamases (MBLs; Ambler class B) represented by the VIM, IMP, SIM families, and the recently discovered New Delhi metallo- $\beta$ -lactamase (NDM).

Among CHDLs in *A. baumannii*, there are four major distinct groups, including OXA-23, OXA-24, OXA-51-like, and OXA-58, which are encoded by the *bla<sub>OXA-23</sub>*, *bla<sub>OXA-24</sub>*, *bla<sub>OXA-51</sub>*-like, and *bla<sub>OXA-58</sub>* genes, respectively (Poirel and Nordmann, 2006; Zavascki et al., 2010). The *bla<sub>OXA-23</sub>* gene has been reported in *A. baumannii* isolates worldwide and

is mediated both by chromosomal integration and plasmids. The *bla*<sub>OXA-24</sub> gene can also be localized to chromosomes or plasmids, appears to be less widespread than the *bla*<sub>OXA-23</sub> gene, and is relatively rare in China (Poirel et al., 2010). The chromosomally located *bla*<sub>OXA-51-like</sub> gene is unique and naturally occurring in *A. baumannii*; thus, this gene is becoming an important genetic marker for the identification of organisms at the species level (Turton et al., 2006, ). The plasmid-mediated *bla*<sub>OXA-58</sub> gene has been reported in *Acinetobacter* sp. from many countries around the world, but is rare in China.

In this research, we found that in all the 145 CRAB isolates, the co-occurrence of *bla*<sub>OXA-51-like</sub> gene and *bla*<sub>OXA-23</sub> gene could be detected in 134 isolates (92.3%). This result indicated that the plasmid-mediated transfer of *bla*<sub>OXA-23</sub> gene might be the most common mechanism by which *A. baumannii* becomes carbapenem resistance in these two comprehensive military hospitals in Beijing. Although the *bla*<sub>OXA-58</sub> gene and *bla*<sub>OXA-24</sub> gene have been reported in many countries, but the prevalence of these two genes is relatively rare in China. That could be explained that only one isolate carried the *bla*<sub>OXA-58</sub> gene and no *bla*<sub>OXA-24</sub> gene was detected in the CRAB isolates. However, 10 isolates could detect none of the plasmid-mediated OXA-carbapenemase genes including *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-24</sub> and *bla*<sub>OXA-58</sub>, which indicated that these isolates might harbored other resistant genes such as MBLs against carbapenem. These data are consistent with other reports from China and other countries (Turton et al., 2006; Woodford et al., 2006; Zavascki et al., 2010; Tiwari et al., 2012a).

In the conventional PCR assay, the *bla*<sub>OXA-51-like</sub> gene could not be amplified from seven clinical isolates. We thought that the low bacterial contents and incorrect pathogen identification may account for the failure to amplify these samples. Thus, the seven strains were analyzed for the presence of the *bla*<sub>OXA-51-like</sub> gene by the LAMP method described here, yielding positive results in each case, which was confirmed using the Vitek 2 system. Our results showed that these clinical isolates were actually *A. baumannii*. These results suggest that occasionally the sensitivity of conventional PCR is not satisfactory and may introduce errors into diagnostic test results.

Compared with traditional methods for pathogen diagnosis, LAMP assays can generate results more easily and rapidly, with high sensitivity and specificity (Notomi et al., 2000). The LAMP reaction does not require temperature cycling, with just a temperature-controlled water bath or a constant-temperature environment being sufficient. LAMP primers are designed to match four or six of the six or eight independent target-sequence regions, so the specificity and sensitivity are enhanced greatly. Under optimum conditions, the LAMP reaction can be completed in 1 h. In this study, we described a LAMP assay based on the *bla*<sub>OXA-51-like</sub> gene for detecting *A. baumannii*. This LAMP method could detect the target DNA within 60 min at an isothermal temperature of 65°C. The detection limit of the LAMP assay was 50 pg/μl, which was about 10-fold greater than that of PCR. Furthermore, the LAMP method described here could distinguish *A. baumannii* from *A. nosocomialis* (genospecies 13TU), *A. pittii* (genospecies 3), and the *A. Baumannii* complex (*A. baumannii*, *A. nosocomialis*, *A. pittii*, and *A. calcoaceticus*).

Previously, there were two reports on the use of an *A. baumannii* LAMP assay, based on targeting the 16S–23S rRNA intergenic spacer sequence (Soo et al., 2013) and the conserved regions of the *pgdA* gene of *A. baumannii* (Wang et al., 2013). However, the former could not effectively distinguish *A. baumannii* from *A. nosocomialis*, and *A. pittii*, and the specificity of *A. baumannii* LAMP assay of the latter was only 75%.

## Conclusion

A rapid, sensitive, specific, and effective LAMP assay was established for the detection of *A. baumannii*. The LAMP assay will be very useful for the rapid detection of pathogens in clinical samples. Meanwhile, this report provides some therapeutic recommendations for the treatment of *A. baumannii* infection and a warning that the growing emergence of carbapenem-resistant *A. baumannii* has led to an inadequacy of therapeutic choices in treating MDR-AB infections among patients in China. Our findings further emphasize that when investigating outbreaks caused by carbapenem-resistant *A. baumannii*, both the detection of carbapenemase-associated genes and PFGE are needed.

## Author Contributions

Puyuan Li and Wenkai Niu wrote the main manuscript text. Puyuan Li, Huan Li, and Wei Liu prepared **Figures 1–4**. Leijing Guo prepared **Figure 5**. Puyuan Li, Wenkai Niu, Hong Lei, Xiangna Zhao, Dayang Zou, Xin Yuan, and Huiying Liu executed the experiments. Changqing Bai and Jing Yuan helped conceive the project and designed the experiments. All authors reviewed the manuscript.

## Funding

This project was supported by the Beijing Natural Science Foundation (Grant No.7142118), a Translational Medicine Project from the Academy of Military Medical Science in 2013, Mega-projects of Science and Technology Research of China Grant 2013ZX10004-203, and Capital Characteristic Clinic project of Beijing (Z121107001012127).

## Acknowledgments

We thank the 309th Hospital of PLA and Institute of Disease Control and Prevention, Academy of Military Medical Sciences for kindly providing clinical isolates and reference strains. We are grateful to Yin Xiuyun from the Department of Clinical Laboratory of the 307th Hospital of PLA for performing the collection of isolates. The analysis of PFGE by Wang Jian from the Institute of Disease Control and Prevention, Academy of Military Medical Sciences (Beijing, China) is gratefully appreciated. We also thank Wei Xiao, Cui Qian, Yang Zhan, Yan Xiabei, and Dong Derong for assistance during the course of this research.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Regulation of the Two-Component Regulator CpxR on Aminoglycosides and $\beta$ -lactams Resistance in *Salmonella enterica* serovar Typhimurium

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### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 15 December 2015

**Accepted:** 11 April 2016

**Published:** 27 April 2016

### Citation:

Huang H, Sun Y, Yuan L, Pan Y,  
Gao Y, Ma C and Hu G (2016)

Regulation of the Two-Component Regulator CpxR on Aminoglycosides and  $\beta$ -lactams Resistance in *Salmonella enterica* serovar Typhimurium. *Front. Microbiol.* 7:604.  
doi: 10.3389/fmicb.2016.00604

The two-component signal transduction system CpxAR is especially widespread in Gram-negative bacteria. It has been reported that CpxAR contributes to the multidrug resistance (MDR) in *Escherichia coli*. CpxR is a response regulator in the two-component CpxAR system. The aim of this study was to explore the role of *cpxR* in the MDR of *S. enterica* serovar Typhimurium. The minimal inhibitory concentrations (MICs) of various antibiotics commonly used in veterinary medicine for strains JS (a multidrug-susceptible standard strain of *S. enterica* serovar Typhimurium), JSΔ*cpxR*, JSΔ*cpxR/pcpxR*, JSΔ*cpxR/pcpxR\**, JSΔ*cpxRΔacrB*, JSΔ*cpxRΔacrB/pcpxR*, JSΔ*cpxRΔacrB/pcpxR\**, 9 *S. enterica* serovar Typhimurium isolates (SH1–9), and SH1–9Δ*cpxR* were determined by the 2-fold broth microdilution method. The relative mRNA expression levels of *ompF*, *ompC*, *ompW*, *ompD*, *tolC*, *acrB*, *acrD*, *acrF*, *mdtA*, *marA*, and *soxS* in strains JS, JSΔ*cpxR*, and JSΔ*cpxR/pcpxR* were detected by real-time PCR. The results showed 2- to 4-fold decreases in the MICs of amikacin (AMK), gentamycin (GEN), apramycin (APR), neomycin (NEO), ceftriaxone (CRO), ceftiofur (CEF), and cefquinome (CEQ) for strain JSΔ*cpxR*, as compared to those for the parental strain JS. Likewise, SH1–9Δ*cpxR* were found to have 2- to 8-fold reduction in resistance to the above antibiotics, except for NEO, as compared to their parental strains SH1–9. Furthermore, 2- to 4-fold further decreases in the MICs of AMK, GEN, APR, and CEF for strain JSΔ*cpxRΔacrB* were observed, as compared to those for strain JSΔ*acrB*. In addition, CpxR overproduction in strain JSΔ*cpxR* led to significant decreases in the mRNA expression levels of *ompF*, *ompC*, *ompW*, *ompD*, *tolC*, *acrB*, *marA*, and *soxS*, and significant increases in those of *stm3031* and *stm1530*. Notably, after all strains were induced simultaneously by GEN to the 15th passage at subinhibitory concentrations, strain JSΔ*cpxR/pcpxR* showed significant increases in mRNA expression levels of the efflux pump *acrD* and *mdtA* genes, as compared to strain JSΔ*cpxR*. Our results indicate that the two-component regulator CpxR contributes to resistance of *S. enterica* serovar Typhimurium to aminoglycosides and  $\beta$ -lactams by influencing the expression level of the MDR-related genes.

**Keywords:** *S. enterica* serovar Typhimurium, CpxR, aminoglycosides,  $\beta$ -lactams, resistance, AcrD

## INTRODUCTION

*Salmonella enterica* serovar Typhimurium is a food-borne pathogen that causes gastroenteritis in humans (Scherer and Miller, 2001) and fowl typhoid in poultry (Barrow et al., 2004). The prevalence of multidrug-resistant (MDR) *S. enterica* species in many parts of the world has become a significant public health concern. Drug resistance in many cases is attributable to synergy between reduced drug intake (mainly due to low outer membrane permeability) (Pagès et al., 2008; Li and Nikaido, 2009) and active drug export (via efflux pumps) (Zgurskaya and Nikaido, 2000; Pagès et al., 2010). Resistance nodulation-cell division (RND)-family efflux systems (including AcrAB, AcrAD, AcrEF, MdtEF, and MdtABC) are especially effective in generating resistance in Gram negative bacteria (Nikaido, 1996) and often have a wide substrate specificity (Nikaido and Pagès, 2012). In *Escherichia coli*, all five RND-family drug exporters confer resistance to β-lactam antibiotics (Nishino et al., 2003), and acrD is also known to participate in the efflux of aminoglycosides (Rosenberg et al., 2000; Nishino and Yamaguchi, 2001a; Aires and Nikaido, 2005; Nishino et al., 2007). Some outer membrane proteins, especially OmpF, OmpC, and OmpW, have been shown to contribute to antibiotic resistance in *E. coli* and *Salmonella* typhimurium (Nikaido, 2003). In addition, OmpD, STM3031, and STM1530 are associated with ceftriaxone (CRO) resistance in *S. enterica* serovar Typhimurium (Hu et al., 2011), and OmpW participates in resistance to neomycin (NEO) and ampicillin (AMP) in *E. coli* (Wu et al., 2012).

Two-component signal transduction systems (TCSs) are key in the sensory response of bacteria (Parkinson and Kofoid, 1992). Studies have elucidated that the TCSs EvgA and BaeR contribute to MDR by modulating production of the multidrug transporter in *E. coli* (Nishino and Yamaguchi, 2001b; Baranova and Nikaido, 2002; Nishino and Yamaguchi, 2002). The Cpx envelope stress response is controlled by a TCS consisting of the membrane localized sensor kinase CpxA and the regulator CpxR. CpxR mediates the output response as a transcriptional regulator through phosphorylation of its receiver domain with an aspartate (D51) moiety (Stephenson and Hoch, 2002; MacRitchie et al., 2008). Phosphorylated CpxR (CpxR-P), which functions as a transcription factor, activates and, in a small number of cases, represses transcription of target genes by binding to the promoter of target genes at the consensus sequence 5'-GTAAAN<sub>5</sub>GTAAA-3' (De Wulf et al., 2002; Price and Raivio, 2009). In addition, the response regulator CpxR is also activated by some signals without the involvement of CpxA. For example, some cytoplasmic or growth signals, as well as excess carbon (glucose or pyruvate) in growth medium both activate CpxR independently of CpxA (Cuny et al., 2007; Wolfe et al., 2008).

In recent years, the CpxAR two-component system conferring resistance to antibacterial agent has received special attention. In *E. coli*, CpxR overproduction was found to confer resistance to β-lactams in an acrB-free background (Hirakawa et al., 2003a). CpxR-P also confers resistance to fosfomycin by directly repressing the expression of two genes, *glpT* and *uhpT*, in the enterohemorrhagic *E. coli* (EHEC) strain O157:H7 (Kurabayashi et al., 2014). Moreover, the CpxAR pathway contributes to

*E. coli* resistance to antimicrobial peptides, such as ApoEdpL-W, polymyxin B, and melittin (Audrain et al., 2013) and protamine (Weatherspoon-Griffin et al., 2014). In *Klebsiella pneumoniae*, CpxR was able to directly bind to the promoter regions of *ompCKP* and *kpnEF*, which contribute to the *K. pneumoniae* MDR phenotype (Srinivasan et al., 2012; Srinivasan and Rajamohan, 2013). In *S. enterica* serovar Typhimurium, studies about the effect of *cpxR* on the resistance are still very limited, only few reports showed CpxAR confers resistance to CRO (Hu et al., 2011) and the antimicrobial peptides protamine, magainin, and melittin (Weatherspoon-Griffin et al., 2011). However, whether CpxAR plays a role in resistance of *S. enterica* serovar Typhimurium especially clinical isolates to aminoglycosides and β-lactams and the molecular mechanisms underlying resistance to aminoglycosides and β-lactams remain unknown. In this study, we systematically investigated the role of *cpxR* in aminoglycoside and β-lactam resistance in both susceptible strains and clinical isolates of *S. enterica* serovar Typhimurium, and also explored the molecular mechanisms of CpxAR that confer resistance to aminoglycosides and β-lactams.

## MATERIALS AND METHODS

### Bacterial Strains, Plasmids, and Bacteriophage

The bacterial strains, plasmids, and bacteriophage used in this study are listed in **Table 1**. *Salmonella enterica* serovar Typhimurium strain CVCC541, a clinical susceptible strain isolated from chicken in Changchun City, China, was supplied by the China Institute of Veterinary Drug Control (Beijing, China) and designated as JS in this report. Strains JSΔ*cpxR* and JSΔ*acrB* were generated from JS using the one-step inactivation of chromosomal genes method. Strain JSΔ*acrB*Δ*cpxR* was constructed by the phage P22-mediated transduction method using strain JSΔ*cpxR* as the donor and JSΔ*acrB* as the recipient. In this study, nine *S. enterica* serovar Typhimurium isolates were isolated from chickens collected from nine different regions of Henan province in China and named SH1-9.

### Construction of the Expression Plasmids pBAD-CpxR and pBAD-CpxR\*

The complete open reading frame of *cpxR* was amplified by PCR with primers *Xba*I-*cpxR*-F/*Hind*III-*cpxR*-R (**Table 2**) from the genomic DNA of strain JS. The mutation sequence *cpxR*\*, which encodes a CpxR variant with an alanine residue at position 51 in place of aspartate, was engineered by overlapping PCR (Urban et al., 1997; Huang et al., 1999). The mutation site was generated through the design of primers Fm and Rm (**Table 2**). Three PCR reactions were performed to obtain the mutation sequence *cpxR*\*. Primers *Xba*I-*cpxR*-F/Rm were used for amplification of the anterior segment of *cpxR*, primers Fm/*Hind*III-*cpxR*-R were used for amplification of the second part of *cpxR*, and the primers *Xba*I-*cpxR*-F/*Hind*III-*cpxR*-R were used for splicing by overlap extension PCR. Finally, the expression plasmids pBAD-CpxR and pBAD-CpxR\* were generated by inserting the target fragment to the multiple cloning site of vector pBAD. The expression level of

**TABLE 1 | Bacterial strains, plasmids, and phage used in this study.**

Strain, plasmid, or phage	Relevant characteristics	References or source
<b>STRAINS</b>		
JS	<i>S. enterica</i> Serovar Typhimurium CVCC541	Supplied by China Institute of Veterinary Drug Control
JSΔcpxR	Derivative of JS that lacks <i>cpxR</i>	Huang et al., 2015
JSΔacrB	Derivative of JS that lacks <i>acrB</i>	Huang et al., 2016
JSΔacrBΔcpxR	Derivative of JS that lacks both <i>cpxR</i> and <i>acrB</i> , Δ <i>cpxR::kan</i>	Huang et al., 2016
SH(1–9)	Clinical isolates from chicken in Henan province in China	This study
SH(1–9) Δ <i>cpxR</i>	Derivative of SH(1–9) that lack <i>cpxR</i> , Δ <i>cpxR::kan</i>	This study
<b>PLASMIDS</b>		
pKD4	Gene knockout help vector: rep <sub>R6Kγ</sub> Ap <sup>R</sup> FRT Km <sup>R</sup> FRT	From <i>E. coli</i> Genetic Stock Center in Yale University
pKD46	Gene knockout help vector: rep <sub>PSC101</sub> <sup>ts</sup> Ap <sup>R</sup> P <sub>araBAD</sub> γ β exo	
pBAD	Expression vector: rep <sub>pBR322</sub> Ap <sup>R</sup> araC P <sub>BAD</sub>	Invitrogen Corporation
pBAD-CpxR	<i>cpxR</i> gene cloned to pBAD; Ap <sup>R</sup>	This study
pBAD-CpxR*	Mutation sequence CpxR* cloned to pBAD; Ap <sup>R</sup>	This study
<b>PHAGE</b>		
P22HT105/int	Transduction medium of <i>Salmonella</i>	Supplied by Microbial Genomics Research Center of Harbin Medical University

target proteins were determined according to the concentration of the inducer L-arabinose (Guzman et al., 1995).

### Construction of *cpxR*-Deficient Mutants of *S. enterica* Serovar Typhimurium Isolates

The generation of strain JSΔ*cpxR::kan* was described in our previous study (Huang et al., 2015). The deletions were then transferred to nine *S. enterica* serovar Typhimurium isolates (SH1–9) by P22HT105/int transductions as previously described (Davis et al., 1980; Mann and Slauch, 1997). Nine *cpxR*-deficient mutants were designated as SH1–9Δ*cpxR* in this study.

### Antibiotic Susceptibility Testing

The minimal inhibitory concentrations (MICs) of selected antibiotics for all strains were determined by the 2-fold broth microdilution method according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2008, 2012). The antibiotics used for susceptibility determination were gentamycin (GEN), amikacin (AMK), apramycin (APR), NEO, CRO, ceftiofur (CEF), CEQ. *E. coli* ATCC 25922 was used for quality control in all susceptibility tests. All tests were performed independently at least three times.

### GEN Induction Testing

A single colony of each tested strain (JS, JSΔ*cpxR*, and JSΔ*cpxR/pcpxR*) was cultured in Luria-Bertani (LB) medium containing a 50% MIC of GEN at 37°C for 18 h. After growth overnight at 37°C, the cultures were diluted 1:100 in LB medium and cultured at 37°C for 18 h, and simultaneously the inducer GEN was added at subinhibitory concentrations. GEN induction testing of the strains was performed for 15 generations in this way.

### Expression Levels of MDR-Related Genes

Total RNA was isolated from bacterial cultures using the MiniBEST Universal RNA Extraction Kit (TaKaRa Bio, Inc.

Shiga, Japan) according to the manufacturer's instructions. OD260/OD280 values of total RNA were detected using a trace nucleic acid protein analyzer spectrophotometer (NanoDrop; Thermo Fisher Scientific, Waltham, MA, USA). Bulk cDNA samples were synthesized from total RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Bio, Inc.). The synthesized cDNA was confirmed by PCR and stored at -20°C until used. Real-time PCR was performed using the LightCycler® 480 System (Roche Diagnostics, Indianapolis, IN, USA) with specific primer pairs (Table 2), cDNA template, and TaKaRa SYBR Premix Ex Taq II (TaKaRa Bio, Inc.). The 16S rRNA gene was chosen as a housekeeping gene. To precisely test the relative expression level of the genes of interest, standard curves of the amplification of all detected genes were individually established. CT values tested came within the linearity range for PCR amplification. Each sample was independently run at least twice. The  $2^{-\Delta(\Delta CT)}$  method was used to calculate altered folds of the gene tested in the mutants, as compared to that in JS. Three independent experiments were performed under the same conditions.

### Statistical Analysis

Statistical analysis was performed using SPSS version 17.0 software (IBM-SPSS, Inc., Chicago, IL, USA). Data were compared using the Student's *t*-test. A probability (*p*) value of > 0.05 was considered statistically significant.

## RESULTS

### Deletion of *cpxR* Increases Susceptibility of JS to aminoglycosides and β-lactams

To examine whether the response regulator CpxR contributes to the drug resistance of *S. enterica* serovar Typhimurium, a *cpxR* deletion mutant, JSΔ*cpxR*, was generated from strain JS, and the complementary strain JSΔ*cpxR/pcpxR* was prepared through the introduction of the expression plasmid pBAD-CpxR into

**TABLE 2 | Sequences of primers used in this study.**

Function	Primer	Sequence (5' → 3')	References or source
Amplification of <i>cpxR</i> gene	<i>Xba</i> I- <i>cpxR</i> -F <i>Hind</i> III- <i>cpxR</i> -R	CGCTCGAGATGAATAAACTCTGTTAGT GCA <u>AGCTT</u> CATGAAGCGGAAACCATCA	This study
Preparation of <i>cpxR*</i>	<i>Xba</i> I- <i>cpxR</i> -F Rm Fm <i>Hind</i> <i>III</i> - <i>cpxR</i> -R	CGCTCGAGATGAATAAACTCTGTTAGT ACTTTTGCTGCCGTATGATGCCGAAG CTTCGGGCATCATGACGGCAAGCAAAGT GCA <u>AGCTT</u> CATGAAGCGGAAACCATCA	This study
<b>REAL-TIME RELATIVE QUANTITATIVE PCR</b>			
<i>ompF</i>	<i>ompF</i> -F <i>ompF</i> -R	CCTGGCAGCGGTGATCC AAATTCTGCTGCCGTTGCG	Tatavarthy and Cannons, 2010
<i>ompC</i>	<i>ompC</i> -F <i>ompC</i> -R	TCGAGCCTGCTGAACCAGAAC ACGGGTTGCGTTAGGCTGAG	Hu et al., 2011
<i>ompD</i>	<i>ompD</i> -F <i>ompD</i> -R	GCAACCGTACTGAAAGCCAGGG GCCAAAGAAGTCAGTGTACGGT	Hu et al., 2011
<i>ompW</i>	<i>ompW</i> -F <i>ompW</i> -R	CAGCAGCAAAGTGCCTCTATGT AGACAGAGGCGCCAATTAACCGAT	Hu et al., 2011
<i>stm3031</i>	<i>stm3031</i> -F <i>stm3031</i> -R	TGCAAGCAGGGAGTAATAACGGGT TCACCTGGATACGCCAGTCCCCT	Hu et al., 2011
<i>stm1530</i>	<i>stm1530</i> -F <i>stm1530</i> -R	CGTCTCGGTTTGCCTGGTTGG GCCGTCATTTTACCTGATACTGC	Hu et al., 2011
<i>acrB</i>	<i>acrB</i> -F <i>acrB</i> -R	CGTGAGCGTTGAGAACGTCT GGCGTCAGTTGGTATTTGGT	Li et al., 2009
<i>acrD</i>	<i>acrD</i> -F <i>acrD</i> -R	TCCGGCCAAATTGAATAGTT TCGGAACCGTCCTGATTAAC	Eaves et al., 2004
<i>acrF</i>	<i>acrF</i> -F <i>acrF</i> -R	TATCTGGCTGGATGCGAATCTGCT ACTTTGCCGAACTTCCGGATCT	Eaves et al., 2004
<i>mdtA</i>	<i>mdtA</i> -F <i>mdtA</i> -R	GAATGCGCTGCTGATCTG TCCAGTTCCCTGACGGGAAAC	Nishino et al., 2007
<i>marA</i>	<i>marA</i> -F <i>marA</i> -R	ATACATCCGCAGCCGTA GTGATTGCGCATGCAATTG	Li et al., 2009
<i>soxS</i>	<i>soxS</i> -F <i>soxS</i> -R	TACGGTAACGCAATCAAACA ACAGGCAGGTGACGGTAAT	Li et al., 2009
16SrRNA	16SrRNA-F 16SrRNA-R	TTAGATAACCCTGGTAGTCCACGC TTGGGGACTTAACCCAAAC	Li et al., 2009

The underlined bases are restriction sites.

*JSΔcpxR*. The MICs of a number of antibiotics for strain JS and *JSΔcpxR* were then determined. As shown in **Table 3**, strain *JSΔcpxR* showed 2–4-fold decreases in the MICs of GEN, AMK, APR, NEO, CRO, CEF, and CEQ, as compared to the parental strain JS. The MICs of the above antibiotics increased by 4-fold for the complementary strain *JSΔcpxR/pcpxR*, as compared to those for *JSΔcpxR*. These results clearly suggest that *cpxR* plays an important role in conferring resistance of *S. enterica* serovar Typhimurium to aminoglycosides and β-lactams. In addition, *JSΔcpxR/pcpxR\** exhibited the same susceptibility as *JSΔcpxR* to the tested antibiotics except for CEQ, which demonstrates that the susceptibility changes of *S. enterica* serovar Typhimurium to the tested antibiotics was mediated by CpxR-P.

## Effects of Deletion of *acrB* on *cpxR*-Mediated Multidrug Resistance

In susceptible *S. enterica* serovar Typhimurium, the AcrAB efflux pump is constitutively expressed and plays a predominant

role in intrinsic and acquired resistance (Mazzariol et al., 2000; Nishino et al., 2006). It has wild substrate spectrum and can capture substrates from the periplasm or the outer leaflet of the cytoplasmic membrane (Yu et al., 2003). Therefore, AcrAB may mask partial function of some efflux pumps located in the cytoplasmic membrane (Hirakawa et al., 2003a,b; Eaves et al., 2004; Nishino et al., 2007). To clarify the role of CpxR in resistance conferred by other efflux pumps, an *acrB* deletion mutant (*JSΔacrB*) and a double deletion mutant (*JSΔacrBΔcpxR*) were generated from strain JS. The *cpxR* complementary strain *JSΔacrBΔcpxR/pcpxR* was prepared as described above. The MICs of various antibiotics for strains *JSΔacrB*, *JSΔacrBΔcpxR*, and *JSΔacrBΔcpxR/pcpxR* were then determined. As shown in **Table 3**, strain *JSΔacrBΔcpxR* showed 2–4-fold decreases in the MICs of GEN, AMK, APR, NEO, and CEF, as compared to strain *JSΔacrB*, while the complementary strain *JSΔacrBΔcpxR/pcpxR* exhibited 2–8-fold increases in the MICs of GEN, AMK, APR, NEO, CRO, CEF, and CEQ, as compared to strain *JSΔacrBΔcpxR*.

**TABLE 3 | Susceptibility of *S. enterica* serovar Typhimurium to several antibiotics.**

Strain	MICs ( $\mu\text{g/mL}$ )								
	AMK	GEN	APR	NEO	CRO	CEF	CEQ	ENR	CIP
JS	0.5	0.25	2	0.4	0.02	0.32	0.08	0.032	0.016
JS $\Delta$ cpxR	0.125	0.0625	1	0.1	0.01	0.08	0.04	0.032	0.016
JS $\Delta$ cpxR/cpcpxR	0.5	0.25	4	0.4	0.04	0.32	0.16	0.032	0.016
JS $\Delta$ cpxR/cpcpxR*	0.125	0.0625	1	0.1	0.01	0.08	0.16	0.032	0.016
JS $\Delta$ acrB	0.25	0.25	2	0.2	0.01	0.0025	0.02	0.001	0.001
JS $\Delta$ acrB $\Delta$ cpxR	0.0625	0.0625	0.5	6.4▲	0.01	0.00125	0.02	0.001	0.001
JS $\Delta$ acrB $\Delta$ cpxR/cpcpxR	0.5	0.25	2	25.6▲	0.02	0.005	0.08	0.001	0.001
JS $\Delta$ acrB $\Delta$ cpxR/cpcpxR▲	0.0625	0.0625	0.5	6.4▲	0.01	0.00125	0.04	0.001	0.001

AMK, Amikacin; GEN, Gentamycin; APR, Apramycin; NEO, Neomycin; CRO, Ceftriaxone; CEF, Ceftiofur; CEQ, Cefquinome; ENR, Enrofloxacin; CIP, Ciprofloxacin.

▲ High NEO resistance is present in strains because of the replacement of cpxR gene by kanamycin-resistant gene ( $\Delta$ cpxR::kan).**TABLE 4 | Susceptibilities of *S. enterica* serovar Typhimurium isolates to antibiotics after cpxR were deleted.**

Strains	MICs ( $\mu\text{g/mL}$ )						
	AMK	GEN	APR	NEO	CRO	CEF	CEQ
SH1	1.25	1	2	0.8	0.5	4	1
SH1 $\Delta$ cpxR	0.625	0.125	0.5	12.8▲	0.5	2	1
SH2	5	32	4	0.4	0.5	4	0.5
SH2 $\Delta$ cpxR	1.25	16	2	6.4▲	0.25	2	0.25
SH3	2	0.2	4	0.4	0.1	0.8	0.05
SH3 $\Delta$ cpxR	0.5	0.1	1	6.4▲	0.05	0.4	0.05
SH4	1	0.4	2	0.4	0.05	0.8	0.1
SH4 $\Delta$ cpxR	0.5	0.1	1	6.4▲	0.05	0.4	0.05
SH5	1	0.4	4	0.4	0.05	0.8	0.1
SH5 $\Delta$ cpxR	0.5	0.2	2	3.2▲	0.05	0.4	0.05
SH6	0.4	0.2	8	0.8	0.1	0.2	0.1
SH6 $\Delta$ cpxR	0.1	0.05	2	12.8▲	0.05	0.2	0.1
SH7	1.6	12.8	1024	1.6	0.1	1.6	0.2
SH7 $\Delta$ cpxR	0.4	3.2	256	12.8▲	0.05	0.8	0.1
SH8	0.4	0.2	4	0.8	0.2	0.4	0.1
SH8 $\Delta$ cpxR	0.2	0.1	2	12.8▲	0.05	0.1	0.05
SH9	0.8	0.4	4	0.8	0.1	0.8	0.05
SH9 $\Delta$ cpxR	0.1	0.05	1	12.8▲	0.05	0.4	0.025

AMK, Amikacin; GEN, Gentamycin; APR, Apramycin; NEO, Neomycin; CRO, Ceftriaxone; CEF, Ceftiofur; CEQ, Cefquinome.

▲ High NEO resistance is present in strains because of the replacement of cpxR gene by the kanamycin-resistant gene ( $\Delta$ cpxR::kan).

These results revealed that CpxR can modulate resistance of *S. enterica* serovar Typhimurium to aminoglycosides and  $\beta$ -lactams in both acrB and  $\Delta$ acrB backgrounds.

### Role of cpxR in drug Resistance of *S. enterica* Serovar Typhimurium Isolates

To determinate the role of cpxR in regulating drug resistance of *S. enterica* serovar Typhimurium isolates, nine cpxR-deficient mutants (SH1–9 $\Delta$ cpxR) derived from nine *S. enterica* serovar Typhimurium isolates (SH1–9) were constructed. The MICs for SH1–9 and SH1–9 $\Delta$ cpxR to the above antibiotics were then determined. As shown in Table 4, among the nine cpxR deletion strains, all showed 2–4-fold decreases in the MICs of GEN,

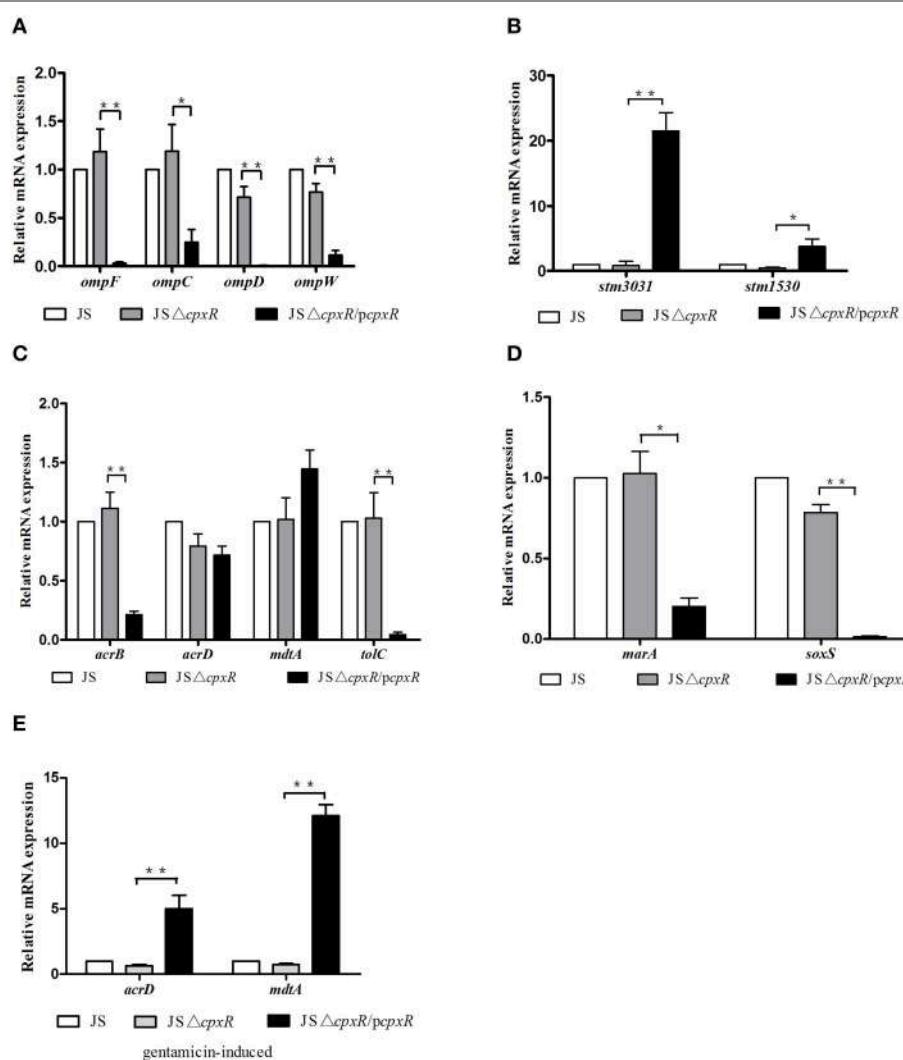
AMK, APR, and CEF, six revealed 2–4-fold decreases in the MIC of CRO, and six revealed 2–4-fold decreases in the MIC of CEQ, compared with their parental strains. These results indicate that cpxR also plays an important role in resistance of *S. enterica* serovar Typhimurium isolates to aminoglycosides and  $\beta$ -lactams.

### Effects of cpxR on the expression Levels of a Series of MDR-Related Genes

In *E. coli*, it has been confirmed that cpxR can modulate the expression of the outer membrane proteins OmpF and OmpC (Batchelor et al., 2005), and the transporter MdtABC (Hirakawa et al., 2005). In *S. enterica* serovar Typhimurium, the expression

of OmpD, STM3031, and STM1530 plays important roles in *cpxR*-mediated CRO resistance (Hu et al., 2011). In order to determine whether the drug resistance mediated by *cpxR* is due to altered expression levels of MDR-related genes, we detected the relative mRNA expression of a series of MDR-related genes. As shown in **Figure 1**, JS $\Delta$ *cpxR* showed no significant differences in the mRNA expression levels of all tested genes, as compared to strain JS, while the mRNA expression levels of *ompF*, *ompC*, *ompD*, and *ompW* genes in strain JS $\Delta$ *cpxR/pcpXR* were significantly decreased ( $p < 0.01$  or  $p < 0.05$ ) relative to strain JS $\Delta$ *cpxR* (**Figures 1A,C,D**) and the mRNA levels of *stm3031* and *stm1530* in strain JS $\Delta$ *cpxR/pcpXR* were significantly increased ( $p < 0.01$  or  $p < 0.05$ ) relative to

strain JS $\Delta$ *cpxR* (**Figure 1B**). There were no significant differences in mRNA expression levels of *acrD* and *mdtA* among strains JS, JS $\Delta$ *cpxR*, and JS $\Delta$ *cpxR/pcpXR* (**Figure 1C**). The expression levels of AcrF in these three strains were all very low and almost undetectable (data not shown). However, after all strains were induced by GEN at subinhibitory concentrations to the 15th passage simultaneously, strain JS $\Delta$ *cpxR/pcpXR* showed significant ( $p < 0.01$ ) increases in the mRNA expression levels of *mdtA* and *acrD*, as compared to JS $\Delta$ *cpxR* (**Figure 1E**). These results suggest that the overexpression of *cpxR* can downregulate the expression levels of OmpF, OmpC, OmpW, OmpD, AcrB, TolC, MarA, and SoxS and upregulate those of STM3031 and STM1530 in susceptible *S. enterica* serovar Typhimurium strains,



**FIGURE 1 | Relative mRNA expression levels (n-fold) determined by real-time PCR.** The expression level of each mRNA in strain JS represents 1-fold. The expression of the 16S rRNA gene was used as an internal control. Each bar represents the average value of three independent experiments. **(A)** Relative mRNA expression levels of the outer membrane genes *ompF*, *ompC*, *ompD*, and *ompW*; **(B)** Relative mRNA expression levels of the outer membrane protein genes *stm3031* and *stm1530*; **(C)** Relative mRNA expression levels of the efflux pumps genes *acrB*, *acrD*, *mdtA*, and *tolC*; **(D)** Relative mRNA expression levels of the transcription factor genes *marA* and *soxS*. **(E)** Relative mRNA expression levels of the efflux pump genes *acrD* and *mdtA* in all tested strains induced by GEN to the 15th passage at subinhibitory concentrations. \* $p < 0.05$ , \*\* $p < 0.01$ .

and it also upregulated the expression levels of the efflux pumps AcrD and MdtA under the pressure of GEN.

## DISCUSSION

In this study, we analyzed the effect of CpxR on the drug resistance of a susceptible strain and nine clinical isolates of *S. enterica* serovar Typhimurium and found 2- to 4-fold decreases in resistance to aminoglycosides and  $\beta$ -lactams by deletion of *cpxR* (Tables 3, 4). These results are similar to those of previous studies reporting that the overexpression of *cpxR* in *E. coli* caused 2-fold increases in resistance to  $\beta$ -lactams (Hirakawa et al., 2003a), but different from the findings that the *cpxA-cpxR* deleted mutant R200( $\Delta cpxAR$ ) showed more remarkable decreases (>2048-fold) than strain JS $\Delta cpxR$  in the MIC of CRO, as compared to their parental strain (Hu et al., 2011). Obviously, R200, generated by a multistep resistance selection method, is a CRO-resistant strain. Therefore, we concluded that the influence of *cpxR* on the drug resistance of resistant strains is greater than that of susceptible strains.

It is known that OmpF and OmpC are the most abundant outer membrane proteins of *S. enterica* serovar Typhimurium. Many antimicrobial agents have been found to alter the expression of these proteins. Moreover, it has been confirmed that decreased level of OmpD, and increased levels of STM3031 and STM1530 are associated with *S. enterica* serovar Typhimurium CRO resistance (Hu et al., 2009, 2011). In this study, we found significant reductions in the levels of OmpF, OmpC, OmpD, and OmpW, and significant increases in levels of STM3031 and STM1530 when *cpxR* was reverted to strain JS $\Delta cpxR$ . Thus, the altered levels of the above-mentioned outer membrane proteins influenced by CpxR may be closely associated with the CpxR-mediated resistance of *S. enterica* serovar Typhimurium to  $\beta$ -lactams.

In Gram-negative bacteria, transporters belonging to the RND family are particularly effective in generating resistance, and MDR often results from the overexpression of multidrug efflux transporters (Grkovic et al., 2002). In this study, before the strains were induced with GEN, CpxR overexpression led to significant reductions in levels of AcrB, TolC, MarA, and SoxS. MarA and SoxS are global regulatory factors (Wall et al., 2009). Once overexpressed, MarA further activates AcrAB/TolC efflux and alters the expression of some membrane proteins (Sulavik et al., 1997). To our knowledge, the influence of CpxR on the mRNA levels of *marA* and *soxS* genes has not been demonstrated. Our results can give two suggestions. One is that the decrease in AcrAB-TolC, mediated by the complementation of *cpxR*, is associated with the decrease of the regulatory factors MarA and SoxS. The other is the expression levels of AcrB and TolC do not play a decisive role in CpxR-mediated resistance of *S. enterica* serovar Typhimurium to aminoglycoside and  $\beta$ -lactams. Our finding that CpxR can influence the susceptibility of *S. enterica* serovar Typhimurium to aminoglycosides and  $\beta$ -lactams in both *acrB* and  $\Delta acrB$  backgrounds also supports the second suggestion. Nevertheless, more studies should be

carried out to elucidate the reciprocal relationship among CpxR, outer membrane protein genes, efflux genes, and regulative genes.

In this study, the up-regulatory effect of CpxR on the expression levels of AcrD and MdtA were observed in the GEN-induced strains. Aminoglycoside uptake in Gram-negative bacteria includes three consecutive steps. The first step is an electrostatic interaction between aminoglycosides and the bacteria cell envelope through displacement of Mg<sup>2+</sup> and Ca<sup>2+</sup> ions that link adjacent lipopolysaccharide molecules, which damages the bacteria outer membrane and enhances its permeability. The second step is energy-dependent phase I of uptake, which leads to a small quantity of antibiotic molecules transversing the cytoplasmic membrane. The third step is energy-dependent phase II of uptake, in which misfolded proteins are produced due to the binding of incoming antibiotics to the ribosome. Some of these proteins are incorporated in the cytoplasmic membrane leading to the loss of membrane integrity. Therefore, additional quantities of aminoglycosides are transported across the damaged cytoplasmic membrane (Taber et al., 1987). Thus, CpxR may be activated by GEN in the inducing experiment *in vitro*. It has been reported that the promoter regions of *acrD* and *mdtABC* harbor binding sites for the response regulator BaeR (Nishino et al., 2007). CpxR also can bind to the *cpxR* box located in the promoter region of target genes. In common, the consensus *cpxR* box includes a tandem repeated GTAAA sequence that is separated by a 5-bp space (Batchelor et al., 2005). The DNA binding feature of CpxR encouraged us to analyze the promoter region of *acrD* and *mdtA* in the chromosome of *S. enterica* serovar Typhimurium LT2 (accession number: AE006468) for the presence of putative CpxR binding sites. Interestingly, our analysis revealed the presence of two similar sequences located 173 bp (site 1: GTAAA-gaacg-GCAAA) and 106 bp (site2: GTAAA-agcgc-ATGAT) upstream of the *acrD* translational start site, respectively. Among them, site 1 was also found 328 bp upstream of the *kpnEF* translational start site. Furthermore, it has been confirmed that purified CpxR from a strain of *K. pneumoniae* can directly bind to site 1 (Srinivasan and Rajamohan, 2013). Because CpxR of *K. pneumoniae* exhibits the highest level of homology to CpxR of *S. enterica* serovar Typhimurium (96%), CpxR of *S. enterica* serovar Typhimurium may directly bind to the promoter region of *acrD*. As we know, AcrD participates in the efflux of aminoglycosides, thus our analysis indicates that CpxR contributes to AcrD-mediated resistance of *S. enterica* serovar Typhimurium to GEN, which belongs to aminoglycosides.

Moreover, in this study, there were no significant differences in the mRNA expression levels of all tested genes in strain JS $\Delta cpxR$ , as compared to strain JS. It has been demonstrated that histidine kinase (HK) also possess response regulator phosphatase activity, which may ensure that the response regulator remains inactive in the absence of activating signals (Raivio and Silhavy, 1997). Therefore, we think that the response regulator CpxR is always in a pre-stimulated resting state and does not modulate mRNA levels at physiological levels. Biochemical data suggest that CpxR can become phosphorylated

by the low-molecular-weight phospho-donor acetyl phosphate and, further, that this form (CpxR-P) has a greater affinity for binding to the promoters of target genes (Pogliano et al., 1997). Similarly, it has been demonstrated that, in the absence of CpxA, CpxR can transcriptionally activate downstream target genes, suggesting that CpxR-P is responsible for transcriptional activation of target genes (Danese et al., 1995; De Wulf and Lin, 2000; Batchelor et al., 2005). In this scenario, we conclude that CpxR overproduction in JS $\Delta$ cpxR encourages the emergence of CpxR-P, which acts as a modulator of gene expression.

In summary, we have reported the first systematical and extensive study about the role of CpxR in aminoglycoside and  $\beta$ -lactams resistance in both susceptible strains and clinical isolates of *S. enterica* serovar Typhimurium. Our results not only clearly confirmed that CpxR contributes to resistance of *S. enterica* serovar Typhimurium to aminoglycoside and  $\beta$ -lactams but also indicated that the effect of CpxR on the expression levels of MDR-related genes is closely associated with CpxR-mediated resistance of *S. enterica* serovar Typhimurium to aminoglycoside and  $\beta$ -lactams. This is the first time that the effect of CpxR on the expression levels of marA and soxS genes have been investigated in *S. enterica* serovar Typhimurium. Further studies are obviously required to investigate the reciprocal relationship among CpxR,

MDR-related outer membrane protein genes, efflux pump genes and regulative genes including marA and soxS.

## AUTHOR CONTRIBUTIONS

HH, YS, and GH conceived of the study, and participated in its design and coordination. YG, CM isolated the *S. enterica* Serovar Typhimurium isolates. HH carried out the antibiotics susceptibility testing and molecular biology studies, including gene deletion, construction of expression vector and RT-PCR. HH and YP performed the statistical analysis. HH drafted the manuscript. YS, LY, and GH revised the manuscript.

## FUNDING

This study was supported by grants from the National Natural Science Foundation of China (31372481) and the Key Research Program of Higher Education of Henan Province (15A230006).

## ACKNOWLEDGMENTS

We thank Professor Chenzhang Wang for assistance with the real-time PCR.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Resistance Mutations in *gyrA* and *parC* are Common in *Escherichia* Communities of both Fluoroquinolone-Polluted and Uncontaminated Aquatic Environments

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## OPEN ACCESS

### Edited by:

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### Specialty section:

This article was submitted to

Antimicrobials, Resistance

and Chemotherapy,

a section of the journal

Frontiers in Microbiology

Received: 13 July 2015

Accepted: 16 November 2015

Published: 09 December 2015

### Citation:

Johnning A, Kristiansson E, Fick J, Weijdegård B and Larsson DGJ (2015) Resistance Mutations in *gyrA* and *parC* are Common in *Escherichia* Communities of both Fluoroquinolone-Polluted and Uncontaminated Aquatic Environments.

Front. Microbiol. 6:1355.

doi: 10.3389/fmicb.2015.01355

Alterations in the target proteins of fluoroquinolones, especially in GyrA and ParC, are known to cause resistance. Here, we investigated environmental *Escherichia* communities to explore the possible link between the abundance of mutations, and the exposure to fluoroquinolones. Sediment samples were collected from a relatively pristine lake, up and downstream from a sewage treatment plant, and from several industrially polluted sites. The quinolone resistance-determining regions of *gyrA* and *parC* were analyzed using amplicon sequencing of metagenomic DNA. Five non-synonymous substitutions were present in all samples, and all of these mutations have been previously linked to fluoroquinolone resistance in *Escherichia coli*. In GyrA, substitutions S83L and D87N were on average detected at frequencies of 86 and 32%, respectively, and 31% of all amplicons encoded both substitutions. In ParC, substitutions S80I, E84G, and E84V were detected in 42, 0.9, and 6.0% of the amplicons, respectively, and 6.5% encoded double substitutions. There was no significant correlation between the level of fluoroquinolone pollution and the relative abundance of resistance mutations, with the exception of the most polluted site, which showed the highest abundance of said substitutions in both genes. Our results demonstrate that resistance mutations can be common in environmental *Escherichia*, even in the absence of a fluoroquinolone selective pressure.

**Keywords:** antimicrobial agents, mechanisms of resistance, antibiotics, microbial communities, next generation sequencing

## INTRODUCTION

Fluoroquinolones are a class of synthetic broad-spectrum antibiotics that target the type II topoisomerases (DNA gyrase and topoisomerase IV) involved in the maintenance of DNA topology. DNA gyrase and topoisomerase IV are both tetrameric enzymes composed of two X and two Y subunits (X<sub>2</sub>Y<sub>2</sub>). DNA gyrase is encoded by the genes *gyrA* and *gyrB*, and topoisomerase

IV is encoded by *parC* and *parE*. The enzymes are homologues, and there is a considerable sequence similarity between *gyrA* and *parC*, and between *gyrB* and *parE*. One of the main mechanisms of fluoroquinolone resistance is amino acid substitutions in the DNA gyrase and topoisomerase IV proteins, in particular in GyrA and ParC (Ruiz, 2003; Hopkins et al., 2005). Several resistance mutations have been characterized in *Escherichia coli*, and the majority of these are located in the quinolone resistance-determining region (QRDR) defined as codons 67–106 in *gyrA* and 56–108 in *parC* (*E. coli* numbering). Certain single mutations in *gyrA* are sufficient to generate high-level resistance to nalidixic acid, a non-fluorinated first generation quinolone. Additional mutations in *gyrA* or other type II topoisomerase genes are, however, necessary for high-level resistance to later generations of fluoroquinolones, such as ciprofloxacin (Hopkins et al., 2005). Indeed, a study of 58 fluoroquinolone-resistant clinical *E. coli* isolates from two hospitals in Houston, TX, USA, found that all isolates had amino acid substitutions in GyrA and approximately 85% had additional substitutions in ParC (Morgan-Linnell et al., 2009), confirming chromosomal mutations as the main mechanism of clinically relevant fluoroquinolone resistance. The frequency of resistant invasive *E. coli* infections in Europe in 2012 ranged from 9.7% in Iceland to 42.0% in Cyprus (ECDC, 2013). Surveillance outside of Europe is less systematic, but a hospital in India reported that as many as 73% of uropathogenic *E. coli* infections were resistant to ciprofloxacin (Mandal et al., 2012).

*Escherichia coli* primarily propagate in the intestines of warm blooded animals, but also, to a limited extent, in the environment (Ishii and Sadowsky, 2008). Because fluoroquinolones are strictly synthetic antibiotics, it would be reasonable to assume that resistance mutations are not as common in environmental *E. coli* isolates as they are among clinical isolates. Accordingly, screening of environmental bacteria from an isolated cave for antibiotic resistance showed very few isolates that were resistant to a high concentration of ciprofloxacin (MIC >20 mg/l), whereas resistance to other classes of antibiotics were more common (Bhullar et al., 2012). Similarly, a recent study from our group found that only <1–6% of bacteria isolated from lakes with no documented history of fluoroquinolone pollution, in Sweden and India, were ciprofloxacin-resistant (MIC >2 mg/l) (Flach et al., 2015). A characterization of the QRDR of *gyrA* from 20 environmental *E. coli* isolates showed that there was considerable variability in the amino acid sequence (Waters and Davies, 1997), and a study of 38 highly ciprofloxacin-resistant (MIC 6–128 mg/l) soil-dwelling bacterial isolates of different species showed that nine isolates (24%) contained amino acid substitutions in this region (D'Costa et al., 2006). This suggests that mutations in the QRDR can be present in environmental bacteria even in the absence of fluoroquinolones. The methods used in previous studies have, however, been low throughput, making the estimation of the abundance of mutations unreliable. Previous studies have also been dependent on the culturability of the bacteria, and often focused on phenotypically fluoroquinolone-resistant bacteria (Adachi et al., 2013; Zurfluh et al., 2014). The characteristics and relative abundance of resistance mutations in the QRDRs

in environmental bacterial communities, have, thus remained unknown.

Recently, concerns have been raised about the risks of resistance development due to antibiotic contamination of the external environment (Gaze et al., 2013; Pruden et al., 2013; Wellington et al., 2013). Antibiotics, including fluoroquinolones, can enter the environment through, for example, human sewage and use in animal farming (Kümmerer, 2009). The highest concentrations of fluoroquinolones detected in the environment are, however, the result of direct discharge from manufacturing (Larsson et al., 2007; Fick et al., 2009; Kristiansson et al., 2011). It is therefore important to assess the risks of the development and spread of resistant bacteria in contaminated environments. Furthermore, because the discharge of antibiotics may be intermittent and difficult to detect, acquired resistance characteristics of environmental bacteria may, given sufficient evaluation, serve as sentinels and biomarkers for antibiotic exposure. The presence and abundance of *qnr*-genes, a group of mobile fluoroquinolone resistance genes, in microbial communities have been shown previously to correlate with fluoroquinolone contamination of stream sediments (Kristiansson et al., 2011). However, horizontally transferred genes are not ubiquitous and may, therefore, not be optimal as biomarkers. In contrast, chromosomal resistance mutations can appear *de novo* and be enriched in a population under sufficient antibiotic selective pressure. Hence, we hypothesize that resistance mutations in *gyrA* and *parC* could serve as biomarkers for fluoroquinolone exposure in different environments.

The aim of the present study was to assess the abundance of chromosomal fluoroquinolone resistance mutations in environmental *Escherichia* communities and to investigate the potential link to fluoroquinolone pollution. Using massively parallel amplicon sequencing of metagenomic DNA, we determined the full genetic resistance characteristics of the QRDR of *gyrA* and *parC* in *Escherichia* communities without any prior culturing of bacteria. The studied environments included a range of selective pressures for fluoroquinolone resistance from a highly polluted Indian stream, samples taken up and downstream from a Swedish sewage treatment plant, and from a remote small Swedish lake under minimal human impact. This allowed us to investigate a possible correlation between fluoroquinolone pollution and the abundance of resistance mutations. Our analysis showed a high abundance of fluoroquinolone resistance mutations in both *gyrA* and *parC* in all investigated environments, but, interestingly, we did not find any significant correlation with the levels of fluoroquinolones detected.

## MATERIALS AND METHODS

### Sample Collection

Samples were taken from an Indian stream and a Swedish stream both upstream (two in India, one in Sweden) and downstream (three in India, one in Sweden) of wastewater treatment plants (WWTPs) as described previously (Kristiansson et al., 2011).

The Indian WWTP is situated in Patancheru near Hyderabad, India, and at the time of sampling, it received industrial effluent from approximately 90 pharmaceutical industries. The Swedish WWTP is located in Skövde and receives municipal wastewater, but with no input from pharmaceutical industries. These seven samples have previously been analyzed for the presence of mobile antibiotic resistance genes using metagenomic sequencing (Kristiansson et al., 2011), and *qnr* genes using qPCR (Rutgersson et al., 2014). For this study, three additional sediment samples were collected from an upland Swedish small lake, Valbergs öga, situated far from habitation, roads and farmland and with no apparent inflow of water (2012-07-25, GPS coordinates 57°51'34.2"N 12°04'58.2"E). Although few places on earth could be referred to as completely pristine, very few people, if any, are likely to walk near this small lake in a given year. Thus, it represents an environment at the very low end in terms of human impact. To determine the fluoroquinolone selective pressure at the samplings sites, the concentrations of ciprofloxacin, difloxacin, enoxacin, enrofloxacin, lomefloxacin, ofloxacin, pefloxacin, and norfloxacin were measured in all samples using liquid chromatography coupled to an ion trap mass spectrometer and electro spray interface (LC-ESI-IT-MSMS) described previously by Kristiansson et al. (2011), where the original chemical analysis data of all of the stream sediment samples can be found. The Swedish lake samples were analyzed at a different time point using the same method. Results were expressed per gram organic matter, rather than per total weight as some samples contained a large proportion of inorganic material (gravel). The detection limit was 0.02 µg/g organic matter for each measured substance.

## DNA Extraction

The total DNA was extracted from the sediment samples and amplified using uniform whole genome amplification. A PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) was used for the DNA extraction with the following modification to the manufacturer's protocol: to complete homogenization and cell lysis, the power bead tubes with sediment and solution C1 were incubated at 70°C for 10 min with a brief vortex after 5 and 10 min. The DNA concentration was measured using a NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the samples were stored at -80°C until the whole genome amplification of purified genomic DNA was performed. Uniform whole genome amplification was done using the REPLI-g mini kit (QIAGEN, Hilden, Germany). The DNA (10 ng) was denatured by adding a denaturation buffer and incubating for 3 min at room temperature. The denaturation was interrupted by a

neutralization buffer and a master mix containing a reaction buffer, and DNA polymerase was added. All buffers and enzyme mixtures were made according to the protocol. The amplification reaction was performed by incubating the samples at 30°C for 16 h in a thermal cycler, after which the reaction was stopped by heating the sample for 3 min at 65°C. The DNA concentration was measured using a NanoDrop, and the samples were stored at -20°C before the PCR amplification of the target genes.

## PCR Amplification of *gyrA* and *parC*

The software Primer3 (Rozen and Skaletsky, 2000) was used through the web interface Primer3Plus (Untergasser et al., 2007) to design PCR primers targeting the regions of *gyrA* and *parC* that include all resistance mutations reported by Ruiz (2003). To avoid placing primers in variable regions, all annotated *gyrA* and *parC* nucleotide sequences belonging to *Escherichia* were downloaded from the Pathosystems Resource Integration Center (PATRIC) (Gillespie et al., 2011) and each gene was aligned using MUSCLE (Edgar, 2004). Because *Shigella* is indistinguishable from *Escherichia* in the targeted gene regions, this genus was also added to the multiple sequence alignment. All variable positions were given as excluded regions in Primer3, and the portions including all resistance mutations were given as the target regions. The product size range was set as 250–350 base pairs (bps) so that the amplicons would, to a large extent, be completely covered by a single 454 sequence read. The top 10 suggested primers were tested experimentally to determine the best primer pair, producing the largest amount of amplicons of the expected length, for each gene. The selected primers, targeted regions, and experimental design are shown in Table 1.

All of the samples were amplified using the selected primer pair for each gene and the set-up described in Table 1. The following was combined in sterile 0.2 ml tubes: 1xGotaq® Reaction buffer, 0.2 mM dNTP Mix, 0.4 µM forward primer, 0.4 reverse primer, 1.25 µ GoTaq® DNA polymerase (Promega, Madison, WI, USA), 50 ng template, and water up to 25 µl. The PCR amplification used an initial denaturation at 95°C for 2 min, denaturation at 95°C for 10 s, annealing at 57°C for 30 s, extension at 72°C for 1 min, and a final extension for 7 min. Each reaction was run for 40 cycles to maximize the amount of amplicon DNA. The PCR products were separated using 1.5% agarose gel electrophoresis and visualized with ethidium bromide. The DNA fragment of interest was excised from the agarose gel and placed in a microcentrifuge tube, and the GeneJET™ gel extraction kit (Fermentas International Inc., Vilnius, Lithuania) was used according to the enclosed protocol to obtain the DNA amplicons. The purified amplicons were pooled to enable sequencing in a single run, and therefore,

**TABLE 1 | Selected primers with target regions and experimental setup.**

Gene	Target region	Product size	Forward primer	Reverse primer	Annealing temperature
<i>gyrA</i>	129–439	311 bp	ggtagacccgtcgctacttt	caaacgaaatcgaccgtctct	57°C
<i>parC</i>	20–306	287 bp	gccttgccgtacatgaattt	accatcaaccagcggataac	57°C

each sample contained amplicons of both *gyrA* and *parC* derived from the same sample site. The amplicons were stored at  $-20^{\circ}\text{C}$  before sequencing.

## DNA Sequencing and Bioinformatics Analyses

The samples were sent to GATC Biotech (Konstanz, Germany) for multiplexed massively parallel pyrosequencing (Margulies et al., 2005) using titanium chemistry on the GS FLX+ system. The sequencing of the Swedish lake samples was performed on a separate time point. The resulting sequence reads were aligned to their respective reference sequence belonging to *E. coli* K-12 MG1655 (RefSeq locus tags, *gyrA*: b2231; *parC*: b3019) using GS Amplicon Variant Analyzer from 454 (v2.5p1). The aligned reads were exported from the software as one FASTA alignment file per gene and sample site. To limit the risk of interpreting sequence variability between species as resistance mutations, reads too dissimilar to the *Escherichia* and *Shigella* sequences were discarded after matching all reads against all sequences annotated with the gene symbol *gyrA* and *parC*, respectively, in the Comprehensive Microbial Resource (CMR) (Peterson et al., 2001) using BLASTn (Altschul et al., 1990). Only reads with a hit against a *gyrA* or *parC* sequence from either *Escherichia* or *Shigella*, and with an *E*-value lower than  $1 \times 10^{-100}$  were retained in the subsequent analysis. To compensate for the issue of homopolymers, which are common in 454 sequencing, any positions in the multiple sequence alignments with insertions in the reference sequence were removed, and any remaining deletions in the reads were substituted with the reference sequence base of that position. The resulting gapless reads were translated using the EMBOSS tool Transeq (reading frame 1 for *gyrA* and 2 for *parC*) (Rice et al., 2000). The relative abundance of non-synonymous substitutions was recorded. The relative abundance of a certain mutation was defined as the percentage of reads encoding that particular amino acid substitution. The average relative abundance of mutations was defined as the percentage of reads encoding any amino acid substitution averaged over all samples. The computations were done for both individual substitutions, and pairs of substitutions occurring

within the same read. The correlation between fluoroquinolone pollution – measured as the total amount of fluoroquinolones detected (log-transformed) – and the abundance of resistance mutations was measured using Pearson's correlation coefficient.

## Nucleotide Sequence Accession Number

The raw sequence data have been submitted to the Short Read Archive (SRA) under BioProject accession number PRJNA239415.

## RESULTS

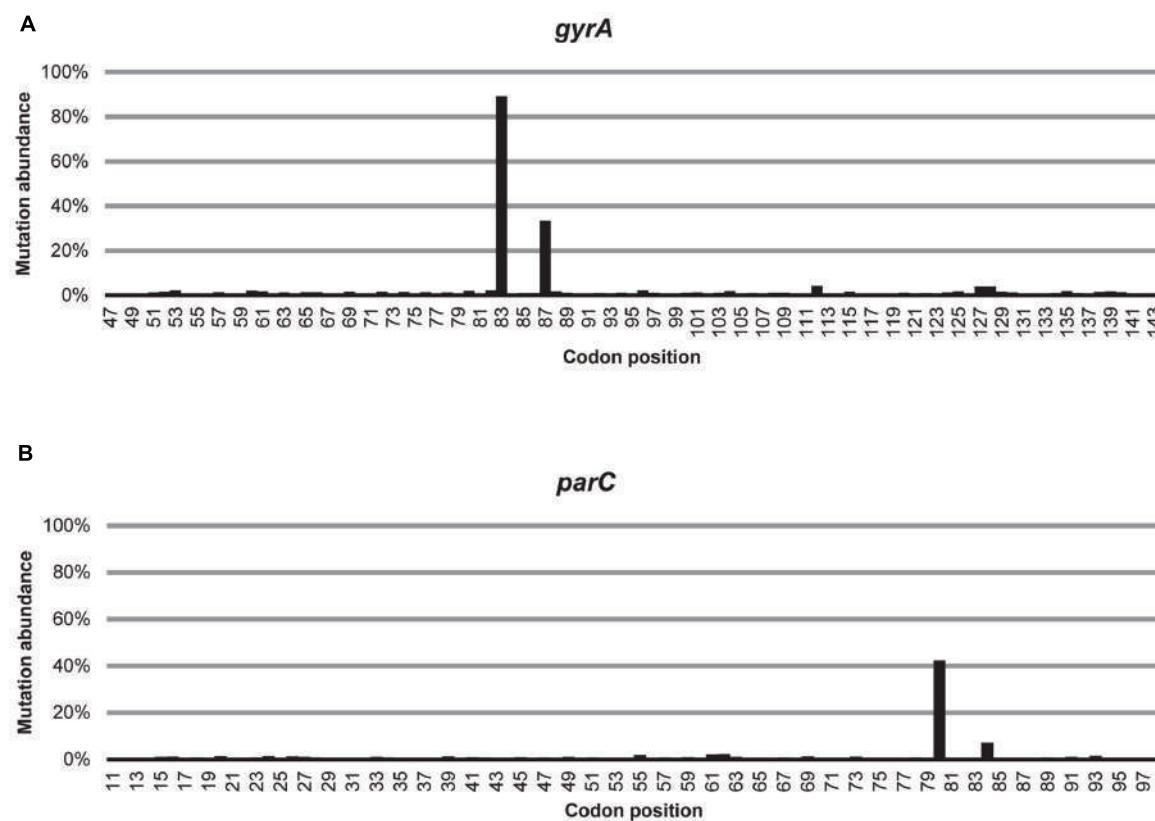
The measurements of the fluoroquinolone concentrations present in the sediments confirmed that the sampled environments represented a range of different fluoroquinolone selective pressures (see Supplementary Table S1). Ciprofloxacin, enrofloxacin, and pefloxacin were detected at all Indian sample sites, ofloxacin was detected only upstream from the Indian WWTP, and lomefloxacin and difloxacin were detected only at the Indian downstream sites. None of the analyzed fluoroquinolones were detected in any of the Swedish samples. The highest level of fluoroquinolones was detected in the Indian downstream samples, while the upstream samples contained moderately high levels, as reported previously (Kristiansson et al., 2011).

A total of 35,417 reads aligned to the *gyrA* reference sequence (1,453–6,339 reads per sample), and 51,933 reads aligned to the *parC* reference sequence (537–15,469 reads per sample) (Table 2). The filtering of reads dissimilar to known *Escherichia* and *Shigella* sequences retained on average 60% of the *gyrA* reads (45–91% per sample) and 78% of the *parC* reads (72–95% per sample), suggesting that the *parC* primer pair is more specific for the targeted genera in the sampled environments compared to the *gyrA* primers. For *gyrA*, a total of 21,295 reads were used in the subsequent analysis (790–3,104 reads per sample), and 40,521 reads were used for *parC* (509–12,006 reads per sample).

Out of all the non-synonymous substitutions detected, the variability in codon 83 and 87 in *gyrA* and in codon 80 in *parC* was considerably higher than at any other codon (Figure 1).

**TABLE 2 |** Number of reads that aligned and that was retained after quality filtering.

Country	Site	Sample	Aligned reads		Filtered reads	
			GyrA	ParC	GyrA	ParC
India	Downstream	1	3,051	712	2,281	658
		2	1,531	3,759	790	3,425
		3	2,788	537	1,753	509
	Upstream	1	3,409	766	3,104	658
		2	3,057	1,919	1,838	1,664
Sweden	Downstream		1,453	2,079	888	1,831
			4,425	2,926	2,773	2,112
	Upstream	1	4,672	15,469	2,438	12,006
		2	4,692	12,446	2,609	9,039
		3	6,339	11,320	2,821	8,619
	Lake	<b>Sum</b>	35,417	51,933	21,295	40,521



**FIGURE 1 | Detected average variability over the amplicon.** The average relative abundance of non-synonymous substitutions in each codon of **(A)** the *gyrA* and **(B)** the *parC* amplicons, averaged over all samples.

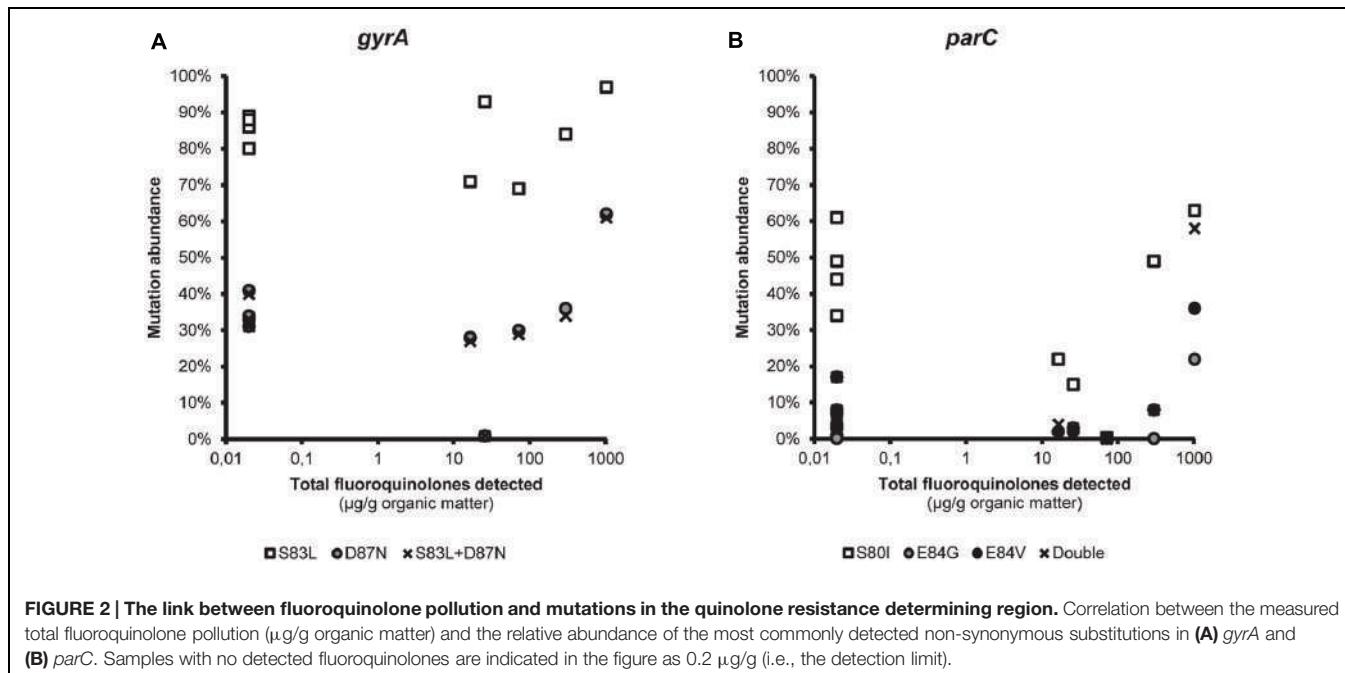
Three non-synonymous mutations in *gyrA* were detected in all samples and they encode the following amino acid substitutions: S83L, D87N, and D82G (see Supplementary Table S2a). The most common substitution, S83L, was detected in a majority of reads in all samples (61–97%), while D87N was less abundant (1–62%). Almost all of the D87N substitutions (97%) occurred in reads also encoding the S83L substitution. Although the D82G substitution was detected in all samples, it was rare ( $\leq 1\%$ ), as were other resistance mutations detected in only some of the samples (0–2%). Three non-synonymous mutations in *parC* were detected in all samples and they encode the following amino acid substitutions: S80I, E84G, and E84V (see Supplementary Table S2b). S80I was the most common substitution encoded by the *parC* amplicons, and was detected in 0.4–63% of the reads in each sample. The mutations in codon 84 were far less common, but 98% of all reads carrying substitutions in codon 84 also carried mutations encoding the S80I substitution. Other resistance mutations in *parC*, which were detected in only some of the samples, were rare (0–7%).

The differences in terms of non-synonymous mutations between the environments were small, and there was no obvious link between the abundance of these mutations and the fluoroquinolone selective pressure for either *gyrA* or *parC* (Figure 2, also see Supplementary Figures S1 and S2). Pearson's correlation coefficient between the log-transformed detected

amounts of total fluoroquinolones (samples with no detected fluoroquinolones were set to the detection limit 0.02  $\mu\text{g/g}$  organic matter) and the abundance of the most commonly detected substitutions were as follows: S83L,  $-0.058$  ( $p = 0.87$ ); D87N,  $0.098$  ( $p = 0.79$ ); and S83L+D87N in GyrA,  $0.088$  ( $p = 0.81$ ); S80I,  $-0.25$  ( $p = 0.49$ ); E84G,  $0.54$  ( $p = 0.10$ ); E84V,  $0.28$  ( $p = 0.42$ ); and S08I+E84G/V in ParC,  $0.34$  ( $p = 0.26$ ). However, the sample taken downstream from and closest to the discharge site of the Indian WWTP showed the highest abundance of all said substitutions (Figure 2, also see Supplementary Figures S1a and S2a).

## DISCUSSION

In this study, we measured the relative abundance of chromosomal fluoroquinolone resistance mutations in *Escherichia* communities residing in both uncontaminated and severely fluoroquinolone polluted aquatic environments. We detected a high abundance of resistance mutations in the chromosomal target genes of fluoroquinolones, *gyrA* and *parC*, in all sampled environments, including environments with no fluoroquinolone pollution or known history thereof. Interestingly, we found no association between the measured concentrations of fluoroquinolones at the samples sites and



the abundance of resistance mutations, except for the most polluted site, which showed the highest abundance of the most commonly detected mutations. This suggests that chromosomal mutations conferring fluoroquinolone resistance only could be considered a potential biomarker for detecting very extensive fluoroquinolone pollution. Additionally, the high abundance of mutations previously linked to fluoroquinolone resistance and the low abundance of other mutations indicate that there could be selective advantages to carry the resistance mutations in the sampled environments, even in the absence of fluoroquinolone selective pressure. Note that the levels of fluoroquinolones detected in the sediment cannot be directly compared to those of water solutions, as some fluoroquinolones are known to sorb to soil particles (Girardi et al., 2011). The exact amount of bioavailable antibiotics is, therefore, unknown, but likely lower than the detected levels.

To our best knowledge, the lowest reported minimal selective concentration for any antibiotic and resistance mutation is 0.1  $\mu\text{g/l}$  for ciprofloxacin and the S83L substitution in GyrA (Gullberg et al., 2011). This concentration is just above the ones found in treated Swedish sewage effluents (Lindberg et al., 2005; Fick et al., 2011). However, the concentration was determined through competition experiments in a lab setting, and therefore, comparisons to environmental conditions should be made with some caution. Although no fluoroquinolones were detected downstream from the Swedish sewage treatment plant in this study, bacteria leaving the plant could theoretically have encountered selective concentrations inside the treatment plant. In the investigated Swedish lake, however, there are no plausible sources for fluoroquinolone exposure, indicating that the mutations found here are not present because of a fluoroquinolone selection pressure. Furthermore, since there is little difference

in mutation abundance between the severely polluted sites and the remote Swedish lake, the data implies that the mutation abundance is not connected to fluoroquinolone selection pressure, but is largely determined by other, unknown factors.

In *gyrA*, mutations encoding the amino acid substitutions S83L and D87N were detected in all samples, with almost all of the D87N substitutions occurring in reads also encoding the S83L substitution. Both substitutions have been associated with fluoroquinolone resistance for *E. coli* and confer higher levels of resistance than any other substitutions in the QRDR (Yoshida et al., 1991). In fluoroquinolone-resistant clinical *E. coli* isolates, S83 is the most frequently altered amino acid, and, moreover, S83L is the most common substitution (Hopkins et al., 2005). A single substitution at position 83 confers higher resistance than at position 87, but additional mutations in *gyrA* or other genes are necessary for the development of high-level fluoroquinolone resistance. The S83L substitution alone, however, confers the same level of resistance to ciprofloxacin for *E. coli* as the combination of S83L and D87N (increased MIC of ciprofloxacin from 0.016 to 0.38  $\mu\text{g/l}$ ), while the D87N substitution alone confers slightly lower resistance (ciprofloxacin MIC = 0.25  $\mu\text{g/l}$ ) (Marcusson et al., 2009; Machuca et al., 2014). In *parC*, mutations causing the substitutions S80I, E84G, and E84V were detected in all samples, and these three substitutions have been previously linked to fluoroquinolone resistance in *E. coli*. In fluoroquinolone-resistant clinical isolates, the most commonly altered amino acid in ParC is S80 followed by E84, and, specifically, S80I is the most common substitution (Hopkins et al., 2005). Substitutions in ParC are often, if not always, found together with substitutions in GyrA in fluoroquinolone-resistant isolates (Hopkins et al., 2005; Morgan-Linnell et al., 2009), and substitutions of S80 or E84 alone do not affect the

susceptibility to fluoroquinolones unless GyrA is also altered (Bagel et al., 1999). Because of the method used in this study, it is not possible to determine to what extent the detected mutations in *gyrA* and *parC* occur in the same bacterium, but it is worth mentioning that a combination of the substitutions S83L and D87N in GyrA with S80I in ParC confers a very high resistance to ciprofloxacin (ciprofloxacin MIC = 32 mg/l) (Marcusson et al., 2009). To set the MICs into perspective, an Enterobacteriaceae isolate is classified as ciprofloxacin-resistant if its MIC is higher than 1 mg/l according to the EUCAST clinical breakpoints (EUCAST, 2014). Isolates with a MIC lower than 0.5 mg/l are classified as sensitive. It is important to note that fluoroquinolone resistance can be caused by a number of different genetic mechanisms and combinations thereof. To extrapolate a level of resistance solely from mutations in the target genes is associated with a high level of uncertainty, therefore, the MICs given above are only included as a rough reference.

The only sampling site that stood out in terms of high abundance of resistance mutations was the site closest to downstream of the discharge site of the Indian WWTP. Here, we observed the highest abundance of the S83L and D87N substitutions in GyrA, the S80I, E84G, and E84V substitutions in ParC, and, consequently, also the highest abundance of the paired substitutions in both proteins. This is indeed the sample site with the highest total concentration of fluoroquinolones, which would be consistent with the hypothesis that there is a selective advantage for resistance mutations in fluoroquinolone-polluted environments, but only at locations with very high concentrations of fluoroquinolones. However, some reads from the most polluted site did not contain any mutations that resulted in amino acid substitutions in either *gyrA* (1.2%) or *parC* (30%), which suggests that some bacteria thriving there may carry additional resistance factors protecting them. Indeed, we have shown previously that mobile fluoroquinolone-resistance genes (*qnr*) are highly abundant in the stream sediment (Rutgersson et al., 2014). The *qnr* genes provide low to moderate resistance to fluoroquinolones [ciprofloxacin MIC up to 2 mg/l in *E. coli* transconjugants (Robicsek et al., 2006; Flach et al., 2013)]. Given the data presented here, it is possible that the presence of *qnr* genes in aquatic bacterial comminutes constitutes a better marker of fluoroquinolone exposure than chromosomal mutations. Or, there could be other mutations, outside the QRDR sequenced in this study, better suited as markers of exposure. Culturing-based methods will be required to fully elucidate the genetic mechanisms for how bacteria have adapted to survive the high concentrations of fluoroquinolones here.

A contributing factor to the high abundance of fluoroquinolone resistance mutations in the environment could be that there is little to no cost for the bacterium to carry these mutations. It has previously been shown that the substitutions S83L and D87N in GyrA and S80I in ParC occurring alone, or all three in the same organism, do not significantly alter the fitness of *E. coli* *in vitro*, nor do the pair of S83L in GyrA and S80I in ParC (Marcusson et al., 2009; Machuca et al., 2014). The pair S83L and D87N in GyrA has been shown to confer a

fitness cost (measured relative fitness 0.97) when introduced in *E. coli* K-12 MG1655 (Marcusson et al., 2009), while the pair substitution was associated with a fitness gain in *E. coli* ATCC 25922 (relative fitness 1.14) (Machuca et al., 2014). In contrast, the pair of D87N in GyrA together with S80I in ParC introduce a slight fitness gain (measured relative fitness 1.02). Competition experiments with wild type *gyrA* in *E. coli* and mutants encoding either the S83L or the D87N substitution have shown that a ciprofloxacin concentration of 0.1 and 2.5 µg/l, respectively, is sufficient for the mutants to have a selective advantage (Gullberg et al., 2011). Furthermore, competitive *in vivo* studies of other species – i.e., *Neisseria gonorrhoeae* in mice (Kunz et al., 2012) and *Campylobacter jejuni* in chickens (Luo et al., 2005) – have even found a fitness benefit associated with *gyrA* mutations corresponding to codon 83 in *E. coli*. In summary, a lack of fitness cost for these substitutions in the sampled environments could explain why they appear to be very common even in environments without any detectable fluoroquinolone pollutants or any known history thereof. As we do not yet understand the possible advantages these genotypes may have in the absence of fluoroquinolones, and because of the limited number and types of environments investigated in this study, we cannot generalize to the point of claiming that resistance mutations in *gyrA* and *parC* are common in all environmental *Escherichia* communities.

The abundance of resistance mutations in the Swedish samples stands in contrast to the frequency of fluoroquinolone-resistant infections in Sweden. On average, the S83L substitution in GyrA was detected in 87% of the Swedish reads, the combination of S83L and D87N in 33%, and the S80I substitution in ParC in 43%. In Sweden, only 11.6% of the clinical invasive *E. coli* isolates tested with ciprofloxacin were resistant in 2013 (ECDC, 2014). This is far below the abundance of resistance mutations detected in both the Swedish lake samples and the samples taken near a sewage treatment plant. The large difference between the abundance of fluoroquinolone resistance mutations in the environment and the frequency of resistant *E. coli* infections in Sweden is likely due to large differences between the pathogenic *E. coli* strains and the strains residing in the sampled environments. Indeed, the genome of *E. coli* is known to be highly variable (Lukjancenko et al., 2010). Furthermore, competition experiments testing the relative fitness of the resistance mutations is typically performed under optimal growing conditions, unlike environmental conditions. It is possible that there is a selective advantage not linked to fluoroquinolones for these mutations in the sampled environments. Indeed, our finding of how abundant the resistance mutations are even in unpolluted environments suggest that the sequence defined as wild-type for GyrA and ParC, respectively, in *E. coli* is only one of a number of common sequences within the species.

The method presented here proved useful to compare the abundances of mutations occurring within a short genomic region in an entire microbial community. Next generation sequencing techniques provide a cost-effective comprehensive approach to study entire bacterial communities without relying on the cultivation of individual isolates. To distinguish between resistance mutations and inter-species variability, it is important

to design the primers as specific for the targeted taxon as possible. If primers are not sufficiently specific, unwanted reads need to be filtered out by matching all reads to the target sequences and discarding reads of low resemblance. However, setting a similarity threshold for such a filtering where you allow for within species variability is not straight-forward. Here, we chose the threshold based on sequence alignments of different *Escherichia* and *Shigella* sequences and closely related genera. Additionally, we propose that amplicon sequencing could also be used to study resistance mutations in the ribosomal rRNA 23S gene that confer resistance to macrolides, lincosamides, and streptogramin B (Vester and Douthwaite, 2001). For future work in finding more suitable markers of fluoroquinolone exposure, we propose a more controlled experimental set up where different environments are dosed with various concentrations of fluoroquinolones for an extended period of time and the effect of the microbiota is studied using metagenomic sequencing.

In this study, we show that there is a significant abundance of fluoroquinolone resistance mutations in environmental *Escherichia* communities residing both in fluoroquinolone-polluted and pristine environments. This suggests that the mutations are not associated with a substantial fitness cost for the bacterium, even where there is no selective pressure for fluoroquinolone resistance. Regardless of whether the detected mutations are sufficient to provide clinically relevant resistance to fluoroquinolones, they are one or two mutations closer to obtaining a high level of resistance.

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## AUTHOR CONTRIBUTIONS

AJ, EK, and JL conceived of the study and participated in its design. AJ collected samples from the Swedish lakes. JF performed the chemical analyses of the sediments. BW prepared DNA from the samples and did the PCR amplifications. AJ designed the PCR primers, performed all data analyses, and drafted the manuscript. All authors read and approved the final manuscript.

## ACKNOWLEDGMENTS

We acknowledge Gamana for help with the sampling in India, and the following funding agencies that supported this study: the Swedish Research Council (K2011-56X-21750-01-6), the Swedish Foundation for Strategic Environmental Research Mistra (2004-147), the Life Science Area of Advance at Chalmers University of Technology, and the Swedish Research Council Formas (244314727).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01355>

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# Serotypes and Antibiotic Susceptibility of *Streptococcus pneumoniae* Isolates from Invasive Pneumococcal Disease and Asymptomatic Carriage in a Pre-vaccination Period, in Algeria

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 31 January 2016

Accepted: 11 May 2016

Published: 14 June 2016

### Citation:

Ziane H, Manageiro V, Ferreira E,  
Moura IB, Bektache S, Tazir M and  
Caniça M (2016) Serotypes and  
Antibiotic Susceptibility of  
*Streptococcus pneumoniae* Isolates  
from Invasive Pneumococcal Disease  
and Asymptomatic Carriage in a  
Pre-vaccination Period, in Algeria.  
*Front. Microbiol.* 7:803.  
doi: 10.3389/fmicb.2016.00803

In Algeria, few data is available concerning the distribution of pneumococcal serotypes and respective antibiotic resistance for the current pre-vaccination period, which is a public health concern. We identified the most frequent *Streptococcus pneumoniae* serogroup/types implicated in invasive pneumococcal disease (IPD;  $n = 80$ ) and carriage ( $n = 138$ ) in Algerian children younger than 5 years old. Serogroup/types of 78 IPD isolates were identified by capsular typing using a sequential multiplex PCR. Overall, serotypes 14, 19F, 6B, 23F, 18C, 1, 5, 7F, 19A, and 3 (55% of PCV7 serotypes, 71.3% of PCV10, and 90% of PCV13) were identified. Additionally, 7.5% of the non-vaccine serotypes 6C, 9N/L, 20, 24F, 35B, and 35F, were observed. In the case of *S. pneumoniae* asymptomatic children carriers, the most common serogroup/types were 6B, 14, 19F, 23F, 4, 9V/A, 1, 19A, 6A, and 3 (42.7% of PCV7 serotypes, 44.2% of PCV10, and 58% of PCV13). For 6.1% of the cases co-colonization was detected. Serotypes 14, 1, 5, and 19A were more implicated in IPD ( $p < 0.01$ ), whereas serotype 6A was exclusively isolated from carriers ( $p < 0.01$ ). Deaths associated with IPD were related to serotypes 19A, 14, 18C, and one non-typeable isolate. Among IPD related to vaccine serotypes, the rates of penicillin non-susceptible isolates were higher in no meningitis cases (80%) than in meningitis (66.7%), with serotypes 14, 19A, 19F, and 23F presenting the highest MIC levels ( $>2\mu\text{g/ml}$ ). Resistance to cefotaxime was higher in isolates from meningitis (40.5%); however, resistance to erythromycin and co-trimoxazole (>40%) was more pronounced in no-meningeal forms. Overall, our results showed that PCV13 conjugate vaccine would cover up to 90% of the circulating isolates associated with IPD in Algeria, highlighting the importance of monitoring the frequency of *S. pneumoniae* serogroups/types during pre- and post-vaccination periods.

**Keywords:** pneumococci serotype, antibiotic susceptibility, invasive disease, pneumococcal carriage, children, vaccine, Algeria

## INTRODUCTION

*Streptococcus pneumoniae* remains the leading cause of bacterial infection among children worldwide, being the most common cause of bacterial pneumonia, and an important cause of meningitis and bacteremia. Approximately 800,000 deaths per year occur among children as a result of pneumococcal infection (Johnson et al., 2010). The management of pneumococcal infections has been aggravated by the rapid worldwide increase of resistance to penicillin and other antibiotics, mostly related to the misuse of these drugs in respiratory pathogens (Song et al., 2012; Ginsburg et al., 2013).

Despite the diversity of capsular types, comprising at least 98 distinct serotypes, only some variants are associated with invasive pneumococcal disease (IPD; Caierão et al., 2014; Richter et al., 2014).

Pneumococcal disease is frequently preceded by asymptomatic nasopharyngeal colonization, which can be quite high in early childhood (Bogaert et al., 2004; Miernyk et al., 2011; Satzke et al., 2013). It is generally agreed that most serotypes recovered from IPD are also frequently identified in colonized healthy children (Bogaert et al., 2004; Dias and Caniça, 2007). However, serotype prevalence can change according to age, geography, time, and antibiotic resistance, among other factors (Finland and Barnes, 1977; Hausdorff et al., 2005; Ingels et al., 2012). IPD has also been related to recent respiratory viral infection (Weinberger et al., 2014).

In some countries, the use of effective pneumococcal conjugate vaccines (PCVs) during infancy have contributed to reduce morbidity and mortality associated to IPD, as well as nasopharyngeal colonization by vaccine serotypes (Ghaffar et al., 2004; Millar et al., 2006; Tan, 2012). Despite the availability of these vaccines, pneumococcal infections remain a global problem due to the replacement of vaccine by non-vaccine serotypes, mostly associated with the emergence of multidrug resistant serotypes, such as the serotype 19A (Dias and Caniça, 2007; Dagan et al., 2009; Richter et al., 2014). Thus, due to the widespread phenomena of serotype replacement, PCVs have been substituted by higher valence pneumococcal vaccines (from PCV7 and PCV10 to PCV13) across the world, according with the recommendations of the Centers for Disease Control and Prevention (CDC, 2013).

The monitoring of antibiotic resistance trends and serotype distribution in the pre- and post-vaccination periods is essential to assess the dynamic change of epidemiology. This way, the impact of vaccines and antibiotic use control programmes should be evaluated across countries.

In this study, we analyzed the frequency of serotypes associated to IPD and *S. pneumoniae* asymptomatic carriage among children younger than 5 years old in Algeria, before the introduction of the pneumococcal vaccine, and correlated the isolates antibiotic susceptibility with vaccine serotypes.

## MATERIALS AND METHODS

### Bacterial Isolates

An overall of 218 *S. pneumoniae* isolates recovered from children <5 years old were collected and studied regarding the serotype.

Briefly, 80 IPD isolates [children <1 year old ( $n = 44$ ), 1–2 years old ( $n = 20$ ), and 3–5 years old ( $n = 16$ )] were collected between the 1st January 2010 and the 31st December 2014. The isolates were recovered from routine microbiological cultures at Laboratory of Clinical Microbiology, at CHU Mustapha Bacha (LCM/CHU), Algiers, Algeria, and at cooperation laboratories in the north ( $n = 50$ ), west ( $n = 12$ ), south ( $n = 2$ ), and northeast ( $n = 16$ ) regions of the country that sent samples and/or isolates to LCM/CHU. Isolates were included if they were obtained from consecutive blood, and cerebrospinal, pleural, peritoneal, ascitic, bone, and joint fluids from patients with symptoms compatible with IPD. Only one isolate per patient was considered.

In addition, all nasopharyngeal cultures ( $n = 130$ ) recovered at LCM/CHU between 2011 and in 2012, from asymptomatic children <5 years old were also included for serotyping evaluation. Each age group of <1 year, 1–2 years, and 3–5 years included 91, 20, and 27 *S. pneumoniae* isolates, respectively. Only one isolate per child was considered, except for eight cultures corresponding to carriers co-colonized with two pneumococcal serogroups/types (in a total of 138 isolates). Authorization for carriage study was approved by “The Direction de la Santé et de la Population de la Wilaya d’Alger,” in Algeria.

Culture of sterile body fluids and nasopharyngeal samples at LCM/CHU was carried out by standard protocols. Pneumococcal isolates were identified using classic microbiological tests: colony morphology, optochin susceptibility, and bile solubility (Figure 1). Thirty seven *S. pneumoniae* isolates expressing different serogroups/types (1, 3, 4, 5, 6B, 6C, 7F, 7C, 8, 9V, 9N, 10A, 10F, 11A, 12F, 13, 14, 15A, 15B, 16F, 17F, 18C, 19F, 19A, 20, 21, 22F, 23A, 23B, 23F, 24F, 31, 33F, 34, 35A, 35B, and 35F), belonging to the collection of the National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections, at National Institute of Health in Portugal, were also used as controls for serotype identification, as previously described (Ziane et al., 2015).

### DNA Extraction and Multiplex PCRs

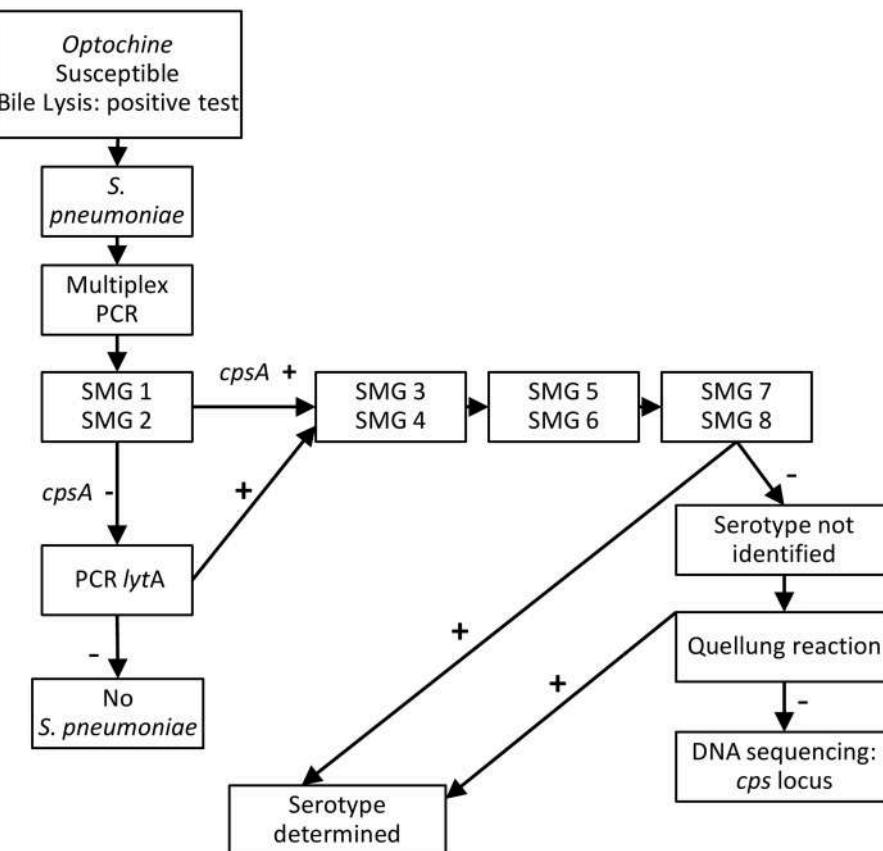
To extract DNA from clinical and control *S. pneumoniae* isolates, several colonies were picked from the culture plates. DNA was extracted by the heat lysis method and stored at  $-20^{\circ}\text{C}$  until further analysis. The serogroups, and whenever possible the serotypes, of 80 *S. pneumoniae* from IPD and of 138 recovered in asymptomatic carriers were determined by sequential multiplex groups (SMGs), as previously described (Ziane et al., 2015; Figure 1).

### Quellung Serotyping

In addition to multiplex PCR (Ziane et al., 2015), serogroups/types of all the 80 invasive isolates were determined at LCM/CHU by Quellung reaction with specific type antiserum [Statens-Serum Institute]. Concerning the isolates recovered from carriers, only those determined as serotype 6A/B or as non-typeable by the multiplex PCR method, were characterized by Quellung reaction (Figure 1).

### PCR Detection of Autolysin (*lytA*) Gene

A primer pair 5'-TCCAGCCTGTAGCCATTTCG-3' and 5'-GC GGTTGAAGTGATTGAAAG-3' that specifically targeted a



SMG, sequential multiplex group; *cpsA*, gene encoding *cps* locus; *lytA*, gene encoding autolysin enzyme.

**FIGURE 1 |** Multiplex PCR scheme used in capsular typing of *S. pneumoniae* isolates from Algeria, and general scheme proposed for usual pneumococcal serotyping.

472 bp internal region of the autolysin (*lytA*) gene was used to the identification of *cps*-negative *S. pneumoniae*. The amplification conditions were: initial denaturation at 94°C for 5 min., followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Positive and negative controls were included in each PCR reaction.

## Antibiotic Susceptibility Testing

Susceptibility testing of 72 IPD vaccine serotype isolates and of 8 IPD non-vaccine serotype was carried out by an agar disk diffusion method for four antibiotics (erythromycin, clindamycin, co-trimoxazole, tetracycline), and minimal inhibitory concentration (MIC) was determined using *E*-test method (AB Biodisk) for four β-lactam antibiotics (penicillin, amoxicillin, cefotaxime, and imipenem). Testing conditions and susceptibility interpretation followed the standards proposed by the Clinical and Laboratory Standards Institute (CLSI, 2014). *S. pneumoniae* ATCC 49619 was used as the quality control strain.

## Statistical Analysis

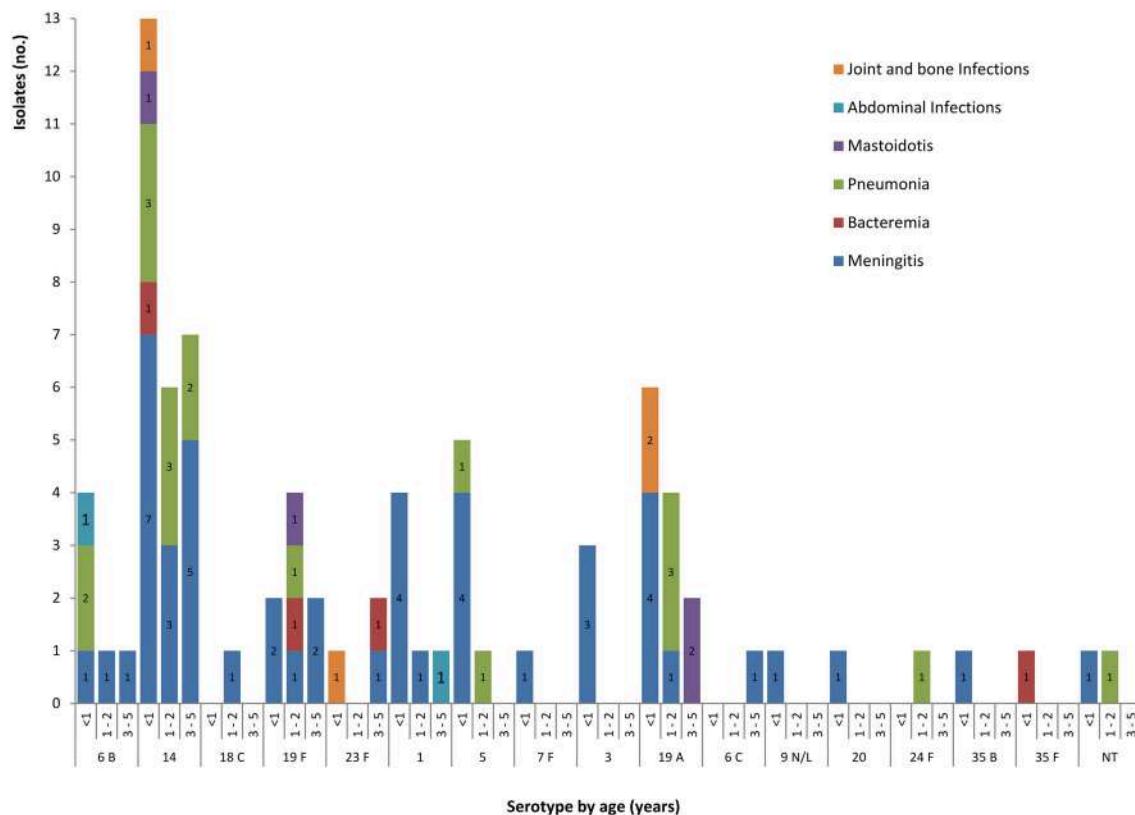
OpenEpi software, version 3.03a (Dean et al., 2015), was used for statistical analysis. Fisher exact test was used to assess differences between IPD and carriers groups. One-tail  $P \leq 0.05$  were considered to be statistically significant.

## RESULTS

### IPD Serogroup/Types

Between 2010 and 2014, a total of 207 episodes of IPD were registered at LCM/CHU. Among those, 80 (38.6%) corresponded to children <5 years old and were retained for this study. These 80 IPD cases (Figure 2) comprised 56.3% of occurrences in males and 43.7% in females, with 64 cases (80%) being reported to infants ( $\leq 2$  years old). Overall, the serotypes were identified in 78 isolates, while two were non-typeable (Table 1).

Forty-eight (60%) of the total IPD episodes included cases of meningitis [of which 62.5% (30/48) were in infants <1 year old], while 40% (32/80) comprised cases of nonmeningitis [56.3% of



**FIGURE 2 | Serogroup/types distribution and age groups in children under 5 years old according to clinical presentations of invasive pneumococcal disease (IPD) episodes.** Abdominal infections, *S. pneumoniae* was isolated from ascitic and peritoneal fluids; NT, non-typeable.

which were pleuropneumonia (18/32), including 55.5% (10/18) in infants from 1 to 2 of age; **Figure 2**. The most frequent serotypes implicated in IPD in this study, which accounted for 87.5% (70/80) of the tested isolates, were in decreasing frequency (**Figure 3**): 14, 19A, 19F, and 1 (from 0 to 5 years old), 5 (from 0 to 2 years old), 6B (from 0 to 5 years old), 3 (from <1 year old), and 23F (from <1 year, and from 3 to 5 years old). Serotypes 14, 1, 19A, 19F, 5, 3, and 6B were the most common in meningitis cases. However, serotypes 14, 19A, 5, and 6B were also implicated in pleuropneumonia, and 19A, 19F, and 14 in bacteremia (**Figure 2**).

Deaths caused by *S. pneumoniae* infection were noticed in 7 IPD cases (7/80, 8.8%) associated with meningitis (6/7, 85.7%), and pneumonia (1/7, 14.3%). Serotypes 19A and 14 were cause of death in 3 (42.8%), and 2 (28.6%) cases, respectively. The two remaining death cases were caused by serotype 18C, and one non-typeable isolate.

A total of 44 pneumococcal isolates expressed the serotypes included in PCV7, which would reflect the coverage of 55% (44/80) (**Figure 4; Table 1**). The coverage of PCV10 would be of 71.3% (57/80), because there were 13 isolates expressing serotypes 1 (6/57, 10.5%), 5 (6/57, 10.5%), and 7F (1/57, 1.8%). The coverage of PCV13 would reach up to 90% (72/80) (**Figure 4; Table 1**). All PCVs would cover the three age groups included in the study. Non-vaccine serogroup/types were expressed in 7.5% (6/80) with one isolate of each: 6C/D, 9N/L, 20, 24F, 35B, and 35F.

## Serogroup/Types Characterization of Nasopharyngeal Isolates

The serogroup/type of 138 isolates, identified in 130 nasopharyngeal cultures from colonized children <5 years old, was determined by multiplex PCR method, using the same scheme as for IPD isolates (**Figure 1**): 117 (83.6%) *S. pneumoniae* were characterized at the serogroup/type level [101 (86.3%) children carried a single serogroup/type, whereas eight children (8/132, 6.1%) were co-colonized with pneumococci belonging to two different serogroup/types]. Furthermore, of the 18 isolates that remained non-typeable by multiplex PCR reactions, 3 were assigned to serogroup/type 15, 19A, and 20 by the Quellung reaction (**Table 1**). Serogroup 6A/B was differentiated by Quellung reaction into serotype 6B (*n* = 16) and serotype 6A (*n* = 13).

The most common serogroup/type was 6 (*n* = 31) [divided in 6A (*n* = 13), 6B (*n* = 16) (both included in PCV13 and PCV7, respectively), and 6C/D (*n* = 2)]; it was followed by serogroup/type 14 (*n* = 13), 19F (*n* = 12), and 23F (*n* = 11; **Figure 4; Table 1**). All these four serogroup/types were identified in the three age groups defined: <1 year, 1–2 years, and 3–5 years (**Figure 3**). The remaining conjugate vaccine serotypes identified in asymptomatic children carriers of *S. pneumoniae* included 19A (*n* = 5), 4 (*n* = 4), 9V/A (*n* = 3), 1 (*n* = 2), and 3 (*n* = 1). Non-conjugate vaccine serotypes accounted for

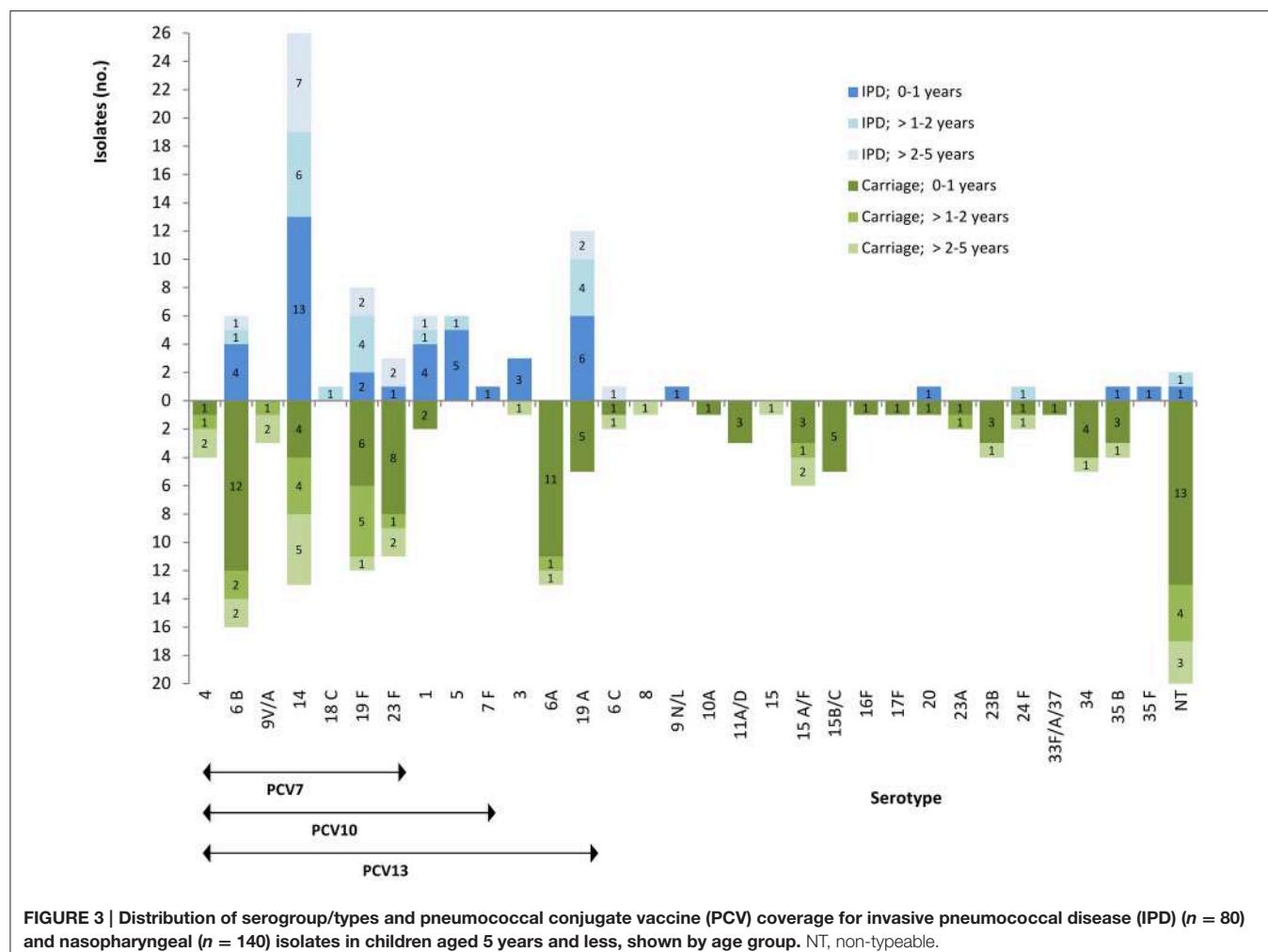
**TABLE 1 |** *S. pneumoniae* conjugate vaccines PCV7, PCV10, and PCV13 and non conjugate vaccine serotypes implicated in IPD cases and in nasopharyngeal carriage, according to the age groups.

Serotypes/Age(years)	IPD				Nasopharyngeal carriage				Global total	<i>P</i> -value <sup>a</sup>
	<1	1–2	3–5	Total	<1	1–2	3–5	Total		
<b>PCV7 SEROTYPES</b>										
4					1	1	2	4	4	0.161
6 B	4	1	1	6	12	2	2	16	22	0.245
9V/A						1	2	3	3	0.256
14	13	6	7	26	4	4	5	13	39	<0.01
18 C		1		1					1	0.364
19 F	2	4	2	8	6	5	1	12	20	0.449
23 F	1		2	3	8	1	2	11	14	0.182
Subtotal	20	12	12	44	31	14	14	59	103	0.045
<b>ADDITIONAL PCV10 SEROTYPES</b>										
1	4	1	1	6	2			2	8	0.028
5	5	1		6					6	<0.01
7 F	1			1					1	0.364
Subtotal	10	2	1	13	2			2	15	<0.01
<b>ADDITIONAL PCV13 SEROTYPES</b>										
3	3			3		1		1	4	0.138
6A					11	1	1	13	13	<0.01
19 A	6	4	2	12	5 <sup>b</sup>			5 <sup>b</sup>	17	<0.01
Subtotal	9	4	2	15	16	1	2	19	34	0.203
<b>NON CONJUGATE VACCINE SEROTYPES</b>										
6 C			1	1	1		1	2	3	0.700
8						1		1	1	0.636
9 N/L	1			1					1	0.364
10A					1			1	1	0.636
11A/D					3			3	3	0.256
15						1 <sup>c</sup>		1 <sup>c</sup>	1	0.636
15 A/F					3	1	2	6	6	0.064
15B/C					5			5	5	0.116
16F					1			1	1	0.636
17F					1			1	1	0.636
20	1			1	1 <sup>c</sup>			1 <sup>c</sup>	2	0.700
23A					1	1		2	2	0.404
23B					3		1	4	4	0.161
24 F	1			1	1		1	2	3	0.700
33F/A/37					1			1	1	0.636
34					4		1	5	5	0.116
35 B	1			1	3		1	4	5	0.401
35 F	1			1					1	0.364
Subtotal	4	1	1	6	29	2	9	40	46	<0.01
Total Typeable	43	19	16	78	78	17	25	120	198	<0.01
Non typeable	1	1		2	13	3	2	18	20 <sup>d</sup>	<0.01
Global Total	44	20	16	80	91	20	27	138	218	

<sup>a</sup>One-tail  $P \leq 0.05$  were considered to be statistically significant.<sup>b</sup>Four isolates were serotyped by Multiplex PCR and one was serotyped by Quellung reaction.<sup>c</sup>Isolates serotyped only by Quellung reaction.<sup>d</sup>Three Multiplex PCR non-typeable isolates were serotyped only by Quellung reaction (serotypes 19A<sup>b</sup>, 15<sup>c</sup>, and 20<sup>c</sup>).

29% (40/138), identified by both PCR ( $n = 38$ ) and Quellung reaction ( $n = 2$ ). These serotypes were mostly identified as 15A/F, 15B/C, 34, 23B, 35B, and 11A/D. Globally, the section of the

study that included the carrier patients allowed the identification of 120 isolates at serogroup/type level, and the evaluation of 18 non-typeable isolates.



**FIGURE 3 |** Distribution of serogroup/types and pneumococcal conjugate vaccine (PCV) coverage for invasive pneumococcal disease (IPD) ( $n = 80$ ) and nasopharyngeal ( $n = 140$ ) isolates in children aged 5 years and less, shown by age group. NT, non-typeable.

Overall, 4.1% (09/218) of the isolates lacked the *cpsA* gene. However, the presence of the *lytA* gene confirmed the identification of *S. pneumoniae* specie. In addition, the sequential multiplex PCR scheme (Figure 1) used in our study demonstrated a specific identification of each serogroup/type in 90.8% (198/218) of the isolates analyzed: among the 80 IPD and the 138 nasopharyngeal *S. pneumoniae* isolates, 97.5% (78/80) and 86.9% (120/138) were positively serotyped by sequential multiplex PCR, respectively ( $p < 0.01$ ; Table 1).

## Antibiotic Susceptibility and Vaccine Serotype

Among *S. pneumoniae* vaccine serotypes ( $n = 72$ ) from meningitis ( $n = 39$ ) we identified 66.7% isolates penicillin non-susceptible ( $\text{MIC} \geq 0.12 \mu\text{g/ml}$ ) with 25.6% showing high level of resistance ( $\text{MIC} 2-4 \mu\text{g/ml}$ ; Table 2). Isolates non-susceptible to cefotaxime and imipenem corresponded to 40.5 and 43.2%, respectively. However, high level of resistance was noticed for imipenem only in 5.4% isolates.

From no meningitis infections ( $n = 25$ ), 80% of the isolates were penicillin non-susceptible with 12% of high level of

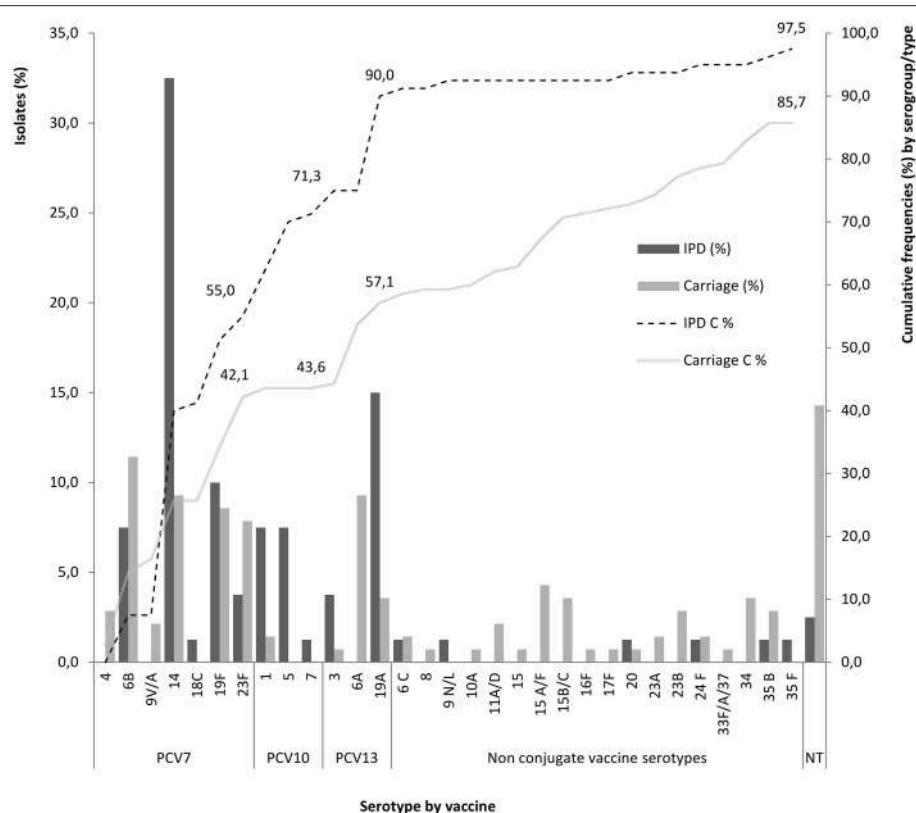
resistance ( $\text{MIC} = 4-8 \mu\text{g/ml}$ ). Intermediate resistance to amoxicillin and cefotaxime was 9.1 and 8.3%, respectively. High level of resistance to amoxicillin was observed in 4.5% isolates ( $\text{MIC} 8 \mu\text{g/ml}$ ).

Non-susceptibility to erythromycin, clindamycin, co-trimoxazole, and tetracycline occurred in lower frequencies in isolates from meningitis (42.5, 40, 53.8, and 27.2%, respectively) than in isolates from no meningitis infections (74.1, 66.7, 75, and 46.2%, respectively).

High level of resistance for vaccine serotypes was observed in serotypes 14, 19A, 19F, and 23F. Overall, the vaccine serotypes 14, 19A, 19F, 23F, 6B, and 5 presented higher rates of resistance (90–100%) than the eight non-vaccine serotypes (6C, 9N, 20, 24F, 35B, 35F, non-typable;  $p < 0.05$ ; data not shown).

## DISCUSSION

*S. pneumoniae* remains the leading cause of bacterial infection among children worldwide, including numerous cases of invasive disease associated high morbidity and mortality rates (Harboe et al., 2010; Adegbola et al., 2014). The pneumococcal



**FIGURE 4 |** Rate of serogroup/type cumulative coverage (C %) in each conjugate vaccine (PCV7, PCV10, PCV13) for *S. pneumoniae* recovered from invasive pneumococcal disease (IPD) and asymptomatic carriers less than 5 years old, from Algeria. NT, non-typeable.

epidemiology regarding capsular types and antibiotic resistance varies geographically and temporally in terms of origin of the isolates (infections or carriage), clinical presentation, pathogenicity (Hausdorff et al., 2005; Harboe et al., 2010; Ingels et al., 2012; Geno et al., 2015), and the methods used for serotyping (Turner et al., 2011; Song et al., 2012; Geno et al., 2015). Although antibiotic susceptibility and serotype data remains insufficient in many countries such as Algeria, its collection and evaluation is essential for the treatment of pneumococcal infections and for the usage of conjugate vaccines (Ramdani-Bouguessa and Rahal, 2003; Tali-Maamar et al., 2012; Ziane et al., 2012; Ramdani-Bouguessa et al., 2015).

Thus, the main goal of this study was to analyze the frequency of serotypes associated to IPD and *S. pneumoniae* asymptomatic carriage in Algeria, in children <5 years old before the introduction of the pneumococcal vaccine. To accomplish our purpose, we used the more recent IPD samples studied in the country (Ramdani-Bouguessa and Rahal, 2003; Tali-Maamar et al., 2012; Ziane et al., 2012; Ramdani-Bouguessa et al., 2015). It is worth mentioning that a molecular approach was here applied for the first time to isolates recovered in this country in the context of a monitoring survey of *S. pneumoniae* serogroup/types (Ziane et al., 2015).

In this study, meningitis represented 60% of the clinical presentation of IPD, with 79.2% registered in children younger

than 2 years old. Pleuropneumonia was reported in 22.5% of the total IPD cases, with the majority being manifested in infants (88.8%, 16/18). Differently from bacteremia, mastoiditis, and abdominal infections, the bone and joint infections were noted exclusively in infants with up to 1 year old. Indeed, IPD occurs mostly in children under the age of 5 years, especially within the subgroup of those under 2 years of age (Tan, 2012). The prevalence of *S. pneumoniae* carriage in healthy children younger than 5 years old can fluctuate between 20 and 93.4% in low income countries, being superior than what is stated for lower-middle income countries (from 6.5 to 69.8%), as reported by Adegbola et al. (2014). In addition, the density of pneumococcal nasopharyngeal carriage seems to decrease in higher age groups, with the children being more competent than adults in the transmission of pneumococci (Roca et al., 2012).

Mortality rate of pneumococcal invasive diseases may range from 10 to 30%, according with the studies of Pebody et al. (2006), and Bravo (2009). Although it has been reported that the highest *S. pneumoniae*-associated morbidity and mortality rates are in Africa and Asia (Johnson et al., 2010; Turner et al., 2011), in our study the case fatality was 8.8%, mainly comprising meningitis related with vaccine serotypes (85.7%).

Overall, we identified serogroups 14, 19, 23, and 6 as the most common among IPD and nasopharyngeal carriers (Figure 2).

**TABLE 2 | Antibiotic susceptibility of 72 isolates of vaccine serotypes<sup>a</sup>, according to site infection.**

Antibiotic <sup>b, c</sup> site of infection	Antibiotic susceptibility (%)				Total no. of isolates (n = 72)	Breakpoint <sup>b, c</sup> (CLSI, 2014)	
	S	I	R	IR		S <sub>≤</sub>	R <sub>≥</sub>
<b>PENICILLIN G</b>							
Meningitis	33.3	–	66.7	66.7	39	0.06	0.12
<b>PARENTERAL PENICILLIN PG</b>							
No meningitis	88.0	8.0	4.0	12.0	25	2	8
<b>ORAL PENICILLIN</b>							
No meningitis	20.0	32.0	48.0	80.0	25	0.06	2
<b>AMOXICILLIN</b>							
No meningitis	86.4	9.1	4.5	13.6	22	2	8
<b>CEFOTAXIME</b>							
Meningitis	59.5	40.5	0.0	40.5	37	0.5	2
No meningitis	91.7	8.3	0.0	8.3	24	1	4
<b>IMIPENEM</b>							
Meningitis	56.8	37.8	5.4	43.2	37	0.12	1
<b>ERYTHROMYCIN</b>							
Meningitis	57.5	0.0	42.5	42.5	40	≥21	≤15
No meningitis	25.9	0.0	74.1	74.1	27	≥21	≤15
<b>CLINDAMYCIN</b>							
Meningitis	60.0	0.0	40.0	40.0	40	≥19	≤15
No meningitis	33.3	0.0	66.7	66.7	27	≥19	≤15
<b>CO-TRIMOXAZOLE</b>							
Meningitis	46.2	2.6	51.2	53.8	39	≥19	≤15
No meningitis	25.0	0.0	75.0	75.0	24	≥19	≤15
<b>TETRACYCLINE</b>							
Meningitis	71.8	2.6	25.6	27.2	39	≥28	≤24
No meningitis	57.7	3.8	42.3	46.2	26	≥28	≤24

<sup>a</sup>Includes serogroups/types: 1 (n = 6), 3 (n = 3), 5 (n = 6), 6B (n = 6), 7 (n = 1), 14 (n = 26), 18C (n = 1), 19A (n = 12), 19F (n = 8), and 23F (n = 3).

<sup>b</sup>Susceptibility testing by E-test method (MIC determination), for penicillin, amoxicillin, cefotaxime, and imipenem.

<sup>c</sup>Susceptibility testing by agar disk diffusion method, for erythromycin, clindamycin, co-trimoxazole, tetracycline.

S, Susceptible; I, Intermediate; R, resistant; IR, non-susceptible.

Among IPD isolates, serotypes 14, 19A, 19F, 6B, 1, and 5 were the most frequent, which shows some differences from data described for other periods in Algeria. For instance, the serogroups/types 1 and 5 were the most common for the period of 1996–2000 (Ramdani-Bouguessa and Rahal, 2003), the serogroups/types 14, 19F, 23F, and 6B for the periods of 2001–2010 and 2005–2011 (Tali-Maamar et al., 2012; Hecini-Hannachi et al., 2014), and serogroups/types 14, 19F, 6B, 1, and 19A for the period of 2005–2012 (Ramdani-Bouguessa et al., 2015). In Tunisia, the most prevalent serogroups for IPD in children were nearly the same: 19, 14, 23, and 4 (Charfi et al., 2012). Internationally, among the 98 known pneumococcal serotypes 11 of them account for more than 70% of IPD, in children younger than 5 years old, with serotypes 1, 5, 6A, 6B, 14, 19F, 23F being the most common (Johnson et al., 2010). Indeed, it has been described that a restricted number of serotypes is in the origin of the majority of the IPD cases worldwide. To summarize, since the report of the first studies concerning the distribution of IPD serotypes in Algeria, there was an emergence of serotype 19A, which is one of most common causes of invasive disease in developed countries in children (Geno et al., 2015). Furthermore,

the studied isolates expressing serotype 19A were assigned to ST276 (M. Caniça, personal communication), which is one of the most predominant sequence-types within this serotype (Reinert et al., 2010; Ramos et al., 2014).

Some of the *S. pneumoniae* serotypes are more prone to successfully colonize the nasopharynx, being in advantage to cause invasive disease. The carriage of *S. pneumoniae* may play an important role in the pathogenesis of IPD and in the transmission of this bacterium (Roca et al., 2012). Indeed, vaccination often brings a decrease in the reduction of vaccine serotype *S. pneumoniae* isolates, and a raise in carriage of non-vaccine serotype isolates (Dias and Caniça, 2007).

In this study, it is essential not only to emphasize the differences in serotype distribution (in IPD and asymptomatic carriage), but also to consider the presence of non-vaccine types (such as 15, 35B, and 34), particularly in carriage, despite the unavailability of pneumococcal vaccines in Algeria (Figure 2). Thus, when comparing serotype rate for IPD and nasopharyngeal carriage, serotypes 14, 1, 5, and 19A were more implicated in IPD than in carriage ( $p < 0.01$ ), whereas serotype 6A was exclusively isolated from carriers ( $p < 0.01$ ; Table 2).

Non-typeable isolates by PCR methods should be typed by Quellung reaction in order to monitor and detect the emergence of *S. pneumoniae* serotype variants, and if this test is negative, the isolates should be sequenced for the *cps* locus to characterize variants (Bentley et al., 2006). In fact, the *cpsA* gene is common within most encapsulated *S. pneumoniae*, being a highly conserved region of the *cps* locus in all known pneumococcal *cps* operons (Bentley et al., 2006; Pai et al., 2006; Jin et al., 2009). However, the absence of *cpsA* amplification in the multiplex PCR scheme has been reported among rough strains, and pneumococci with mutated capsular genes, or without the *cps* locus (Pai et al., 2006; Ahn et al., 2012; Richter et al., 2013). Previous studies have also reported absence of *cpsA* gene by PCR-based serotyping of *S. pneumoniae* in 1–3% of the cases, particularly among serotypes 38 and 25F (da Gloria Carvalho et al., 2010; Jourdain et al., 2011). In this study, 4.1% of the isolates lacked the *cpsA* gene.

Among the *cpsA* negative isolates, the detection of the *lytA* gene resolved the *S. pneumoniae* specie (Moreno et al., 2005). Thus, we propose the scheme in **Figure 1** for pneumococcal serotyping that will extend the application of multiplex PCR in laboratories, and concentrate the use of conventional method in reference laboratories only.

Similarly to other countries (Flasche et al., 2011; Ahn et al., 2012; Steens et al., 2013; von Gottberg et al., 2014), we noticed in Algeria an increase of serotypes covered by the different PCVs, particularly among IPD (**Figure 4**). These new data suggests an increase in the frequency of serotypes included in PCV13 in Algeria, in comparison with the 74.2% registered between 2005 and 2011 (Hecini-Hannachi et al., 2014), but similar to what was observed from 2005 to June 2012 (86.8%; Ramdani-Bouguessa et al., 2015).

Concerning the antibiotic resistance of *S. pneumoniae* from vaccine serotypes, the rates obtained were higher in no meningitis infections probably due to the large use of these antibiotics in the treatment of respiratory or urinary tract infections in Algeria. Tetracycline resistance rate might be explained by a lesser use of this antibiotic.

Rates of *S. pneumoniae* non-susceptible to penicillin vary in the country according to the study periods. Therefore, Algerian studies reported 34.6% of penicillin non-susceptible *S. pneumoniae* in 2003 (Ramdani-Bouguessa and Rahal, 2003), 25.2% in 2001–2010, mostly from meningitis, and 4.4% of high level resistance to cefotaxime (Tali-Maamar et al., 2012). A later study, conducted on invasive and non-invasive pneumococcal disease in children, identified 48% of penicillin non-susceptible isolates, mainly in no meningeal infections (Ramdani-Bouguessa et al., 2015). Rates of 48.5% and 45% have been reported in Morocco and Tunisia, respectively (Charfi et al., 2012; Elmdaghri et al., 2012).

In our study, the decreased susceptibility to penicillin is significantly associated with both vaccine serotypes and non-vaccine serotypes ( $p < 0.05$ ; data not shown), but the vaccine

serotypes 14, 19A, 19F, and 23F, showed the highest levels of penicillin resistance (MIC Peni G  $> 2\mu\text{g/ml}$ ). These data is in agreement to earlier reports from Algeria (Ramdani-Bouguessa and Rahal, 2003; Hecini-Hannachi et al., 2014), Tunisia (Charfi et al., 2012), Morocco, (Elmdaghri et al., 2010), and France (Varon, 2012), where the serotypes 14, 19F, 23F, 6B were described as the most resistant. However, in France, after the introduction of PCV13, the serotypes 19A, 19F, 15A, 35B, and 24F emerged as those with higher resistance (Varon et al., 2015).

The fluctuations of *S. pneumoniae* circulating serotypes and its relation with antibiotic resistance and the PCVs coverage, reinforces the importance of *S. pneumoniae* serogroup/type identification and studies of antibiotic susceptibility, in pre- and post-vaccination periods, particularly in countries with few data, such as Algeria. These will help guide the treatment and will motivate the implementation of strategies for prevention of pneumococcal disease.

## AUTHOR CONTRIBUTIONS

HZ designed the study, performed experiments, analyzed the data, and wrote the manuscript. VM analyzed the data and reviewed the manuscript. EF performed experiments and reviewed the manuscript. IM performed experiments and reviewed the manuscript. SB performed experiments. MT reviewed the manuscript. MC designed the study, analyzed the data and reviewed the manuscript. All authors read and approved the final manuscript.

## FUNDING

HZ was supported by a grant from Ministère de l'Enseignement Supérieur et de la Recherche Scientifique in Algeria. VM was supported by FCT fellowship (grant SFRH/BPD/77486/2011), financed by the European Social Funds (COMPETE-FEDER), and national funds of the Portuguese Ministry of Education and Science (POPH-QREN).

## ACKNOWLEDGMENTS

The authors thank D. Jones-Dias for her suggestions for the manuscript. Additionally, the authors thank to R. Touati, F. Z. Iles, N. Abdelaziz, S. Benada, N. Haddadi, and A. Mertani, for their technical assistance and support during the study. The authors also thank to all collaborators in the north, western, south and northeast regions of the country who sent isolates to LCM/CHU (S. Bekhoucha, S. Zouagui, Z. Guechi, S. Mahrane, A. Azzam, D. Haouchine, L. Oussadou, D. Touati, S. Azzoug, M. Naim, F. Z. Henniche, N. Aggoune, K. Lassas, S. Azrou, F. Lalaoui, F. Sahli) as well as to the clinical collaborators (T. Annane, N. Bouhafs, A. Laraba, S. Kermani, S. Bouziane, and M. Neggazi).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Specialty section:**

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 06 March 2015

**Accepted:** 01 June 2015

**Published:** 22 June 2015

**Citation:**

Kuang X, Hao H, Dai M, Wang Y,  
Ahmad I, Liu Z and Yuan Z (2015)  
Serotypes and antimicrobial  
susceptibility of *Salmonella* spp.  
isolated from farm animals in China.  
*Front. Microbiol.* 6:602.  
doi: 10.3389/fmicb.2015.00602

# Serotypes and antimicrobial susceptibility of *Salmonella* spp. isolated from farm animals in China

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*Salmonella* spp. can indirectly infect humans via transfer from animals and animal-derived food products, and thereby cause potentially fatal diseases. Therefore, gaining an understanding of *Salmonella* infection in farm animals is increasingly important. The aim of this study was to identify the distribution of serotypes in *Salmonella* samples isolated from chickens ( $n = 837$ ), pigs ( $n = 930$ ), and dairy cows ( $n = 418$ ) in central China (Henan, Hubei, and Hunan provinces) in 2010–2011, and investigate the susceptibility of strains to antimicrobial agents. *Salmonella* isolates were identified by PCR amplification of the *invA* gene, serotypes were determined by using a slide agglutination test for O and H antigens, and susceptibility to 24 antimicrobials was tested using the agar dilution method. In total, 248 *Salmonella* strains were identified: 105, 105, and 38 from chickens, dairy cows, and pigs, respectively. Additionally, 209 strains were identified in diseased pigs from the Huazhong Agricultural University veterinary hospital. Among these 457 strains, the dominant serotypes were Typhimurium in serogroup B, IIIb in serogroup C, and Enteritidis in serogroup D. In antimicrobial susceptibility tests, 41.14% of *Salmonella* spp. were susceptible to all antimicrobial agents, 48.14% were resistant to at least one, and 34.72% were resistant to more than three classes. Strains were highly resistant to sulfamethoxazole-trimethoprim (39.61%), nalidixic acid (39.17%), doxycycline (28.22%), and tetracycline (27.58%). Resistance to cephalosporins and fluoroquinolones ranged from 5.25 to 7.44% and 19.04 to 24.51%, respectively. Among penicillin-resistant and cephalosporin-resistant strains, 25 isolates produced extended-spectrum β-lactamases (ESBLs). The multidrug-resistant and ESBL-producing *Salmonella* strains identified in healthy animals here will present a challenge for veterinary medicine and farm animal husbandry, and could also pose a threat to public health. The level of antibiotic resistance observed in this study further highlights the need for careful and selective use of antibiotics.

**Keywords:** *Salmonella*, farm animals, serotype, antibiotics, antimicrobial resistance

## Introduction

*Salmonella* spp. are a common source of foodborne diseases that cause morbidity and mortality worldwide (Chiu et al., 2010). Treating *Salmonella* infection in humans is expensive; for example, in the USA, it causes illness in ~1.2 million patients annually, resulting in estimated medical costs of \$365 million (Centers for Disease Control and Prevention, 2014). In the European Union (EU), salmonellosis is the second most commonly reported gastrointestinal infection, with a confirmed case rate of 20.4 cases per 100,000 individuals in 2011 (European Centre for Disease Prevention and Control, 2013). In China, *Salmonella* causes an estimated 22.2% of foodborne diseases (Wang et al., 2007), and salmonellosis ranks fourth among the most prevalent foodborne diseases caused by microbial agents (Zhu et al., 2012). Many *Salmonella* serotypes exist, with >2600 serovars classified based on the reactivity of antisera to O and H antigens (Stevens et al., 2009), and ~292 identified in China between the 1980s and the end of 2000 (Yang, 2010). *Salmonella* also affects farm animals, and infections on farms can cause substantial economic damage in relation to, for example, loss of poultry stocks and costly animal husbandry. Numerous serotypes of isolated *Salmonella* have been found to overlap between farm animals and humans (Alcaine et al., 2006). Indeed, *Salmonella* not only directly infects humans but also causes indirect infections via transfer from animals and animal-derived food products such as pork and milk.

The use of antimicrobials is important for the control and treatment of *Salmonella*. However, since the early 1990s, antimicrobial- and multidrug-resistant *Salmonella* strains have emerged, leading to treatment failure. Researchers have reported a link between the use of antimicrobials in food animals and the emergence of antimicrobial resistance in pathogenic bacteria (Gong et al., 2013). Multidrug-resistant bacteria pose a severe threat to public health, particularly those that are resistant to  $\beta$ -lactams and fluoroquinolones (Lai et al., 2014). The increasing number of multidrug-resistant *Salmonella* strains is a global concern, with some countries and international organizations creating surveillance systems which include collaboration between human health, veterinary, and food-related sectors to monitor the spread of these and other foodborne bacteria. Examples include the Danish Integrated Antimicrobial Resistance Monitoring and Research Program, the European Food Safety Authority, the National Antimicrobial Resistance Monitoring System in the USA, and the Global Foodborne Infections Network run by the World Health Organization. These surveillance systems are also employed to monitor antimicrobial resistance, antimicrobial consumption in livestock, and serotype distribution, and data describing the current trend of increasing resistance to multiple drugs has been made available (European Centre for Disease Prevention and Control, 2013). In contrast, surveillance reports are unavailable in China; however, the National Monitoring Network for Bacteria of Animal Origin was launched as a surveillance system in 2008. Travel, migration, and the distribution of food between countries can also contribute to the spread of foodborne diseases and multidrug-resistant bacteria. Therefore, monitoring the

distribution of *Salmonella* serotypes and levels of antibiotic resistance in animals and animal-food products is also important for maintaining safe travel and the commercial trade in food animals (Lai et al., 2014; Russell et al., 2014).

In central China, Henan, Hubei, and Hunan provinces are important residential and trade centers. These three areas are also the main producers of animal-derived foods in China; in 2012, their output of meat, eggs, and milk accounted for 19.14%, 19.45%, and 9.17% of total Chinese production, respectively (China Agriculture Statistical Report, 2012). Previous studies documented a phenotype for *Salmonella* serovars that was discovered in chickens and pigs, and in animal-derived foods, in China (Gong et al., 2013; Li et al., 2013; Lai et al., 2014; Wang et al., 2014). However, specific knowledge of the *Salmonella* serovars that exist in dairy cows and other farm animals in central China is currently lacking. Therefore, the aim of this study was to evaluate the prevalence of *Salmonella* in chickens, pigs, and dairy cows on farms in central China. Additionally, the diversity of *Salmonella* serovars on these farms was identified, and their susceptibility and resistance to antimicrobial agents was investigated. A diverse range of serotypes was observed in healthy and diseased farm animals, and several multidrug-resistant and extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Salmonella* strains were identified.

## Materials and Methods

### Samples and *Salmonella* Isolation

Cloacal and anal swabs were collected from healthy animals on farms in Henan, Hubei, and Hunan provinces between March 2010 and July 2011. Each sampling site was visited only once. In total, 2185 samples were collected in a random manner from chickens ( $n = 837$ ), pigs ( $n = 930$ ), and dairy cows ( $n = 418$ ). Farms were chosen based on their scale with the following requirements: for pigs, annual sales were >10,000 heads; for chickens, the breeding stock was >100,000 heads; and for dairy cows, the breeding stock was >1000 heads. The owners of each farm gave permission for swab samples to be collected. The animals from which samples were extracted remained alive, did not undergo any surgery, and were not administered any drugs. Therefore, ethical approval was not required for the study because the sampling process did not harm the animals; however, their distress was considered and minimized at all times. All collected samples were stored at 4 °C and cultured at least 24 h before the isolation experiments were conducted.

The procedure for culture and isolation was based on standard laboratory protocol (WHO Global Foodborne Infections Network, 2010). Briefly, the samples were mixed with 5 mL of 0.85% saline solution for 30 min, and 1 mL of this mixture solution was added to buffered peptone water (BPW; Hopebiol, Qingdao, China) at a volume ratio of 1:10. The resultant mixture was incubated for 10 h at 37 °C for pre-enrichment. Approximately 500  $\mu$ L of this BPW mixture was then added to 5 mL selenite cystine broth (Hopebiol, Qingdao, China) and incubated at 37 °C for 20 h. The broth was streaked onto CHROMagar *Salmonella* (CHROMagar, France) and

incubated at 37 °C for 24–48 h. Potential *Salmonella* colonies were transferred into Luria–Bertani agar for purification and enrichment, and then incubated for 20 h at 37 °C to facilitate identification.

Additionally, 209 strains of *Salmonella* were isolated from samples collected from diseased pigs in the Huazhong Agricultural University veterinary hospital (HAUvh) between 2008 and 2010.

### Salmonella Identification

Biochemical testing and the *invA* gene were used to confirm the identity of isolates with typical *Salmonella* phenotypes. Biochemical testing was performed using a biochemical tube (Hangzhou Microbe Reagent Co., Ltd., China), and the results were interpreted based on Bergey's Manual of Systematic Bacteriology (Garrity et al., 2004). The *invA* gene was amplified by polymerase chain reaction (PCR) (Malorny et al., 2003). Positive results were randomly selected for sequencing. The obtained sequences were compared with the same gene registered in GenBank by using BLAST.

### Determination of *Salmonella* Serogroup and Serotype

The serogroup and serovars of the *Salmonella* isolates were determined by slide agglutination tests with O antigen and H antigen antiserums obtained from Lansheng, Lanzhou Institute of Biology, China. The results were interpreted according to the Kauffmann-White scheme (Grimont and Weill, 2007).

### Antimicrobial Susceptibility Testing

The agar dilution method with Mueller-Hinton agar (Oxoid Ltd., England) was used to test the susceptibility of *Salmonella* to 24 antibiotics (Table 1). These antimicrobials were classified based on their importance to human medicine (Government of Canada, 2005). *Escherichia coli* ATCC 25922 was used as the control microorganism. The results of the tests for ampicillin (AMP), amoxicillin (AMX), amoxicillin-clavulanic acid (AMC), ceftriaxone (CRO), imipenem (IPM), aztreonam (ATM), gentamicin (GEN), amikacin (AMK), tetracycline (TET), doxycycline (DOX), ciprofloxacin (CIP), levofloxacin (LEV), nalidixic acid (NAL), sulfamethoxazole-trimethoprim (SXT), chloramphenicol (CHL), and fosfomycin (FOS) were interpreted based on CLSI M100-S22 (Clinical and Laboratory Standards Institute, 2012), whereas those for ceftiofur (CEF), enrofloxacin (ENO), and florfenicol (FFC) were interpreted based on CLSI M31-A3 (Clinical and Laboratory Standards Institute, 2008). Interpretive CLSI criteria were not available for cefquinome (CEQ), polymyxin B (PB), azithromycin (AZM), olaquindox (OLA), and mequindox (MEQ); therefore, results with the following minimum inhibitory concentration (MIC) values were considered resistant for these antibiotics: CEQ ≥ 8 µg/mL (using CEF as a reference); PB ≥ 4 µg/mL (Kwa et al., 2007); AZM ≥ 16 µg/mL (Sjölund-Karlsson et al., 2011); OLA and MEQ ≥ 64 µg/mL (Sørensen et al., 2003).

Strains that were resistant to AMP, AMX, ATM, and/or CRO were examined for ESBLs by using the MIC values of ceftazidime, cefazime-clavulanic acid, cefotaxime, and cefotaxime-clavulanic

**TABLE 1 | Antibiotics and the range of concentrations tested.**

Antibiotic	Abbreviation	Concentration range (µg/mL)
Ampicillin	AMP	0.06 ~ 256
Amoxicillin	AMX	0.06 ~ 256
Amoxicillin-clavulanic acid	AMC	0.06/0.03 ~ 128/256
Ceftriaxone	CRO	0.06 ~ 512
Ceftiofur	CEF	0.06 ~ 512
Cefquinome	CEQ	0.015 ~ 256
Imipenem	IPM	0.03 ~ 128
Aztreonam	ATM	0.06 ~ 512
Gentamicin	GEN	0.5 ~ 512
Amikacin	AMK	0.5 ~ 512
Tetracycline	TET	0.5 ~ 512
Doxycycline	DOX	0.5 ~ 512
Ciprofloxacin	CIP	0.015 ~ 512
Enrofloxacin	ENO	0.06 ~ 512
Levofloxacin	LEV	0.06 ~ 512
Nalidixic acid	NAL	0.06 ~ 512
Sulfamethoxazole-trimethoprim	SXT	0.25/4.75 ~ 128/2432
Chloramphenicol	CHL	0.5 ~ 512
Florfenicol	FFC	0.5 ~ 512
Fosfomycin	FOS	1 ~ 2,048
Polymyxin B	PB	0.5 ~ 512
Azithromycin	AZM	0.5 ~ 512
Olaquindox	OLA	0.25 ~ 128
Mequindox	MEQ	0.25 ~ 128

acid. Results were interpreted based on CLSI M100-S22 (Clinical and Laboratory Standards Institute, 2012), and *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as the control organisms.

### Statistical Analysis

The isolated strains were categorized as sensitive (S), intermediary (I), or resistant (R) based on the MIC values and the CLSI interpretive criteria. MIC<sub>50</sub> and MIC<sub>90</sub> were calculated using previously described methods (Schwarz et al., 2010), and 95% confidence intervals were calculated using SPSS 16.0 software (IBM, USA).

## Results

### Salmonella Isolates and Serotypes

In total, 248 bacterial isolates taken from healthy chickens, pigs, and dairy cows, and 209 isolates from diseased pigs, were verified as *Salmonella* spp., and 457 strains identified as serotypes were tested for antimicrobial susceptibility. In all samples taken from healthy animals, the prevalence of *Salmonella* spp. was 11.35% (248/2185). The specific prevalence of *Salmonella* spp. in chickens, pigs, and dairy cows was 12.55% (105/837), 4.09% (38/930), and 25.12% (105/418), respectively.

After serotyping, all isolates were divided into three groups (Table 2). Thirty-four isolates did not cluster for

**TABLE 2 | Serovar distribution of isolated *Salmonella* (*n* = 457).**

Serotype (N) <sup>a</sup>	Serovars	Chicken (n <sup>b</sup> )	Pig (n <sup>b</sup> )	Dairy cow (n <sup>b</sup> )
Unable to self-agglutination (34)		6	28	0
Group A (3)	Unidentified types	3	0	0
Group B (205)	Unidentified subtypes	31	19	45
	Typhimurium	20	11	26
	Agona	3	0	12
	Derby	1	2	6
	II	1	1	4
	Kunduchi	0	0	4
	Schwarzengrund	0	3	0
	Lagos	0	2	1
	Fyris	0	0	2
	Agama	1	1	0
	Farsta	1	0	0
	Gloucester	1	0	0
	Kingston	0	0	1
	Kubacha	1	0	0
	Saintpaul	1	0	0
	Stanley	0	1	0
	Travis	0	1	0
	Tumodi	0	0	1
	Uppsala	0	1	0
Group C1 (141)	Unidentified subtypes	0	90	1
	IIIb	0	22	0
	Typhisuis	0	7	0
	II	0	6	0
	Schwabach	0	5	0
	Irumu	0	3	0
	Kaduna	0	2	0
	Birkenhead	0	1	0
	Cotonou	0	1	0
	Hissar	0	1	0
	Paratyphic	0	1	0
	Thompson	0	1	0
Group C2-C3 (5)	Unidentified subtypes	0	4	0
	Quiniela	0	1	0
Group D1 (16)	Unidentified subtypes	4	0	0
	Enteritidis	9	0	1
	II	1	0	0
	Berta	1	0	0
Group D3 (1)	II	0	1	0
Group E1 (4)	Meleagridis	1	0	0
	Newlands	0	2	0
	Simi	1	0	0
Group E4 (20)	Unidentified subtypes	2	9	0
	Kouka	4	0	0
	Gnesta	0	3	0
	Visby	0	1	0
	Rideau	1	0	0
Unidentified subgroups (28)		11	16	1

<sup>a</sup>N, the total number of *Salmonella* isolates per serogroup.<sup>b</sup>n, number of isolates with a given serotype of animals.

self-agglutination in sterile saline. Twenty-eight isolates could self-agglutination in sterile saline, but such activity was not observed with O antigen. The serogroups of the remaining 395 strains were determined. Eight distinct serogroups and 41 distinct serotypes were identified, as shown in **Table 2**. Group B ( $n = 205$ , 44.86%) and group C1 ( $n = 141$ , 30.85%) were identified as the dominant serogroups. Typhimurium 12.47% ( $n = 57$ ) and IIIb 4.81% ( $n = 22$ ) were the dominant serovars. Detailed serotypes could not be determined for 208 *Salmonella* isolates using the available H antigen in the different serogroups. According to the species of animal tested, the dominant serotypes were Typhimurium and Enteritidis in chickens, IIIb and Typhimurium in pigs, and Typhimurium and Agona in dairy cows (**Table 2**).

### Antimicrobial Susceptibility Testing

Results of antimicrobial susceptibility testing are summarized in **Table 3**. Among the 457 isolates, 188 (41.14%) were susceptible to all tested antimicrobials and 220 (48.14%) were resistant to at least one antibiotic. All strains showed susceptibility or intermediate susceptibility to imipenem. Strains were most commonly resistant to nalidixic acid (39.17%), sulfamethoxazole-trimethoprim (39.61%), doxycycline (28.22%), and tetracycline (27.58%). They also showed resistance to all specialist drugs used only in veterinary medicine, including enrofloxacin (24.51%), florfenicol (20.13%), ceftiofur (7.44%), and cefquinome (5.25%), and to drugs used in both veterinary and human medicine, such as ciprofloxacin (24.07%), gentamicin (19.69%), ceftriaxone (6.34%), and amoxicillin/clavulanate (4.38%). Resistance to azithromycin, which is one of the macrolides often used against gram-positive bacteria, was expressed at 16.19%. Although they are rarely used in clinical applications, resistance to polymyxin B (18.82%) and fosfomycin (12.04%) was also observed.

When antibiotic resistance was analyzed according to animal species, the resistance rates of *Salmonella* were different for the majority of the drugs tested (**Table 4**). Excluding imipenem, resistance to the other 23 drugs ranged from 1.90 to 49.52% in chickens and 0.40 to 48.18% in pigs. In comparison, *Salmonella* from dairy cows showed resistance to 12 types of drug, and resistance rates ranged from 0.95 to 10.48%.

In total, 159 strains (34.72%) exhibited varying degrees of multidrug resistance (MDR) (**Table 5**), defined as resistance to at least three different classes of antimicrobials. The *Salmonella* spp. that exhibited MDR consisted of 104 strains from pigs (including 15 and 89 strains from healthy and diseased pigs, respectively), 51 strains from healthy chickens, and 4 strains from healthy dairy cows. MDR isolates showed diverse resistance to different classes of antibiotics, but were most frequently resistant to penicillins, fluoroquinolones, and quinolones (each occurring 11 times).  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations appeared with the lowest frequency (five times) in *Salmonella* strains with MDR.

Based on analysis of MIC values and CLSI, 25 of the 457 isolates (5.47%) were confirmed as ESBL producers, with three suspected strains (**Table 6**). The ESBL-producing strains were obtained from different animals and areas as follows: two strains from chickens in Hubei, 15 strains from chickens in Henan, and eight strains from diseased pigs. Because the differences in MIC

values between CAZ, CAZ/C and/or CTX, and CTX/C were not  $\geq 3$ -fold based on CLSI, three strains from diseased pigs were suspected to be ESBL-producing strains.

The *Salmonella* serotypes of isolates differed in terms of their sensitivity to drugs (**Table 7**). Among all drug-sensitive isolates ( $n = 188$ ), 80 isolates were identified in 17 specific serotypes. Typhimurium ( $n = 28$ ) was the major serotype. Isolates of *Salmonella* strains with MDR showed 26 specific serotypes and the major serotypes were Enteritidis and IIIb (both  $n = 10$ ). All serotypes were resistant to nalidixic acid, but differences were observed in the resistance profiles of *Salmonella* serotypes that exhibited MDR. The most common resistance profiles in *Salmonella* IIIb ( $n = 10$ ) and Enteritidis isolates ( $n = 10$ ) were AMP-AMX-SXT-NAL and SXT-OLA-MEQ-NAL, respectively. The resistance profile was AMP-AMX-TET-DOX-ENO-SXT-CHL-NAL in Typhimurium ( $n = 6$ ), TET-DOX-CIP-ENO-SXT-CHL-FFC-NAL in Schwabach ( $n = 4$ ), and PB-OLA-MEQ-NAL in Kouka ( $n = 4$ ).

### Discussion

The prevalence of *Salmonella* spp. in the center of China were in agreement with those obtained from Sichuan province farm animals (Li et al., 2013), but differed from other areas. The results were higher than in EU (European Food Safety Authority, 2014), where chicken 2.7%, pig 6.3%, cattle 2.4%, but were lower than in USA (National Antimicrobial Resistance Monitoring System, 2011), where chicken 52.56%  $\sim$  47.95%, pig 10.34%  $\sim$  8.79%, cattle 23.02%  $\sim$  33.20%. Differences in isolation rates can be interpreted based on differences in region, sample types, collection seasons, culture methods, isolation methodologies, culture media, and local environmental conditions. For example, salmonellosis cases increased over the summer months, peaking in August and September, began decreasing thereafter (European Centre for Disease Prevention and Control, 2013). Therefore, a global perspective should ideally be adopted during sample collection, isolate separation, and, in particular, long-term monitoring.

The prevalence of *Salmonella* serotypes differed among animals and regions. Serogroup B and C1 were dominant, this result was consistent with in USA (Antunes et al., 2011). Serotype differ from other study. The dominant in chicken, pig, dairy cattle were Typhimurium and Enteritidis, IIIb and Typhimurium, Typhimurium and Agona in this study, respectively. The dominant serotype from chicken were Derby and Typhimurium in Sichuan province (Li et al., 2013), from pig were Enteritidis Indianain in Shandong province (Lai et al., 2014). The dominant from chicken, pig, cattle were Kentucky and Enteritidis, Adelaide and Johannesburg, Montevideo and Dublin in USA (National Antimicrobial Resistance Monitoring System, 2011); and from chicken, pig, cattle were Enteritidis and Infantis, Typhimurium and Typhimurium (monophasic), Typhimurium and Dublin in EU (European Food Safety Authority, 2014). This difference suggests serogroups and serotypes are varying according to geographical regions, and diversities and complexities. Certain serovars can emerge within a country or region for a certain period and then disappear with no evident cause or intervention.

**TABLE 3 | Susceptibility of *Salmonella* isolates (*n* = 457) to antimicrobials.**

Drug	Distribution of MICs (μg/mL) <sup>a</sup>															S(%)	I(%)	R(%)	MIC <sub>50</sub>	MIC <sub>90</sub>	95%CI	Category <sup>d</sup>
	2048 <sup>b</sup>	1024	512 <sup>b</sup>	256 <sup>b</sup>	128	64	32	16	8	4	2	1	0.5 <sup>c</sup>	0.25 <sup>c</sup>	0.125 <sup>c</sup>	0.06 <sup>c</sup>	0.03 <sup>c</sup>	0.015 <sup>c</sup>				
AMP	95	15	7	4	3	2	3	34	163	4	1	1	127	72.87	0.67	26.46	1	>256	59.40-79.42	=		
AMX	99	15	2	2	5	1	3	1	64	137	1	1	126	73.09	1.09	25.82	0.5	>256	64.55-85.37	=		
AMC	11	1	1	6	13	61	18	14	5	198	3	127	92.78	2.84	4.38	0.5/0.25	128/64	12.07-19.39	-			
CRO	8	9	5	1	4	2	1	1	1	1	26	400	93.44	0.22	6.34	<0.06	2	7.60-16.82	-			
CEF	9	6	10	4	1	0	3	21	47	106	132	4	1	113	87.96	4.60	7.44	0.5	8	14.46-29.93	-	
CEQ <sup>e</sup>	2	6	13	3	1	3	15	10	34	35	190	37	108	94.53	0.22	5.25	0.06	1	1.03-2.82	-		
IPM	1	1	16	18	33	15	0	3	47	169	148	119	8	97.16	2.84	0	0.25	1	0.34-0.44	-		
ATM	7	1	16	18	33	15	10	8	4	11	3	17	401	97.15	2.18	0.67	<0.06	0.125	0.11-7.73	-		
GEN	7	7	1	1	8	41	38	200	17	145	130	130	80.31	0	19.69	1	64	19.25-32.35	=			
AMK	7	5	38	55	27	0	1	5	92	120	16	98	71.33	1.09	27.58	4	128	4.84-16.36	=			
TET	5	14	37	42	16	14	54	131	13	130	130	130	59.96	11.82	28.22	4	128	40.65-57.47	III			
DOX	6	6	60	9	10	2	9	14	9	14	28	20	5	271	64.77	11.16	24.07	<0.015	32	30.16-44.54	III	
CIP	45	4	3	28	8	5	9	10	19	32	12	8	274	64.33	11.16	24.51	<0.06	128	22.29-36.35	-		
ENR	45	46	6	11	23	6	16	8	26	30	11	274	79.65	1.31	19.04	<0.06	64	6.10-9.65	-			
LEV	165	4	4	5	1	2	64	97	7	1	4	79	23	60.83	-	39.17	8	>512	1.68-2.13	III		
SXT	127	15	3	22	5	9	90	69	5	112	60.39	-	39.61	>38	>38	>38	>38	>38	>38	>38		
CHL	26	73	7	5	3	2	1	21	179	42	98	74.62	0.44	24.94	2	256	59.68-85.76	III				
FFC	38	18	21	11	2	2	4	27	141	66	127	78.99	0.88	20.13	2	256	48.66-75.67	III				
FOX	24	18	12	5	6	1	2	1	30	358	86.87	1.09	12.04	<1	512	1.19-2.10	-					
PBf	56	1	1	10	18	5	161	205	205	205	205	205	18.82	1	>512	48.86-79.73	III					
AZM <sup>g</sup>	22	14	28	11	2	13	24	56	287	287	287	287	16.19	<0.5	32	21.44-41.77	II					
OLA <sup>h</sup>	80	41	28	107	114	8	79	79	79	79	79	79	26.48	16	128	31.84-40.25	IV					
MEQ <sup>i</sup>	76	39	26	61	159	13	2	81	81	81	81	81	25.38	8	128	29.94-38.35	IV					

AMP, Ampicillin; AMX, Amoxicillin; AMC, Amoxicillin-clavulanic acid; CRO, Ceftazidime; CEQ, Cefotetan; CEF, Ceftriaxone; CIP, Ciprofloxacin; ENO, Enrofloxacin; LEV, Levofloxacin; NAL, Nalidixic acid; SXT, Sulamethoxazole-trimethoprim; CHL, Chloramphenicol; FFC, Fluorfenicol; FOS, Fosfomycin; PB, Polymyxin B; AZM, Aztreonam; GEN, Gentamicin; AMK, Amikacin; TET, Tetracycline; DOX, Doxycycline; OIP, Ofloxacin; OLA, Olaparib; MEC, Mequindox.

<sup>a</sup>Susceptibility breakpoints are indicated by black vertical bars and resistance breakpoints are double vertical bars. CLSI breakpoints used when available. CLSI breakpoints do not exist for cefquinome, polymyxin B, aztreonam, olaparib, and mequindox.

<sup>b</sup>Including higher than this tested MIC value.

<sup>c</sup>Including lower than this tested MIC value.

<sup>d</sup>Category I: antimicrobials of very high importance in human medicine, essential to the treatment of serious bacterial infections, no alternatives for resistant infections; Category II: antimicrobials of high importance in human medicine, used as first-line drugs, alternatives for resistance are generally available; Category III: antimicrobials of medium importance in human medicine, used to treat a variety of infections, alternatives for resistance to category II antimicrobials.

<sup>e</sup>CLSI interpretative criteria for this bacterium/antimicrobial combination are not currently available. Isolates with an MIC  $\geq 4 \mu\text{g/mL}$  were reported as resistant.

<sup>f</sup>CLSI interpretative criteria for this bacterium/antimicrobial combination are not currently available. Isolates with an MIC  $\geq 16 \mu\text{g/mL}$  were reported as resistant.

<sup>g</sup>CLSI interpretative criteria for this bacterium/antimicrobial combination are not currently available. Isolates with an MIC  $\geq 64 \mu\text{g/mL}$  were reported as resistant.

**TABLE 4 | Antibiotic resistance of *Salmonella* isolated from different food-producing animals unit: %.**

Drug	Total (n = 457)	Chicken (n = 105)	Pig (n = 247)	Dairy cow (n = 105)
AMP	26.46	31.43	35.22	0.95
AMX	25.82	30.48	34.41	0.95
AMC	4.38	2.86	6.88	0
CRO	6.34	20.00	3.24	0
CEF	7.44	20.95	4.45	0.95
CEQ	5.25	15.24	3.24	0
IPM	0	0	0	0
ATM	0.67	1.90	0.40	0
GEN	19.69	17.14	29.15	0
AMK	1.53	6.67	0	0
TET	27.58	22.86	41.30	0
DOX	28.22	23.81	42.11	0
CIP	24.07	25.71	32.39	2.86
ENO	24.51	26.67	32.79	2.86
LEV	19.04	19.04	27.13	0
NA	39.17	46.67	48.18	10.48
SXT	39.61	49.52	47.77	10.48
CHL	24.94	26.67	34.41	0.95
FFC	20.13	20.95	27.94	0.95
FOS	12.04	6.67	19.43	0
PB	18.82	23.81	24.70	0
AZM	16.19	15.24	23.08	0.95
OLA	26.48	40.00	30.77	2.86
MEQ	25.38	40.95	28.34	2.86

AMP, Ampicillin; AMX, Amoxicillin; AMC, Amoxicillin-clavulanic acid; CRO, Ceftriaxone; CEF, Cefotiofur; CEQ, Cefquinome; IPM, Imipenem; ATM, Aztreonam; GEN, Gentamicin; AMK, Amikacin; TET, Tetracycline; DOX, Doxycycline; CIP, Ciprofloxacin; ENO, Enrofloxacin; LEV, Levofloxacin; NAL, Nalidixic acid; SXT, Sulfamethoxazole-trimethoprim; CHL, Chloramphenicol; FFC, Florfenicol; FOS, Fosfomycin; PB, Polymyxin B; AZM, Azithromycin; OLA, Olaquindox; MEQ, Mequindox.

For instance, compared with data in 2010, the number of Enteritidis and Typhimurium decreased by 6% and 9% in 2011, respectively, (European Centre for Disease Prevention and Control, 2013); similar, compared with results in 2009, Enteritidis decreased, even disappeared from chicken and pig in 2012 from Shandong province (Lai et al., 2014).

The *Salmonella* serotypes isolated from farm animals overlapped with those that cause illnesses in humans, further highlighting the fact that *Salmonella* could be transmitted from animals to humans via the food chain (Alcaine et al., 2006; de Jong et al., 2009), such as Typhimurium and Enteritidis had identified from human in Henan and Hubei provinces (Cui et al., 2009; Xia et al., 2009). Some researchers have suggested that the differences in the dominant serotypes between animals and humans might be due to variations in their pathogenicity and corresponding resistance profiles (Volf et al., 2010), the strain containing Typhimurium and Enteritidis could acquire resistance to a large number of different antimicrobial compounds. At least two different serotypes existed from multiple-two strains to multiple-eleven strains, especially Enteritidis and Typhimurium, which were similar with other study from animals (Li et al., 2013; European Food Safety

**TABLE 5 | The number of multidrug-resistance *Salmonella* strains identified in sampled animals unit: strain.**

Resistance pattern	Chickens	Pigs	Dairy cows
3	3	6	1
4	16	7	2
5	1	4	0
6	3	7	1
7	6	13	0
8	7	18	0
9	4	3	0
10	9	13	0
11	2	22	0
12	0	7	0
13	0	4	0
Total	51	104	4

Authority, 2014; Lai et al., 2014), also with human (Cui et al., 2009; Xia et al., 2009). This may be related to the presence of the genetic structure known as *Salmonella* genomic island 1 (SGI1) and resistance genes (European Food Safety Authority, 2014). Therefore, monitoring of *Salmonella* should preferably focus more strongly on serovars that could, for example, be involved in a large outbreak.

Based on our MIC analysis, antibiotic-resistant bacteria, including multidrug-resistant bacteria, appeared in both healthy and diseased farm animals. These results were similar with other study (Gong et al., 2013; Li et al., 2013; Lai et al., 2014). One potential explanation is the use of antibiotics during breeding. Disease control and prevention in China during the breeding process mainly depends on the use of antibiotics (Gong et al., 2013). This arguably irrational use of antibiotics has contributed to the emergence of multidrug-resistant bacteria under selective antimicrobial pressure. Huazhong area (Henan, Hubei, and Hunan provinces) is one of the more concentrated areas of farm animals (China Agriculture Statistical Report, 2012), the demands and uses of drugs are huge, then inevitable there would be unreasonable use of drugs. These drugs, or the raw materials of the drugs, are inexpensive and easy to obtain; they have been widely used on animals in China. However, some steps have been taken to prevent the emergence of drug-resistant bacteria and promote food safety and public health with the Ministry of Agriculture of the People's Republic of China having formulated plans to ban or reduce the use of specific antimicrobials (Ministry of Agriculture of the People's Republic of China, 2002).

After the therapeutic effects of antimicrobials were confirmed in humans in the mid-1940s, they were soon introduced to veterinary medicine (McEwen, 2006). Even though some drugs were exclusively designed for veterinary use, the compounds administered are identical or very similar to those used in human medicine because they belong to the same antimicrobial classes (Heuer et al., 2009) such as  $\beta$ -lactams, cephalosporins, aminoglycosides, macrolides, tetracycline, sulfonamides, fluoroquinolones. Most of the antibiotic resistance to different classes has appeared in humans, animals, and/or animal

**TABLE 6 | MICs used to verify ESBL-producing *Salmonella*.**

Sample code	Ceftazidime ( $\mu\text{g/mL}$ )	Ceftazidime-clavulanic acid ( $\mu\text{g/mL}$ )	Cefotaxime ( $\mu\text{g/mL}$ )	Cefotaxime-clavulanic acid ( $\mu\text{g/mL}$ )	Source
147	2	0.5	64	<0.25	Chicken/Henan
151	4	0.5	>64	<0.25	Chicken/Henan
157	2	0.25	64	<0.25	Chicken/Henan
165	4	0.5	>64	<0.25	Chicken/Henan
171	4	0.5	>64	<0.25	Chicken/Henan
173	4	0.5	>64	<0.25	Chicken/Henan
174	4	0.5	>64	<0.25	Chicken/Henan
175	2	0.25	64	<0.25	Chicken/Henan
176	4	0.25	64	<0.25	Chicken/Henan
199	4	0.5	>64	<0.25	Chicken/Hubei
209	2	<0.25	32	<0.25	Chicken/Henan
211	2	<0.25	32	<0.25	Chicken/Henan
216	64	0.5	>64	<0.25	Chicken/Henan
217	2	<0.25	64	<0.25	Chicken/Henan
218	64	0.5	>64	<0.25	Chicken/Henan
264	2	0.5	32	<0.25	Chicken/Henan
268	128	2	32	1	Chicken/Hubei
4p	0.5	<0.25	16	<0.25	Pig/HAUvh
78p	0.5	<0.25	32	<0.25	Pig/HAUvh
127p	0.5	0.5	4	0.25	Pig/HAUvh
128p	0.5	0.5	64	4	Pig/HAUvh
151p	>128	128	16	8	Pig/HAUvh (suspected)
153p	4	0.5	64	<0.25	Pig/HAUvh
159p	4	0.5	>64	<0.25	Pig/HAUvh
167p	4	0.5	>64	<0.25	Pig/HAUvh
187p	>128	>128	16	8	Pig/HAUvh (suspected)
201p	128	64	>64	>64	Pig/HAUvh (suspected)
212p	0.5	0.5	64	0.5	Pig/HAUvh
212p	0.5	0.5	64	0.5	Pig/HAUvh

HAUvh, Huazhong Agricultural University veterinary hospital.

products. Ceftriaxone had showed resistance rates  $\leq 13\%$  and 4% from imported foods and humans (Akiyama and Khan, 2012; Wong et al., 2014). Similar, gentamicin had shown resistance in animals and humans (Lai et al., 2014; Wong et al., 2014). Overlapping resistance not only can lead to treatment failure, but also have negative consequences in both humans and animals.

ESBL-producing *Salmonella* strains were isolated from chickens and pigs in different areas in this study. Currently, the resistance genes were still on attempting to identify and sequencing from the samples. The emergence of producing-ESBLs bacteria in animals and animal products is of particular concern to public health, it allow bacteria to become resistant to a wide variety of penicillins and cephalosporins. ESBL-producing bacteria have appeared in poultry, pig, and cattle farms (Horton et al., 2011; European Food Safety Authority, 2014). In *Salmonella*, acquisition of resistance genes was likely to have occurred by conjugation, usually with other Enterobacteriaceae through the transfer of plasmids (European Food Safety Authority, 2014). Bacteria that develop resistance via ESBLs could become a reservoir of resistance genes, which may enter the food chain (European Food Safety Authority, 2012). When antibiotic resistance occurs during infection, the

remaining treatment option is usually an antibiotic from the carbapenem family. While carbapenems were previously drugs of last resort, their use is now also contributing to resistance (Centers for Disease Control and Prevention, 2014). They are used to treat highly resistant infections in humans, and are not used in food-producing animals. However, carbapenemase-producing organisms have been isolated from farm animals (Fischer et al., 2012). In China, monitoring strategies do not currently contain testing and identification of ESBL- or AmpC-producing organisms in isolates from animals and/or animal-derived food products. It suggest that this should be established within surveillance procedures to anticipate possible changes in the status of ESBL enzymes.

*Salmonella* isolates showed multiple-resistance in results, it suggested the emergence of co-resistance and/or cross-resistance (Cantón and Ruiz-Garbajosa, 2011). For example, amikacin had showed low levels of resistance (0.67%); this may be due to cross-resistance among aztreonam, ciprofloxacin, and  $\beta$ -lactams (Livermore, 2002). Co-resistance to fluoroquinolones and third- and fourth-generation cephalosporins has also been identified in *Salmonella* isolated from humans (Zhang et al., 2014). Considering these results, the prudent use of antibacterial

**TABLE 7 | Distribution of resistant *Salmonella* isolates according to serovars unit: strain.**

Serogroup, serotype	Pan-susceptible	Intermediary	Number of antimicrobial classes in resistance pattern						Total
			1~2	3~4	5~6	7~8	9~10	11~13	
B, Typhimurium	28	18	5	0	0	5	1	0	57
B, Agona	13	0	1	1	0	0	0	0	15
B, Derby	5	0	2	2	0	0	0	0	9
B, II	4	0	1	0	0	0	0	1	6
B, Kunduchi	4	0	0	0	0	0	0	0	4
B, Schwarzenground	0	0	0	0	0	1	1	1	3
B, Lagos	1	0	0	0	0	1	0	1	3
B, Agama	0	1	0	0	0	0	0	1	2
B, Fyrirs	2	0	0	0	0	0	0	0	2
B, Farsta	1	0	0	0	0	0	0	0	1
B, Gloucester	0	1	0	0	0	0	0	0	1
B, Kingston	1	0	0	0	0	0	0	0	1
B, Kubacha	0	0	0	0	0	1	0	0	1
B, Saintpaul	0	1	0	0	0	0	0	0	1
B, Stanley	0	0	0	0	0	0	1	0	1
B, Travis	0	0	0	0	0	0	1	0	1
B, Tumodi	0	0	1	0	0	0	0	0	1
B, Uppsala	0	0	0	0	0	0	1	0	1
C1, III b	7	0	5	5	1	0	2	2	22
C1, Typhisuis	6	0	1	0	0	0	0	0	7
C1, II	3	0	1	0	0	1	1	0	6
C1, Schwabach	0	0	1	0	0	3	1	0	5
C1, Irumu	1	0	0	0	0	1	0	1	3
C1, Kaduna	0	0	0	0	0	0	2	0	2
C1, Birkenhead	0	0	1	0	0	0	0	0	1
C1, Cotonou	0	0	0	0	0	1	0	0	1
C1, Hissar	0	0	0	0	1	0	0	0	1
C1, Paratyphic	1	0	0	0	0	0	0	0	1
C1, Thompson	1	0	0	0	0	0	0	0	1
C2-C3, Quiniela	1	0	0	0	0	0	0	0	1
D1, Enteritidis	0	0	0	7	2	1	0	0	10
D1, Berta	0	0	0	1	0	0	0	0	1
D1, II	0	0	0	0	0	1	0	0	1
D3, II	1	0	0	0	0	0	0	0	1
E1, Newlands	0	0	0	0	0	2	0	0	2
E1, Meleagridis	0	0	0	0	0	1	0	0	1
E1, Simi	0	0	0	0	0	0	1	0	1
E4, Gnesta	0	0	3	0	0	0	0	0	3
E4, Kouka	0	0	0	3	0	1	0	0	4
E4, Rideau	0	0	0	1	0	0	0	0	1
E4, Visby	0	0	1	0	0	0	0	0	1
A, Unidentified subtypes	0	0	0	0	0	1	1	1	3
B, Unidentified subtypes	43	23	6	2	4	12	2	3	95
C1, Unidentified subtypes	39	3	14	6	5	5	4	15	91
C2-C3, Unidentified subtypes	2	0	0	0	0	0	1	1	4
D1, Unidentified subtypes	0	0	0	1	0	2	1	0	4
E4, Unidentified subtypes	0	1	8	2	0	0	0	0	11
Unidentified subgroup	10	0	2	3	1	2	6	4	28
Without-self-curing	14	1	8	1	1	3	2	4	34
Total	188	49	61	35	15	45	29	35	457

agents should be strongly recommended in clinical, veterinary, and agricultural settings in order to preserve antibiotic activity and avoid the development of cross-resistance.

In previous studies, researchers have demonstrated the relationship between the increased prevalence of antimicrobial-resistant bacteria and (a) the increased use of antimicrobials in human and veterinary medicine, (b) greater movement of people and animals, and (c) increased industrialization (Cheng et al., 2012). Huazhong is central to China; this area not only meets the needs of its own people via the production of animals and food but also supplies other areas and imports animals and food from other regions. This movement is a potential contributor to the spread of antibiotic-resistant bacteria. The environment itself also contains a variety of bacteria that potentially represent an immense pool of antibiotic-resistance genes, which if transferred between bacteria could cause human and animal disease (D'Costa et al., 2006). For example, wild animals that are carriers of antimicrobial-resistant bacteria could pass on colonies of resistant strains when they encounter humans, farming areas, and/or waste (Literak et al., 2011). A limitation of our study was that the samples were only collected from farm animals. For complete consideration in a surveillance system, samples should also be collected from, for example, the farm environment and farm workers.

Despite bans and legislation, some antibiotics are still routinely fed to livestock prophylactically to increase profits and to limit potential bacterial infections in stressed and crowded livestock (Ndi and Barton, 2011). Antibiotic-resistant bacteria will inevitably follow wherever antimicrobials are used; therefore, a coordinated multi-disciplinary approach will be required to address this issue (Smith et al., 2009). Various programs around the world have been established to monitor and control antibiotic-resistant bacteria. Recently, an official document, "National Medium- and Long-Term Planning for

Prevention and Control of Animal Epidemics (2012 to 2020)," (General Office of State Council of China, 2012) was issued in China. The government outlined plans to control diseases by detection and purification using a medium- or long-term program, with the aim of reducing the antimicrobials used in animals and in symptomatic treatment. In future, the safest way to prevent the problem may be reducing antimicrobial usage with strict policies and legislation to control the development and spread of resistant bacteria in animals.

## Funding

This research is supported by the Ministry of Science and Technology of the People's Republic of China (2012 BAK01B02), National Natural Science Foundation of China (31101856/31302143), Grants from National Basic Research program of China (2013CB127200), National Key Technology R&D Program (2012BAK01B00), Fundamental Research Funds for the Central Universities (2662015PY035) and project supported by the morning program of Wuhan in China (2015070404010191).

## Author Contributions

XK, HH, MD, YW, and ZY designed the study. XK, HH, IA, and ZL collected the antimicrobial susceptibility data. XK, HH, YW, and ZL collected the serotype data. XK, HH, MD, and ZY analyzed the data. XK, HH, IA, and YW wrote the manuscript. XK, HH, MD, and ZY revised manuscript.

## Acknowledgments

We thank Professor Aizhen Guo, who provided some of the *Salmonella* spp. isolates from diseased pigs.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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# Susceptibility of Austrian Clinical *Klebsiella* and *Enterobacter* Isolates Linked to Patient-Related Data

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 28 October 2015

Accepted: 11 January 2016

Published: 05 February 2016

### Citation:

Badura A, Pregartner G, Holzer JC,  
Feierl G and Grisold AJ (2016)  
Susceptibility of Austrian Clinical  
*Klebsiella* and *Enterobacter* Isolates  
Linked to Patient-Related Data.  
*Front. Microbiol.* 7:34.  
doi: 10.3389/fmicb.2016.00034

The aim of the study was to analyze the antimicrobial susceptibility of Austrian clinical *Klebsiella* sp. and *Enterobacter* sp. isolates linked to patient-related data over a time period from 1998 to 2014. The main findings of this study were (i) a marked difference of antibiotic susceptibility rates between different infection sites for both *Klebsiella* sp. and *Enterobacter* sp., (ii) significantly greater percentages of resistant isolates among both *Klebsiella* sp. and *Enterobacter* sp. in male patients compared to female patients and (iii) significantly greater percentages of resistant isolates among both *Klebsiella* sp. and *Enterobacter* sp. from hospital-derived samples compared to samples from the community. In conclusion, our statistical data analysis clearly indicated a strong association of patient-related data and *Klebsiella* sp. and *Enterobacter* sp. susceptibility profiles.

**Keywords:** antibiotic resistance, *Klebsiella*, *Enterobacter*, Austria, statistical analysis

## INTRODUCTION

Whereas the antibiotic resistance crisis in Gram-positive pathogens largely seems to be kept under control, the emergence and dissemination of antibiotic resistance in Gram-negative pathogens risk getting out of control (Arya et al., 2008; Slama, 2008; Bush et al., 2011; Livermore, 2012). To gain useful information on overall trends in a specific region and alert for more effective prevention and control measures, surveillance studies based on large data sets are of particular importance in this field (World Health Organization [WHO], 2014). Multivariate statistical analysis further allows studying possible relationships between antimicrobial resistance data and patient-related factors; however, there are only a limited number of studies up to now on that issue mainly dealing with *Escherichia coli*. The main findings of these studies are that susceptibilities to several antimicrobial agents are strongly depending on infection site as well as patient location (hospital/community), gender and age (Livermore et al., 2003; Miguel Sahuquillo-Arce et al., 2011; Badura et al., 2015). Under the hypothesis that the same is true for *Klebsiella* sp. and *Enterobacter* sp., the aim of this study is to analyze a large amount of antibiotic resistance data (almost 39.000 isolates) from our geographical region according to patient-related factors. The application of multivariate statistical analysis enables us to detect significant associations between patient's characteristics and the percentages of resistant isolates what is of key importance to further identify possible risk factors related to antibiotic-resistant *Klebsiella* and *Enterobacter* infections.

**TABLE 1 | Data basis for the statistical analysis.**

	Patient gender (%)		Patient location (%)		Number of isolates
	Female	Male	Community	Hospital	
Klebsiella sp.	59.4	40.5	52.3	47.7	24024
Enterobacter sp.	52.8	47.1	44.0	56.0	14969
Total					38993

**TABLE 2 | Overall resistance percentages of Klebsiella sp. and Enterobacter sp.**

Antibiotic	Klebsiella sp.		Enterobacter sp.		
	%R	n/total	%R	n/total	
AN	3.47	366/10550	0.96	78/8094	
AMC	9.44	2264/23979			
CAZ	7.19	1230/17102	20.55	2341/11389	
CIP	6.43	1543/23995	5.2	778/14961	
CTX	5.76	1370/23765	19.87	2943/14811	
NF	24.43	2297/9404	30.71	1502/4891	
GM	6.66	1458/21889	2.76	392/14216	
IPM	0.89	60/6747	0.54	26/4839	
MEC	3.73	120/3220			
MEM	0.67	70/10511	0.28	22/7720	
PT	6.98	1270/18186	20.17	2505/12418	
SXT	10.82	2595/23990	6.22	930/14958	

AN, amikacin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; NF, nitrofurantoin; GM, gentamicin; IPM, imipenem; MEC, mecinilam; MEM, meropenem; PT, piperacillin/tazobactam; SXT, trimethoprim/sulfamethoxazole; %R, percentages of non-susceptible isolates.

## MATERIALS AND METHODS

A total of 24.024 *Klebsiella* sp. and 14.969 *Enterobacter* sp. isolates were included in the present analysis. The study design of this retrospective observational study (data origin, laboratory methods, and statistical analysis) is largely similar to our previous report on *E. coli* (Badura et al., 2015). Data were retrieved from the Laboratory of Medical Bacteriology and Mycology of the Medical University of Graz, Austria from January 1998 to March 2014. In the event of multiple isolates from one person in a year, only the first one was considered. For each isolate, patient-related data (age, gender), patient location (community/hospital) and culture site were obtained. Culture sites were subdivided into the following categories: urinary tract (UT), genital tract (GT), wounds (WOU), respiratory tract (RES), and blood (BLO); patient age was categorized as follows: <1, 1–9, 10–19, 20–29, 30–39, 40–49, 50–59, 60–69, 70–79, ≥ 80 years.

All isolates had been tested for antibiotic susceptibilities in the routine microbiology laboratory in the same way as reported in a previous study using the disk diffusion method or a VITEK 2 system (bioMérieux, Marcy l'Etoile France) (Badura et al., 2015). From January 1st, 1998 to May 31st, 2011 results were interpreted using the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards (NCCLS)

(CLSI, 2012). From June 1st, 2011 results were interpreted using the criteria recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST breakpoint tables v1.2, 2011) in its respective current version. For this study, resistance to the following 12 antibiotic agents was analyzed: amikacin (AN), amoxicillin-clavulanic acid (AMC; analyzed for *Klebsiella* sp. only), ceftazidime (CAZ), ciprofloxacin (CIP), cefotaxime (CTX), nitrofurantoin (NF; analyzed for urinary tract isolates only), gentamicin (GM), imipenem (IPM), mecinilam (MEC; analyzed for *Klebsiella* sp. urinary tract isolates only); meropenem (MEM), piperacillin/tazobactam (PT), and trimethoprim/sulfamethoxazole (SXT). Resistant and intermediate resistant isolates were combined.

## Statistical Analysis

For each of the two microbes and for each antibiotic under consideration, the absolute and relative frequencies of resistant samples were determined in total, per culture site, patient location, age group, gender and year of acquisition. In order to assess the influence of the covariates on antibiotic resistance, multivariable logistic regression analysis was performed. For binary covariates, odds ratios and 95% confidence intervals are presented and are adjusted for year of acquisition, patient gender, patient age, patient location and resistance phenotype; for culture site a model containing the aforementioned covariates as well as cultures site has been tested against a model without culture site and p values from the respective Chi-square test are presented. Statistical analysis was performed using the R package, version 3.1.1 and SPSS, version 21.0 (R Development Core Team, 2008; IBM Corp, 2012).

## Ethics Statement

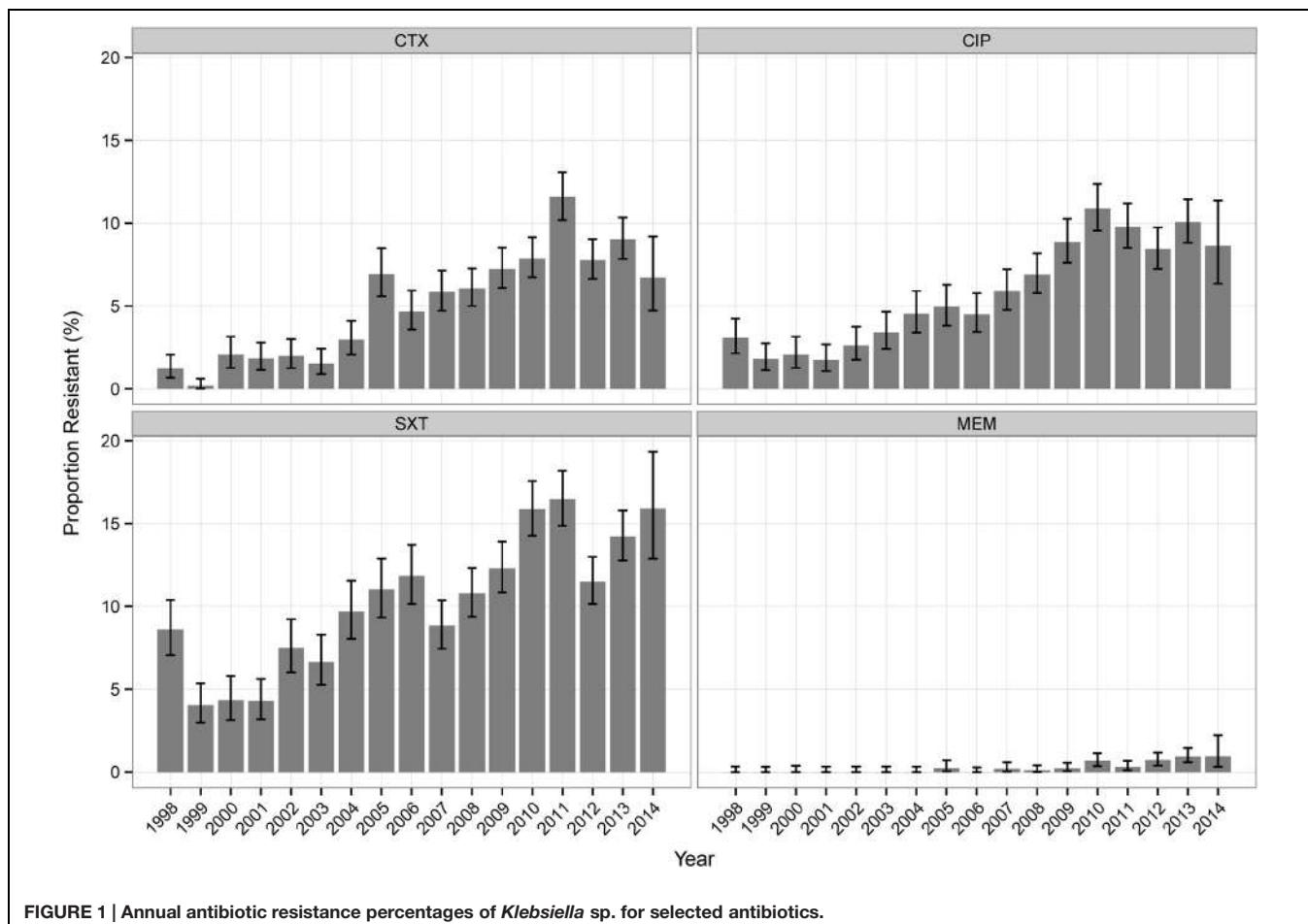
The study was approved by the Ethics Committee of the Medical University of Graz (26-502 ex 13/14); patient records were anonymized prior to analysis.

## RESULTS

The underlying data basis for the present analysis is shown in Table 1. In total, 38.993 clinical isolates of *Klebsiella* sp. and *Enterobacter* sp. were included in the statistical analysis.

## Species Distribution

Within the *Klebsiella* samples, *K. pneumoniae* (61%) and *K. oxytoca* (27.1%) were the most frequently recovered species;



**FIGURE 1 |** Annual antibiotic resistance percentages of *Klebsiella* sp. for selected antibiotics.

within the *Enterobacter* samples, *E. cloacae* complex (59.2%) and *E. aerogenes* (15.4%) were the most frequently recovered species.

## Overall and Annual Resistance Percentages

**Table 2** shows the overall resistance percentages of all tested isolates. For *Klebsiella* sp. isolates, NF (analyzed for UT isolates only) and SXT showed the highest values, for *Enterobacter* sp. the highest values were found for NF, CAZ, PT, and CTX. For *Klebsiella* sp. isolates, most antibiotics (CAZ, CTX, NF, CIP, SXT, IPM, and MEM) showed increasing percentages of resistant isolates over time (**Figure 1, Supplemental Table S1**). The percentages of resistant *Enterobacter* sp. isolates did not change considerably over the investigational period for the majority of antibiotic agents (Supplemental Table S1).

## Resistance Percentages According to Patient-Related Data

Results of the multivariate analysis of the antibiotic resistance patterns according to culture sites for both pathogens are presented in **Table 3**. The findings reveal the lowest resistance

percentages from samples originating from the male or female genital tract (GT). For *Klebsiella* sp. isolates, the highest resistance percentages for almost all antibiotics analyzed occurred in blood infections. For *Enterobacter*, the highest resistance percentages were found in isolates originating from blood (CAZ, CTX, GM, PT, SXT), and urinary tract infections (AN, CIP) (**Table 3**).

Antibiotic resistance patterns in relation to patient gender are shown in **Table 4**. Resistance percentages for almost all antibiotic agents analyzed are higher in male patients. A significant difference can be shown for all antibiotics except for AN for *Klebsiella* sp. isolates and for AN, GM, IPM, MEM, and PT for *Enterobacter* sp., respectively. Antibiotic resistance patterns in relation to patient location are shown in **Table 5**. Resistance percentages for almost all antibiotic agents analyzed are significantly higher in hospital treated patients. This is true for both *Klebsiella* sp. and *Enterobacter* sp. isolates.

Regarding the association between antibiotic resistance percentages of *Klebsiella* sp. and *Enterobacter* sp. isolates and patient age, the majority of antibiotics show a balanced distribution except for CIP where a constant increase with age can be shown for both pathogen groups (**Figure 2**).

**TABLE 3 |** Resistance percentages of *Klebsiella* sp. and *Enterobacter* sp. according to culture sites.

	Klebsiella sp. %R (n/total)						Enterobacter sp. %R (n/total)					
	UT	GT	WOU	RES	BLO	p-value <sup>a</sup>	UT	GT	WOU	RES	BLO	p-value <sup>a</sup>
AN	4.2 (32/766)	0.1 (1/1648)	1.4 (37/2657)	1.7 (48/2867)	0 (0/103)	0.022	5.7 (22/388)	0 (0/767)	0.5 (13/2629)	0.4 (10/2467)	0 (0/68)	<0.001
AMC	6.9 (845/12264)	2.7 (63/2363)	9.7 (288/3086)	10.7 (357/3347)	14.3 (17/119)	<0.001	21.8 (61/2806)	6.4 (56/871)	17.9 (513/2869)	19.9 (553/2778)	36 (31/86)	<0.001
CAZ	7.9 (486/6156)	0.8 (16/1893)	5.2 (152/2923)	5.9 (190/3228)	10.1 (12/119)	<0.001	7.4 (436/5887)	0.7 (7/1075)	5 (153/3039)	4 (115/2874)	4.7 (4/86)	<0.001
CIP	7.6 (937/12262)	1.6 (39/2365)	6.9 (213/3088)	5.9 (198/3354)	10.1 (12/119)	<0.001						
CTX	4.3 (518/12044)	1 (24/2364)	5.2 (161/3084)	5.6 (189/3350)	10.9 (13/119)	0.011	19.3 (111/5747)	6.8 (73/1076)	18.2 (553/3038)	19.7 (564/2865)	36 (31/86)	<0.001
GM	3.5 (351/10162)	0.7 (17/2363)	3.9 (119/3087)	5.9 (198/3352)	4.2 (5/119)	<0.001	1.7 (86/5144)	0.1 (1/1076)	1.2 (37/3039)	1.3 (36/2872)	2.3 (2/86)	<0.001
IPM	0.2 (2/1110)	0 (0/814)	0.8 (12/1495)	1.1 (20/1755)	3.7 (3/81)	0.013	0.2 (1/583)	0 (0/306)	0.5 (7/1380)	0.3 (5/1476)	0 (0/54)	0.025
MEM	0.2 (5/2101)	0 (0/1124)	0.7 (15/2286)	1 (24/2414)	3.7 (4/108)	<0.001	0.3 (3/931)	0 (0/580)	0.2 (4/2344)	0.1 (3/2076)	0 (0/80)	0.003
PT	5.9 (421/7102)	1.4 (30/2121)	7.8 (230/2948)	9.1 (288/3156)	12.7 (15/118)	<0.001	16.3 (805/4934)	22.7 (204/899)	22.6 (539/2390)	23.4 (567/2426)	38 (27/71)	<0.001
SXT	12.6 (1551/12263)	4.2 (100/2365)	8 (248/3085)	7.2 (242/3354)	9.2 (11/119)	<0.001	6 (354/5881)	6.2 (67/1076)	6 (182/3041)	6.3 (182/2873)	14 (12/86)	0.221

UT, urinary tract; GT, genital tract; WOU, wounds; RES, respiratory tract; BLO, blood; AN, amikacin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, ceftaxime; NF, nitrofurantoin; GM, gentamicin; IPM, imipenem; MEM, meropenem; PT, piperacillin/tazobactam; SXT, trimethoprim/sulfamethoxazole. <sup>a</sup>The p-values stem from a goodness-of-fit test comparing a model including all variables against a reduced model including all variables except for culture site.

## DISCUSSION

We here report an observational study whose aim is to analyze the susceptibility of Austrian clinical *Klebsiella* sp. and *Enterobacter* sp. isolates linked to patient-related data. The underlying database for this statistical analysis covers the period from 1998 to 2014. A major limitation of the present study is that it was not possible to link the data to severity of disease and patient outcome as these data were not available. Additionally, information about the patient's length of the hospital stay was not included in the analysis which would have been useful for the classification as hospital or community acquired infections. In the present work, infections were categorized depending on the patient's location at the moment the clinical sample was obtained. The passage from CLSI to EUCAST guidelines in 2011 in Austria may further bias data comparability. Several authors have reported an increase of antibiotic resistance rates for certain *Enterobacteriaceae* species/drug combinations following EUCAST breakpoints implementation (Hombach et al., 2012; Wolfensberger et al., 2013). However, we did not observe any artificial rise of antibiotic resistance at that time indicating that the change of interpretation criteria does not apparently impact the results of the large data set analyzed.

In general, surveillance is a substantial basis in the combat against antimicrobial resistance in both community and hospital settings; a large number of resistance surveillance reports thus exist about *Klebsiella* sp. and *Enterobacter* sp. isolates from various parts of the world describing the current resistance situation (Jones et al., 2004; Bedenic et al., 2010; Gales et al., 2012; Badal et al., 2013; Kaye and Pogue, 2015). Furthermore, studies dealing with the association of antimicrobial resistance data and specific factors depending on the host are of major importance with regard to the identification of possible risk factors related to antibiotic-resistant infections. Several studies aimed to find various patient demographics and clinical factors associated with the presence of antibiotic-resistant *Enterobacteriaceae* isolates, however, most of them involve particular infections, patient cohorts, anatomic sites or specific resistance problems. These studies thus mostly cover a small amount of isolates consequently limiting statistical data processing (O'Fallon et al., 2010; Metan et al., 2013; Ivady et al., 2015; Moini et al., 2015; Oliveira et al., 2015). Here we present a comprehensive statistical analysis on associations between patient-related data and the percentages of antibiotic resistant *Klebsiella* sp. and *Enterobacter* sp. isolates referring on a large database with almost 39.000 isolates from Austria. The main findings of this study are (i) a marked difference of antibiotic susceptibility rates between different infection sites for both *Klebsiella* sp. and *Enterobacter* sp., (ii) significantly greater percentages of resistant isolates among both *Klebsiella* sp. and *Enterobacter* sp. in male patients compared to female patients and (iii) significantly greater percentages of resistant isolates among both *Klebsiella* sp. and *Enterobacter* sp. isolates from hospital-derived samples compared to samples from the community.

**TABLE 4 | Antibiotic resistance percentages of Klebsiella sp. and Enterobacter sp. according to patient gender.**

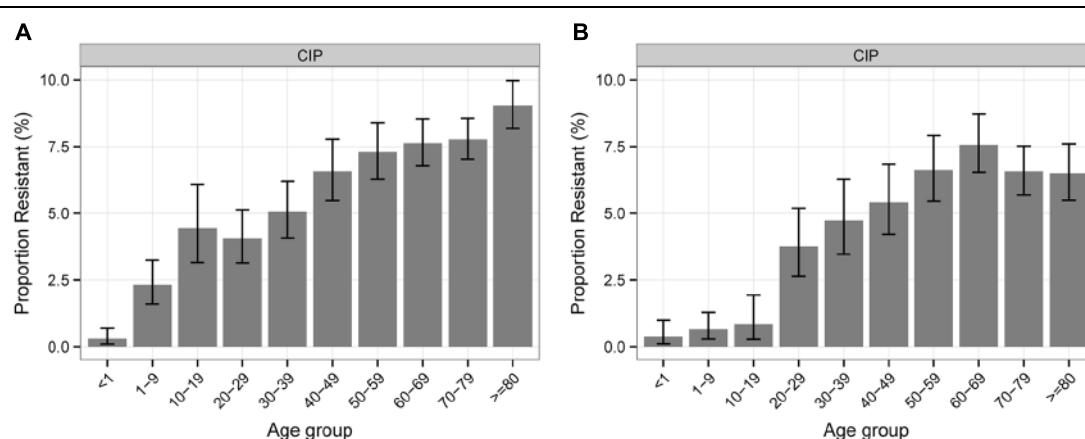
Antibiotic	Klebsiella sp. %R (n/total)			Enterobacter sp. %R (n/total)		
	Female	Male	OR	Female	Male	OR
AN	3.2 (174/5396)	3.7 (192/5141)	1.16 [0.95, 1.44]	0.8 (28/3731)	1.1 (50/4355)	1.43 [0.89, 2.34]
AMC	7.4 (1053/14245)	12.5 (1210/9715)	1.78 [1.63, 1.94]			
CAZ	5.9 (552/9398)	8.8 (677/7688)	1.55 [1.38, 1.74]	17.7 (990/5598)	23.3 (1350/5782)	1.13 [1.03, 1.25]
CIP	4.5 (647/14248)	9.2 (895/9728)	2.13 [1.92, 2.37]	3.3 (263/7899)	7.3 (515/7050)	2.2 [1.88, 2.57]
CTX	4.3 (611/14093)	7.9 (758/9653)	1.88 [1.68, 2.1]	17.4 (1361/7812)	22.6 (1580/6987)	1.1 [1.01, 1.2]
NF	22.4 (1392/6208)	28.3 (905/3196)	1.37 [1.24, 1.51]	28.2 (837/2972)	34.7 (665/1919)	1.23 [1.09, 1.4]
GM	4.8 (613/12791)	9.3 (844/9079)	2.04 [1.83, 2.27]	2.3 (170/7416)	3.3 (222/6788)	1.11 [0.9, 1.38]
IPM	0.5 (17/3464)	1.3 (43/3273)	2.7 [1.57, 4.87]	0.5 (12/2255)	0.5 (14/2578)	0.8 [0.37, 1.76]
MEC	3 (60/2012)	5 (60/1208)	1.62 [1.11, 2.36]			
MEM	0.4 (19/5174)	1 (51/5326)	2.07 [1.24, 3.61]	0.3 (9/3509)	0.3 (13/4206)	1 [0.43, 2.43]
PT	5.3 (545/10257)	9.2 (724/7912)	1.35 [1.19, 1.52]	19.7 (1286/6523)	20.7 (1216/5887)	1.01 [0.93, 1.11]
SXT	9.3 (1321/14245)	13.1 (1273/9726)	1.3 [1.19, 1.43]	5.8 (457/7897)	6.7 (472/7049)	1.16 [1.01, 1.33]

AN, amikacin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; NF, nitrofurantoin; GM, gentamicin; IPM, imipenem; MEC, mecillinam; MEM, meropenem; PT, piperacillin/tazobactam; SXT, trimethoprim/sulfamethoxazole. OR, odd ratio

**TABLE 5 | Antibiotic resistance percentages of Klebsiella sp. and Enterobacter sp. according to patient location.**

Antibiotic	Klebsiella sp. %R (n/total)			Enterobacter sp. %R (n/total)		
	Outpatient	Hospital	OR	Outpatient	Hospital	OR
AN	0.3 (10/2894)	4.6 (356/7656)	4.18 [2.24, 8.71]	0.6 (11/1782)	1.1 (67/6312)	1.39 [0.74, 2.83]
AMC	4.8 (602/12557)	14.6 (1662/11422)	2.43 [2.18, 2.71]			
CAZ	4.5 (303/6703)	8.9 (927/10399)	1.32 [1.04, 1.69]	10.2 (369/3607)	25.3 (1972/7782)	2.97 [2.63, 3.37]
CIP	5.6 (700/12558)	7.4 (843/11437)	1.08 [0.96, 1.23]	4.5 (297/6587)	5.7 (481/8374)	1.22 [1.05, 1.43]
CTX	2.7 (335/12552)	9.2 (1035/11213)	2.39 [1.8, 3.18]	11.8 (775/6584)	26.4 (2168/8227)	2.67 [2.44, 2.93]
NF	21 (1376/6557)	32.3 (921/2847)	1.77 [1.59, 1.96]	26.1 (872/3339)	40.6 (630/1552)	1.82 [1.6, 2.07]
GM	2 (205/10465)	11 (1253/11424)	2.86 [2.4, 3.42]	0.7 (40/5847)	4.2 (352/8369)	4.48 [3.23, 6.39]
IPM	0.2 (4/1863)	1.1 (56/4884)	2.55 [1.01, 8.59]	0 (0/1032)	0.7 (26/3807)	*
MEC	3.3 (78/2393)	5.1 (42/827)	1.3 [0.87, 1.93]			
MEM	0.2 (4/2508)	0.8 (66/8003)	5.14 [2.09, 17.09]	0.1 (1/1534)	0.3 (21/6186)	5.79 [1.18, 104.62]
PT	3.4 (266/7892)	9.8 (1004/10294)	2.71 [2.34, 3.14]	18.3 (956/5219)	21.5 (1549/7199)	1.2 [1.09, 1.32]
SXT	9.5 (1190/12556)	12.3 (1405/11434)	0.95 [0.86, 1.05]	5.9 (387/6582)	6.5 (543/8376)	1.05 [0.91, 1.21]

AN, amikacin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; NF, nitrofurantoin; GM, gentamicin; IPM, imipenem; MEC, mecillinam; MEM, meropenem; PT, piperacillin/tazobactam; SXT, trimethoprim/sulfamethoxazole. \*Not estimable because of 0 resistant isolates in outpatient group. OR, odd ratio

**FIGURE 2 | Percentages of antibiotic resistance of Klebsiella sp. (A) and Enterobacter sp. (B) to CIP according to patient age.**

To our knowledge, this is the first report to compare percentages of antibiotic resistant *Klebsiella* sp. and *Enterobacter* sp. isolates from different infection sites within a specific region. The highest resistance percentages for the majority of antibiotics analyzed can be shown for both pathogen groups originating from blood infections. This is in accordance with previous statistical analyses on the association of the infection site and *Escherichia coli* susceptibility profiles (Miguel Sahuquillo-Arce et al., 2011; Badura et al., 2015). As reported for *Escherichia coli*, *Klebsiella* sp. and *Enterobacter* sp. isolates originating from the GT generally show the lowest resistance percentages indicating that detection from this culture site often represents the normal patient flora. Interestingly, antibiotic-resistant *Klebsiella* sp. and *Enterobacter* sp. infections within our study collection derive mostly from men. The same association between patient sex and antibiotic resistance was previously shown for *Escherichia coli*, however, the underlying cause remains as yet largely unclear (Livermore et al., 2003; Miguel Sahuquillo-Arce et al., 2011; Badura et al., 2015). Similar to the results from several other studies dealing with antibiotic resistance percentages of *Enterobacteriaceae* in community and hospital settings we found significantly greater resistance rates for both pathogen groups amongst the hospital-derived isolates suggesting that the problem of antibiotic resistance amongst Gram-negative pathogens is still focused on hospital settings in our region (Boyd et al., 2008; Ferjani et al., 2015; McKay and Bamford, 2015).

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## CONCLUSION

Our statistical data analysis clearly indicates a strong association between patient characteristics (gender, localization, infection site) and *Klebsiella* sp. and *Enterobacter* sp. susceptibility profiles. To gain further insight in the correlation of bacterial antibiotic resistance and its host, additional studies analyzing large databases of microbiological information are of crucial importance ultimately influencing local antimicrobial therapy guidelines.

## AUTHOR CONTRIBUTIONS

AB: general conception, study design, data acquisition, data analysis, data interpretation, writing of the manuscript; GF: data interpretation, revising of the manuscript; JH and GP: statistical analysis, data interpretation, revising of the manuscript; AG: data interpretation, revising of the manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00034>

**TABLE S1 | Annual resistance percentages of *Klebsiella* sp. and *Enterobacter* sp.**

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The Risk of Some Veterinary Antimicrobial Agents on Public Health Associated with Antimicrobial Resistance and their Molecular Basis

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
Frontiers in Microbiology

**Received:** 07 December 2015

**Accepted:** 29 September 2016

**Published:** 18 October 2016

### Citation:

Hao H, Sander P, Iqbal Z, Wang Y, Cheng G and Yuan Z (2016) The Risk of Some Veterinary Antimicrobial Agents on Public Health Associated with Antimicrobial Resistance and their Molecular Basis. *Front. Microbiol.* 7:1626.  
doi: 10.3389/fmicb.2016.01626

The risk of antimicrobial agents used in food-producing animals on public health associated with antimicrobial resistance continues to be a current topic of discussion as related to animal and human public health. In the present review, resistance monitoring data, and risk assessment results of some important antimicrobial agents were cited to elucidate the possible association of antimicrobial use in food animals and antimicrobial resistance in humans. From the selected examples, it was apparent from reviewing the published scientific literature that the ban on use of some antimicrobial agents (e.g., avoparcin, fluoroquinolone, tetracyclines) did not change drug resistance patterns and did not mitigate the intended goal of minimizing antimicrobial resistance. The use of some antimicrobial agents (e.g., virginiamycin, macrolides, and cephalosporins) in food animals may have an impact on the antimicrobial resistance in humans, but it was largely depended on the pattern of drug usage in different geographical regions. The epidemiological characteristics of resistant bacteria were closely related to molecular mechanisms involved in the development, fitness, and transmission of antimicrobial resistance.

**Keywords:** antimicrobial agents, food-producing animal, antimicrobial resistance, public health, molecular basis

## INTRODUCTION

Antimicrobial agents have been used in food animal production since the 1950s. Antimicrobial agents have contributed significantly to the prevention and treatment of infectious diseases in food animals and some of them have played a very important role in the promotion of animal growth and feed efficiency (Dibner and Richards, 2005; Niewold, 2007). Since many classes of antimicrobial agents used in food animals are also used in human medicine, there is the potential for selection, and spread of antimicrobial resistant bacteria in animals to humans through the food supply. Human health consequences have been raised concerning whether the use of antimicrobial agents in food animals may minimize the effectiveness of the same classes of medically important antimicrobial agents to treat antimicrobial resistant infectious diseases in humans (Salisbury et al., 2002). In this respect, the administration of low doses (5–40 mg/kg.feed) of antimicrobial growth-promoters in animal feed were banned by Europe (EU) in 2006 to protect public health, and this

ban drew a great attention of other countries and international organizations (Marshall and Levy, 2011) since this low dose drug exposure over a long period of time could elicit selective pressure leading to the emergence of resistant bacteria.

For comprehensive surveillance monitoring of antimicrobial resistance in food-borne pathogens, many countries have established antimicrobial resistance monitoring systems, such as the National Antimicrobial Resistance Monitoring System (NARMS) in United States of America (USA) and Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP). The Food and Agriculture Organization (FAO), World Health Organization (WHO), and World Organization for Animal Health (OIE) have organized expert workshops on the risk assessment and management of non-human antimicrobial usage and their resistance (FAO/OIE/WHO, 2003, 2004, 2006). WHO/FAO/OIE have jointly carried out systematic evaluation of veterinary antimicrobial resistance for the impact on public health (FAO/OIE/WHO, 2006). The European Medicines Agency (EMA), European Food Safety Authority (EFSA) and European Center for Disease Prevention and Control (ECDC) have also work together for the Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) and have also published reports on antimicrobial use in food animals and antimicrobial resistance recently (ECDC/EFSA/EMA, 2015).

In the present review, results of risk assessments based on data from different antimicrobial resistance monitoring systems are reported to evaluate the stewardship programs of antimicrobial use in food animals. Resistance to some representative antimicrobial agents (e.g., cephalosporins, tetracyclines, fluoroquinolones, macrolides, glycopeptides, and streptogramins) in some selected pathogens (e.g., *Enterococci* spp, *Campylobacter* spp, *Salmonella* spp, and *Escherichia coli*) were taken as examples to describe the relationship between antimicrobial resistance and drug usage in food animals. The molecular mechanism involved in the development, fitness, and transmission of antimicrobial resistance was also integrated into the review to provide a comprehensive understanding of the antimicrobial resistance and to identify the need for risk management of antimicrobial drugs.

## AVOPARCIN AND GLYCOPEPTIDE-RESISTANT ENTEROCOCCI

Avoparcin, a vancomycin analog, was effective against gram-positive bacteria by disturbing their cell wall synthesis. Avoparcin had been widely used as a feed additive in food animals during 1940s–1990s. As a member of glycopeptides, there was concern that the misuse of avoparcin may confer cross-resistance to glycopeptides and in particular vancomycin which is known as one of the important last-line antimicrobials in human medicine. In 1993, isolation and frequency of vancomycin-resistant *Enterococci* (VRE) from food-producing animals in Great Britain drew public health concern about the consequences of wide use of avoparcin as a growth promoter in animals.

From 1995 to 2000, Denmark, United Kingdom, EU members, Japan and China gradually banned the use of avoparcin in food-producing animals.

It is important to keep in mind that the term of VRE includes several combinations of bacterial species (e.g., *Enterococcus faecium* and *Enterococcus faecalis*) and resistance genes (*vanA*, *B*, *C*, *D*, *E*, *G*, *L*, *M*, and *N*). The characteristics of *van* A-N genes are summarized in Table 1. The *vanA*, *B*, *D*, *E*, *G*, *L*, *M*, and *N* are acquired genetic determinants in *E. faecium* and/or *E. faecalis*, while *vanC* is intrinsic gene present in *E. gallinarum* and *E. casseliflavus/E. flavescentis* (Cetinkaya et al., 2000; Boyd et al., 2008). Among those, *E. faecium* with *vanA* type vancomycin resistance is clinically most important because *vanA* is an acquired and transferable gene which is resistant to both vancomycin and teicoplanin (Nilsson, 2012). The *vanB* and *vanN* are transferable but is susceptible to teicoplanin (Nomura et al., 2012). The *vanM* is newly found in China and confirmed to have transferability and resistance to both vancomycin and teicoplanin (Xu et al., 2010; Chen et al., 2015).

Extensive use of avoparcin for animal growth promotion in most parts of Europe may be the reason for high prevalence of VRE in the intestinal microbiota of farm animals in Europe during the 1990s (Aarestrup, 1995; Klare et al., 1995). Once the use of avoparcin was prohibited, prevalence of VRE among farm animals decreased in some EU countries. According to DANMAP report, substantial reductions (from 80 to 0%) in the prevalence of VRE were observed between 1995 and 2013, after the ban on avoparcin as growth promoter in Denmark (DANMAP, 2013). Very few vancomycin resistant enterococci have been isolated from Danish livestock and produced meat during 2003–2013 (DANMAP, 2013).

However, it was noteworthy that ban on avoparcin did not effectively reduce incidence of vancomycin-resistant *E. faecium* in avian feces. A paper published in 2008 showed that vancomycin-resistant *E. faecium* was still highly prevalent in poultry in Europe (Werner et al., 2008). Even after 15 years of the EU ban on avoparcin, vancomycin-resistant *E. faecium* was still present in the food chain and could be detected in 47% of the broiler feces (Garcia-Migura et al., 2007; DANMAP, 2010). The proportion of broilers colonized with vancomycin-resistant *E. faecium* increased from less than 1% in 2000 to over 40% in 2005, even though Sweden had forbidden the use of avoparcin as growth promoter since 1986 (Nilsson et al., 2009). The high prevalence of vancomycin-resistant *E. faecium* in EU countries may be due to the transferability of *E. faecium* with the *vanA* gene (Nilsson et al., 2009). The *vanN* gene with transferability was also found in vancomycin-resistant *E. faecium* isolated from chicken meat (Nomura et al., 2012).

The most important concern is not only that VRE are present among farm animals but also their potential to transfer resistance genes to vancomycin susceptible enterococci and other Gram-positive bacteria that may be transmitted via food products to humans. Some earlier reports showed that hospital isolates of *E. faecium* generally clustered in subgroups which were different from those found in animals (Top et al., 2004; Willems et al., 2005). In contrast, some *in vivo* transfer studies indicated that *vanA* gene was located in transposon Tn1546 and may be

**TABLE 1 | Characteristics of glycopeptide-resistant genes in Enterococci.**

Characteristics	<i>vanA</i>	<i>vanB</i>	<i>vanC</i>	<i>vanD</i>	<i>vanE</i>	<i>vanG</i>	<i>vanL</i>	<i>vanM</i>	<i>vanN</i>
Enterococcus species	<i>E. faecium</i> ; <i>E. faecalis</i>	<i>E. faecium</i> ; <i>E. faecalis</i>	<i>E. gallinarum</i> , <i>E. casseliflavus</i> , <i>E. flavescentis</i>	<i>E. faecium</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. faecium</i>
Vancomycin MIC ( $\mu\text{g/mL}$ )	64–1024	4–1024	2–32	16–64	16–32	16–32	8	>256	16
Teecloplanin MIC ( $\mu\text{g/mL}$ )	16–512	<1	<1	2–16	<0.5	<0.5	<0.5	96	0.5
Genetic determinant	acquired	acquired	intrinsic	acquired	acquired	acquired	acquired	acquired	acquired
transferable	Yes	Yes	No	No	No	No	No	Yes	Yes
New precursor of ligase	D-ala-D-lac	D-ala-D-lac	D-ala-D-ser	D-ala-D-lac	D-ala-D-ser	D-ala-D-ser	D-ala-D-ser	D-ala-D-lac	D-ala-D-ser
Expression	Inducible	Inducible	constitutive	constitutive	Constitutive or inducible	Constitutive or inducible	Constitutive or inducible	Inducible	constitutive

transferred between animal and human adapted enterococci (Jensen, 1998; Lester et al., 2006; Lester and Hammerum, 2010). The *vanM* gene was also located in transferable element and could transfer by conjugation (Xu et al., 2010). Similar strains of VRE have been isolated from both farm animal and human, indicating that some of those strains may adapt to farm animals and cause infectious diseases in humans (Freitas et al., 2011). However, the origin of Tn1546 element encoding *vanA* resistance in US hospitals was still unknown (Jensen, 1998; Lester et al., 2006; Lester and Hammerum, 2010). It was difficult to determine to what extent did the presence of VRE among farm animals actually affect public health (Nilsson, 2012).

Notably, a controversy was perceived in the geographical distribution of VRE among humans. In the United States, about 20,000 (or 40%) of *Enterococcus* healthcare-associated infections in 2013 were vancomycin resistant, including 77% of vancomycin resistant *E. faecium* (CDC, 2013). In Denmark, vancomycin resistance was detected in only 3.4% of *E. faecium* isolates from bloodstream infections in 2013 (DANMAP, 2013). In other Nordic countries, the level of vancomycin resistant *Enterococcus* was even lower than in Denmark in recent years (EARS-Net, 2012). Therefore, VRE was more common in US hospitals than that in European hospitals, although in the USA, avoparcin had never been approved for use in food animals (Gambarotto et al., 2000; Bonten et al., 2001). It was likely that the serious problem of VRE in US hospitals was not related to the use of avoparcin in food animals but to that of vancomycin use in human medicine, because therapeutic vancomycin treatment was much higher in the USA when compared with that in Europe (Acar et al., 2000).

## VIRGINIAMYCIN AND STREPTOGRAMIN-RESISTANT ENTEROCOCCI

Virginiamycin is a streptogramin antimicrobial which could block the transpeptidation or translocation of protein synthesis in bacteria. It has been used for the prevention of *Clostridial enteritis*

and enhancement of growth and feed efficiency in poultry, swine and cattle for more than 30 years in Japan, Canada, U.S.A, and other countries. However, EU prohibited its use in food-producing animals in 1999 because it was assumed to select for streptogramin-resistant *Enterococci* (SRE) and lead to treatment failure of patients in hospitals with pristinamycin and Synergic (Quinupristin-Dalfopristin) resistant *Enterococcus faecium* infections. Mechanisms conferring resistance to streptogramin in *E. faecium* was mediated via related acetyltransferases (VatD and VatE), erythromycin ribosomal methylase B (ErmB) and staphylococcal-type lactonase (VgbA) (Werner et al., 2002).

Some epidemiological investigations showed that streptogramin-resistant genes (*vat*) were detected in 25% of virginiamycin-resistant *E. faecium* isolated from pigs and chickens, and in 29% of isolates from farm workers in Denmark (Hammerum et al., 1998; Haroche et al., 2000). The NARMS report revealed that long-term use of virginiamycin for growth promotion was likely to result in the emergence of streptogramin-resistant *Enterococcus* which was present in 30–70% of poultry products purchased from supermarkets (NARMS, 2012).

Transferability of these *vat* genes was observed among *E. faecium* isolates from food-animals (Sørensen et al., 2001). The resistant strains may also spread indirectly to human beings from farms through the environment including raw manure or surface/ground water (Smith et al., 2003). Sørensen et al. (2001) found that streptogramin-resistant *E. faecium* from food animals was able to establish transient populations in the gut after experimental ingestion. Additionally, similar genetic patterns of *vanA* containing *Enterococci* isolates, from poultry and human, were reported in Spain (Robredo et al., 2000).

However, streptogramin-resistant *E. faecium* from poultry may be well-adapted to cloaca, but difficult to survive against the gastric barrier and colonization resistance in the gut (Smith et al., 2003). Despite the high rate of exposure in contaminated meat, prevalence of streptogramin resistant *E. faecium* remained low and therefore, bacteria with high resistance could rarely establish in human beings (McDonald et al., 2001). By using pulsed-field gel electrophoresis (PFGE) technique, Hershberger

et al. (2005) found distinct gene cluster of streptogramin-resistant *E. faecium*, isolated from poultry and human beings. Additionally, numerous studies found the different genetic profiles of streptogramin-resistant *E. faecium*, isolated from food-animals (poultry and pork) and human beings, indicating that the bacterial strains might be highly host specific (Smith et al., 2003; Hershberger et al., 2005; Hammerum et al., 2010).

The US Food and Drug Administration and Center for Veterinary Medicine (FDA-CVM) reported that there was not enough data to show the transmission of SRE between animals and humans (FDV-CVM, 2004). A quantitative risk assessment performed by US FDA-CVM concluded that risk of SRE, caused by virginiamycin use in food animals, was about 1–14 heads per billion due to the treatment failure of VRE infections (FDV-CVM, 2004). A quantitative human health risk and benefits assessment for virginiamycin showed that human health benefits of virginiamycin withdrawal ranged from zero to less than one statistical life and withdrawal of the drug may cause more human illnesses than it would prevent (Cox and Popken, 2004; Cox, 2005). Phillips (2007) showed that human health risk from resistance among enterococci selected by virginiamycin was small (Phillips, 2007). Some scientists have disagreed with the conclusion that the ban of virginiamycin in EU could lead to an increased prevalence of streptogramin-resistant enterococci (Hammerum et al., 2007). Although an investigation showed that the continued use of virginiamycin as growth promoter in poultry may increase the potential for streptogramin-resistant *E. faecium* infection in humans (Kieke et al., 2006).

## FLUOROQUINOLONE AND RESISTANCE IN CAMPYLOBACTER

Fluoroquinolones, a family of synthetic broad-spectrum antimicrobial agents, played an important role in the treatment of bacterial infection in both veterinary medicine and in human medicine. Fluoroquinolones can inhibit the DNA synthesis of bacteria by selectively inhibiting their DNA gyrase and/or topoisomerase (Suto et al., 1992). The fluoroquinolone resistance in *Campylobacter* was mediated by mutations in the drug target enzyme (e.g., Thr-86-Ile mutation in GyrA) and/or by overexpression of efflux pumps (e.g., CmeABC) (Griggs et al., 2005).

Both enrofloxacin and ciprofloxacin were the second generation of fluoroquinolones. They had similar structural and antimicrobial activity. The enrofloxacin was approved to treat bacterial infections in poultry in the USA before 2005, while ciprofloxacin was used in human medicine to treat foodborne infections such as *Campylobacter*, *Salmonella*, *E. coli*, and *Shigella*. When enrofloxacin was administrated into some food producing animal, it could be metabolized to ciprofloxacin (Gratacós-Cubarsí et al., 2007). The close relationship between fluoroquinolone drug in veterinary medicine and its use in human medicine may raise the risk of fluoroquinolone resistance from animal to human.

There was evidence that the use of enrofloxacin in poultry production would induce fluoroquinolone resistance in *Campylobacter jejuni* and these fluoroquinolone resistant bacteria transferred to humans and contributed to the treatment failure of campylobacteriosis in humans via poultry exposure (FDA, 2002; Nelson et al., 2007). Some *in vitro* and *in vivo* studies have demonstrated that FQ-resistant strains would rapidly emerge when *Campylobacter* was exposed to FQs (e.g., enrofloxacin). The frequencies of emergence may range between approximately  $10^{-6}$ – $10^{-8}$ /cell/generation in culture media, indicating that resistant bacteria would inevitably emerge when cell population was sufficiently larger than  $10^6$  (Yan et al., 2006; Han et al., 2008). FQ-susceptible *C. jejuni* in chicken could rapidly attain FQ-resistance within 24 h after the initiation of treatment with enrofloxacin (McDermott et al., 2002; Luo et al., 2003; van Boven et al., 2003; Farnell et al., 2005; Griggs et al., 2005). The FQ-resistant *Campylobacter* population could eventually colonize into intestinal tract of birds and may be transmitted to human via the contaminated poultry meat (Luangtongkum et al., 2009).

Due to above reasons, US FDA withdrew the use of enrofloxacin in poultry in 2005 (USFDA, 2005). After withdrawal of enrofloxacin from poultry, the rate of FQ-resistance in *C. jejuni* decreased in chicken during 2005–2007 (NARMS, 2010). Human clinicians also observed a reduction in domestically acquired *Campylobacter* infections with decreased susceptibility to fluoroquinolones, and it was thought to be a great achievement regarding public health (Nelson et al., 2007). However, during 2008–2011, the positive rate of ciprofloxacin-resistant *C. jejuni* from retail chicken was again increased (14.6–22.7%) in the USA (NARMS, 2012). These studies suggest that the policy on the ban of fluoroquinolone use in poultry did not reduce or eliminate reservoirs of FQ-resistant *C. jejuni* with subsequent reemergence and persist in poultry products in the USA.

The spread of FQ-resistant *C. jejuni* in USA might result from high mutation rate and enhanced fitness of the bacteria in chicken reservoirs (Luangtongkum et al., 2009). Previous studies have demonstrated that resistant *C. jejuni*, carrying Thr-86-Ile mutation in GyrA, could colonize chicken caecum in the absence of antimicrobial selection pressure (Luo et al., 2005; Nelson et al., 2007; Han et al., 2012; Zeitouni et al., 2012). The Thr-86-Ile mutation in GyrA can modulate DNA supercoiling homeostasis and result in better survivability of FQ-resistant *C. jejuni* in chicken host (Han et al., 2012). Due to the enhanced fitness, it will be difficult to reduce the prevalence of FQ-resistance in *C. jejuni*, even though farmers have not used these antimicrobials in poultry. Additionally, the NARMS human data showed that ciprofloxacin resistant *C. jejuni* in USA kept increasing from 16.7 in 1997 to 25.3% in 2012 (NARMS, 2012).

Contrary to the situation in USA, the ECDC/EFSA/EMA JIACARA reported data showed no associations between the consumption of fluoroquinolones in food-producing animals and the occurrence of resistance in *Campylobacter spp* from cases of human infection (ECDC/EFSA/EMA, 2015). Although the growth promoting agents of fluoroquinolone were withdrawn in European countries earlier in the Twenty first century, the incidence of FQ-resistant *C. jejuni* in broilers raised from 5.3 in

2001 to 26% in 2013 (DANMAP, 2013). The relationship between fluorquinolone use in food animals and antimicrobial resistance in humans may be different based on the geographic region, poultry production environment and fluoquinolone surveillance monitoring protocols.

## MACROLIDES AND RESISTANCE IN *CAMPYLOBACTER*

Macrolides inhibit bacteria protein biosynthesis by preventing peptidyltransferase location and/or inhibiting ribosomal translation (Tenson et al., 2003; Liang and Han, 2013). Macrolides were the first choice for treatment infections caused by Gram-positive bacteria in animal and campylobacteriosis in human. Macrolide resistance develops in *Campylobacter* by point mutation in target genes of 23S rRNA, ribosomal protein and by overexpression of efflux pumps (Hao et al., 2013).

In food-producing animals, macrolide drugs, such as tylosin and tilmicosin, have been used as growth promoters for decades in USA and Canada. However, two macrolide members, tylosin, and spiramycin were banned for their use as animal growth promoters in Finland and EU since 1995 because exposure of animals to these drugs was implicated as a possible cause of treatment failure in *Campylobacter* infections in humans.

From the ECDC/EFSA/EMA JIACARA report, positive associations were noted for total consumption of macrolides in food-producing animals in 2011 and 2012 and the occurrence of resistance in *C. jejuni* from cases of human infection (ECDC/EFSA/EMA, 2015). However, the data obtained from European antimicrobial resistance monitoring systems, like DANMAP, revealed that macrolide resistance in *C. jejuni* isolates from Danish broilers kept at a relatively low level (lower than 1%) and there was no significant temporal variation during 2000–2012 (DANMAP, 2013).

Contrary to the ECDC/EFSA/EMA JIACARA report, the USA risk assessment data showed that tylosin and tilmicosin in food animals did not result in a risk to public health in relationship to the development and dissemination of macrolide resistant *Campylobacter* (Hurd et al., 2004). The probability of treatment failure of drug resistant *Campylobacter* infections was only one person in 2.36, 14, and 53 billion per year due to consumption of beef, poultry, and pork, respectively (Hurd et al., 2004). In other words, the probability of therapy failure of human campylobacteriosis due to resistant bacteria in food animals exposed to tylosin or tilmicosin, was much less than the mortality due to automobile accidents (1/7,000), shootings (1/10,000), motorcycle and car accidents (1/500,000), aircraft accidents (1/1,000,000), lightning strikes (1/3,000,000) and shark attacks (1/100,000,000) in USA (Hurd et al., 2004). Therefore, the two veterinary macrolide drugs (tylosin and tilmicosin) may have negligible risk for human health (Casewell et al., 2003; Phillips et al., 2004a,b; Turnidge, 2004). Data obtained from NARMS also revealed that macrolide resistance in *C. jejuni* isolates from American chicken breast kept at a relatively low level (lower than 1%) and there was no

significant temporal variation during 2000–2012 (NARMS, 2012; DANMAP, 2013).

The mutation frequency for macrolide resistance in *Campylobacter* was reported to be about  $10^{-10}$ /cell/generation which is approximately 10,000-fold lower than that of FQ resistance (Yan et al., 2006; Lin et al., 2007). The mutants obtained by single-step selection tend to have low-to-intermediate levels of macrolide resistance (Erythromycin MIC = 8–64  $\mu$ g/ml) (Kim et al., 2006; Lin et al., 2007; Caldwell et al., 2008). These mutants generally had fitness cost in the absence of macrolide drugs (Kim et al., 2006; Caldwell et al., 2008). Acquisition of mutations in 23S rRNA, which conferred a high level of resistance to erythromycin (MIC  $\geq$  512  $\mu$ g/ml), appeared to require stepwise selection and/or prolonged exposure to macrolide drugs (Lin et al., 2007; Caldwell et al., 2008). In the absence of macrolide selection pressure, most of the 23S rRNA mutations could be stably maintained without competition (Gibreel et al., 2005; Caldwell et al., 2008), but due to their fitness burden, they could be rapidly outcompeted by the erythromycin susceptible *C. jejuni* in both *vitro* or *vivo* environment (Hao et al., 2009; Almofti et al., 2011a,b; Luangtongkum et al., 2011; Zeitouni et al., 2012).

Since *Campylobacter coli* is the most common *Campylobacter* species in pigs, we also discuss the topic of macrolide resistant *C. coli* in this review. The ban on tylosin as growth promoter had a remarkable effect on the level of erythromycin resistance in *C. coli* from pigs, as it decreased from 66 to 20% in Denmark between 1998 and 2005 (Hammerum et al., 2007). However, DANMAP data showed that during 2006–2010, the macrolide resistance in *C. coli* varied within the range of 10–20% without significant reduction (DANMAP, 2010). The macrolide resistance in *C. coli* isolates, although more prevalent than that in *C. jejuni* isolates, also did not increase over the past 10 years since the beginning of the monitoring in the U.S.A (NARMS, 2010). A previous study demonstrated that *C. coli* were not intrinsically more mutable than *C. jejuni*, because no elevated mutation frequency for erythromycin was observed in *C. coli* (Lin et al., 2007).

## TETRACYCLINES AND RESISTANCE IN *SALMONELLA*

Tetracyclines are protein synthesis inhibitors. They bind to the 30S ribosomal subunit and prevent the binding of aminoacyl-tRNA to mRNA-ribosome complex (Bassetti et al., 2013). Bacteria could develop resistance to tetracycline by efflux pumps (TetA, B, C, D, E, F, G, H, I, J, K, L, P(A), P(B), V, Y, Z, 30, OtrB, TcrC), ribosomal protection (TetM, O, Q, S, T, U, W, OtrA,), and enzymatic inactivation of drugs (TetX) (Linkevicius et al., 2015). Moreover, the tetracycline resistance genes are often located in some transferable elements including plasmids and integrons (Szmolka et al., 2015).

Tetracyclines have widely been used in human and veterinary medicine since the first discovery of tetracycline in 1948. In food animal production, tetracyclines, like oxytetracycline, and chlorotetracycline, were broadly used for growth promotion

and prophylaxis (Chopra and Roberts, 2001). The data from antimicrobial resistance monitoring programs showed that tetracycline resistance was commonly detected in foodborne pathogens (DANMAP, 2014; EFSA, 2015). EU countries have banned the use of tetracyclines for growth promotion since 2006.

However, withdrawal of tetracycline growth promoters did not alter the epidemiology of tetracycline resistance in EU countries. DANMAP reported that tetracycline resistant *Salmonella* Typhimurium isolated from pigs had increased from less than 30% in 2001 to 47% in 2013 (DANMAP, 2013). It was also stated that 29,797 kg (active compound) of tetracyclines were sold to the pig industry in Denmark during 2013 and total consumption of the drugs in pig industry was increased by 2-fold in 2013 as compared to that in 2001 (DANMAP, 2013). The increased therapeutic use of tetracyclines might be a primary reflection of the increased occurrence of drug resistance in *S. Typhimurium* and *E. coli* isolates from pigs (DANMAP, 2010). Using logistic regression analysis, the results from a previous study also showed that both the *S. Typhimurium* phage type ( $p < 0.0001$ ) and the increase in tetracycline consumption ( $p = 0.0007$ ) were significantly associated with the antimicrobial resistance (Embong et al., 2007). The ECDC/EFSA/EMA JIACARA reported positive associations for total consumption of tetracyclines and the occurrence of resistance in *Salmonella spp* from cases of human infection in 2011 and 2012 (ECDC/EFSA/EMA, 2015). Additionally, tetracycline-resistant *S. Typhimurium* also became more prevalent in human cases and reported as domestically acquired sporadic (36%) and outbreak related (21%) (Embong et al., 2007).

The high prevalence of tetracycline resistance in zoonotic pathogens could be explained by the transfer of already established or new resistant clones rather than the conversion of well-established susceptible clones into resistant ones by uptake of resistance genes (Szmolka et al., 2015). The serotype of *Salmonella* may also decide the prevalence of resistance to tetracyclines. It is known that *Salmonella Enteritidis* and *S. Typhimurium* are two general serotypes of *Salmonella* associated with public health. However, the resistance to tetracycline in *S. Enteritidis* (5%) from human was much lower than that in *S. Typhimurium* (DANMAP, 2010). The drug resistance among *S. Typhimurium* isolates might be due to the transferrable ability of tetracycline resistance determinants (e.g., class 1 integron and *Salmonella* genomic island 1) and the possible fitness of these determinants in pig host (Anjum et al., 2011).

## CEPHALOSPORINS AND RESISTANCE IN *SALMONELLA* AND *E. COLI*

The cephalosporins are a class of  $\beta$ -lactams which can disrupt the synthesis of the peptidoglycan layer forming the bacterial cell wall. Cephalosporins have great significance in the treatment of bacterial infection in human medicine. The third and fourth generation cephalosporins are the most common antimicrobial

drugs used as human medicine worldwide. However, the third generation cephalosporins, such as ceftiofur, has also been extensively used in many different food animals and the fourth-generation cephalosporin, like cefquinome, was approved by the US Food, and Drug Administration (US FDA) in 2007 soon after its approval by the European Union.

Gram-negative bacteria may develop resistance to  $\beta$ -lactams by producing  $\beta$ -lactamase to inactivate the drugs. The major public health concern is that use of third and fourth generation of cephalosporins in food animals might result in resistance development in foodborne pathogens (e.g., *Salmonella* and *E. coli*). Some evidence, provided by Keep Antibiotic Working (KAW), Union of Concerned Scientists (UCS), the American Medical Association (AMA) and the Infectious Diseases Society of America (IDSA), showed that approval of cefquinome might induce the development of resistance in foodborne pathogens and enhance the transfer risk of resistance to human which may compromise public health. Recently the FDA ordered to prohibit extra label use of cephalosporin drugs in food-producing animals (FDA, 2015).

Interestingly, NARMS data showed that there was no considerable change of cephalosporins resistance in *E. coli* and *Salmonella* isolates from ground turkey, ground beef and pork chop during 2002–2012 (NARMS, 2012). Although there was a transient increase in cephalosporin resistance of *Salmonella* from ground turkey during years of 2007–2009, yet it decreased again in the year 2012. During 2002–2012, antimicrobial resistance was increased (10–35%) in *Salmonella* isolates from chicken breast and the resistance rate was considerably lower in *E. coli* isolates than that in *Salmonella* isolates. In the case of *E. coli* from chicken breast, ground turkey and pork chop, resistance was lower than 10% (except for 11.7% in chicken breast in 2009) and even less than 1% for the ground beef bacteria from 2002 to 2010 (NARMS, 2010).

Additionally, the NARMS human data showed that cephalosporins resistance in *Salmonella* and *E. coli*O157 kept at a very low level (less than 1%) during 2000–2012 (NARMS, 2012). From the ECDC/EFSA/EMA JIACARA report, no associations were observed between the consumption of 3rd—and 4th—generation cephalosporins in food-producing animals and the occurrence of resistance to this sub-class in selected bacteria from human (ECDC/EFSA/EMA, 2015).

Concern of resistant bacteria carrying extended-spectrum  $\beta$ -lactamases (ESBLs) has been raised after the use of third and fourth generation of cephalosporins. The ESBLs encoding genes (e.g., *blaOXA*, *blaPSE*, *blaSHV*, *blaTEM*, *blaCTX-M*), as well as the plasmid-mediated AmpC  $\beta$ -lactamases (PMA $\beta$ , such as *blaCMY* and *blaFOX*), and carbapenemases (e.g., *blaIMP*, *blaKPC*, *blaVIM*) could be involved in the resistance to extended-spectrum  $\beta$ -lactams (Liebana et al., 2013; Rubin and Pitout, 2014; Bae et al., 2015). There were many reports about the transmission of ESBL carrying bacteria (Hasman et al., 2005; Collignon and Aarestrup, 2007), but the possible zoonotic spread of ESBL is still controversial. Some investigators found that there was similarity between ESBL genes and bacterial properties in isolates from human, livestock and companion animal populations, indicating that exchange of ESBL genes and ESBL bacterial between these

reservoirs (Valentin et al., 2014; Dahms et al., 2015). A review paper by Ewers et al. (2012) showed that the European, American and Asian countries (e.g., Japan) shared a similar population of ESBL subtypes, but ESBL subtype (*blaCTX-M*) from human was similar to that from pets but significantly different with that from food-producing animals (Ewers et al., 2012). Largely unknown environmental factors might impact the spread of resistant pathogens and increase the complexity of development and transfer of resistance enzymes. For example, wild animals like waterfowl, prey and rodents carry similar subtype of ESBL *E. coli* to humans, indicating that wildlife could be an environmental reservoir and melting pot for enzymatic resistance. The bacteria might re-infect humans through the omnipresent bird feces. Similar to humans traveling, the birds migration might also

contribute to the worldwide spread of the resistant organism (Guenther et al., 2009, 2010, 2011; Dolejska et al., 2011).

## CONCLUSIONS

The relationship between use of antimicrobial agents in food animals and antimicrobial resistance associated with human public health is a complex and controversial subject (Table 2). The risk of the use of some antimicrobial agents in food-producing animals with consequences on human public health is still problematic because there are so many factors to consider from an antimicrobial resistance perspective (Table 2). There is not enough of compelling evidence to assert that the prevalence

**TABLE 2 | Summary of risk assessment of some veterinary antimicrobial drugs on human public health associated with antimicrobial resistance and their molecular basis.**

Veterinary drug Use and ban	Associated Public health	Resistance monitoring data in animals	Resistance monitoring data in humans	Risk assessment; Risk association	Molecular basis
Avoparcin, Used 1940s–1990s; Banned 1995–2000; Not approved in USA for use in animal.	Vancomycin-resistant <i>Enterococci</i> (VRE), and <i>E. faecium</i> (VREF)	In EU, high prevalence of VRE in 1990s and in poultry after 2000; In Denmark, VRE reduced from 1995 to 2013; few VRE in livestock during 2003–2013.	In U. S. A, 40% VRE infections in 2013; In EU, <5% VRE in 2013.	Positive risk; Still some controversy questions	vanA gene located in transferable transposon Tn1546
Virginiamycin, Used as GP for 30 years; Banned in 1999 in EU	streptogramin-resistant <i>Enterococci</i> (SRE), and <i>E. faecium</i> (SREF)	In Denmark, 25% SREF from pigs and chickens; In USA, 30–70% SREF from poultry products in 2012.	Very rare in human hospital.	FDA-CVM: risk is little weight	VatD, VatE; ErmB; VgbA; Hard resistance development
Veterinary fluoroquinolones, e.g., enrofloxacin Banned use on poultry in 2005 in USA	fluoroquinolones resistant <i>Campylobacter jejuni</i>	In USA, high prevalence of FQ-resistant <i>C. jejuni</i> in poultry before 2005, resistance reduced during 2005–2007, resistance increased during 2008–2011.	In USA, ciprofloxacin resistant <i>C. jejuni</i> kept increasing from 16.7% in 1997 to 25.3% in 2012.	FDA-CVM: Positive risk ECDC/EFSA/EMA JIACARA: no risk associations	Thr-86-Ile mutation in GyrA; high mutation rate and enhanced fitness in chicken
Veterinary Macrolides e.g., tylosin, tilmicosin. EU banned tylosin and spiramycin as GP since 1995	Macrolide resistant <i>Campylobacter spp</i>	Resistant <i>C. jejuni</i> kept low level (<1%) in USA and Denmark; Resistant <i>C. coli</i> in pig reduced during 1998–2005 and kept at about 10% during 2006–2010 in Denmark; kept at a stable level in USA in the past decade.	Erythromycin resistant <i>C. jejuni</i> is rare in human	FDA-CVM: negative risk ECDC/EFSA/EMA JIACARA: positive risk associations	point mutation in target genes of 23S rRNA; low mutation frequency and fitness cost of resistance
Veterinary tetracyclines EU banned tetracyclines as growth promotor since 2006	Tetracycline resistance in <i>Salmonella Typhimurium</i>	In Denmark, resistant <i>S. Typhimurium</i> from pigs had increased from less than 30% in 2001 to 47% in 2013.	High prevalent of tetracycline-resistant <i>S. Typhimurium</i> in human	ECDC/EFSA/EMA JIACARA report: positive associations	<i>Tet</i> genes were normally located in some transferable elements
Veterinary Cephalosporins	Cephalosporins resistance in <i>E. coli</i> and <i>Salmonella</i>	In USA, no significant change of resistance from animal product during 2002–2012;	Resistance kept at a very low level (<1%) during 2000–2012 in USA.	ECDC/EFSA/EMA JIACARA report: no risk associations	Complex distribution of ESBLs in animal, human and environment.

GP was Growth promotor; ESBL was extended-spectrum  $\beta$ -lactamases; ECDC/EFSA/EMA JIACARA was European center for disease prevention and control/European food safety authority/European Medicines Agency. Joint Interagency Antimicrobial Consumption and Resistance Analysis report. Risk association means the association between consumption of veterinary antimicrobial drugs in food-producing animal and the occurrence of resistance bacterial from human infection. The FDA-CVM risk means the relationship between the use of antimicrobial agents in food-producing animal and human public health associated with antimicrobial resistance in special foodborne pathogens.

of some resistant bacteria in humans was due to antimicrobial agents used in food animals (NAMI, 2010; Horigan et al., 2016). The zoonotic spread of antimicrobial resistant bacteria or resistant genes is also questionable because resistant pathogens could be found in soil, water and environment and long-term occurrence of antimicrobial resistant genes in nature was even known before the antimicrobial era (Casewell et al., 2003; Aminov and Mackie, 2007; Kobayashi et al., 2007; Aminov, 2010). Therefore, it is not wise to oversimplify the opinion that the resistant bacteria from food producing animal is a major origin of human infection and neglect the highly complex environment scenario.

Furthermore, the ban on some antimicrobial usage has not altered or decreased the incidence of resistance in foodborne pathogens. This may be due to the enhanced fitness or high transferability of some resistant determinants. To control the increased animal disease, therapeutic levels of some antimicrobial drugs (e.g., fluroquinolones and tetracyclines) has been increased. This may also be a reason for the increased detection of resistance to some therapeutic drugs in food borne pathogens isolated from food animals after the ban of growth promoters (Koluman and Dikici, 2013).

On the concept of “one health one world,” international governments need to cooperate to establish an international antimicrobial resistance surveillance monitoring program and monitor the antimicrobial resistance trends in human and

animals for a long time. Both the benefit and risk outcomes should be considered into the risk assessment and management. To find wise strategy to control antimicrobial resistance, it is necessary to considerate the chemotherapeutic medicine, microbiology and agricultural environment and fully understand molecular basis involved in the emergence of antimicrobial resistance.

## AUTHOR CONTRIBUTIONS

The Idea and designation: HH, and ZY; Data collection and analysis: HH, PS, and YW; The writing and modification of this manuscript: HH, ZI, PS, and GC.

## FUNDING

This article is financially supported by grants from National Basic Research Program of China (2013CB127206), National Key Research and Development Program (2016YFD0501302), Fundamental Research Funds for the Central Universities (2662015PY035), Morning Program of Wuhan in China (2015070404010191), National Program for Risk Assessment of Quality and Safety of Livestock and Poultry Products (GJFP2016008), National Natural Science Foundation of China (31101856/31302143/31272614), National Key Technology R&D Program (2012BAK01B00).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# A Designed Experiments Approach to Optimizing MALDI-TOF MS Spectrum Processing Parameters Enhances Detection of Antibiotic Resistance in *Campylobacter jejuni*

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 30 January 2016

**Accepted:** 13 May 2016

**Published:** 31 May 2016

### Citation:

Penny C, Grothendick B, Zhang L, Borror CM, Barbano D, Cornelius AJ, Gilpin BJ, Fagerquist CK, Zaragoza WJ, Jay-Russell MT, Lastovica AJ, Ragimbeau C, Cauchie H-M and Sandrin TR (2016) A Designed Experiments Approach to Optimizing MALDI-TOF MS Spectrum Processing Parameters Enhances Detection of Antibiotic Resistance in *Campylobacter jejuni*. *Front. Microbiol.* 7:818.

doi: 10.3389/fmicb.2016.00818

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MALDI-TOF MS has been utilized as a reliable and rapid tool for microbial fingerprinting at the genus and species levels. Recently, there has been keen interest in using MALDI-TOF MS beyond the genus and species levels to rapidly identify antibiotic resistant strains of bacteria. The purpose of this study was to enhance strain level resolution for *Campylobacter jejuni* through the optimization of spectrum processing parameters using a series of designed experiments. A collection of 172 strains of *C. jejuni* were collected from Luxembourg, New Zealand, North America, and South Africa, consisting of four groups of antibiotic resistant isolates. The groups included: (1) 65 strains resistant to cefoperazone (2) 26 resistant to cefoperazone and beta-lactams (3) 5 strains resistant to cefoperazone, beta-lactams, and tetracycline, and (4) 76 strains resistant to cefoperazone, teicoplanin, amphotericin B and cephalothin. Initially, a model set of 16 strains (three biological replicates and three technical replicates per isolate, yielding a total of 144 spectra) of *C. jejuni* was subjected to each designed experiment to enhance detection of antibiotic resistance. The most optimal parameters were applied to the larger collection of 172 isolates (two biological replicates and three technical replicates per isolate, yielding a total of 1,031 spectra). We observed an increase in antibiotic resistance detection whenever either a curve based similarity coefficient (Pearson or ranked Pearson) was applied rather than a peak based (Dice) and/or the optimized preprocessing parameters were applied. Increases in antimicrobial resistance detection were scored using the jackknife maximum similarity technique following cluster analysis. From the first four groups of antibiotic resistant isolates, the optimized preprocessing parameters increased detection respective to the aforementioned groups by: (1) 5% (2) 9% (3) 10%, and (4) 2%. An additional second categorization was created from the

collection consisting of 31 strains resistant to beta-lactams and 141 strains sensitive to beta-lactams. Applying optimal preprocessing parameters, beta-lactam resistance detection was increased by 34%. These results suggest that spectrum processing parameters, which are rarely optimized or adjusted, affect the performance of MALDI-TOF MS-based detection of antibiotic resistance and can be fine-tuned to enhance screening performance.

**Keywords:** **MALDI-TOF MS, *Campylobacter jejuni*, antimicrobial resistance, antibiotic resistance, designed experiments, spectrum processing**

## INTRODUCTION

MALDI-TOF MS has revolutionized the field of molecular microbial diagnostics in recent years (Sauer and Kliem, 2010; Welker, 2011; Welker and Moore, 2011; Kliem and Sauer, 2012). This approach has been implemented in biomedical, veterinary and environmental routine procedures for bacterial identification at the genus, species, and sometimes at the subspecies level (e.g., Wieser et al., 2011; Croxatto et al., 2012; Koubek et al., 2012; Lartigue, 2013; Sandrin et al., 2013; Randall et al., 2015). While microbial identification using MALDI-TOF MS is rapid and reliable, the taxonomic resolution obtained from the mass spectra is not always sufficient, or the bioinformatics software pipeline is not optimized or adapted, for typing the candidate bacteria below the species level (Sandrin et al., 2013; Zhang et al., 2014). However, some studies have shown that categorization of strains of bacteria with respect to their membership in nucleic acid-based subgroups, pathogenicity traits or antimicrobial resistance (AMR) identification is indeed feasible, but depends on the level of variability inside a given taxon as well as on the precise identification of characteristic biomarkers using bioinformatics tools (Sandrin et al., 2013). Fine-tuning of mass spectrum analysis is evidently mandatory.

Rigorous analysis of spectra has permitted successful detection of AMR (e.g., Hrabák et al., 2013; Kostrzewska et al., 2013; Pulido et al., 2013), but further development is needed to render MALDI-based approaches a more routine, reliable, and effective alternative to traditional methods. As has been shown in several MALDI applications to discriminate bacterial strains (Mitchell et al., 2015), high reproducibility is required for reliable AMR detection. Although many portions of the MALDI-TOF MS workflow such as sample preparation and data acquisition have been optimized with regard to spectrum reproducibility and method performance (e.g., Freiwald and Sauer, 2009; Goldstein et al., 2013), no standardization for mass spectrum processing parameters has been proposed. Processing parameters are used to calculate, define, and resolve acquired spectra into interpretable data. Baseline subtraction, a common processing parameter, establishes a baseline from the spectrum, leaving a clearer picture of the remaining peaks. Another processing parameter, smoothing, reduces background noise, and increases signal-to-noise ratio. The manner in which the data are translated by processing parameters may affect the ability of MALDI-TOF MS-based fingerprinting to detect antibiotic resistance. In addition, different software packages that use distinct spectrum processing workflows and parameters are often used. Many of

these programs do not offer the ability to alter and optimize spectrum processing parameters. Such optimization, though, may be necessary to enhance method performance, particularly with regard to resolving strain-level differences, such as AMR.

The issues regarding the ever-increasing bacterial resistance to large categories of antimicrobial compounds are particularly of public health concern for the world's leading bacterial gastroenteritis agent *Campylobacter* (World Health Organization [WHO], 2013). The species *Campylobacter jejuni* is recognized as the major food- and waterborne pathogen inside this taxon, and is a major threat to public health (Kaakoush et al., 2015; Wagenaar et al., 2015). AMR in *Campylobacter* is steadily increasing (e.g., Luangtongkum et al., 2009; Ge et al., 2013; Iovine, 2013; Wieczorek and Osek, 2013). Of particular concern is also the increasing incidence of AMR of *Campylobacter* spp. other than *C. jejuni*, whose disease potential is not fully appreciated at present (Lastovica, 2006; Lastovica et al., 2014). *Campylobacter* easily undergoes DNA transformation by foreign exogenous DNA resulting in many different antibiotic-resistant strains (Bae et al., 2014). Also, antibiotic resistance mutations in *C. jejuni* continue to develop (Iovine, 2013). For example, a single mutation in the gyrase subunit A (*gyrA*) gene, resulting in an amino acid substitution, is sufficient for conferring resistance to quinolones (Wang et al., 1993; Payot et al., 2006).

In diagnostic and clinical microbiology, as well as epidemiological surveillance, the need for implementation of early and precise information retrieval concerning AMR has been raised (Laxminarayan et al., 2013). This could greatly improve treatment of infectious diseases and help limit the spread of multiple resistant strains of harmful bacteria. In this context, the evolution toward microbial characterization, and more specifically AMR prediction through whole-genome sequencing (WGS) has been described in recent years including the identification of AMR-specific signatures in *Campylobacter* (e.g., Didelot et al., 2012; Zhao et al., 2016). But also, the potential of various MALDI-TOF MS applications for the prediction of AMR mechanisms has been identified (Hrabák et al., 2013; Kostrzewska et al., 2013). Most importantly, the use of mass spectrometry toolkits for the diagnosis of AMR in *Campylobacter* is emerging (Wieser et al., 2011; Lartigue, 2013; Schubert and Kostrzewska, 2015), and a new microbial typing method relying on mass spectrometry-based phyloproteomics (MSPP), permitting biomarker, and genetic features characterization in *Campylobacter*, has recently been published (Zautner et al., 2015). Still, the MALDI-TOF MS-based workflow remains in the need of optimization and simplification of robust, reliable and

reproducible workflows, especially regarding data handling after automated routine acquisition of mass spectra.

Consequently, the overarching objective of this study was to determine whether MALDI-TOF mass spectrum processing parameters could be optimized to enhance the detection of antibiotic resistance in clinically relevant environmental, animal, and human isolates of *C. jejuni*. Therefore, 172 isolates of *C. jejuni* were collected from four continents, some of which share antibiotic resistances within four different groups (Table 1). Special emphasis was put on *C. jejuni* resistance to beta-lactams, as this antibiotics group is considered among the most important and widespread treatment with resistance issues (Wieser et al., 2011; Lartigue, 2013; Schubert and Kostrzewska, 2015). A designed experiments approach was employed (Zhang et al., 2014), in which spectrum processing parameters were varied to optimize detection of AMR. Translation of the genetic and phenotypic characteristics of *C. jejuni* might identify useful and straightforward information collection in a global One Health context (Maloy and Atlas, 2014). Our results suggest that a designed experiments approach allows optimization of mass spectrum analysis and enhances detection of AMR in *C. jejuni*.

## MATERIALS AND METHODS

### *Campylobacter jejuni* Strains and Culture Conditions

A collection of 16 *C. jejuni* isolates were used as a model system for the designed experiments and a total of 172 *C. jejuni* strains from various geographical and animal host origins were used in application of the model spectrum processing parameters (Table 1).

Antibiotic resistance profiles were established by a non-exhaustive series of phenotypic and genomic attribute tests of the *C. jejuni* isolate collection, depending on specific culture media used and availability of whole genome sequence data (WGS) of the strains. Genomics-based AMR potential of part of the

**TABLE 1 | Characteristics of the *Campylobacter jejuni* isolates used in this study.**

#### *C. jejuni* isolate collection

Number of strains	172
Geographic origin	Luxembourg, New Zealand, South Africa, USA
Sources	Alpaca, bovine, chicken, feral swine, goat, goose, human, milk product, ovine, raccoon, surface water, turkey, vole, wastewater, wildfowl
Antibiotic resistance profiles (phenotypically or genotypically confirmed resistances)	Group 1 (65 isolates): Cefoperazone Group 2 (26 isolates): Beta-lactams, cefoperazone Group 3 (5 isolates): Beta-lactams, cefoperazone, tetracycline Group 4 (76 isolates): Cefoperazone, teicoplanin, amphotericin B, cephalotin

collection was obtained through screening using the ResFinder bioinformatics platform<sup>1</sup> (Zankari et al., 2012).

For each strain, chocolate agar plates (Remel Microbiology Products, Lenexa, KS, USA) were inoculated with stock suspensions stored at  $-80^{\circ}\text{C}$  in FBP medium (Gorman and Adley, 2004), and incubated for  $40 \pm 4$  h at  $42^{\circ}\text{C}$  under microaerobic conditions in gastight jars (2.5 L, Remel) using CampyGen 2.5 L gaspacks (Remel). For biological replicates, the same stock suspension was streaked onto two to three separate chocolate agar plates on different days.

## Sample Preparation

A previously described protein extraction sample preparation method was employed with minor modifications (Freiwald and Sauer, 2009). Briefly, cells from  $40 \pm 4$  h cultures were pelleted by centrifugation ( $17,000 \times g$  for 5 min) and washed with sterile double-distilled water (ddH<sub>2</sub>O) (Millipore Corp.; Bedford, MA, USA). Cells were re-suspended in sterile ddH<sub>2</sub>O, and the cell density of each suspension was adjusted to  $0.8 \pm 0.1$  OD<sub>600</sub>. Each 1 mL cell suspension was pelleted by centrifugation ( $17,000 \times g$  for 5 min), and the supernatant was carefully removed. Cell pellets were inactivated by resuspension in 800  $\mu\text{L}$  of absolute ethanol and 300  $\mu\text{L}$  sterile ddH<sub>2</sub>O. Inactivation was verified by streaking a loopful of the inactivated suspension onto chocolate agar and verification of absence of colony formation after 72 h of incubation under the conditions described above. Each sample was centrifuged ( $17,000 \times g$  for 5 min), and the resulting supernatant was discarded. A washing step with 1 mL ddH<sub>2</sub>O was performed on each cell pellet. Twenty-five microliters of 70% formic acid (Sigma-Aldrich, St. Louis, MO, USA) and 25  $\mu\text{L}$  acetonitrile (Alfa Aesar, Ward Hill, MA, USA) were mixed with the pellet by vortexing thoroughly. Each sample was centrifuged ( $17,000 \times g$  for 5 min), and the supernatant containing the protein extract was transferred into a sterile 1.5 mL microcentrifuge tube. Protein extract (1.0  $\mu\text{L}$ ) was pipetted onto a polished steel 96-well MALDI target plate (Bruker Daltonics, Billerica, MA, USA) and allowed to air-dry for 10 min. Samples were spotted onto predetermined, randomly distributed locations on the target plate. After the sample had dried, it was overlaid with 1.0  $\mu\text{L}$  of  $\alpha$ -cyano-4-hydroxycinnamic acid (ACROS, Fair Lawn, NJ, USA) matrix prepared in 50% acetonitrile and supplemented with 2.5% trifluoroacetic acid (ACROS, Fair Lawn, NJ, USA). Each isolate was spotted in three technical replicates per biological replicate.

## MALDI-TOF MS Data Acquisition

MALDI-TOF MS analyses were performed using a Bruker Microflex LRF MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a nitrogen laser ( $\lambda = 337$  nm) under the control of FlexControl software (v. 3; Bruker Daltonics). Each spectrum was obtained in a linear, positive ion mode and calibrated externally using ACTH (1–17) (2094.427 Da), ACTH (18–39) (2466.681 Da), insulin oxidized B (3494.651 Da), insulin (5734.518 Da), cytochrome C (12360.974 Da), and myoglobin (16952.306 Da). Data acquisition was performed automatically in

<sup>1</sup><https://cge.cbs.dtu.dk//services/ResFinder/>

**TABLE 2 | (A)** Factors and levels used in the designed experiments for processing MALDI-TOF MS spectra of the *C. jejuni* collection.

Processing steps (factors)	Processing options (levels)
Baseline Subtraction 1:	Binned Baseline, Monotone Minimum, Moving Bar,
Binned Baseline	Rolling Disk
Smoothing: Kaiser Window	Gaussian, Kaiser window, Moving Average, Savitzky–Golay
Baseline Subtraction 2:	Binned Baseline, Monotone Minimum, Moving Bar, Moving Bar
Moving Bar	Rolling Disk
Similarity Coefficient	Dice, Pearson, Ranked Pearson

**TABLE 2 | (B)** Factors and levels used in the designed experiments approach with center points for highest scoring in predicted settings.

Processing options (factors)	Processing settings (levels)
Binned Baseline	Bin Size of 4, 77, 150
Kaiser Window	Window Size of 1, 21, or 40
Moving Bar	Bar Width of 3, 102, or 201

steps of 100 shots for a total of 500 shots. Laser power was set to the necessary minimum power for ionization of selected samples before starting the analyses. The signal-to-noise threshold was set at two, the minimum intensity threshold at 100, and the maximum number of peaks to 500. Peak width was set at 10 m/z and a height of 80%.

## Spectrum Cluster Analysis

Mass spectra were exported from FlexAnalysis (version 3.0; Bruker Daltonics) as .txt files and imported into BioNumerics (version 7.1; Applied Maths, Sint-Martens-Latem, Belgium). Spectra were initially pre-processed using the default program settings (Baseline Subtraction, 1; Rolling Disk, 200). For cluster analysis, spectra were compared pairwise using the Pearson correlation coefficient. The Dice similarity coefficient, in which lists of peaks containing only binary values (present or absent) were generated from spectra, was also evaluated. A dendrogram was generated using the unweighted pair group method with an arithmetic mean (UPGMA) algorithm. Multidimensional scaling (MDS) analysis was performed as previously described to visualize the similarity between spectra (Goldstein et al., 2013). Jackknife analysis was performed as described previously using maximum similarities to quantify rates of correct classification with regard to AMR (Giebel et al., 2008).

## Processing Steps and Settings Selection: Designed Experiments

Processing steps chosen as factors were based on those commonly cited in literature and an initial descriptive analysis of their effects on the response (jackknife score). These were found to be important in prior work in our lab, because of their ability to affect number of peaks and spectrum quality. The steps chosen were baseline subtraction, smoothing, and similarity coefficient. The levels, or different methods of baseline subtraction, smoothing, and similarity coefficient, were considered categorical variables, and each step was considered as a categorical variable

(**Table 2A**). The highest scoring levels from each category were then selected and further optimized based on their numerical settings (**Table 2B**).

## Statistical Analyses

Each of the two/three biological replicates contained three technical replicates. Each set of biological replicates was composed of sixty runs of calculations with varying processing step settings. All 180 experimental runs were carried out in 3 days in random order, and grouped into blocks by both day and biological replicate. Blocking, or grouping together, based on the aforementioned variables, helped to reduce sources of variability and increase precision (Montgomery, 2012). The datasets were subjected to analysis of main effects, interaction of factors, best, and worst combination of settings, significant factors affecting spectrum qualities, and *post hoc* tests on factors. Main effects and interactions of factors on reproducibility were analyzed based on analysis of variance (ANOVA) and *t*-tests using a 5% level of significance. *Post hoc* tests were performed using Tukey's test (Minitab Inc., PA, USA).

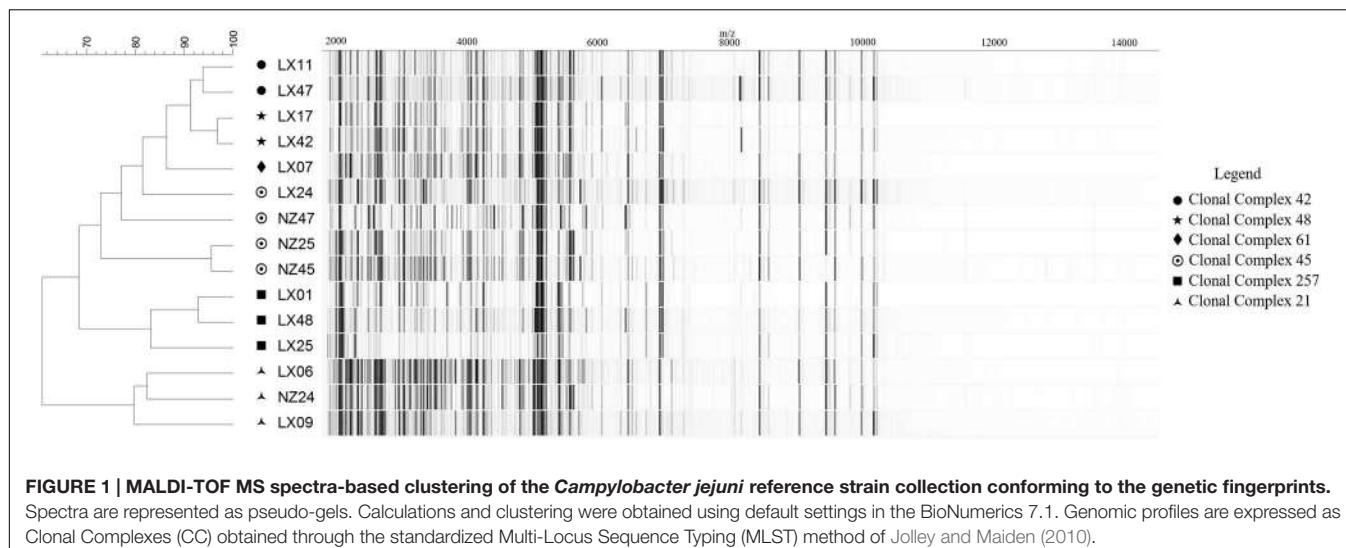
## Parameter Optimization

An optimized setting for preprocessing parameters and similarity coefficient was determined using ANOVA. The optimized setting was applied to each dataset. The jackknife score (response) from each dataset was reported using either the default settings or optimized preprocessing settings with combinations of three different similarity coefficients: Dice, Pearson, and ranked Pearson. Jackknife analysis was used as described previously (Goldstein et al., 2013) to evaluate the extent to which MALDI-TOF MS profiles were assigned to particular AMR categories. Differences in spectrum quality and jackknife scores before and after optimization were identified using *t*-tests with a 5% level of significance. A second dendrogram and MDS were visualized following optimization. Both optimal and default preprocessing settings were applied to larger sets of *C. jejuni*. In addition, varying similarity coefficients, and jackknife scores were used to measure the effectiveness of each processing parameter combination.

## RESULTS

A total of 172 different strains of *C. jejuni* were analyzed for this study. The sample collection was representative of diverse environments and hosts from four continents (**Table 1**). The MALDI-TOF MS profile of each strain was constituted using two sets of biological replicates, and each biological replicate was analyzed using three technical replicates (LX-32 is an exception due to the corruption of a single spectrum in biological replicate B2). Thus, the total number of spectra generated for this analysis was 1,031 spectra.

Within the strain collection, a model set of 16 *C. jejuni* isolates was used for optimizing spectrum preprocessing methods at levels below the species. Organisms for the model set were selected based on (i) their genetic fingerprints, considering six



**TABLE 3 | Effect of MALDI-TOF MS spectrum processing parameters on AMR detection in strains of *C. jejuni*.**

Antibiotic resistance group	Preprocessing	Similarity coefficient	Score
1	Default	Dice	89 ± 5
2	Default	Dice	87 ± 6
3	Default	Dice	73 ± 9
4	Default	Dice	93 ± 3
1	Default	Pearson	98 ± 1
2	Default	Pearson	97 ± 3
3	Default	Pearson	100 ± 5
4	Default	Pearson	100 ± 1
1	Default	Ranked	85 ± 14
2	Default	Ranked	73 ± 6
3	Default	Ranked	73 ± 28
4	Default	Ranked	98 ± 2
1	Optimized	Dice	92 ± 5
2	Optimized	Dice	88 ± 7
3	Optimized	Dice	80 ± 9
4	Optimized	Dice	96 ± 3
1	Optimized	Pearson	99 ± 0
2	Optimized	Pearson	98 ± 3
3	Optimized	Pearson	97 ± 5
4	Optimized	Pearson	100 ± 0
1	Optimized	Ranked	97 ± 3
2	Optimized	Ranked	97 ± 4
3	Optimized	Ranked	100 ± 0
4	Optimized	Ranked	100 ± 1

Scores represent mean ( $\pm SD$ ) jackknife values calculated using maximum similarities.

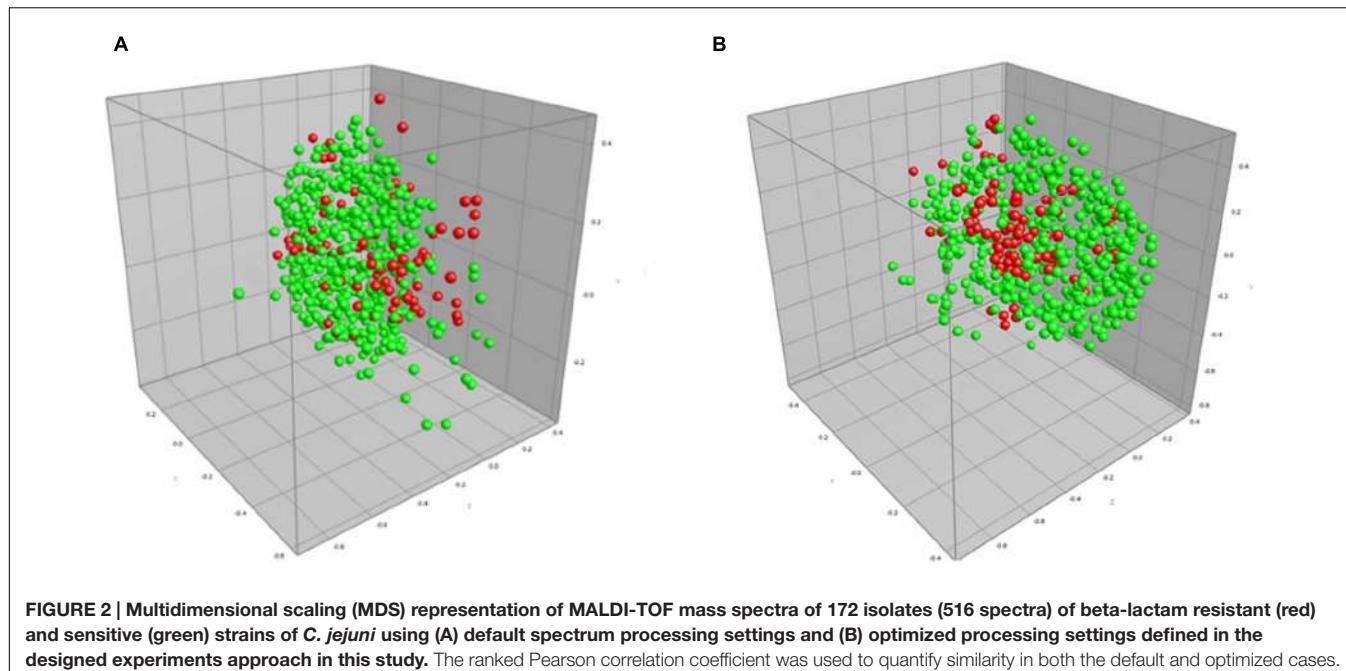
major Clonal Complexes (CCs) obtained through MLST analysis and represented by one to four isolates each (with varying host sources and origins), and (ii) their respective MALDI-TOF MS spectrum profiles with regard to shared base peaks and varying degrees of peak intensity (Figure 1). For the model set, we

utilized three biological replicates with three technical replicates each and generated a total of 144 spectra.

Relying on the example of the model set of isolates (excluding isolate LX-41 whose CC affiliation was not confirmed), it could be demonstrated that MALDI-TOF MS-based clustering of *C. jejuni*, based on peak mass and intensity ranges within the spectrum, was concordant with the genotype profiles expressed as CCs (Figure 1). All strains, except two isolates from CC-45, clustered together with their genetically closest neighbors from the same CC. Genetic diversity information on *Campylobacter* isolates could therefore be transcribed through MALDI-TOF MS fingerprinting profiles. This highlighted the potential of mass spectrometry for clonality prediction inside bacterial taxa. Then, characteristic traits such as AMR can individually be screened within each isolate, following optimization in the bioinformatics workflow for spectrum processing and MS-based clustering and typing.

In this context, we subsequently analyzed our model set of *C. jejuni* using the initial designed experiments approach for enhancing AMR detection and strain clustering into their respective groups of AMR (Table 1). The parameters for optimization were split into factors and then their individual components were referred to as levels (Tables 2A,B). The most optimal combination of the four parameters was found to be the following: (i) Binned Baseline (Bin Size of 77), (ii) Kaiser Window (Window Size of 33), (iii) Moving Bar (Bar Width of 129), and (iv) ranked Pearson similarity coefficient.

Following the model set analysis, these optimized parameters were applied to all spectra from the collection. Increases in rates of correct classification with regard to AMR, when switching from default settings to optimized settings, were observed (Table 3). Considering all four groupings of antibiotic-resistant *C. jejuni*, we observed a 5% increase in group 1, a 9% increase in group 2, a 10% increase in group 3, and finally, a 2% increase in group 4. Overall, use of optimized settings yielded a significant 7% increase ( $t$ -test,  $p = 0.05$ ) in detection of AMR when compared to use of default settings. In each of the



antibiotic resistance groups, the Pearson correlation coefficient outperformed the Dice similarity coefficient ( $p = 0.0002$ ). In only one instance was there a decrease in the rate of correct classification when using optimized settings. When the settings for group 3 were swapped from default to optimal with the Pearson coefficient, we observed a decrease from 100 to 97% (Table 3).

We also performed a direct comparison between default processing settings and the optimized processing settings when applied to isolates exhibiting beta-lactam resistance (Figure 2; Table 4). The MDS representing optimized parameters shows tighter grouping compared to the default parameter MDS, thus representing an increase in AMR detection, and here specifically beta-lactam resistance. Use of optimized spectrum processing settings increased the rate of correct classification from 63.5 to 95.7%.

## DISCUSSION

Workflows to rapidly characterize bacteria using MALDI-TOF MS typically include four components: (i) culturing, (ii) sample preparation, (iii) data acquisition, and (iv) data analysis (Sandrin et al., 2013). Each of the first three components of this common workflow have been shown previously to affect the ability of MALDI-TOF MS to reliably and accurately characterize bacteria at the strain level (Schumaker et al., 2012; Goldstein et al., 2013). Results presented here show clearly that the fourth component of this workflow, data analysis (spectrum processing parameters), affects the ability of MALDI-TOF MS to detect AMR in *C. jejuni*. To our knowledge, this work represents the first report of enhancing MALDI-TOF performance to detect AMR in *C. jejuni* through optimization of spectrum processing parameters.

Designed experiments have been used previously to enhance MALDI-TOF MS-based characterization of bacteria (Zhang et al., 2014). In that work, the third component of the MALDI-TOF MS workflow, data acquisition, was enhanced by systematically adjusting parameters (e.g., threshold base peak, S-N resolution, etc.) in an algorithm commonly used for automated spectrum acquisition. Similar to our work here applying designed experiments to data analysis, Zhang et al. (2014) reported increases in method performance (reproducibility) with optimization afforded by designed experiments. Furthermore, Zhang et al. (2014) reported that optimized data acquisition parameters obtained with one bacterium (*Pseudomonas aeruginosa*) were useful in increasing reproducibility of spectra of other bacteria (*Klebsiella pneumoniae* and *Serratia marcescens*). Further research is warranted to determine whether the optimized settings we identified here to enhance detection of AMR in *C. jejuni*, will enhance detection of AMR in other bacteria. Currently, the necessity for adapting the settings of variables and parameters for each individual microorganism or

**TABLE 4 |** Optimized spectrum preprocessing parameters enhance beta-lactam-resistance detection in *C. jejuni*.

Preprocessing	Similarity coefficient	Score
Default	Dice	86 ± 5
Default	Pearson	97 ± 3
Default	Ranked	74 ± 18
Optimized	Dice	89 ± 8
Optimized	Pearson	98 ± 2
Optimized	Ranked	98 ± 3

Scores represent mean ( $\pm SD$ ) jackknife values calculated using maximum similarities.

taxon is rigorously being evaluated, but in that case optimization is facilitated with the use of the designed experiments approach described here.

Following spectrum processing (i.e., baseline subtraction, smoothing, etc.), the similarity of processed spectra is often quantified using previously described similarity coefficients including the Dice similarity coefficient, the Pearson correlation coefficient, or the ranked Pearson correlation coefficient (Dieckmann et al., 2008; Schmidt et al., 2009; Sandrin et al., 2013). Each of these coefficients has been used previously to compare spectra and characterize diverse bacteria using MALDI-TOF MS. Our work here suggests that the Dice correlation coefficient underperforms in comparison to Pearson correlation coefficients, particularly the ranked Pearson correlation coefficient. This is in accordance with our prior work with *Enterococcus* (Giebel et al., 2008), in which we reported the importance of considering peak intensity information, which is included in the Pearson correlation coefficient calculations but not the Dice coefficient. For this reason, future efforts to detect AMR using MALDI-TOF MS should use correlation coefficients that include peak intensity, such as the Pearson correlation coefficient.

Most likely in complement to current tools for *Campylobacter* fingerprinting, MALDI-TOF MS spectra indeed reflect the genetic diversity (Zautner et al., 2013), but more importantly reflect the actual genetic expression profile and AMR potential of the strain candidates upon isolation and culture. The designed experiments approach described here appears in this way as a convenient bioinformatics tool for the optimization of information retrieval from MALDI-TOF MS spectra. BioNumerics currently represents the most versatile and flexible routinely used software package for screening optimal processing variable values and parameters such as described in **Table 2**.

Within the next steps, further AMR profiling of *Campylobacter* based on MALDI-TOF MS should be undertaken, in order to obtain more complete AMR profiles in the future. Using extended bioinformatics and proteomics tools, AMR signature identification is likely to be pursued, e.g., by characterizing specific beta-lactamase biomarker(s) within the resistant *C. jejuni* mass spectra (Sparbier et al., 2012; Kostrzewska et al., 2013). Here, bottom-up and top-down proteomics approaches could be deployed in order to complement and supplement existing tools (e.g., Fagerquist et al., 2005, 2009; Sandrin et al., 2013). Also, further comparisons with AMR prediction through WGS data or MSPP phyloproteomics screening will certainly be fruitful (Zankari et al., 2012; Zautner et al., 2015).

The features and potential of MALDI-TOF MS will continue to contribute to significant scientific and technological

advances in the fields of functional characterization and fingerprinting of microorganisms. As shown here, optimized bioinformatics workflows inside MALDI-TOF MS analysis will, among others, allow enhanced AMR detection for improved decision-making and healthcare through implementation in microbial subspecies typing and diagnostics. It would be interesting to adapt the analysis software inside the routine workflow by adding an AMR detection module fed with the optimized parameter settings for data acquisition, processing, and biomarker screening. Constitution of AMR profiles using *Campylobacter*-specific, sensitive and reproducible analysis parameters, such as those set-up here, will greatly add value to limiting the ongoing emergence of multi-drug resistances in *Campylobacter* sp.

## AUTHOR CONTRIBUTIONS

CP, H-MC and TS designed the study. CP, BG, LZ, CB, DB, and TS were responsible for data acquisition and analysis. CP, AC, BG, AL, MJ-R, CR, CK, and WZ contributed to the *C. jejuni* strain collection set-up and the AMR profiling and genetic fingerprinting of the isolates. All authors contributed to the writing and/or critical reviewing of the manuscript, and have approved the manuscript.

## FUNDING

CP received specific funding from the National Research Fund of Luxembourg (FNR; grant reference INTER/MOBILITY/14/7356524). This study was supported by the Arizona State University's New College undergraduate research program, NCUIRE (New College Undergraduate Inquiry and Research Experiences) and the Salt River Project (SRP, USA).

## ACKNOWLEDGMENTS

William G. Miller from USDA (USA), Stephen L.W. On from Lincoln University (NZ) and J. Mossong from the National Health Laboratory (LNS, Luxembourg) are acknowledged as facilitators of the project consortium constitution and strain collection assembling. We wish to acknowledge the assistance of Mary H. Chapman and Anna H. Bates from USDA. The authors warmly thank Cécile Walczak, Delphine Collard, Angela Tavares Furtado from LIST (Luxembourg) and Regina Diaz from ASU (USA) for technical and experimental support in the study.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Complete Nucleotide Sequence of pGA45, a 140,698-bp IncFIIy Plasmid Encoding bla<sub>IMI-3</sub>-Mediated Carbapenem Resistance, from River Sediment

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### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 27 September 2015

Accepted: 03 February 2016

Published: 24 February 2016

### Citation:

Dang B, Mao D and Luo Y (2016)  
Complete Nucleotide Sequence of pGA45, a 140,698-bp IncFIIy  
Plasmid Encoding bla<sub>IMI-3</sub>-Mediated Carbapenem Resistance, from River  
Sediment. *Front. Microbiol.* 7:188.  
doi: 10.3389/fmicb.2016.00188

Plasmid pGA45 was isolated from the sediments of Haihe River using *Escherichia coli* CV601 (*gfp*-tagged) as recipients and indigenous bacteria from sediment as donors. This plasmid confers reduced susceptibility to imipenem which belongs to carbapenem group. Plasmid pGA45 was fully sequenced on an Illumina HiSeq 2000 sequencing system. The complete sequence of plasmid pGA45 was 140,698 bp in length with an average G + C content of 52.03%. Sequence analysis shows that pGA45 belongs to IncFIIy group and harbors a backbone region which shares high homology and gene synteny to several other IncF plasmids including pNDM1\_EC14653, pYDC644, pNDM-Ec1GN574, pRJF866, pKOX\_NDM1, and pP10164-NDM. In addition to the backbone region, plasmid pGA45 harbors two notable features including one bla<sub>IMI-3</sub>-containing region and one type VI secretion system region. The bla<sub>IMI-3</sub>-containing region is responsible for bacteria carbapenem resistance and the type VI secretion system region is probably involved in bacteria virulence, respectively. Plasmid pGA45 represents the first complete nucleotide sequence of the bla<sub>IMI</sub>-harboring plasmid from environment sample and the sequencing of this plasmid provided insight into the architecture used for the dissemination of bla<sub>IMI</sub> carbapenemase genes.

**Keywords:** carbapenem resistance, plasmid, pGA45, T6SS, antibiotic resistance

## INTRODUCTION

The overuse and misuse of antibiotics have contributed to the emergence and spread of antibiotic resistance genes (ARGs) and multidrug resistance pathogens (Zhang and Zhang, 2011; He et al., 2014). Now ARGs have been recognized as a new type of pollutants (Pruden et al., 2006). Among various ARGs, carbapenem resistance genes, especially plasmid mediated carbapenem resistance genes, have raised worldwide concern, leading to the extensive research on some of these genes and related plasmid architecture (McGann et al., 2012; Tiwari et al., 2012; Villa et al., 2012, 2013; Lo et al., 2013; Tiwari and Moganty, 2014). Acquired carbapenem resistance can be resulted from carbapenemases of Amber class A (IMI, GES and KPC), Amber class B (metallo β-lactamases including IMP, VIM and NDM) or Amber class D (OXA-48 and OXA-181) (Nordmann et al.,

2012). The *bla*<sub>KPC</sub> gene of Amber class A and the metallo  $\beta$ -lactamase genes have been the research focus but there is rare reports about the other carbapenem resistance genes especially little is known about the *bla*<sub>IMI</sub> genes and the plasmid architecture involved in the dissemination of this type of genes (Aubron et al., 2005; Yu et al., 2006; Rojo-Bezares et al., 2012; Teo et al., 2013; Chen et al., 2015).

The *bla*<sub>IMI-1</sub> gene was first identified in 1996 and found to be located in the chromosome of *Enterobacter cloacae* isolates whereas *bla*<sub>IMI-2</sub> was first identified in 2005 in *Enterobacter asburiae* isolates and found to be related to plasmids (Rasmussen et al., 1996; Aubron et al., 2005). In 2009, a new variant of *bla*<sub>IMI-1</sub>, *bla*<sub>IMI-3</sub>, was identified in Hong Kong in *Enterobacter cloacae* isolates (Chu et al., 2011). The *bla*<sub>IMI-3</sub> was also located in the conjugative plasmids. The *bla*<sub>IMI</sub>-mediated carbapenem resistance is an infrequent mechanism but it has been reported both in clinical strains and environmental bacteria from rivers. Horizontal transfer may occur between environmental bacteria and clinical strains. With the horizontal transfer, the *bla*<sub>IMI</sub> genes would broaden their hosts and inevitably pose serious risks to the public health. For further research on the dissemination of *bla*<sub>IMI</sub> genes, the full sequence of *bla*<sub>IMI</sub>-related plasmid is needed. Here we report the first complete nucleotide sequence of *bla*<sub>IMI</sub>-carrying conjugative plasmid from the environment sample.

## MATERIALS AND METHODS

### Studying Sites and Sample Collection

Sediment sample was collected under JinGang Bridge of Haihe River. JinGang Bridge was located in densely populated urban areas with frequent human activities. Sediment sample was collected with a grab sampler and then put into sterile containers. The sample was immediately taken to the laboratory and stored in  $-20^{\circ}\text{C}$  for subsequent experiments after sampling was completed.

### Isolation of the Conjugative Plasmids Conferring Resistance to Imipenem

In order to obtain conjugative plasmids which confer resistance to imipenem, ampicillin resistant plasmids were first isolated by filter mating assays and these ampicillin resistant plasmids were then subjected to antibiotic susceptibility testing against imipenem and other antibiotics. The filter mating assays were applied using *Escherichia coli* CV601 (*gfp*-tagged, kanamycin and rifampicin resistant) as recipients and sediment samples as donors (Heuer et al., 2002). Transconjugants were selected by Mueller-Hinton agar plates supplemented with ampicillin ( $100 \text{ mg L}^{-1}$ ), kanamycin ( $50 \text{ mg L}^{-1}$ ), rifampicin ( $50 \text{ mg L}^{-1}$ ) and cycloheximide ( $100 \text{ mg L}^{-1}$ ). *E. coli* CV601 recipient culture was plated on the same selective plates as controls. The procedure used for filter mating assays was described by Heuer et al. (2002) with slight modification. Briefly, the sediment samples from which the indigenous bacteria were used as donors were doubled to 2 g and the Luria-Bertani (LB) broth used for resuspending the sediment samples was accordingly doubled to 18 ml. After

incubation for 2 days, transconjugants were determined by green fluorescence which is resulted from green fluorescence protein (GFP) gene. All the ampicillin resistant transconjugants were then streaked on the ampicillin selective plates. Overnight culture of these transconjugants were stored in  $-80^{\circ}\text{C}$  for further study.

### Antibiotic Susceptibility Testing of the Ampicillin Resistant Transconjugants

Kirby-Bauer disk diffusion method was applied to determine which ampicillin resistant transconjugants confer resistance to imipenem. According to the criteria of the Clinical and Laboratory Standards Institute (CLSI), the disks used in this study are as follows: imipenem ( $10 \mu\text{g}$ ), ampicillin ( $10 \mu\text{g}$ ), gentamicin ( $10 \mu\text{g}$ ), streptomycin ( $10 \mu\text{g}$ ), tetracycline ( $30 \mu\text{g}$ ), ciprofloxacin ( $5 \mu\text{g}$ ), sulfamethoxazole ( $300 \mu\text{g}$ ) and erythromycin ( $15 \mu\text{g}$ ). *E. coli* ATCC25922 was used as quality control strain. In this study, one transconjugant designated GA45 was found to confer resistance to imipenem and ampicillin. The conjugative plasmid harbored by GA45 was named pGA45 and stored for further analysis.

### Conjugative Transfer Experiments and the Role Determination of pGA45 in Recipient Strains

To assess the conjugative frequency of plasmid pGA45, liquid mating assays were employed using *E. coli* CV601 (pGA45) as donor strains and *E. coli* J53 (azide and nalidixic acid resistance) as recipient strains. For liquid mating assay, overnight cultures of donor and recipient strains were centrifuged, washed and adjusted to the optical density of 0.6 at the wavelength of 600 nm ( $\text{OD}_{600}$ ) with LB broth. Then 0.5 ml cultures of each donor and recipient strains were mixed and make up to the volume of 5 ml with LB broth. After incubation of 16 h in  $37^{\circ}\text{C}$ , transconjugants were selected on LB plates containing azide ( $200 \text{ mg L}^{-1}$ ), nalidixic acid ( $20 \text{ mg L}^{-1}$ ) and ampicillin ( $100 \text{ mg L}^{-1}$ ). Conjugative frequency was determined by the following formula: conjugative frequency = transconjugants (CFU/ml)/recipients (CFU/ml). The *E. coli* J53 transconjugants were then tested against imipenem to confirm the role of pGA45 in recipient strains. The results showed that pGA45 also conferred resistance to imipenem in recipient strains.

### Plasmid Sequencing and Bioinformatics

Plasmid DNA from the *E. coli* J53 transconjugants was extracted using a Qiagen plasmid midikit (Qiagen, Inc). The plasmid DNA was sequenced on an Illumina HiSeq 2000 sequencing system. Sequencing reads were *de novo* assembled into contigs using the SOAPdenovo 2.04 software (Li et al., 2008, 2010). Gaps between contigs were closed by PCR with standard Sanger sequencing. Glimmer 3.02 was used to predict putative open reading frames (ORFs) (Salzberg et al., 1998; Delcher et al., 1999, 2007). All ORFs were translated and aligned with different protein databases including NR (version: 20121005), KEGG (version: 59), COG (version: 20090331), SwissProt (version: 201206) and GO (version: 1.419).

## Nucleotide Sequence Accession Number

The complete nucleotide sequence of pGA45 was deposited in GenBank under accession no. KT780723.

## RESULTS

Sequencing of plasmid pGA45 generated 237,937,000 reads in total. Reads either of low quality or representing the host chromosome contamination through comparison with the sequence of reference strain *E. coli* MG1655 were filtered. In the end, 34 contigs were obtained and then assembled into 10 scaffolds. Through PCR and Sanger sequencing, gaps between contigs and scaffolds were closed. The complete sequence of plasmid pGA45 was 140,698 bp in length with an average G + C content of 52.03% (Figure 1). Analysis of the sequence identified 157 ORFs, 64 of which were transcribed in the opposite direction. The backbone of this plasmid included the replication region, stability region 1, and transfer region (51524 bp), making up 36.6% of the total sequence. This backbone region shared high homology and gene synteny to several other IncF plasmids including pNDM1\_EC14653 (Wu et al., 2015), pYDC644 (GenBank accession no.: KR351290), pNDM-Ec1GN574 (GenBank accession no.: KJ812998), pRJF866 (Qu et al., 2015), pKOX\_NDM1 (Huang et al., 2013) (86% query coverage and 97% nucleotide identity, NCBI database) and pP10164-NDM (Sun et al., 2015) (86% query coverage and 94% nucleotide identity, NCBI database). Notably, all these plasmids were recently sequenced and four of them were found in China. Thus, to our best of knowledge, pGA45 and other recently sequenced plasmids represent a new IncF subtype and this type of plasmids exhibit a high prevalence in China. By contrast, the rest parts of pGA45 [including the variable region, the stability region 2 and the type VI secretion system (T6SS) region] showed no significant similarities with other sequenced plasmids in GenBank.

The replication region (1,431 bp) of pGA45 (positions 75496–76926), including the replication initiation protein gene *repA* and replication regulatory protein gene *repA2*, shared 93% nucleotide similarity with the six IncF plasmids mentioned above with 100% query coverage. Plasmid pGA45 was further assigned to the IncFIIy incompatibility group through sequence queries against the plasmid MLST databases<sup>1</sup>.

Plasmid pGA45 contained one transfer region (position 42643–73867 bp) that comprised 21 *tra* genes, 3 *trb* genes (ordered as follows: *traM*, *traY*, *traA*, *traL*, *traE*, *traK*, *traB*, *traV*, *traP*, *traC*, *trbI*, *traW*, *traU*, *trbC*, *traN*, *traF*, *traQ*, *trbB*, *traH*, *traG*, *traT*, *traD*, *traI*, *traX*) and *finO*. Mating out experiments demonstrated that this region made pGA45 self-transmissible at a relatively high frequency of  $(7.81 \pm 7.15) \times 10^{-3}$  transconjugants per recipient between *E. coli* CV601 and *E. coli* J53.

The genes on plasmid pGA45 that are responsible for plasmid stability and maintenance included *umuC*-*umuD* genes

which confer resistance to UV light, *relE*-*relB* genes encoding a toxin-antitoxin system, *ardA* gene with antirestriction function, *parA*-*parB* genes for partition, *psiA*-*psiB* genes involved in the bacterial SOS inhibition and *ssb* gene involved in recombination and repair.

The variable region of plasmid pGA45 contained two resistance genes including one ARG *bla<sub>IMI-3</sub>* and one copper resistance gene *copC* (with 54% coverage and 94% amino acid identity to *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104, NCBI database). Plasmid pGA45 also harbored a T6SS region (position 82987–114739bp) which may be related to bacterial pathogenesis.

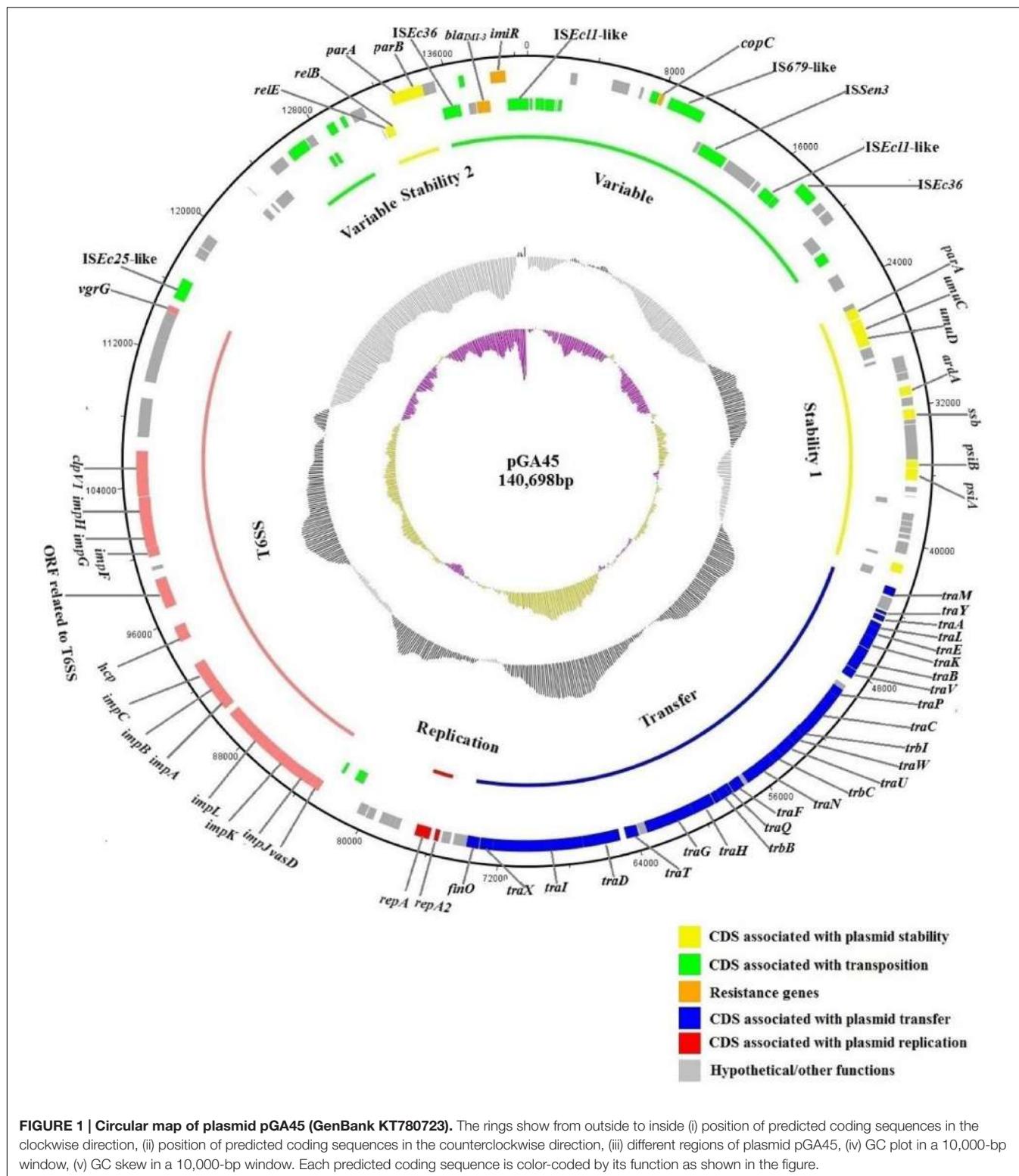
## DISCUSSION

The *bla<sub>IMI-3</sub>* gene from the variable region was the only ARG harbored by pGA45. The *bla<sub>IMI-3</sub>*-containing region (position 58145–74139 bp) was bracketed by one copy of insertion sequence *ISEc36*<sup>2</sup> and one copy of insertion sequence highly similar to *ISEc1*<sup>2</sup> (with 100% coverage and 94% nucleotide identity to *ISEc1*) in the same orientation (Figure 2). *ISEc36* was first identified in a *bla<sub>IMI-2</sub>*-bearing *E. coli* W635 strain (Rojo-Bezares et al., 2012). In this strain, *bla<sub>IMI-2</sub>* was detected upstream the IMI-2R gene. However, in plasmid pGA45, *bla<sub>IMI-3</sub>* and IMI-3R changed positions with each other and IMI-3R was located upstream the *bla<sub>IMI-3</sub>* gene. Another well characterized *bla<sub>IMI</sub>*-containing structure was from *Enterobacter cloacae* plasmid pT103 (GenBank accession no.: NG\_036022.1) (Yu et al., 2006). In this partially sequenced plasmid, the *bla<sub>IMI</sub>*-containing region comprises two *ISEc13*<sup>2</sup> (one is partial) elements flanking the *bla<sub>IMI-3</sub>* and *bla<sub>IMI-3R</sub>* genes in the opposite directions and one partial *ISEc36* located downstream of this region. In all these characterized *bla<sub>IMI</sub>*-containing regions, *bla<sub>IMI</sub>* genes have close relationships with insertion sequence *ISEc36*. Therefore, *ISEc36* may play an essential role in the dissemination of *bla<sub>IMI</sub>* genes between different plasmids. It is also noteworthy to point out that the six plasmids mentioned above similar to pGA45 in backbones mainly have two different bacterial hosts, *Klebsiella pneumoniae* and *Enterobacter cloacae*. In addition, the identified *bla<sub>IMI</sub>* genes were mostly from *Enterobacter* species. In view of this, the most probable hosts for plasmid pGA45 were *Enterobacter* species.

Another notable feature harbored by pGA45 was the T6SS region. Compared to other similar IncFII plasmids, the T6SS region was unique to pGA45. This region was most closely related to the T6SS system of plant pathogen *Erwinia amylovora* not only in nucleotide identity (71% coverage and 82% nucleotide identity) but also in gene organization. Previous studies showed that *bla<sub>IMI-2</sub>* and *bla<sub>IMI-3</sub>* genes were located on plasmids with sizes ranging from 48.5 to 80 kb (one of these plasmids had been identified to belong to IncF group). In this study, pGA45 was much bigger than these plasmids. This perhaps resulted from the

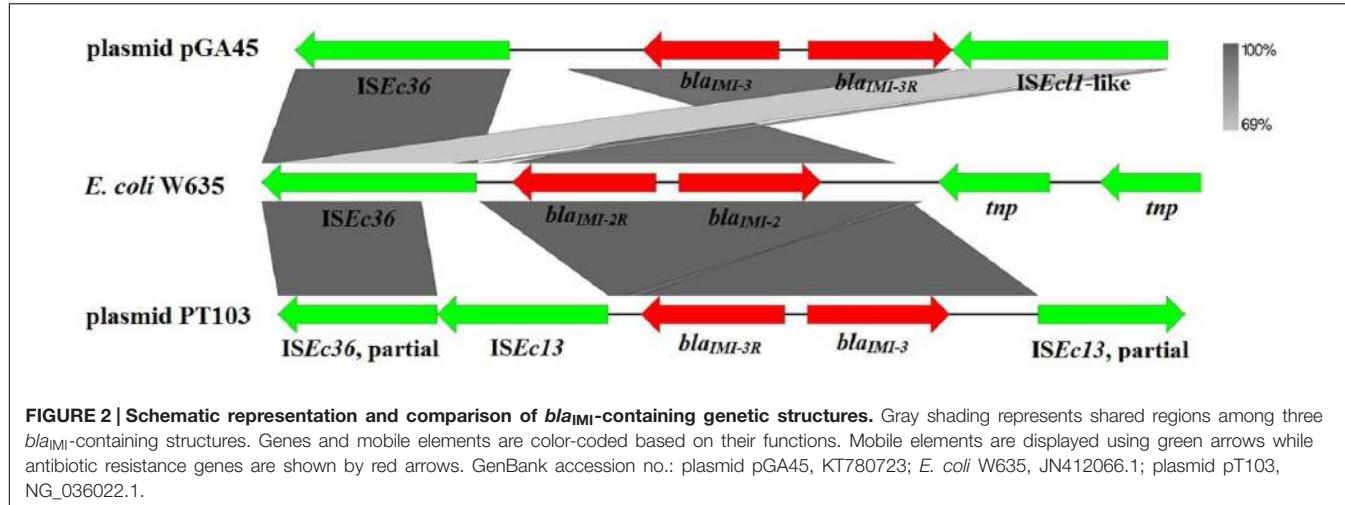
<sup>1</sup><http://pubmlst.org/plasmid>

<sup>2</sup><https://www-is.biotoul.fr/>



integration of this T6SS region (31753 bp). The T6SS region of plasmid pGA45 was flanked by a copy of ISEc25-like element (with 100% coverage and 85% nucleotide identity to ISEc25) downstream and two transposase genes (with weak

amino acid identity to known transposase) upstream. The T6SS region of plasmid pGA45 comprised 14 T6SS-related genes including *vasD* (with 93% coverage and 64.46% amino acid identity to *Erwinia amylovora* ATCC 49946, KEGG database),



*impJ* (with 100% coverage and 82.55% amino acid identity to *Erwinia pyrifoliae* Ep1/96, KEGG database), *impK* (with 99% coverage and 84.26% amino acid identity to *Erwinia pyrifoliae* Ep1/96, KEGG database), *impL* (with 100% coverage and 84.38% amino acid identity to *Erwinia tasmaniensis*, KEGG database), *impA* (with 99% coverage and 77.45% amino acid identity to *Erwinia amylovora* ATCC 49946, KEGG database), *impB* (with 97% coverage and 86.52% amino acid identity to *Erwinia amylovora* ATCC 49946, KEGG database), *impC* (with 100% coverage and 94.99% amino acid identity to *Erwinia billingiae*, KEGG database), *hcp* (with 99% coverage and 86.79% amino acid identity to *Enterobacter cloacae* subsp. *cloacae* ATCC 13047, KEGG database), an ORF encoding a FHA-domain containing protein (with 97% coverage and 70.38% amino acid identity to *Erwinia pyrifoliae* Ep1/96, KEGG database), *impF* (with 99% coverage and 85.03% amino acid identity to *Erwinia amylovora* ATCC 49946, KEGG database), *impG* (with 100% coverage and 87.5% amino acid identity to *Erwinia pyrifoliae* Ep1/96, KEGG database), *impH* (with 99% coverage and 76.95% amino acid identity to *Erwinia tasmaniensis*, KEGG database), *clpV* (with 99% coverage and 89.64% amino acid identity to *Erwinia amylovora* ATCC 49946, KEGG database) and *vgrG* (with 100% coverage and 96.92% amino acid identity to *Kosakonia radicincitans* DSM 16656, NR database).

The T6SS was a recently discovered phage-like secretion apparatus. First reported in *Vibrio cholerae* and *Pseudomonas aeruginosa*, T6SS was likely to be involved in bacterial pathogenesis through acting like a potential nano-syringe for the translocation of effector proteins into the host cell (Pukatzki et al., 2006; Sarris et al., 2011). The Hcp (haemolysin co-regulated protein) and VgrG (valine-glycine repeat G protein) proteins are putative effectors for T6SS (Russell et al., 2014) and the genes encoding these two proteins are also found to be located in the T6SS region of plasmid pGA45. T6SS-related genes are harbored by many kinds of Gram-negative bacterial pathogens which can result in human or animal diseases. In this study, plasmid pGA45 was isolated from

river sediment which was collected from urban section of Haihe River. This area was densely populated and strongly affected by human activities. In previous published literatures, most of the bla<sub>IMI</sub> isolates were from clinical settings. Therefore, the occurrence of T6SS and bla<sub>IMI-3</sub>-containing plasmid pGA45 in the river environment are a potential risk for human health and the horizontal transfer of this plasmid between *Enterobacteriaceae* bacteria may aggravate this situation.

## CONCLUSION

This report demonstrated the complete nucleotide sequence of the bla<sub>IMI</sub>-harboring plasmid. The sequencing of this plasmid provided insight into the architecture used for the dissemination of bla<sub>IMI</sub> carbapenemase genes. In addition to the bla<sub>IMI</sub> gene, plasmid pGA45 also harbored a T6SS cluster probably involved in bacteria virulence. Notably, this plasmid was isolated from environment sample, which will increase the risks of obtaining infections resulted from various types of pathogens carrying this plasmid.

## AUTHOR CONTRIBUTIONS

DM and YL designed experiments; BD carried out experiments and analyzed experimental results; BD wrote the manuscript.

## ACKNOWLEDGMENTS

We are greatly thankful to Professor Holger Heuer (Julius Kühn-Institut) for providing *E. coli* CV601 and *E. coli* J53. This work was supported by the National Natural Science Foundation of China (Grants 41473085 and 31470440, 31270542), the Ministry of Education, People's Republic of China as an innovative research team project (grant No. IRT13024).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Downregulation of host tryptophan–aspartate containing coat (TACO) gene restricts the entry and survival of *Leishmania donovani* in human macrophage model

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 16 June 2015

Accepted: 27 August 2015

Published: 13 October 2015

### Citation:

Gogulamudi VR, Dubey ML, Kaul D,  
Atluri VSR and Sehgal R (2015)  
Downregulation of host  
tryptophan–aspartate containing coat  
(TACO) gene restricts the entry  
and survival of *Leishmania donovani*  
in human macrophage model.  
*Front. Microbiol.* 6:946.  
doi: 10.3389/fmicb.2015.00946

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*Leishmania* are obligate intracellular protozoan parasites of mammalian hosts. Promastigotes of *Leishmania* are internalized by macrophages and transformed into amastigotes in phagosomes, and replicate in phagolysosomes. Phagosomal maturation arrest is known to play a crucial role in the survival of pathogenic *Leishmania* within activated macrophages. Recently, tryptophan–aspartate containing coat (TACO) gene has been recognized as playing a central role in the survival of *Mycobacterium tuberculosis* within human macrophages by arresting the phagosome maturation process. We postulated that a similar association of TACO gene with phagosomes would prevent the vacuole from maturing in the case of *Leishmania*. In this study we attempted to define the effect of TACO gene downregulation on the entry/survival of *Leishmania donovani* intracellularly, by treatment with Vitamin D<sub>3</sub> (Vit.D<sub>3</sub>)/Retinoic acid (RA) and chenodeoxycholic acid (CDCA)/RA combinations in human THP-1 macrophages (*in vitro*). Treatment with these molecules downregulated the TACO gene in macrophages, resulting in reduced parasite load and marked reduction of disease progression in *L. donovani* infected macrophages. Taken together, these results suggest that TACO gene downregulation may play a role in subverting macrophage machinery in establishing the *L. donovani* replicative niche inside the host. Our study is the first to highlight the important role of the TACO gene in *Leishmania* entry, survival and to identify TACO gene downregulation as potential drug target against leishmaniasis.

**Keywords:** leishmaniasis, TACO gene, Coronin-1A, host-parasite interactions, intracellular parasite, vitamin D, retinoic acid, chenodeoxycholic acid

## Introduction

Leishmaniasis is a major public health issue in many parts of the world, affecting over 20 million people worldwide. About 350 million people are at risk of being infected with leishmaniasis, and 1.5–2 million children and adults develop the disease each year (Desjeux, 2004). This disease is caused by a parasite, which belongs to the genus *Leishmania*. All species of the genus *Leishmania*

are obligate intracellular parasites that pass their life cycle in two hosts: the mammalian host, and the insect vector, the female sandfly. In human and other mammalian hosts, they multiply within macrophages, in which they occur exclusively in the amastigote form. Intracellular parasites have evolved through diverse mechanisms to enhance their survival and replication within host cells (Hackstadt, 2000). These mechanisms greatly involve adaptations for survival in different intracellular compartments that permit the parasites to avoid lysosomal killing. Although functions of most of these strategies remain unclear, the majority is expressed early on infectious process, suggesting that manipulation of the vacuole is critical to the outcome of the host-parasite interaction.

Tryptophan-aspartate containing coat (TACO) protein (a coat protein of phagosomes), also known as Coronin 1A, was shown to restrict the delivery of *Mycobacteria* to lysosomes (Ferrari et al., 1999). TACO/Coronin-1A belongs to the tryptophan-aspartate (WD) repeat containing family proteins (Neer et al., 1994; Suzuki et al., 1995), some of which are implicated in cytoskeletal organization, signal transduction, motility (Burrows et al., 1995), cytokinesis and vesicle formation (Pryer et al., 1993). Specifically, TACO is a 57 kDa polypeptide that binds to actin and is involved in cytoskeletal modulation (Gatfield et al., 2005), cytokinesis and intracellular membrane transport (Rybakin and Clemen, 2005). TACO is present on the cytoplasmic face of the plasma membrane and is retained by vacuoles carrying mycobacteria through phagocytosis, results in shielding of the mycobacteria within phagosomes of the host molecule by inhibiting its fusion with any other organelle, including lysosomes (Ferrari et al., 1999). Therefore, the active retention of TACO around the mycobacterial phagosome prevents the delivery of this machinery, and the pathogen continues to survive and replicate within the TACO armored phagosome. Several lines of evidence have been developed to show that TACO becomes activated in *Mycobacterium tuberculosis* infected cells, and this contributes to disease pathogenesis. Recent studies have shown that the downregulation of TACO leads to the suppression of mycobacteria infectivity and multiplication rate (Anand and Kaul, 2005).

However, the possible contribution of TACO in the inhibition of fusion of *Leishmania donovani*-containing phagosomes was not studied. Both *Mycobacterium* and *Leishmania* are intracellular organisms with macrophages as their primary target cells. These interference mechanisms are the main focus of this study. Dermine et al. (2000) suggested that it would be of interest to validate whether this TACO protein is also coupled with *Leishmania*-containing phagosomes. In addition to their limited availability, anti-leishmanial drugs have disadvantages such as toxicity, required prolonged treatment, challenge of drug resistance and high cost (Sundar et al., 2007). Attempts to develop newer synthetic compounds have met with a limited success. Thus, there is an urgent need to develop better anti-leishmanial drugs to supplement those in present use. Simple compounds like vitamins have not yet been explored as sources for possible anti-leishmanial agents. Vitamin D<sub>3</sub> (Vit.D3), retinoic acid (RA), and chenodeoxycholic acid (CDCA) have been explored against *M. tuberculosis*; however, no scientific data

is available on the utility of these endogenous molecules with regard to their anti-leishmanial activity *in vitro* or *in vivo*.

Taking all these findings into consideration, the aim of this study was to assess the effect of downregulation of TACO gene expression on entry and survival of *L. donovani* in human macrophages. The present study was designed to explore the inherent capacity of isoprenoid (CDCA, derived from mevalonate pathway) and vitamins to regulate TACO gene transcription, and their effect on entry and survival of *Leishmania* intracellularly. Based upon its previously described effects, this study has been designed to address these significant gaps in evidence that downregulation of TACO gene expression will lead to inhibition of *Leishmania* entry and survival in host macrophages. Such approach may help in the development of new, safe, effective, and inexpensive drug molecules, which can act at preventive and therapeutic levels against *Leishmania* infection.

## Materials and Methods

### Parasites

The standard strain of *L. donovani*: MHOM/IN/80/DD8 being maintained in the Department of Parasitology, PGIMER, Chandigarh, by serial *in vitro* culture and passaged through BALB/c mice for maintenance of virulence was used in the study.

### Macrophage Cell Line

THP-1, a human monocytic leukemia cell line was procured from NCCS, Pune, India. THP-1 monocytic cells were cultured in complete culture medium containing RPMI-1640 and HEPES (Invitrogen) supplemented with 10% fetal calf serum and 1% Penicillin (5000 U/ml)-streptomycin (5000 µg/ml; Sigma-Aldrich). The cells were kept in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. Cells were seeded in six-well plates (1 × 10<sup>7</sup> cells/ml) in the complete medium. The cells were incubated with 30 nM/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 72 h (Tsuchiya et al., 1982; Verstraelen et al., 2008), which induced differentiation of these cells into adherent macrophages. Plates were washed twice with pre-warmed RPMI-1640 to remove PMA, and 2000 µl of complete RPMI-1640 medium was then added. Differentiated cells were maintained until experiments were conducted.

### Conventional RT-PCR

After differentiation of THP-1 cells into macrophages as explained above, culture medium was aspirated and cells were rinsed with PBS. These cells were processed for total RNA extraction using TRI Reagent solution (Ambion, USA) based on acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Quantity and quality of RNA were determined by reading absorbance at 260 and 280 nm. First-strand cDNA was synthesized from 1.5 µg of total cellular RNA using random hexamer primers and Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) in a 20 µl reaction volume. Reagents were obtained from RevertAid<sup>TM</sup> first strand cDNA

synthesis kit (Fermentas, USA). 1  $\mu$ l of cDNA was used as a template in each PCR reaction. cDNA of each sample was amplified using primers specific for TACO gene and carried out using glyceraldehyde phosphate dehydrogenase (GAPDH) as a control for RT efficiency. PCR reagents were obtained from MBI Fermentas and specific primers were obtained from MWG-Biotech. PCR was performed in a 25  $\mu$ l reaction volume containing 1x PCR buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.5  $\mu$ M of each primer and 0.625 U of Taq (*Thermus aquaticus*) DNA polymerase. The primer sequences are described below. TACO (512 bp) Forward: 5'-CCAGTGCTATGGATGTGCGCG-3' Reverse: 5'-GACACGACTCGCTTGTACGGC-3' GAPDH (270 bp) Forward: 5'-AAGGCACAGACATGGTTGGT-3' Reverse: 5'-TGGAAAGCAAATGCCCTGA-3'.

### TACO Gene Sequencing

Polymerase chain reaction amplicons were detected by electrophoresis using 1.5% agarose gels and visualized using ethidium bromide. The amplified TACO gene PCR product of the expected size 512 bp was extracted from the agarose gel with a Mini Gel Extraction Kit (Qiagen). The PCR products were purified using Microspin columns (Amersham Pharmacia) prior to sequencing. Sequencing was done using specific primers and ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit. The sequences were analyzed on an ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The sequence of amplicon was confirmed by single direction using TACO primers.

### Determination of TACO mRNA Level

GAPDH gene was used as a control. The relative levels of TACO gene mRNA in Vit.D<sub>3</sub>/RA and CDCA/RA treated cells were determined by real-time PCR. The concentrations of molecules showing the maximum gene downregulation were used in further studies.

After treating the THP-1 cells with PMA as explained above, culture medium was aspirated and cells were rinsed with PBS. At the end of the incubation, the cells were incubated for 24 h in the presence of various stimuli: one set of cultures were pre-treated with Vit.D<sub>3</sub>/RA (Sigma-Aldrich, USA) at two doses (0.5/0.5, 1.0/1.0  $\mu$ M), and another set were pre-treated with CDCA/RA in a dose dependent manner (25, 50, 75, and 100  $\mu$ M of CDCA) in the constant presence of RA at 0.5  $\mu$ M for 24 h at 37°C in 5% CO<sub>2</sub>. After 24 h of incubation at 37°C, real time PCR (relative quantification) was performed using LightCycler 480 SYBR Green I master mix (Roche) detection method to confirm the quantitative expression of TACO gene. GAPDH was used as internal control. Reaction mixture containing 1  $\mu$ l (20-30 ng) of purified cDNA, 10  $\mu$ l of 2X master mix (SYBR Green1 Dye), 10 picomoles of each primer of TACO and GAPDH genes was prepared and total volume was made up to 20  $\mu$ l in nuclease free water. The cDNA synthesis reaction was performed with RNA along with all the other components but without RT enzyme. A "no-RT" control would yield a PCR product in case of genomic DNA contamination of the RNA sample. In order to nullify the variation in samples,

we have presented the data of our quantitative analysis as  $\Delta Cp$  ( $\Delta Cp = Cp$  Target gene -  $Cp$  GAPDH).  $Cp$  (crossing point) value is inversely proportional to the gene level, thus  $Cp$  has an indirect relationship with the relative levels of the target gene; the greater the value, the lower the expression level.

Relative gene expression of TACO mRNA was performed by real-time PCR using SYBR Green-I master detection method using specific primers. To minimize the loading variations, GAPDH, an internal control gene, was run in all the samples from different groups. The real-time PCR analysis of transcripts for GAPDH in the Vit.D<sub>3</sub>/RA and CDCA/RA in different doses showed similar amplification and dissociation curves. In order to nullify the due variation in samples, the data was normalized to GAPDH, which served as internal control ( $\Delta Cp = Cp$  Target gene -  $Cp$  GAPDH), then again normalized to control ( $\Delta\Delta Cp = \Delta Cp$  Test sample -  $\Delta Cp$  Control sample), and final data was presented in the form of relative fold change by  $2^{-\Delta\Delta Cp}$ .

### Western Blot Analysis

After differentiation of THP-1 cells into macrophages as explained above, culture medium was aspirated and cells were rinsed with PBS. Adhered macrophages were detached by adding 1 ml acutase (PAA Laboratories, UK) at 37°C for 10 min. The cells were pelleted and the supernatant was discarded. The cells were washed gently by resuspending in 1 ml of PBS, and pelleted at low speed. Cell pellet was lysed in 1 ml of lysis buffer [50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA), 0.1% Triton X-100, 0.3% w/v β-mercaptoethanol, 0.28 TIU/ml aprotinin, 2 mM phenylmethylsulphonyl fluoride (PMSF), leupeptin 0.5 mg/ml and containing 100  $\mu$ l protease inhibitor cocktail (Roche, Mannheim, Germany)] for 3 min at 4°C. Western blotting was carried out using a Mini Trans-blot electrophoretic transfer assembly (Bio-Rad Laboratories, USA; Towbin et al., 1979). After the transfer, PVDF membrane was blocked with 5% PBS-skimmed milk for 2 h and incubated with primary antibody (Novus Biologicals, USA) diluted in 5% PBS-skimmed milk overnight at 4°C. The membrane was then washed twice with PBST, once with PBS and incubated with Horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., USA) diluted in 5% PBS-skimmed milk, against the respective primary antibodies for 2 h. Protein bands were visualized by enhanced chemiluminescence (ECL) plus detection system with UV-Pro Transilluminator.

### Immunofluorescence Assay

For the localization of TACO protein, immunofluorescence was done on THP-1 cells by growing cells on cover slips and exposing them to PMA for 48 h at 37°C and in the presence of 5% CO<sub>2</sub> to permit cell differentiation into macrophages. Two sets of cells were fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 20 min, permeabilized and blocked with 0.2% (w/v) BSA/0.1% (w/v) saponin in PBS (blocking solution) for 20 min. The cells were subsequently incubated with rabbit Coronin 1A antibody (Novus biological, USA) for 60 min at room

temperature. After washing three times with PBST, cells were incubated with goat anti rabbit IgG FITC-conjugated secondary antibody (Immunology Consultants Laboratory, Inc., USA), for 60 min at 37°C. After incubation, cells were washed thrice with PBS and immediately examined by fluorescence microscopy (Olympus BX51, Japan) equipped with a UV filter system. Images were obtained with a CCD camera (Olympus, model no.E330-ADU1.2X, Japan).

### **Effect of Macrophage TACO Gene Downregulation on *L. donovani* Infection *In Vitro***

Once the maximal downregulation dose of TACO mRNA was determined by treatment with the above molecules, the same dose was applied during the parasite infection study. One set of THP-1 macrophages were treated with the maximal downregulating dose of Vit.D<sub>3</sub>/RA and another set with CDCA/RA. After 24 h of treatment, the cells were washed two times with plain medium. Infection of macrophage was carried out using a virulent strain (DD8) of *L. donovani* grown in axenic culture. These promastigotes were added to macrophages at a ratio of 10:1. This culture was incubated for 1 h at 37°C in 5% CO<sub>2</sub> incubator. Non-adherent cells were washed out and macrophages were cultivated in complete culture medium supplemented with 10% heat inactivated FBS, streptomycin (200 ug/ml), benzyl penicillin (200 U/ml) and gentamicin (40 ug/ml). Parasite load and replication rate was determined at different time intervals: early (2 and 4 h), middle (24 and 48 h) and late (72 and 96 h) stages. Infected macrophages on the cover slips were fixed and stained with Giemsa. Parasite loads were examined microscopically by counting at least 500 target macrophages for each cover slip. Efficacy of the molecules was determined by calculating the number of amastigotes per 100 macrophages at each time point.

### **Statistical Analysis**

Data was expressed as the mean  $\pm$  SD values of triplicate samples. The statistical significance of the differences between and within groups was determined by analysis of variance (ANOVA) to define the multiple comparisons amongst different

groups. The statistical analyses were performed using SPSS software. Differences were considered statistically significant for  $p < 0.05$ .

## **Results**

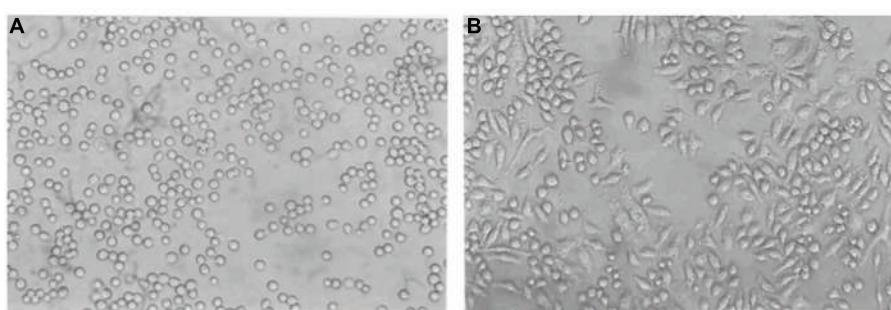
### **Optimum Concentration of PMA for Differentiation of THP-1 Monocytes to Macrophages**

According to the results presented in **Figure 1**, monocytes in suspension were converted to matured macrophages in 72 h after treatment with PMA. Treatment with 30 nM/ml PMA for 72 h induced changes in morphology; their rounded shape became elongated and flattened. However, attachment of cells at 20 nM/ml was unstable, and the cells were easily detached by washing. This indicated that the concentrations below 30 nM/ml PMA were not enough to differentiate the THP-1 cells stably. Hence, the minimal concentration of PMA for stable differentiation was determined to be 30 nM/ml. Cell adhesion and spreading, which are hallmarks of macrophages, were then examined under a microscope. Macrophage cells grew in a distinct morphological pattern, showing a significant raise in adhesion and spreading patterns to the growth substratum.

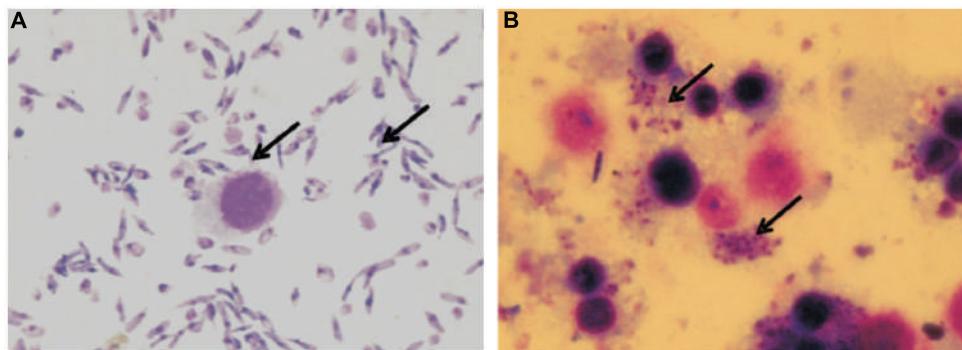
### **Infectivity of *L. donovani* Promastigotes in Differentiated THP-1 Macrophages and TACO Gene Expression**

Differentiated THP-1 macrophages were infected with virulent *L. donovani* promastigotes for 24 h. As demonstrated in **Figure 2**, the *L. donovani* promastigotes exhibited a marked infectivity to macrophages 24 h after infection. The uptake of parasites by mammalian cells was determined by Giemsa staining.

In order to determine the expression of TACO gene expression in *Leishmania* infected macrophages, RT-PCR was performed using specific primers with equal concentration of cDNAs. The house-keeping gene GADPH was amplified and used as a loading control. Gels containing RT-PCR products were photographed by UV-Pro Transilluminator camera and bands were analyzed (**Figure 3**). This assay confirmed the 512 bp TACO gene



**FIGURE 1 | Phase contrast micrographs of THP-1 cells in culture induced by PMA.** THP-1 cells were incubated with PMA (30 nM/ml) for 72 h. Morphological changes in THP-1 (40X) on (A) Day 1 (B) Day 3 are shown. Their morphology changed from a rounded shape to elongated and flattened.



**FIGURE 2 |** Giemsa stained micrograph showing human macrophage cell line (THP-1) infected with *L. donovani* promastigotes. **(A)** Promastigotes and Macrophages; **(B)** Macrophages showing intracellular parasite converted to amastigotes after internalization (100X; indicated by arrowheads).

expression, when the PCR product showing ethidium bromide-stained bands obtained after electrophoresis along with the marker (Figure 3) and 240 bp of GAPDH gene.

Sequencing of PCR product of TACO gene (512 bp band) from agarose gel electrophoresis and BLAST search for homologous nucleotides in the database showed that 512 bp of TACO gene expression showed the highest (100%) homology to other TACO mRNAs in many organisms including humans. This confirmed that the PCR amplified gene was the exact product of TACO/Coronin 1A. This TACO gene sequence has been deposited in GenBank under the accession number GQ2141031.

### Localization and Expression of TACO in THP-1 Macrophages

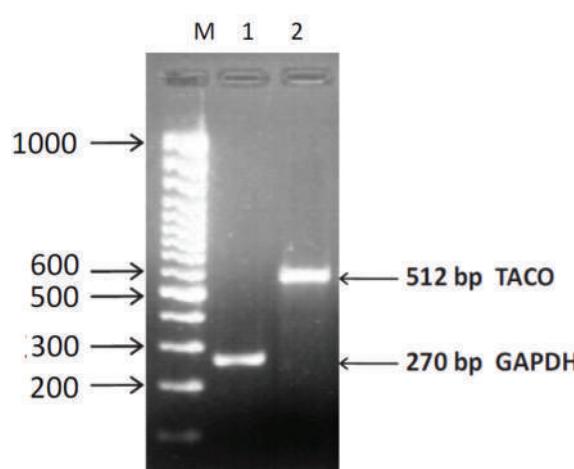
TACO protein localization in human THP-1 macrophages were determined by indirect immunofluorescence using an affinity-purified antibody. Strong punctuate staining

of TACO protein was observed in the cytoplasmic face of activated macrophages (Figure 4). Observation of this assay showed TACO protein localization on the cytoplasmic face of plasma membranes of activated THP-1 human macrophages.

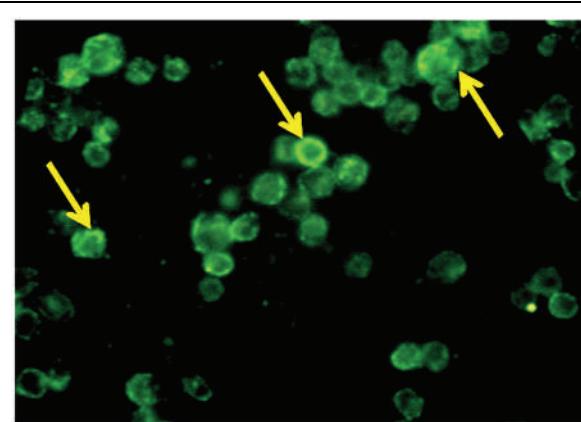
Expression of TACO protein in human macrophages was confirmed by Western blot analysis using a specific antibody. Results showed that in lysates of THP-1 macrophages (Figure 5), anti-TACO/Coronin 1A antibody recognized a single 57 kDa protein in Western blots, confirming the expression of TACO protein.

### Vitamin D<sub>3</sub>/Retinoic Acid Regulates TACO Gene Expression in THP-1 Macrophages

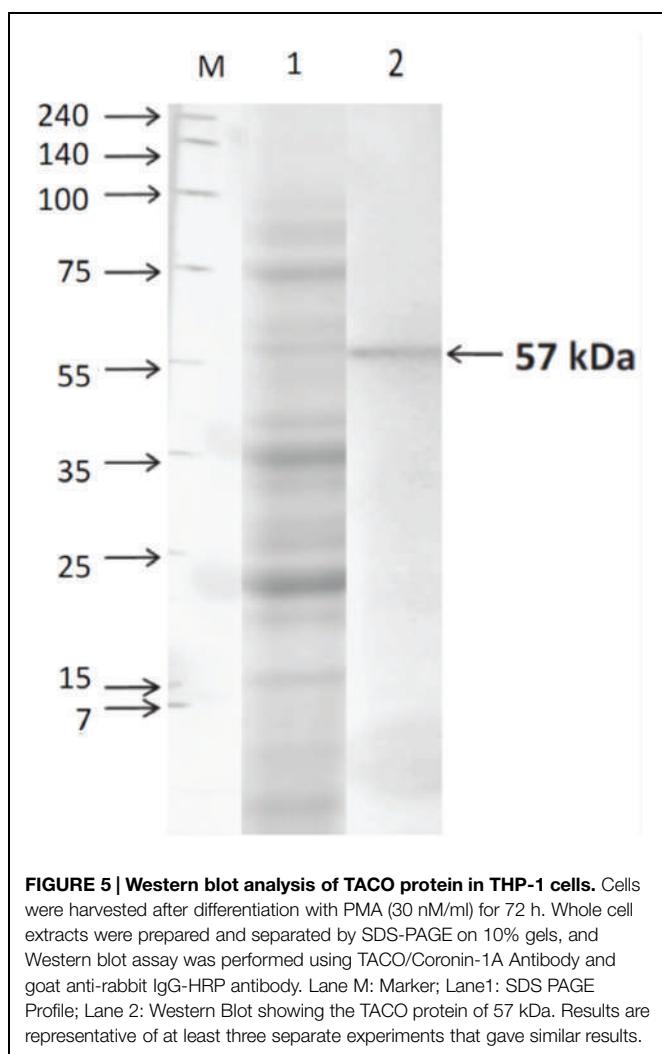
During this phase, to study the transcriptional regulation of TACO gene by Vit.D<sub>3</sub>/RA and CDCA/RA, experiments were designed to determine the optimum dose of molecules used



**FIGURE 3 |** Agarose gel photograph showing ethidium bromide stained bands of RT-PCR products of TACO and GAPDH genes. Lane M: 100 bp DNA ladder. Lane 1: GAPDH gene; Lane2: TACO gene.



**FIGURE 4 |** Localization of TACO/Coronin1A in differentiated THP-1 macrophages. Monocytes cultured on glass coverslips were differentiated into macrophages in the presence of PMA, fixed, permeabilized and stained with primary antibody, TACO/Coronin 1A Antibody, for 2 h followed by FITC labeled secondary antibody. Cells were observed under fluorescent microscope equipped with a UV filter system using 100X; indicated by arrowheads.



for downregulation of TACO mRNA. Compounds from two different categories, i.e., isoprenoids derived from mevalonate pathway and vitamins (A, D), were evaluated for their effect on TACO gene expression. Exposure of THP-1 differentiated macrophages to Vit.D<sub>3</sub> and RA for 24 h resulted in significant dose dependent downregulation of TACO mRNA expression. TACO mRNA level was significantly downregulated by 0.686 fold ( $p < 0.0001$ ) in cells treated with 0.5  $\mu$ M/0.5  $\mu$ M Vit.D<sub>3</sub>/RA compared to controls. Whereas cells treated with a dose of 1.0  $\mu$ M/1.0  $\mu$ M Vit.D<sub>3</sub>/RA, mRNA level was highly downregulated by 0.949 fold ( $p < 0.0001$ ) when compared to control groups. The effective dose on TACO mRNA downregulation of this combination was 1.0  $\mu$ M/1.0  $\mu$ M as compared with control groups (Figures 6A,B).

### Chenodeoxycholic Acid/Retinoic Acid Regulates TACO Gene Expression in a Dose Dependent Manner

Further, exposure of THP-1 macrophages to a combination of CDCA 0–100  $\mu$ M and RA (0.5  $\mu$ M) caused a further reduction

in TACO mRNA expression levels (Figure 6C). Surprisingly, a combined dose of 25  $\mu$ M CDCA 0.5  $\mu$ M RA resulted in moderate upregulation by 0.33 fold ( $p = 0.132$ ) of TACO mRNA compared to control groups (Figure 6D). In additional doses, TACO mRNA expression was downregulated at the doses of 50  $\mu$ M/0.5  $\mu$ M by 0.165 fold ( $p = 0.498$ ), 75  $\mu$ M/0.5  $\mu$ M by 0.441 fold ( $p < 0.002$ ) and 100  $\mu$ M/0.5  $\mu$ M by 0.717 fold ( $p < 0.0001$ ) as compared to control cells. In this combination of molecules (CDCA/RA) 100  $\mu$ M/0.5  $\mu$ M dose was most effective in downregulation of TACO gene as compared to control groups (Figure 6D).

### Vitamin D<sub>3</sub>/Retinoic Acid and Chenodeoxycholic Acid/Retinoic Acid Inhibit Entry and Survival of *Leishmania* promastigotes in a Time Dependent Manner

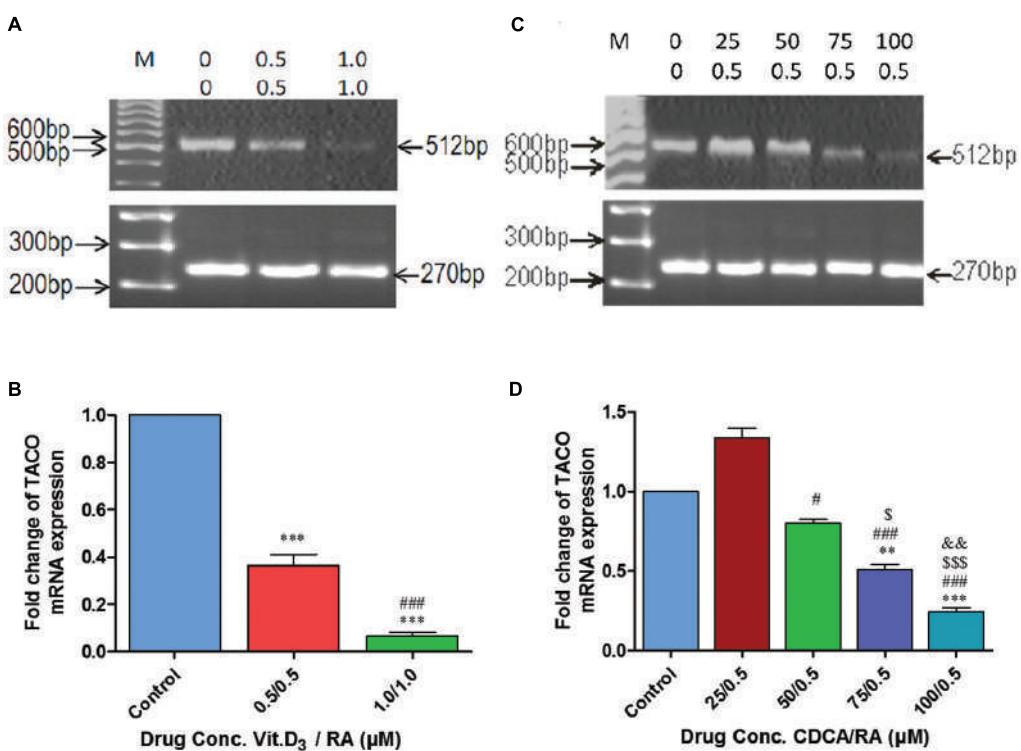
To examine the entry and intracellular survival of *L. donovani* in TACO downregulated macrophages, cells were pre-treated with maximum TACO mRNA downregulated doses of Vit.D<sub>3</sub>/RA and CDCA/RA (treatment before parasite infection) for 24 h. Further, to investigate the parasite survival when TACO gene is downregulated, macrophages were infected with promastigotes at 1:10 ratio (Figure 7), and subsequently time kinetics of THP-1 cell infection by *L. donovani* were assessed at early (2 and 4 h), middle (24 and 48 h), and late (72 and 96 h) stages. The rate of entry and survival was observed at different time intervals.

Treatment with Vit.D<sub>3</sub>/RA before *Leishmania* promastigote infection revealed that the entry and survival of parasites within these cells varied at different time intervals. At 2 h, 31% ( $p = 0.506$ ) reduction and at 4 h, 13% ( $p = 0.387$ ) reduction in parasite load was observed, but there was no significant difference between control and treated cells. At 24 h, we observed 47% ( $p < 0.0001$ ) reduction, at 48 h 57% ( $p < 0.002$ ) reduction, at 72 h 60% ( $p < 0.0001$ ) reduction and at 96 h maximum reduction, i.e., 80% reduction in parasite load ( $p < 0.001$ ; Figure 8) compared to the control.

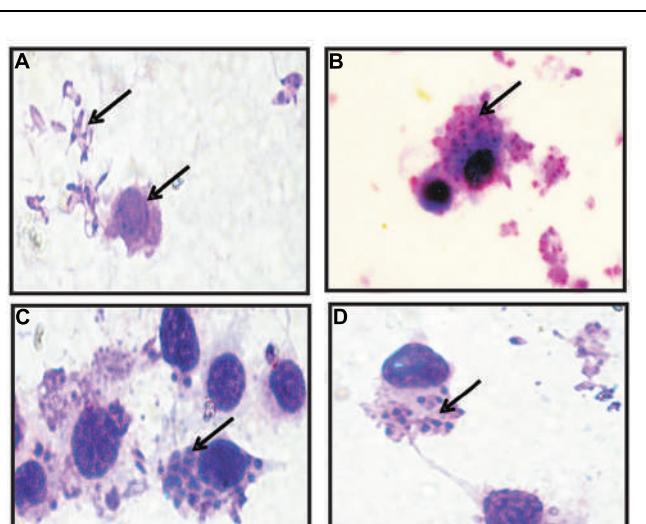
Similarly, in cells after CDCA/RA pre-treatment, there was no significant difference in parasite load at 2 h (15% reduction;  $p = 0.517$ ), and at 4 h it showed 5% reduced parasite load ( $p = 0.553$ ). However, significant reduction in parasite loads at 24 (28%,  $p < 0.04$ ), 48 (30%,  $p < 0.016$ ), 72 (26%,  $p < 0.014$ ), and 96 h (50%,  $p < 0.004$ ) were observed compared to controls (Figure 8). The results showed that Vit.D<sub>3</sub>/RA displayed a maximum 80% ( $p < 0.001$ ) suppression of parasite multiplication and entry of promastigote in THP-1 macrophages, while CDCA/RA treatment showed 50% reduction ( $p < 0.004$ ; Figure 8).

### Discussion

Intracellular infection by the *Leishmania* parasite depends on a sequence of events including attachment, internalization, amastigote differentiation and intracellular survival. Understanding the impact of intracellular pathogens on the behavior of their host cell is the key to designing new



**FIGURE 6 |** Polymerase chain reaction (PCR) amplification showing synergistic action of Vitamin D<sub>3</sub>/Retinoic acid (Vit.D<sub>3</sub>/RA) and chenodeoxycholic acid/Retinoic acid (CDCA/RA) on mRNA expression of TACO gene. **(A,C)** Representative agarose gel photographs showing ethidium bromide stained Real Time PCR products of TACO and GAPDH genes. **(B,D)** The relative levels of TACO mRNA expression represented in fold change. Each data point in the graph represents mean  $\pm$  SD of three independent experiments. \*\*\* $p$  < 0.001 (Control Vs 0.5/0.5 μM; 1.0/1.0 μM), ### $p$  < 0.001 (0.5/0.5 μM Vs 1.0/1.0 μM), \*\*\* $p$  < 0.001 (Control Vs 75/0.5 μM; 100/0.5 μM), # $p$  < 0.05, ### $p$  < 0.001 (25/0.5 μM Vs 50/0.5 μM; 75/0.5 μM; 100/0.5 μM), \$ $p$  < 0.05, \$\$\$ $p$  < 0.001 (50/0.5 μM Vs 75/0.5 μM; 100/0.5 μM), && $p$  < 0.05 (75/0.5 μM Vs 100/0.5 μM).

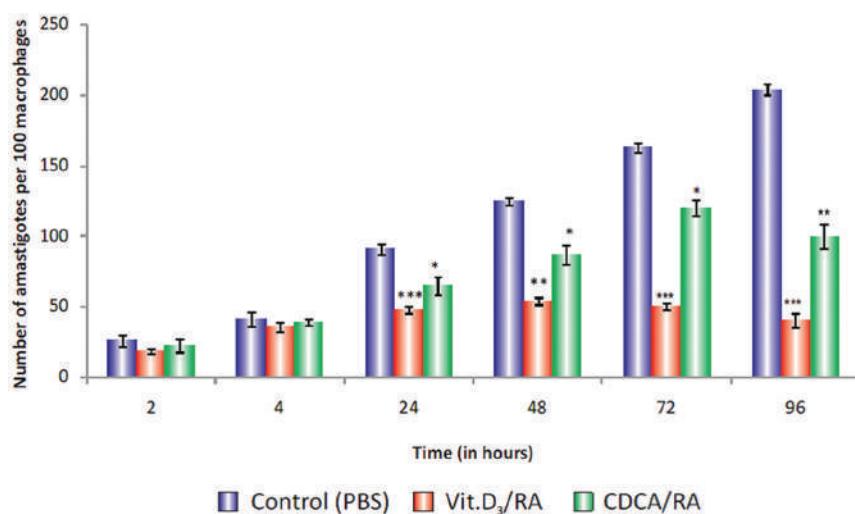


**FIGURE 7 |** Giemsa Stained micro-photographs of Vit.D<sub>3</sub>/RA and CDCA/RA pre-treated THP-1 macrophage infected with promastigotes. **(A)** Promastigotes and Macrophages; **(B–D)** Conversion to amastigotes after internalization (indicated by arrowheads).

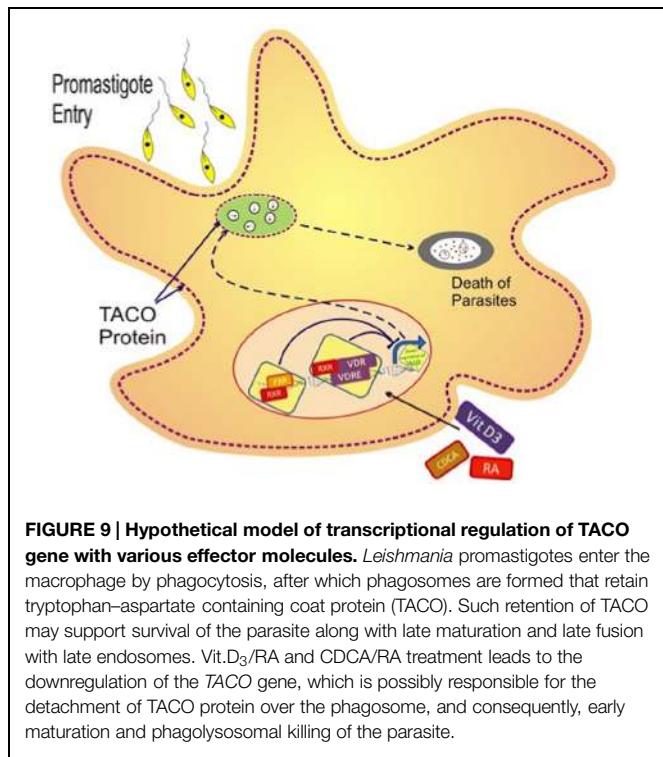
interventions. The present study was based on the hypothesis that the transcriptional manipulation of the TACO gene leads to phagosome (carrying parasite) maturation, which will affect the survival and replication of intracellular pathogens.

In the present study, Vit.D<sub>3</sub> and RA combination showed significant TACO mRNA downregulation in a dose dependent manner (Figure 6). Biological effects of Vit.D<sub>3</sub> are mediated by vitamin D receptor (VDR), a member of a superfamily of nuclear hormone receptors (Carlberg, 2003). Similarly RA modulates transcription through the ligand dependent transcription factor RXR, which binds to a particular response element in the promoter region of target genes (Giguere, 1999). The heterodimeric complex VDR/RXR then binds to a different set of response elements within effector genes. RXR ligands have been reported to synergize with Vit.D<sub>3</sub> to activate the 25 hydroxy-vitamin D<sub>3</sub>-24-hydroxylase promoter (Zou et al., 1997), whereas VDR/RXR ligands that have been reported to inhibit transcriptional activity of rat osteocalcin gene (MacDonald et al., 1993; Anand and Kaul, 2003) showed similar results with these molecules. Therefore, it is reasonable to speculate that Vit.D<sub>3</sub> and RA act synergistically to downregulate TACO mRNA expression (Anand and Kaul, 2003).

In the present study, mevalonate-pathway derived isoprenoids, i.e., CDCA along with RA, were also used to evaluate



**FIGURE 8 | Parasite load in Vit.D<sub>3</sub>/RA and CDCA/RA pre-treated THP-1 macrophage cells infected with promastigotes.** THP-1 human macrophage cell lines were pre-treated with Vit.D<sub>3</sub>/RA, CDCA/RA for 24 h to downregulate the TACO gene, then infected with *L. donovani* promastigotes. Parasite loads were determined by calculating the total number of amastigotes per 100 macrophages at different time intervals (2, 4, 24, 48, 72, and 96 h) post-infection. The values listed are mean  $\pm$  SD of three independent cultures. Significant differences are indicated by asterisks: \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.



**FIGURE 9 | Hypothetical model of transcriptional regulation of TACO gene with various effector molecules.** *Leishmania* promastigotes enter the macrophage by phagocytosis, after which phagosomes are formed that retain tryptophan-aspartate containing coat protein (TACO). Such retention of TACO may support survival of the parasite along with late maturation and late fusion with late endosomes. Vit.D<sub>3</sub>/RA and CDCA/RA treatment leads to the downregulation of the TACO gene, which is possibly responsible for the detachment of TACO protein over the phagosome, and consequently, early maturation and phagolysosomal killing of the parasite.

TACO mRNA expression in a dose dependent fashion. Initially, a moderate increase in TACO mRNA expression was observed (Figure 6). An increased dose combination with CDCA/RA was observed to be more potent in downregulating TACO mRNA expression, initiating a pronounced downregulatory effect on TACO gene transcription (Figure 6). It is pertinent to note that CDCA is a potent activator of FXR, which is ligand-dependent

transcription factor (Giguere, 1999). On activation, the receptor translocates into the nucleus and regulates a number of genes, either alone or in combination with its heterodimeric partner retinoid-X-receptor (RXR; Grober et al., 1999; Edwards et al., 2002). This raises the possibility that CDCA and RA, when used in combination, show a synergistic effect by activating the ligand-dependent heterodimeric complex FXR/RXR.

In support of this, Anand and Kaul (2003) demonstrated the mechanism of transcriptional repression by Vit.D<sub>3</sub>/RA and CDCA/RA through a planned bioinformatic approach coupled with reporter assay technology that was used to pinpoint the promoter region of the TACO gene. This region was evaluated for the presence and functional status of response elements corresponding to VDR/RXR and FXR/RXR. It has been noted that the three compounds that showed transcriptional repression of TACO mRNA expression are potent activators/inhibitors of these transcription factors (Anand and Kaul, 2003).

Keeping in view the positive role of TACO in *Leishmania* entry and survival, it is likely that downregulation of TACO mRNA could be accompanied by inhibition of leishmanial entry and survival within the macrophage. Consequently, investigation was carried out to discover whether or not treatment with these molecules suppress TACO mRNA, and their affects on *Leishmania* entry and survival. THP-1 macrophages were pre-treated with different effector molecules followed by *Leishmania* promastigote infection. The results of entry and survival of the parasite were analyzed by Giemsa staining at different time-periods.

Vit.D<sub>3</sub>/RA pre-treatment revealed 80% inhibition of parasite load as compared to untreated control groups (Figure 8). It was observed at 96 h as analyzed in *Leishmania* infected macrophages. Previously, Vit.D<sub>3</sub> and RA had been reported to restrict the intracellular growth of *M. tuberculosis* (Wilkinson et al., 2000;

Anand and Kaul, 2003). Similarly, Sly et al. (2001) reported that Vit.D<sub>3</sub> treatment of *M. tuberculosis* infected THP-1 cells activated PI3K. PI3K was shown to be essential for the formation of the phagosomal cup (class-1A PI3K) and its fusion with lysosomes in normal cells (Fratti et al., 2001). In our study, we also observed that Vit.D<sub>3</sub>/RA acts synergistically and downregulates TACO gene expression significantly (Figure 8). Anand and Kaul (2003) reported that the effect of Vit.D<sub>3</sub>/RA on TACO gene expression was not evident when either was used alone, and explained that this mode of synergistic action was due to the activation of VDR/RXR transcription factors.

Similarly, CDCA/RA pre-treatment restricted *Leishmania* entry by 50% as observed at 96 h after exposure compared to control groups (Figure 8). Accumulating evidence suggests that bile acids modulate various signal transduction pathways including the protein kinase A, protein kinase C, c-Jun N-terminal kinase and MAP-kinase dependent cascades (Bouscarel et al., 1999; Alpini et al., 2004; De Fabiani et al., 2004). Further, no previous study has shown a direct link between CDCA and *Leishmania*. The results of the experiments in this study indicate a potential role of CDCA in killing *Leishmania*.

Altogether, the results reveal that Vit.D<sub>3</sub>/RA and CDCA/RA suppress TACO mRNA expression to significant levels. Such expression has been reported to operate through the activity of transcription factors VDR/RXR and FXR/RXR. Consequently, TACO gene suppression with these molecules led to inhibition of *Leishmania* entry and survival, thus proving Vit.D<sub>3</sub>/RA and CDCA/RA combinations, we found

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- that Vit.D<sub>3</sub>/RA combination showed highly effective levels of parasite suppression compared with the CDCA/RA combination. These observations suggest that TACO gene downregulation may modulate the invasion of parasite phagolysosomal maturation in macrophages following phagocytosis of *L. donovani* carrying promastigotes (Figure 9). The current study provides evidence that TACO gene downregulation modulates parasite entry and survival in macrophages following the phagocytosis of *L. donovani* promastigotes.
- The conclusions derived from this study suggest that the effect of TACO gene downregulation can help elucidate the pathophysiology of host and parasite interactions, thereby unraveling the probable potential preventive and therapeutic alternatives against leishmaniasis. To the best of our knowledge, this study is the first to report the effect of TACO gene downregulation at the transcriptional level using vitamins and isoprenoids on *L. donovani* infection, which could prove to be a basis for the development of novel approaches in the control of leishmaniasis.

## Acknowledgments

This work was supported by a grant received from the Indian Council of Medical Research (ICMR), New Delhi, India in the form of Senior Research Fellowship to Dr. VG (First author). We thank Loula Burton for proofreading assistance.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Draft Genomic Analysis of an Avian Multidrug Resistant *Morganella morganii* Isolate Carrying *qnrD1*

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

**Received:** 25 May 2016

**Accepted:** 05 October 2016

**Published:** 25 October 2016

### Citation:

Jones-Dias D, Clemente L,  
Moura IB, Sampaio DA,

Albuquerque T, Vieira L, Manageiro V  
and Caniça M (2016) Draft Genomic  
Analysis of an Avian Multidrug  
Resistant *Morganella morganii* Isolate  
Carrying *qnrD1*. *Front. Microbiol.* 7:1660.  
doi: 10.3389/fmicb.2016.01660

*Morganella morganii* is a commensal bacterium and opportunistic pathogen often present in the gut of humans and animals. We report the 4.3 Mbp draft genome sequence of a *M. morganii* isolated in association with an *Escherichia coli* from broilers in Portugal that showed macroscopic lesions consistent with colisepticemia. The analysis of the genome matched the multidrug resistance phenotype and enabled the identification of several clinically important and potentially mobile acquired antibiotic resistance genes, including the plasmid-mediated quinolone resistance determinant *qnrD1*. Mobile genetic elements, prophages, and pathogenicity factors were also detected, improving our understanding toward this human and animal opportunistic pathogen.

**Keywords:** *qnrD1*, plasmid, multidrug resistance, *Morganella morganii*, WGS

## INTRODUCTION

The Gram negative *Morganella morganii* belongs to the tribe *Proteaceae* of the family *Enterobacteriaceae* (O'Hara et al., 2000). This species, along with other elements of *Proteus* and *Providencia* genera can be found in the normal flora of humans, reptiles and in the wider environment (O'Hara et al., 2000; Lee and Liu, 2006; Dipinetto et al., 2014). However, *M. morganii* isolates also constitute clinically relevant opportunistic pathogens, which can cause a variety of infections. Nosocomial outbreaks have been reported, suggesting that infections caused by *M. morganii* can lead to major clinical problems, such as wounds, urinary tract infections and septicemia (Nicolle, 2001; Tsanaktsidis et al., 2003; Falagas et al., 2006; Lee and Liu, 2006; Lin et al., 2015).

This bacterium has also been associated with infections in animals and with human animal bite wound infections, which suggests that *M. morganii* may also cause zoonotic infectious diseases (Ono et al., 2001; Choi et al., 2002; Abrahamian and Goldstein, 2011; Zhao et al., 2012; Di Ianni et al., 2015).

Several factors can affect the progression and severity of an infection. The presence of pathogenicity determinants is essential to the success of *M. morganii* in any environment,

particularly in food animal farms, where the pressure caused by antibiotic treatments and the lack of prophylactic measures to avoid the spread of infectious diseases are usually noteworthy (Chen et al., 2012; Lin et al., 2015). It is globally accepted that horizontal gene transfer plays an important role in the dissemination of antibiotic resistance genes and pathogenicity factors (Huddleston, 2014). Considering that *M. morganii* may share the habitat with other clinically relevant pathogens, the investigation of any multidrug resistant isolate recovered from poultry is an important assignment.

Resistance to quinolones and fluoroquinolones has been increasingly reported among human and veterinary isolates, very likely as a consequence of the great usage of those antibiotics (Tamang et al., 2011). The *qnrD* gene, now denominated *qnrD1* due to the report of a second variant of the gene (Abgottspoon et al., 2014), is a relatively uncommon antibiotic resistance gene, which has been described in members of the *Proteobacteria* family from different origins (Mazzariol et al., 2012; Zhang et al., 2013; Nasri Yaiche et al., 2014). This plasmid-mediated quinolone resistance (PMQR) determinant encodes a protein that protects DNA gyrases and topoisomerases from quinolone inhibition (Cavaco et al., 2009; Jacoby et al., 2014). Carriage of PMQR-encoding genes frequently confers modest increases to the minimum inhibitory concentrations (MIC) of fluoroquinolones (Poirel et al., 2012). Current studies have identified the environment, particularly animals and aquatic habitats, as a reservoir of PMQR genes (Poirel et al., 2012).

The aim of this study was to investigate the molecular background sustaining the multidrug resistance and pathogenicity of a *M. morganii* isolate. In this study, we report the antibiotic susceptibility and the draft genome sequence of a *qnrD1*-harboring avian isolate. The data gathered from bioinformatics analysis may improve our understanding toward this opportunistic pathogen.

## MATERIALS AND METHODS

### Bacterial Isolation, Antibiotic Susceptibility, and Molecular Characterization

*Morganella morganii* INSRALV892a was recovered in association with *E. coli* INSRALV892b in 2012 from a 13-days old broiler, recovered from a poultry industrial unit in Portugal. Samples consisted of organs (macerates of liver and spleen) collected during post-mortem examination that were submitted for bacteriological analysis. During post-mortem examination, the birds showed macroscopic lesions consistent with colisepticemia: aerosaculitis, acute enteritis, perihepatitis, and fibrinous peritonitis. Suspected *Enterobacteriaceae* colonies obtained in MacConkey agar plates were isolated in non selective media and identification was performed using API 20E strips (BioMérieux, Marcy-l'Étoile).

Minimum inhibitory concentrations were determined for both isolates by agar dilution method to ten antibiotics: ampicillin, cefotaxime, ceftazidime, meropenem, ciprofloxacin,

gentamicin, chloramphenicol, trimethoprim, colistin, and tigecycline. To assess non-susceptibility, interpretation of results was performed according to the clinical breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST)<sup>1</sup>.

Plasmid-mediated quinolone resistance [QnrA, QnrB, QnrC, QnrD, QnrS, QepA, OqxAB, and Aac(6')-Ib-cr]-,  $\beta$ -lactamase (TEM, SHV, OXA-G1, and CTX-M)-, and integrase (class 1, 2, and 3)-encoding genes were identified by PCR and confirmed by sequencing using DNA of both isolates, as previously described (Clemente et al., 2013).

The transference ability of specific antibiotic resistance genes from *M. morganii* INSRALV892a and *E. coli* INSRALV892b was assessed by broth mating out assays using *E. coli* J53 NaN3<sup>R</sup> as recipient strain, as described elsewhere (Jones-Dias et al., 2016). Resistant *E. coli* J53 transconjugants were then selected on MacConkey agar plates containing amoxicillin (100 mg/l) or ciprofloxacin (0.06 mg/l) together with sodium azide (200 mg/l), according with the antibiotic susceptibility profile of the donor isolates. To confirm the acquisition of the antibiotic resistance genes, we detected and identified the determinants in the transconjugants, following the methodology described above in this section.

### Genome Sequencing and Analysis

Genomic DNA of *M. morganii* INSRALV892a was extracted using DNeasy Blood and Tissue Kit (Qiagen, Aarhus), and DNA quantification was performed by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's instructions. Libraries were prepared from 1 ng of genomic DNA using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA), also following manufacturer's instructions. Whole Genome Sequencing (WGS) was performed using 150 bp paired-end reads on a MiSeq (Illumina, San Diego, CA, USA).

Sequence reads were then trimmed and filtered according to quality criteria. Briefly, reads were assembled *de novo* using CLC genomics workbench version 8.5 (Qiagen, Aarhus), which is based on Smith and Waterman algorithm. The raw FASTQ reads were first processed by quality score trimming (quality score limit = 0.05), removing all reads containing more than two ambiguous nucleotides or shorter than 50 bp. Trimmed reads were then *de novo* assembled with automatic bubble, word size and paired distance detection, using mapping mode "map reads back to contigs" (including scaffolding, and minimum contig length of 400 nucleotides). The NCBI prokaryotic genome automatic annotation pipeline (PGAAP) was used for annotation<sup>2</sup>. All *de novo* contigs were BLAST searched against the GenBank's non-redundant nucleotide collection (nr/nt). PathogenFinder 1.1, ResFinder 2.1, and PlasmidFinder 1.3 were used to estimate the number and type of pathogenicity determinants, antibiotic resistance genes and plasmids, respectively, within the genome (Zankari et al., 2012; Cosentino et al., 2013; Carattoli et al., 2014). PHAST search web

<sup>1</sup><http://www.eucast.org>

<sup>2</sup>[http://www.ncbi.nlm.nih.gov/genome/annotation\\_prok/](http://www.ncbi.nlm.nih.gov/genome/annotation_prok/)

tool was used to identify and annotate any prophage sequence present in the draft genome (Zhou et al., 2011). ISSaga semi-automatic annotation system was also applied to detect the presence of insertion sequences (IS) (Varani et al., 2011).

Contigs containing antibiotic resistance genes were searched for identity through blastn<sup>3</sup> against the nr/nt NCBI database, and further mapped against the closest bacterial plasmids or genomes using CLC Genomics Workbench version 8.5.

## Nucleotide Sequence GenBank Accession Numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LGYC000000000. The version described in this paper is the LGYC010000000<sup>4</sup>.

## RESULTS AND DISCUSSION

*Morganella morganii* INSRALV892a was found to be non-susceptible to ampicillin (>64 mg/L), cefotaxime (>4 mg/L), ceftazidime (2 mg/L), ciprofloxacin (>8 mg/L), chloramphenicol (16 mg/L), gentamicin (>32 mg/L), trimethoprim (>32 mg/L), colistin (>16 mg/L), and tigecycline (0.5 mg/L). However, it is important to highlight that *M. morganii* is intrinsically resistant to colistin, while tigecycline has also been shown to have poor activity against this species<sup>5</sup>. Among the antibiotics tested, the isolate was susceptible only to meropenem (0.125 mg/L). The *E. coli* INSRALV892b was also characterized with regard to antibiotic susceptibility and found to be non-susceptible to ampicillin (>64 mg/L), cefotaxime (>4 mg/L), ceftazidime (2 mg/L) and trimethoprim (>32 mg/L), and susceptible to meropenem ( $\leq$ 0.03 mg/L), ciprofloxacin (0.125 mg/L), chloramphenicol ( $\leq$ 8 mg/L), gentamicin ( $\leq$ 0.5 mg/L), colistin ( $\leq$ 1 mg/L), and tigecycline (0.5 mg/L). The molecular characterization of the isolates showed the presence of *qnrD1* and a class 2 integron in *M. morganii* INSRALV892a, and *bla<sub>CTX-M-1</sub>* gene flanked by an *ISEcp1* and *orf477*, as well as a class 1 integron in *E. coli* INSRALV892b. Conjugation experiments only revealed the transference of *bla<sub>CTX-M-1</sub>* from *E. coli* INSRALV892b to isogenic *E. coli* J53 strain.

The WGS assembly of *M. morganii* INSRALV892a yielded 74 contigs (each >200 bp long and >100-fold coverage), which together comprised 4,267,817 bp, showing a GC content of 50.6%. The largest contig was 523,676 bp long and the N50 statistic, which stands for the minimum contig length of at least 50% of the contigs, was 342,352 bp. The average length of the obtained contigs was 34,190 bp. Among the obtained data, six contigs, ranging from 802 to 8,575 in length and showing a minimum coverage of 117.7-fold, matched plasmid sequences of different species. Overall, the genome sequence comprised 4,116 putative genes, among which 3,950 consisted of protein encoding sequences.

*In silico* analysis of the antibiotic resistance genes (90% identity and 40% minimum length) revealed the presence of loci for acquired resistance to aminoglycosides [*aadA1y*, *aph(3')-Ic*, and *strA-strB*],  $\beta$ -lactam (*bla<sub>OXA-1</sub>*), fluoroquinolones (*qnrD1*, *aac(6')-Ib-cr*), phenicols (*cata2* and *catB3*), rifampicin (*Δarr*), sulphonamides (*sul2*), trimethoprim (*dfrA1*), tetracycline (*tetY*), and streptothricin (*sat2*). Non-susceptibility to third generation cephalosporins such as cefotaxime and ceftazidime was not associated to any extended-spectrum  $\beta$ -lactamase, suggesting the involvement of inducible or stably derepressed *M. morganii* chromosomal *ampC* gene, the *blaDHA-type* gene (Harris and Ferguson, 2012).

The *dfrA1*, *catB2*, *sat2*, and *aadA1y* genes were enclosed in an In2-17 class 2 integron that has already been described, for instance, in *Proteus vulgaris* isolates from China (HQ386830) (Figure 1A). Genes encoding resistance to tetracycline (*tetY*) and streptomycin (*strA-strB*) were detected in association with each other, and with proteins linked to DNA transfer processes, such as *ISAb14* and an incomplete Tn5393 (Figure 1B). The sulphonamide resistance gene *sul2* was flanked upstream by a *glmM*-containing region and *ISCR2*, while the downstream region consisted of a chromosomal region typical of *M. morganii* (Figure 1C); the *glmM* gene (formerly called *ureC*) encodes a phosphoglucosamine mutase that is considered a housekeeping gene essential for the cell wall synthesis (Tavares et al., 2003). The aminoglycoside resistance gene *aph(3')-Ic* was associated to an IS26 IR and a unknown *orf* (Figure 1D), and the genes *Δarr*, *catB3*, *bla<sub>OXA-1</sub>*, and *aac(6')-Ib-cr* were enclosed together, as gene cassettes of an integron variable region that has been previously found, for instance, in *S. enterica* from livestock (Figure 1E). However, in the latter, the array was flanked up and downstream by truncated inverted repeats of IS26 while no conserved integron regions were found. The genetic regions where antibiotic resistance genes were incorporated were highly similar to other plasmid-borne structures, previously described in different Gram-negative bacteria, suggesting acquisition of resistance determinants through horizontal gene transfer (Figure 1).

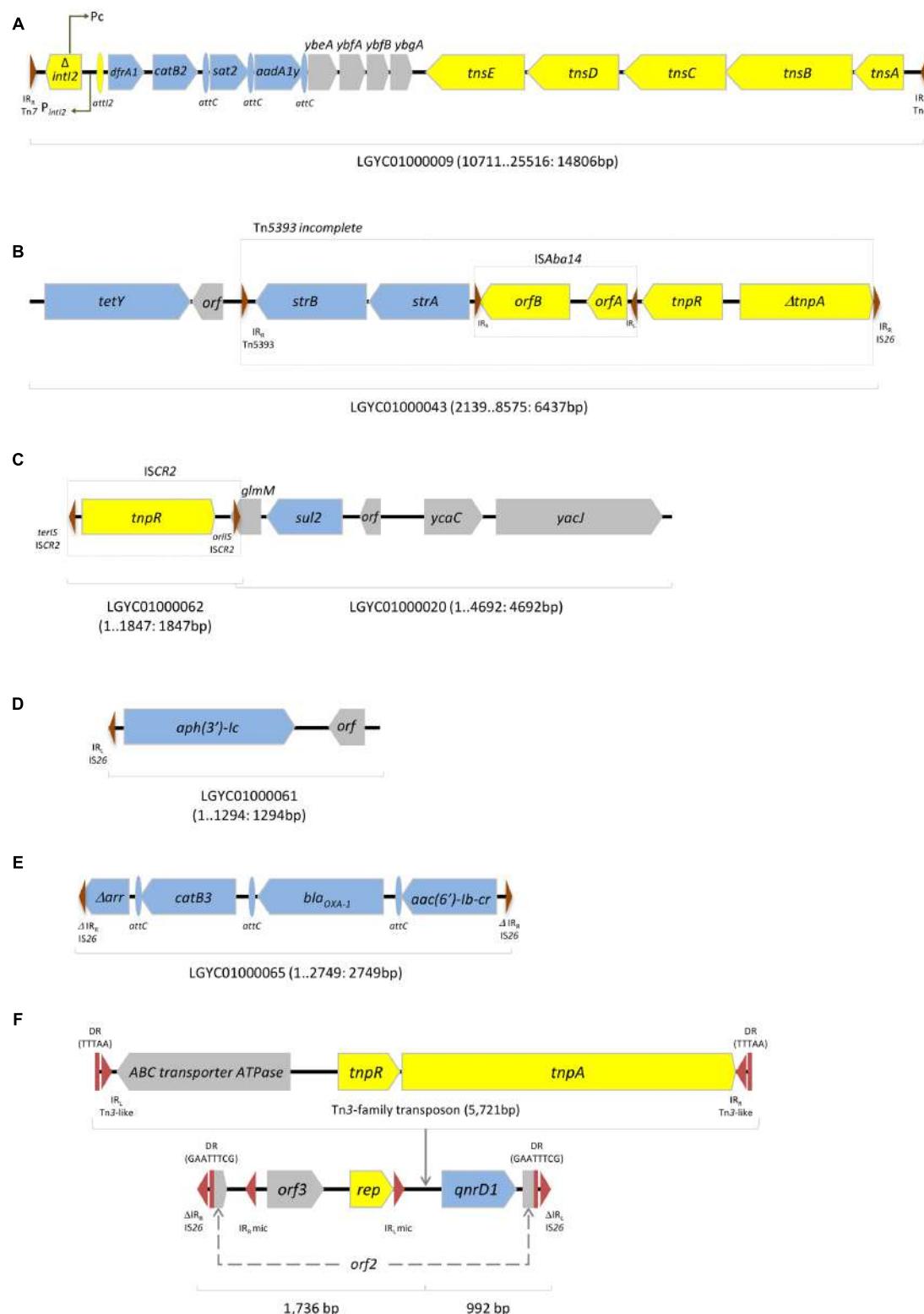
The *qnrD1* gene was enclosed in an 8,449 bp length contig (LGYC01000051: mean coverage of 183.9-fold and a total read count of 13,382), matching a Col3M plasmid. Indeed, the *qnrD1* gene is frequently located on small non-conjugative plasmids harbored by *Proteaceae*, which was corroborated by our conjugation assay (Zhang et al., 2013). Furthermore, the *qnrD1* gene has been located in plasmids showing similarities with a specific *Providencia vermicola* plasmid, suggesting that these small non-conjugative plasmids might be the product of recombination between an unknown *qnrD*-bearing region and a native plasmid from *Proteaceae*.

This contig accommodated a 2,683 bp sequence showing 99% identity with previously described *qnrD1*-harboring plasmids, such as pGHS09-09a (HQ834473) and pCGS49 (JQ776507), reported in France and China, respectively. The three *qnrD1*-encoding sequences shared a *rep* gene (also reported as *orf4*) and two additional *orfs* (Figure 1F) (Guillard et al., 2012; Zhang et al., 2013). Six single nucleotide variants (SNVs) were detected between *qnrD1*-INSRALV892a and the pGHS09-09a plasmid,

<sup>3</sup><http://blast.ncbi.nlm.nih.gov/Blast.cgi>

<sup>4</sup><http://www.ncbi.nlm.nih.gov/nuccore/LGYC00000000.1>

<sup>5</sup><http://www.eucast.org>



**FIGURE 1 | Examples of contigs containing antibiotic resistance genes in *Morganella morganii* INSRALV892a. (A)** In2-17 class 2 integron encoding *dfrA1*, *catB2*, *sat2*, and *aadA1y*; **(B)** *tetY* and *strA-strB* associated to transposable elements; **(C)** *sul2* flanked upstream by *ISCR2*; **(D)** *aph(3')-lc* associated to an *IS26* IR; **(E)**  $\Delta\text{arr}$ , *catB3*, *bla\_{OXA-1}*, and *aac(6')-lb-cr* are bracketed by truncated *IS26* IRs. **(F)** *qnrD1*-containing region. Blue, antibiotic resistance genes; Yellow, mobile genetic elements; Gray, other genes. Right and left inverted repeats ( $\text{IR}_R$  and  $\text{IR}_L$ ) and direct repeats (DR) are indicated as red triangles and squares, respectively; mic, mobile insertion cassette.

two within the *rep* gene. Only one SNP was found with relation to pCGS49 in a non-coding region. The *qnrD1* gene was located within a mobile insertion cassette (mic) element bracketed by two inverted repeats, as previously described (Guillard et al., 2014) (**Figure 1F**).

Comparative bioinformatics analyses revealed the disruption of *orf2* caused by the insertion of IS26 left and right inverted repeats flanking a region containing *orf3* and *rep*, within a mic, followed by *qnrD1*. In fact, this shows that it is possible that LGYC01000051 contig could be either a Col3M plasmid missing an IS26-flanked region or a *qnrD1*-containing region that has become incorporated into a larger plasmid.

In addition, this region (**Figure 1F**) included three additional open reading frames: besides an ABC transporter-encoding gene perfectly matching a protein from *Aeromonas hydrophila*, this region harbored Tn3-like resolvases- and transposase-encoding genes, displaying *E. coli* plasmid pH226B (KX129784) as its best blast hit.

Mobile genetic elements are crucial tools for the acquisition of genetic diversity (Huddleston, 2014). Thus, we decided to search for and characterize the elements detected in the *M. morganii*'s genome. We identified 10 prophage regions, among which six were incomplete and four were intact, comprising 381 prophage-related genes. Intact prophage regions presented between 24.2 and 41.7 Kb and harbored 13–56 coding DNA sequences. The intact phages showing highest scores were assigned to Enterobacteria phage SfV, which is associated with O-antigen modification and serotype conversion in *Shigella flexneri*, and Enterobacteria phage mEp235 that consists of an unclassified Lambda-like virus (Sun et al., 2013). The bioinformatics detection of IS resulted in the identification of seven transposable elements: IS3, Tn3, ISL3, IS256, IS6, IS91, and ISAs1. Besides the already mentioned Col3M no other typable plasmids were detected within the *M. morganii* genome, according with the PlasmidFinder tool.

Based on the probability scores assigned by PathogenFinder web-server (Cosentino et al., 2013), the isolate has a probability of acting as a human pathogen of 68.9%, which is in line with the opportunistic nature of this species. *M. morganii*'s genome matched 22 pathogenic families and 5 non-pathogenic families. Pathogenic factors showed diversity of functions and hosts, and included, for instance, transposase *insA* from IS91 of *Salmonella enterica*, transposition protein *tnsE* of the Tn7 transposon of *S. flexneri*, and transcriptional regulator LysR family protein from *S. enterica*.

Multidrug resistant *M. morganii* isolates are rare and normally associated with non invasive nosocomial opportunistic infections in humans (Nicolle, 2001; Falagas et al., 2006). The detection

of an avian *M. morganii* isolate harboring multiple and mobile antibiotic resistance genes and pathogenicity factors raises concerns regarding the dissemination of infection in birds and potential risk of zoonotic transmission. Several factors may affect the susceptibility of poultry to bacterial diseases, namely environmental stressors and previous antibiotic treatments, which are crucial to the development of infections involving different *Enterobacteriaceae* (Burkholder et al., 2008). The detection of an avian *M. morganii* isolate harboring multiple and mobile antibiotic resistance genes and pathogenicity factors raises concerns regarding the dissemination of infection in birds and potential risk of zoonotic transmission.

*M. morganii* is a well characterized opportunistic pathogen (Lee and Liu, 2006). However, its detection in poultry flocks, co-habiting the same hosts as other clinically important pathogens, makes it susceptible to the acquisition and donation of pathogenicity factors by horizontal gene transfer (Huddleston, 2014). To the best of our knowledge this report represents the first genome analysis of an isolate from animal origin carrying *qnrD1*. This genome sequence represents a valuable resource for studies on the epidemiology of zoonotic *M. morganii* isolates, and its features may be used as markers for the study of antibiotic resistance.

## AUTHOR CONTRIBUTIONS

DJ-D designed the study, performed molecular experiments, analyzed the data and wrote the manuscript. LC performed the microbiological experiments and reviewed the manuscript. IM analyzed the data and reviewed the manuscript. TA, acquired laboratory data. DS and LV performed genome sequencing experiments. VM designed the study, analyzed the data and reviewed the manuscript. MC designed the study, reviewed and edited the manuscript. All authors read and approved the final manuscript.

## FUNDING

DJ-D has received research funding from Fundação para a Ciência e a Tecnologia (FCT, grant number SFRH/BD/80001/2011). VM was supported by FCT fellowship (grant SFRH/BPD/77486/2011), financed by the European Social Funds (COMPETE-FEDER) and national funds of the Portuguese Ministry of Education and Science (POPH-QREN). We thank the support of FCT grant number PEst-OE/AGR/UI0211/2011-2014 and UID/MULTI/00211/2013.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Effect of Photodynamic Therapy on the Virulence Factors of *Staphylococcus aureus*

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 19 November 2015

Accepted: 18 February 2016

Published: 07 March 2016

### Citation:

Bartolomeu M, Rocha S, Cunha Â, Neves MGPMS, Faustino MAF and Almeida A (2016) Effect of Photodynamic Therapy on the Virulence Factors of *Staphylococcus aureus*. *Front. Microbiol.* 7:267.

doi: 10.3389/fmicb.2016.00267

*Staphylococcus aureus* is a Gram-positive bacterium that is present in the human microbiota. Nevertheless, these bacteria can be pathogenic to the humans. Due to the increasing occurrence of antibiotic-resistant *S. aureus* strains, new approaches to control this pathogen are necessary. The antimicrobial photodynamic inactivation (PDI) process is based in the combined use of light, oxygen, and an intermediary agent (a photosensitizer). These three components interact to generate cytotoxic reactive oxygen species that irreversibly damage vital constituents of the microbial cells and ultimately lead to cell death. Although PDI is being shown to be a promising alternative to the antibiotic approach for the inactivation of pathogenic microorganisms, information on effects of photosensitization on particular virulence factors is strikingly scarce. The objective of this work was to evaluate the effect of PDI on virulence factors of *S. aureus* and to assess the potential development of resistance of this bacterium as well as the recovery of the expression of the virulence factors after successive PDI cycles. For this, the photosensitizer 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py<sup>+</sup>-Me) and six strains of *S. aureus* [one reference strain, one strain with one enterotoxin, two strains with three enterotoxins and two methicillin resistant strains (MRSA) – one with five enterotoxins and the other without enterotoxins] were used. The effect of photosensitization on catalase activity, beta hemolysis, lipases, thermonuclease, enterotoxins, coagulase production, and resistance/susceptibility to methicillin was tested. To assess the development of resistance after successive cycles of treatment, three strains of *S. aureus* (ATCC 6538, 2065 MA, and SA 3 MRSA) were used. The surviving colonies of a first cycle of PDI were collected from the solid medium and subjected to further nine consecutive cycles of PDI. The results indicate that the expression of some external virulence factors is affected by PDI and enterotoxin producing strains were more susceptible to PDI than non-toxigenic strains. The surviving bacteria did not develop resistance. PDI, contrarily to traditional antibiotics, inhibited the expression of virulence factors, efficiently inactivating either highly virulent strains and low virulent *S. aureus* strains, inactivating also antibiotic susceptible and resistant strains, without development of photoresistance after at least 10 consecutive cycles of treatment, and so this therapy may become a strong promising alternative to antibiotics to control pathogenic microorganisms.

**Keywords:** *Staphylococcus aureus*, photodynamic inactivation (PDI), virulence factors, coagulase, enterotoxins, antibiotic/methicillin resistance

## INTRODUCTION

*Staphylococcus aureus* is a Gram-positive bacterium that resides on the surface of the skin and on mucous membranes of warm-blooded animals (Morikawa et al., 2001; Costa A.R. et al., 2013) as a commensal microorganism, asymptotically colonizing the host (Bronner et al., 2004). Nevertheless, due to its invasiveness and taking advantage of host immune weaknesses, *S. aureus* is able to cause a wide spectrum of infections affecting different organs (Bronner et al., 2004; Baptista et al., 2015), from infections of superficial lesions to intoxications and life threatening systemic conditions (Bien et al., 2011). This opportunistic bacterium is a major human pathogen not only associated with community-acquired bacteremia but also nosocomial bacteremia (Morikawa et al., 2001; Cheung et al., 2004; Bien et al., 2011), being readily able to acquire antibiotic resistance (Morikawa et al., 2001). Its ability to survive under stressful conditions, such as those imposed by host immune system, is due to the activation of stress response mechanisms (Morikawa et al., 2001; Bronner et al., 2004; Cheung et al., 2004). These mechanisms involve the action of an interactive regulatory network that includes the accessory gene regulator (*agr*) and staphylococcal accessory element (*sae*) (Bronner et al., 2004; Novick and Geisinger, 2008; Costa A.R. et al., 2013). These two components of the regulatory system regulate the expression of several exoproteins and cell wall-associated proteins related to virulence (Costa A.R. et al., 2013). The regulatory network also includes the staphylococcal accessory regulator A (*sarA*) and its homologs that regulate the expression of some virulence factors; the sigma factors ( $\sigma$ ), as the primary sigma factor,  $\sigma^A$ , that may function in living process through the housekeeping genes expression and the alternative sigma factor  $\sigma^B$ , which may participate on the bacterial response to stress conditions by regulating the expression of several genes involved on stress response (Morikawa et al., 2001; Cheung et al., 2004; Costa A.R. et al., 2013).

The *S. aureus* pathogenicity involves a wide array of cell wall and extracellular components orderly expressed during the different stages of infection: colonization, avoidance, or invasion of the host immune defense, growth, and cellular division culminating in bacterial dissemination, causing toxic effects to the host (Cheung et al., 2004; Bien et al., 2011; Costa A.R. et al., 2013; Ebrahimi et al., 2014). Some of the cell wall components are responsible for the recognition of adhesive matrix molecules, such as the clumping factor proteins (Clf) that mediates the adherence to fibrinogen (Costa A.R. et al., 2013) and the carotenoid pigment staphyloxanthin that acts as virulence factor once it is able to perform an antioxidant action against oxidant-based reactions (Clauditz et al., 2006; Liu and Nizet, 2009; Costa A.R. et al., 2013). The extracellular components include the superantigen molecules such as the staphylococcal enterotoxins (SE), a family of a single chain proteins with small molecular-weight (24–30 kDa; Johnson et al., 1991; Baptista et al., 2015); the cytolytic  $\beta$ -hemolysin, the clotting factor coagulase, besides more exoenzymes as lipases and nucleases, in which their main function is to disrupt the host cells/tissue and the inactivation of host immune mechanisms of defense (Costa A.R. et al., 2013).

Additionally to the virulence factors already described, *S. aureus* has a notorious capacity to acquire antibiotic resistance (Guillemot, 1999; Morikawa et al., 2001; Ito et al., 2003; Chambers and DeLeo, 2009; Costa A.R. et al., 2013; Theuretzbacher, 2013), by a bacterial gene mutation and horizontal transfer of resistance genes from external sources (Ito et al., 2003; Chambers and DeLeo, 2009). The resistance to penicillin emerged in the mid-1940s, only a few years after the introduction of this antibiotic in the clinical practice (Chambers and DeLeo, 2009; Costa A.R. et al., 2013). Later, in 1959, the semi-synthetic antibiotic methicillin was introduced for the treatment of infections caused by penicillin-resistant *S. aureus* (Enright et al., 2002; Costa A.R. et al., 2013). Yet, in 1961 the first cases of methicillin-resistant *S. aureus* (MRSA) isolates (Chambers and DeLeo, 2009; Costa A.R. et al., 2013) were reported and currently, only few compounds are still effective in the treatment of MRSA infections (Chambers and DeLeo, 2009; Theuretzbacher, 2011).

With the knowledge that the development of new classic antibiotics is not likely to solve the resistance drug problem for too long (Chambers and DeLeo, 2009), non-traditional antimicrobial approaches to treat MRSA infections will be needed. Ideally, the new antimicrobial methods should be non-invasive and non-toxic to the hosts, but efficient and with fast action, avoiding the development of resistance (Calin and Parasca, 2009; Kossakowska et al., 2013; Alves et al., 2014; Almeida et al., 2015). In this context, the photodynamic inactivation (PDI) arises as a photo chemotherapeutic approach with forthcoming applications as antimicrobial therapy (Almeida et al., 2009, 2015; Carvalho et al., 2009; Costa et al., 2012; Alves et al., 2013, 2014, 2015b; Melo et al., 2013). This technology has already proved to be effective against Gram-positive and Gram-negative bacteria, viruses, fungi, and parasites (Almeida et al., 2011, 2015; Costa et al., 2012). The photodynamic effect is based on the use of visible light and an agent (photosensitizer, PS) capable to absorb energy from light and transfer it to molecular oxygen, originating highly cytotoxic species, namely reactive oxygen species (ROS) as singlet oxygen ( ${}^1\text{O}_2$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), peroxide anion radical ( $\text{O}_2^{\bullet-}$ ), and hydroxyl radical ( $\text{OH}^{\bullet}$ ; Alves et al., 2008, 2013, 2014; Calin and Parasca, 2009; Melo et al., 2013). These reactive cytotoxic species can cause irreversible damages to molecular cell constituents or even its destruction (Alves et al., 2014). Initially, the PS adheres to the microbial external structures and later, during the irradiation process, the PDI is initiated and cellular components, such as proteins and lipids will be exposed to oxidizing reactions which will alter their structure and, subsequently, affect the biological function in which they are involved (Alves et al., 2014).

As PDI acts via ROS, a high number of microbial targets are simultaneously affected, thus preventing the development of resistance (Costa et al., 2008; Tavares et al., 2010), and allowing the inactivation of a broad-spectrum of microorganisms, independently of their resistance profiles to classic antimicrobials (Tavares et al., 2010; Arrojado et al., 2011; Costa et al., 2011; Almeida et al., 2014). In addition, PDI affects the expression of virulence factors, also causing their degradation (Kömerik et al., 2000; Tubby et al., 2009; Kossakowska et al., 2013). The effects of PDI on virulence factors is of extreme importance as they may be

present during the infection process, when the microorganism is present, but they can also be present when the microorganism is not present already, such as in the case of intoxications, causing severe damage to the host.

Clinical trials using light based therapies to evaluate the potential use of photodynamic therapy in the clinical field have been conducted along the last years, with positive results, including studies against viral infections, such as the ones caused by human papilloma virus, with systemic and topical applications (Buji et al., 1993; Shikowitz et al., 1998) and to treat herpes simplex lesions, with topical applications (Moore et al., 1972). Non-viral infections by *Propionibacterium acnes*, a natural porphyrin producer, were already efficiently inactivated (Kawada et al., 2002; Elman et al., 2003), among others, as *acne vulgaris* (Ormond and Freeman, 2013). Nowadays the miniaturization of the light devices such as low-power lasers, light emitting diodes, or conventional lamps able to activate the antimicrobial molecules place this technology closer to be used in clinical application. Superficial skin infections like wounds and burns can be easily treated (Ebrahimi et al., 2014). Using fiber-optic technology, most regions of the anatomy are also accessible. *Loci* of infection could be managed endoscopically, allowing local application both of the PS agent and light (Dai et al., 2010; Kharkwal et al., 2011). Even for deep-seated infections a transcutaneous needle could deliver both drug and light via fibers.

Some *in vitro* studies showed that the biological activity of lipopolysaccharides from *Escherichia coli* and proteases from *Pseudomonas aeruginosa* were successfully reduced by toluidine blue (TBO)-mediated PDI (Tubby et al., 2009). Additionally, light-activated methylene blue (MB) showed to inhibit the expression of staphylococcal V8 protease, alpha-hemolysin and sphingomyelinase (Tubby et al., 2009). However, the information about the effects of PDI on virulence factor is still scarce. The objectives of this work were the evaluation of the effect of PDI on some virulence factors of *S. aureus* – catalase activity, beta hemolysis, lipases, thermonuclease, enterotoxins, coagulase – and the assessment of development of resistance to PDI treatment. For that, a cationic porphyrin 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py<sup>+</sup>-Me) was used as PS against six *S. aureus* strains – ATCC 6538, 2153 MA, 2065 MA, 2095 M1A1, DSM 25693 MRSA, and SA 3 MRSA.

## MATERIALS AND METHODS

### Experimental Design

An experimental procedure was established in order to study the effects of PDI on some virulence factors expression/activity of *S. aureus* strains and to test the potential development of resistance to PDI by *S. aureus* strains after successive photodynamic cycles of treatment, testing also the recovery of the expression/activity of the virulence factors after the successive photodynamic cycles of treatment. Six different strains of *S. aureus*, including methicillin resistant and susceptible strains, as well as, enterotoxin and non-enterotoxin producing

strains were tested. The effect of PDI on the expression/activity of virulence factors was tested in all strains. The potential development of resistance to PDI and the recovery of the expression/activity of the virulence factors after the successive cycles of treatment were tested only in three of the strains. For each of these three strains, a total of ten cycles of treatments were performed.

### Characterization of Bacterial Strains and Culture Conditions

Six strains of *S. aureus* were used in this study: ATCC 6538, a non-enterotoxic strain; 2153 MA, the only strain used that does not ferment mannitol (Baptista et al., 2015), producing SE A; 2065 MA, with SE A, G, I, and *S. aureus* 2095 M1A1 with SE C, G and I – the three strains isolated from food products and characterized in the Centre of Biotechnology and Fine Chemistry of the Faculty of Biotechnology of the Catholic University, Porto, Portugal; *S. aureus* DSM 25693, a methicillin-resistant (MRSA) strain, positive for SE A, C, H, G, and I; and a staphylococcal strain isolated from a biological sample from the lower respiratory tract of an hospitalized individual, a non-enterotoxic MRSA strain (SA 3 MRSA; Gonçalves et al., 2014).

All the strains were grown in Brain-Heart Infusion (BHI, Liofilchem, Italy) at 37°C for 18 h at 170 rpm, in order to reach the stationary phase, corresponding approximately to a concentration of 10<sup>8</sup>–10<sup>9</sup> colony forming units per mL (CFU mL<sup>-1</sup>). Before each PDI assay, a colony of *S. aureus* was transferred to 30 mL of BHI and incubated as previously described. Subsequently, an aliquot was transferred to fresh medium, and grown in the same conditions. This procedure was repeated twice.

### Photosensitizer

The PS 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py<sup>+</sup>-Me) used in this study was prepared according to the literature (Carvalho et al., 2010). Their <sup>1</sup>H NMR and UV-vis spectra were consistent with the literature data. Their purity was confirmed by thin layer chromatography and <sup>1</sup>H NMR. <sup>1</sup>H NMR (DMSO-d<sub>6</sub>): -3.12 (s, 2H, NH), 4.73 (s, 12H, CH<sub>3</sub>), 9.00 (d, J = 6.5 Hz, 8H, Py-o-H), 9.22 (s, 8H, β-H), 9.49 (d, J = 6.5 Hz, 8H, Py-m-H). UV-vis (DMSO)  $\lambda_{\text{max}}$  (log ε): 425 (5.43), 516 (4.29), 549 (3.77), 588 (3.84), 642 (3.30) nm. The stock solutions (500 μM) of this porphyrin were prepared using the polar aprotic solvent dimethyl sulfoxide (DMSO).

### Antimicrobial Photodynamic Therapy (PDI) Treatments

Bacterial cultures in stationary phase were 10-fold diluted in phosphate buffered saline (PBS) and this bacterial suspension was distributed in sterilized glass beakers. The appropriate quantity of the PS Tetra-Py<sup>+</sup>-Me was added to achieve a final concentration of 5.0 μM. The total volume of final solution was 10 mL per beaker. During the experiments, light and dark controls were also performed: in the light control the beaker without Tetra-Py<sup>+</sup>-Me was exposed to light; in the dark control the beaker containing 5.0 μM Tetra-Py<sup>+</sup>-Me was protected from light with

aluminum foil during the experiment. During the pre-irradiation period, the samples were incubated for 10 min with stirring, at room temperature, in order to promote the binding of the porphyrin to *S. aureus* cells. The samples were exposed to an artificial white light (PAR radiation, 13 OSRAM 21 lamps of 18 W each, 380–700 nm) with an irradiance of  $40 \text{ W m}^{-2}$  for 60 min, under stirring. During the experiment, aliquots of treated and control samples were collected at times 0, 5, 10, 15, 30, and 60 min.

## Enumeration of Viable Cells

From each treated and control samples 10-fold serial dilutions were prepared in sterile PBS ( $10^{-1}$  to  $10^{-6}$ ). Aliquots of  $100 \mu\text{L}$  were pour-plated, in duplicate, in Plate Count Agar medium (PCA, Liofilchem, Italy). The plates were incubated at  $37^\circ\text{C}$  for 48 h and the number of colonies was counted. Three independent assays were performed.

## PDI Resistance Assays

In order to verify the development of resistance to PDI treatment with Tetra-Py<sup>+</sup>-Me, ten cycles of PDI were performed. After each cycle of a total irradiation time of 60 min, a new set of bacterial cultures were prepared from an isolated colony, surviving to the previous cycle of PDI (at  $37^\circ\text{C}$ , 18 h, 170 rpm). The PDI treatment was repeated under similar conditions. Three independent assays were performed.

## Virulence Factors, Mannitol Fermentation, and Methicillin Susceptibility

To assess if PDI treatments affected the virulence factors of *S. aureus*, treated and controls samples were tested for the presence or activity of virulence factors, according to literature (Baptista et al., 2015). One typical colony of *S. aureus* was selected from each strain. The  $\beta$ -hemolysin activity was detected by streaking Blood Agar Plates (Sheep Blood 7%; BAP, Liofilchem, Italy) and observing the development of a clear/yellow zone surrounding *S. aureus* colonies. Lipase and lecithinase activities were assessed by streaking Baird Park Agar (BPA, Liofilchem, Italy) in which *S. aureus* colonies appear in black, with an opaque precipitation zone (lipase activity) and a clear zone surrounding it (lecithinase activity). The mannitol fermentation was evaluated using Mannitol Salt Agar (MSA, Liofilchem, Italy), being the positive results detected by a change of color of the medium from pink to yellow. Catalase activity was assessed using Catalase/Oxy Test (Liofilchem, Italy), interpreting the formation of gas bubbles as a positive result. The activity of bound coagulase (clumping factor) was determined using Pastorex Staph Plus (Bio-Rad, USA) and the activity of free coagulase was detected using BBL Coagulase Plasma Rabbit (BD, USA). For this, 1.0 mL of supernatant of treated and non-treated samples was collected by centrifugation at  $13,000 \times g$  for 10 min, at  $4^\circ\text{C}$ , and 0.5 mL of BBL Coagulase Plasma reagent was added and the mixture was incubated at  $37^\circ\text{C}$  for 24 h. The results were considered positive when the agglutination occurred. The rate of clot formation was evaluated according to the manufacturer's instruction. Thermanuclease activity was

determined by D.N.A. Toluidine Blue Agar (Bio-Rad, USA) and positive results were detected as a change of color of the halos from blue to pink. The presence of SE was determined using SET-RPLA Kit Toxin Detection Kit (Thermo Scientific, UK), a kit based on reversed passive latex agglutination technique, according to the manufacturer's instruction. Treated and non-treated samples were centrifuged at  $900 \times g$  for 20 min at  $4^\circ\text{C}$ . The presence of enterotoxins H, G, and I was not tested since SET-RPLA Kit Toxin Detection Kit only covers SE A, B, C, and D, which are the most common enterotoxins of *S. aureus* (Baptista et al., 2015). The susceptibility to methicillin was determined using the cefoxitin disk screen test, accordingly to the Clinical and Laboratory Standards Institute [CLSI] (2013). Cultures with halos  $\geq 22$  mm were considered methicillin susceptible and cultures with halos  $\leq 21$  were classified as methicillin resistant (Clinical and Laboratory Standards Institute [CLSI], 2013). Carotenoid pigments (staphyloxanthin) were determined using a protocol adapted from Morikawa et al. (2001). Each strain was cultured in BHI medium at  $37^\circ\text{C}$  for 72 h. Twenty milliliters of the culture were harvested by centrifugation ( $10,000 \times g$ , 10 min) and washed with purified water. The cells were suspended in 5.0 mL of methanol and heated in a bath at  $55^\circ\text{C}$  for 15 min, until visible pigments have been extracted. Cellular debris was removed by centrifugation at  $15,000 \times g$  for 10 min. The absorbance at 465 nm of the methanol extracts were measured in a quartz cuvette in a spectrophotometer (Dynamica Halo DB-20, UK).

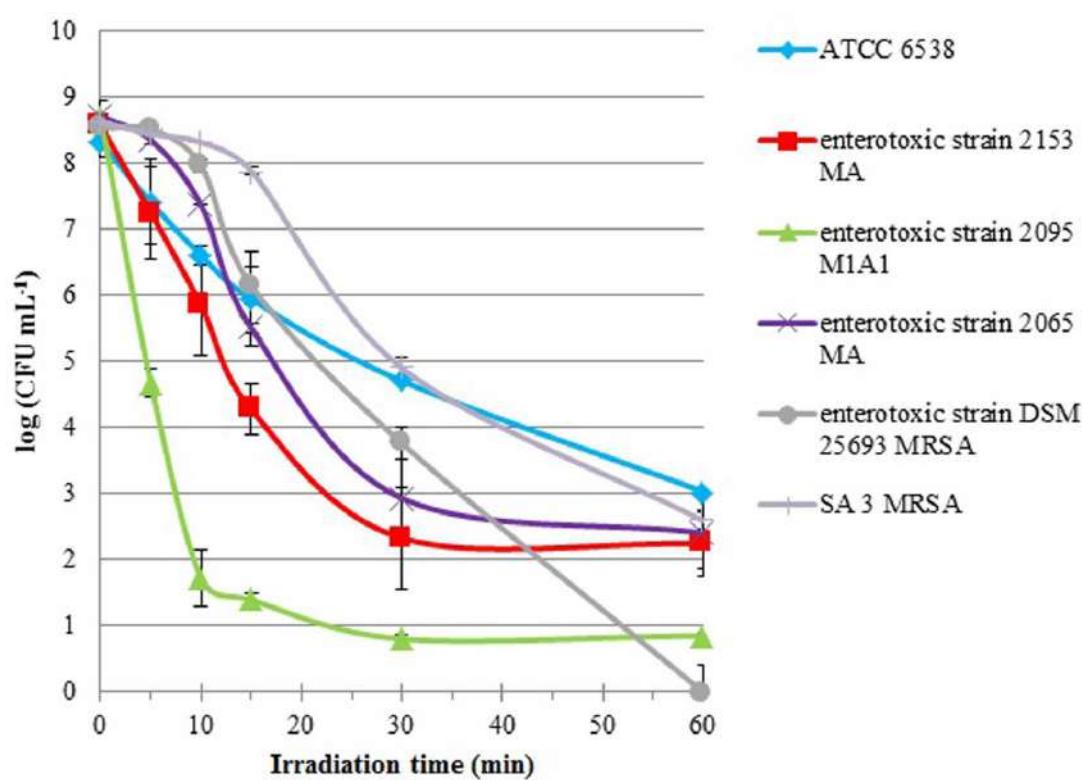
The  $\beta$ -hemolysis, lipase and lecithinase, catalase, bound coagulase and thermonuclease activities, mannitol fermentation and methicillin susceptibility were tested in the PDI surviving cells, after plating and incubation at  $37^\circ\text{C}$ . Methicillin susceptibility was inferred from the diameter of the inhibition zone around cefoxitin disks, and the results represent the average of the inhibition zones from three independent tests. Free coagulase and SE A and C activities were assessed in the supernatant of treated samples and controls after PDI assays.

The effect of PDI on the isolated toxins was also individually assessed. Purified SE A and C (available on SET-RPLA Kit Toxin Detection Kit as SE A and C controls) were subjected to the PDI treatment. The concentration of the PS was the same used before:  $5.0 \mu\text{M}$ ; the amount of SE used was  $63 \mu\text{L}$  from the reconstituted control reagents (the kit control solution contains 25 ng of purified enterotoxin reconstituted in 0.5 mL of diluent from the kit as showed in Figure in section "Quantity of Enterotoxins (ng) Per Kit" of the Supplementary Material), and PBS was added to make up a total volume of 2.0 mL of solution, which was irradiated. Light and dark controls were included. At times 0, 5, 10, 15, 30, and 60 min aliquots of  $25 \mu\text{L}$  were collected and the activity of the SE was tested using the SET-RPLA Kit Toxin Detection Kit.

## RESULTS

### Bacterial Inactivation by PDI

Cells suspensions of *S. aureus* strains were subjected to 60 min of PDI treatments ( $5.0 \mu\text{M}$  of Tetra-Py<sup>+</sup>-Me and an irradiance



**FIGURE 1 |** Survival curves of six *S. aureus* strains (ATCC 6538, enterotoxic strains 2153 MA, 2095 M1A1, 2065 MA, and DSM 25693 MRSA and SA 3 MRSA) incubated with 5.0  $\mu\text{M}$  of Tetra-Py $^{+}\cdot\text{Me}$  and irradiated with white light (380–700 nm) with an irradiance of 40  $\text{W m}^{-2}$  for 60 min. Lines representing light (LC) and dark (DC) controls viability were omitted, since cell viability in the controls was not affected. Values represent the mean of three independent experiments with two replicates each; error bars indicate the standard deviation.

of 40  $\text{W m}^{-2}$ ), and aliquots were taken before (0 min) and after 5, 10, 15, 30, and 60 min of treatment. All the strains were efficiently inactivated by PDI (Figure 1). After 60 min of treatment under the tested conditions reductions higher than 5 log CFU  $\text{mL}^{-1}$  were observed for all the tested strains. However, in general, the pattern of photoinactivation was different among the *S. aureus* strains as showed by the log<sub>10</sub> reduction rates (Figure 1).

The enterotoxic strains were more efficiently inactivated than the non-enterotoxic ones. The toxigenic *S. aureus* strain 2095 M1A1 was particularly susceptible to PDI showing a reduction of 7 log within the first 10 min of irradiation. With equivalent irradiation time, the enterotoxic strain 2153 MA showed a reduction of only 2.5 log and all the other strains were inactivated less than 2 log. For DSM 25693 MRSA strain, also an enterotoxic, a linear decrease was observed after 15 min of treatment, reaching complete inactivation after 60 min of treatment (8.5 log). After 60 min of treatment, the inactivation factors for the other strains were 5.3 log for ATCC 6538, 6.0 log for SA 3 MRSA, 6.3 log for 2065 MA, 6.3 log for 2153 MA, and 7.8 log for 2095 M1A1. In the controls, the concentration of viable cells did not vary, indicating that the viability of bacterial cells was neither affected by light alone (light control), nor by the direct toxicity of the PS (dark control).

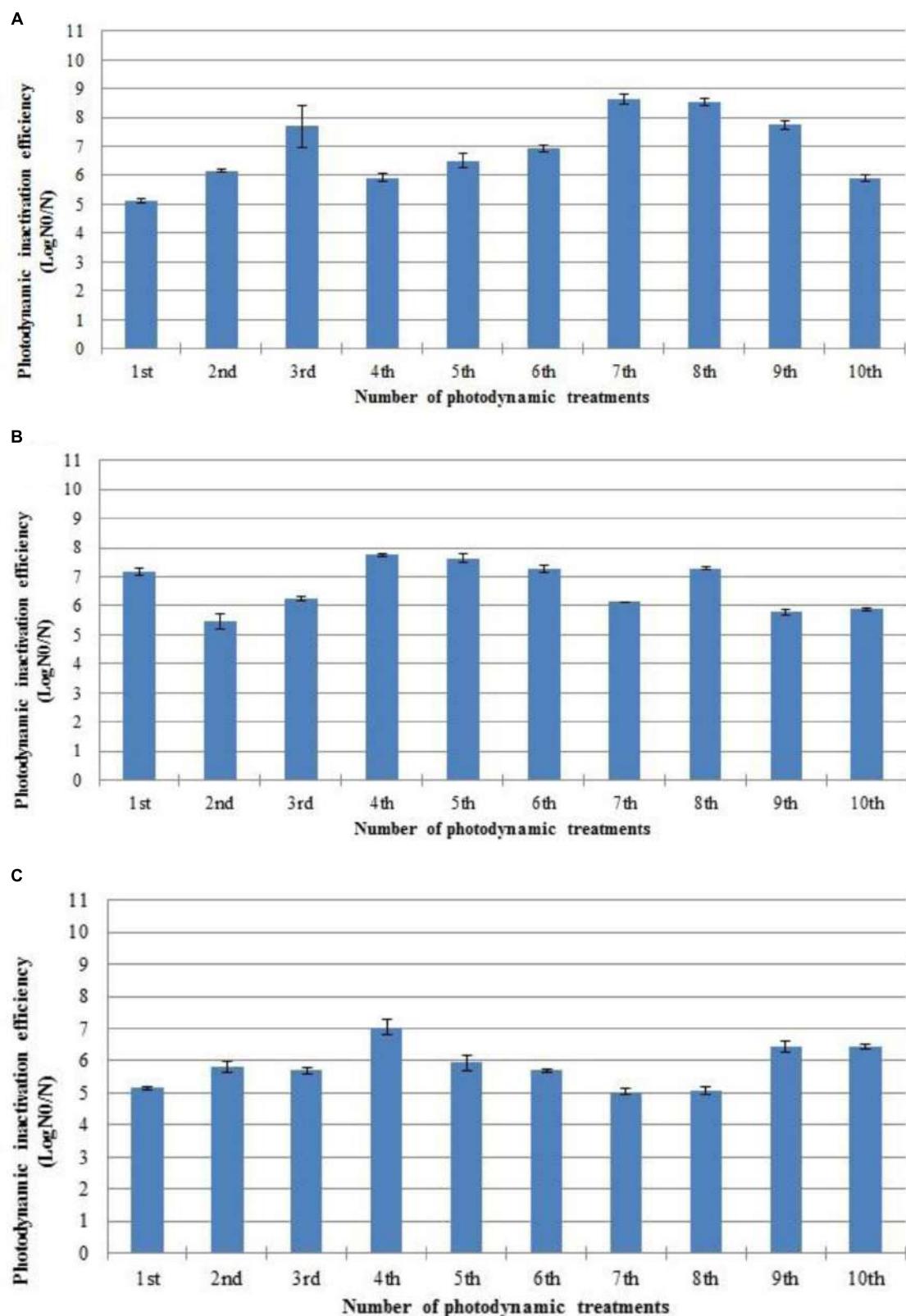
## Development of Resistance after Repeated PDI Cycles and Recovery of Viability Between Cycles

Three *S. aureus* strains (ATCC 6538, 2065 MA, and SA 3 MRSA) were subjected to ten consecutive PDI cycles (Figure 2). The authors decide to test 10 cycles having into account previous results of the research group (Tavares et al., 2010; Costa et al., 2011). The PDI efficiency of photosensitization with Tetra-Py $^{+}\cdot\text{Me}$  was not affected during the sequence of 10 PDI cycles.

## Effect of PDI on *S. aureus* Virulence Factors, Methicillin Susceptibility, and Mannitol Fermentation

After each PDI assay, the activity and presence of virulence factors, mannitol fermentation, and methicillin susceptibility were tested (Table 1).

The surviving cells to PDI treatments retained the capacity to express all the virulence factors and to ferment mannitol. However, the activity of the extracellular virulence factors free coagulase and enterotoxins, assessed in the supernatant of treated samples, was affected (see section “Testing the Presence of Free Coagulase” of Supplementary Material and Table 1). For the test of free coagulase, the BBL Coagulase Plasma reagent was



**FIGURE 2 |** Photodynamic inactivation efficiency of ten consecutive cycles of *S. aureus* ATCC 6538 (A), 2065 MA (B), and SA 3 MRSA (C) by 5.0  $\mu\text{M}$  of Tetra-Py<sup>+</sup>-Me after 60 min of irradiation with white light (40  $\text{W m}^{-2}$ ).  $N_0$  represents the plaque counts of bacterial cells before the irradiation; N represents the plaque counts after the cycle treatment; error bars indicate the standard deviation.

**TABLE 1 |** The activity of the virulence factors, mannitol fermentation, and susceptibility to methicillin were tested, after PDI treatments, in presence of 5.0  $\mu\text{M}$  of Tetra-Py<sup>+</sup>-Me and irradiated with white light (380–700 nm) with an irradiance of 40  $\text{W m}^{-2}$  for 60 min.

Staphylococcus aureus strains	Samples	Surviving cells							Supernatant		
		$\beta$ -Hemolysis	Lipase and lecithinase	Mannitol fermentation	Catalase	Bound Coagulase	Thermonuclease	Susceptibility to methicillin (mm)	Free coagulase	SEs	
		A	C							A	C
ATCC 6538	S	+	+	+	+	+	+	30	–	#	#
	LC	+	+	+	+	+	+	29	4 <sup>+</sup>	#	#
	DC	+	+	+	+	+	+	29	4 <sup>+</sup>	#	#
2153 MA	S	+	+	–	+	+	+	25	–	–	#
	LC	+	+	–	+	+	+	26	3 <sup>+</sup>	+	#
	DC	+	+	–	+	+	+	26	3 <sup>+</sup>	+	#
2065 MA	S	+	+	+	+	+	+	28	–	–	#
	LC	+	+	+	+	+	+	28	3 <sup>+</sup>	+	#
	DC	+	+	+	+	+	+	28	3 <sup>+</sup>	+	#
2095 M1A1	S	+	+	+	+	+	+	27	–	#	–
	LC	+	+	+	+	+	+	26	3 <sup>+</sup>	#	+
	DC	+	+	+	+	+	+	27	3 <sup>+</sup>	#	+
DSM 25693 MRSA	S	+	+	+	+	+	+	<21	–	–	–
	LC	+	+	+	+	+	+	<21	3 <sup>+</sup>	+	+
	DC	+	+	+	+	+	+	<21	3 <sup>+</sup>	+	+
SA 3 MRSA	S	+	+	+	+	+	+	<21	–	#	#
	LC	+	+	+	+	+	+	<21	4 <sup>+</sup>	#	#
	DC	+	+	+	+	+	+	<21	4 <sup>+</sup>	#	#

These tests were performed in three independent assays for each strain. Cardinal symbol in the “Supernatant, SE” columns means that such tests have not been performed for the respective strains once these strains do not produce the mentioned virulence factors.

added to an aliquot of supernatant and the resulting samples was incubated for 24 h. Clot formation was not detected in photosensitized samples. The SE test, performed in the supernatant of photosensitized cells by the SET-RPLA Kit Toxin Detection Kit test, revealed the formation of a tight button, interpreted as absence of SE or presence at a concentration below the detection limit. These two virulence factors persisted in light and dark controls.

## Susceptibility of Staphylococcal Isolated Enterotoxins to PDI

The isolated enterotoxins A and C were directly treated by PDI [see section “Purified SE A (A) and C (B) Subjected to Photodynamic Treatment” of Supplementary Material and **Table 2**]. The positive result corresponds to agglutination, leading to the formation of a lattice structure and negative correspond to the formation of a tight button, which occurs if SE are absent or present in a concentration bellow the detection level (TD0900, SET-RPLA, 2015). Before the treatment, enterotoxins A and C were still detected (formation of a lattice structure). During the PDI, the formation of the lattice structure decreases (as seen in the first well of 15 and 30 min test, see section “Purified SE A (A) and C (B) Subjected to Photodynamic Treatment” of Supplementary Material) and the formation of a tight button (as seen in the first well of 60 min test), begins to occur, indicating the shift to a negative result. After 60 min of irradiation, the

**TABLE 2 |** The purified SE A and C were subjected to PDI for 60 min.

Irradiation time	Isolated and purified SEs		
	A	B	C
0	S	+	+
	LC	+	+
	DC	+	+
60	S	–	–
	LC	+	+
	DC	+	+

The SE activity was assessed before (0 min), during (5, 10, 15, and 30 min) and after 60 min of treatment for treated (S) and non-treated samples (light control, LC; dark control, DC). Three independent assays were performed.

inactivation was >68% for both SE [SE A and C; see section “Calculating the Decrease (%) of Enterotoxins After PDI Assays” of Supplementary Material].

## Carotenoid Pigments Content

Carotenoid pigments were detected in all the tested strains. The strains SA 3 MRSA with an absorbance at 465 nm of  $0.995 \pm 0.001$  and the 2065 MA with an absorbance of  $0.911 \pm 0.017$ , display the highest concentration of pigments, followed by DSM 25693 MRSA (Abs of  $0.788 \pm 0.021$ ), ATCC

6538 (Abs of  $0.701 \pm 0.005$ ), 2153 MA (Abs of  $0.480 \pm 0.002$ ), and 2095 M1A1 (Abs of  $0.411 \pm 0.013$ ).

## DISCUSSION

According to literature, the susceptibility of *S. aureus* to PDI is strain-dependent and MRSA strains seem to have a lower susceptibility to PDI than methicillin-sensitive counterparts (Grinholc et al., 2008). The lower susceptibility of MRSA strains to PDI has been attributed to slime production by the MRSA strains used (Grinholc et al., 2008).

In this study, all the strains tested were susceptible to PDI, with a survival reduction above  $5 \log \text{CFU mL}^{-1}$ , which according to American Society of Microbiology is higher than the minimum required (reduction  $> 3 \log \text{CFU mL}^{-1}$ ) for a new approach to be termed as antimicrobial (ASM, 2015). However, as observed in previous studies, a strain-dependent efficiency of inactivation was observed (Grinholc et al., 2008). Nonetheless PDI does not seem to be antibiotic-susceptible dependent, since DSM 25693, an MRSA and enterotoxic strain, was the only strain that was inactivated to the limit of detection of the method, with a survival reduction of  $8.5 \log$ . Another MRSA strain, SA 3 MRSA, was not as efficiently inactivated, but the inactivation profile was similar to that of the reference, ATCC 6538 strain. The four enterotoxic strains were more efficiently inactivated than the two non-enterotoxic strains (see Figure 1), which could suggest that the enterotoxic strains are more susceptible to PDI. However, as showing the  $\log_{10}$  reduction rates differences in PDI inactivation between enterotoxic strains were observed, which means that other cell factors may contribute to these differences in PDI efficiency. Whereby, further studies should be performed exploiting the factors which may contribute to the difference in PDI susceptibility among the bacterial strain.

Carotenoid pigment content is related with the resistance of *S. aureus* to several stress factors, such as external oxidative stress. Several studies have shown that mutant strains that are unable to produce carotenoids are more susceptible to those stress factors (Liu et al., 2005; Claditz et al., 2006; Cebrián et al., 2007). In an attempt to verify if carotenoid pigments were able to attenuate oxidative damages and consequent cellular inactivation, the pigment content of each strain was measured. Although a direct relation between carotenoids content and PDI efficiency was not observed for all the strains, the least susceptible strains to PDI (ATCC 6538 and SA 3 MRSA) show a high relative concentration of this pigment, with absorbances of 0.701 and 0.995, respectively.

Once the photodynamic action occurs through ROS generation (formed along the irradiation process), the damages (through oxidative process) can affect a variety of cellular components with great importance in the maintenance of bacterial stability, such as molecular components (proteins and lipids) of external structures, and enzymes (Alves et al., 2014; Almeida et al., 2015). The majority of virulence factors of *S. aureus* are proteins or enzymes that are prone to photodynamic oxidative damage. The results of this study show that the

phenotypic expression of the of  $\beta$ -hemolysin, lipase, lecithinase, catalase and bound coagulase by surviving cells cultivated on PCA medium for 48 h at  $37^\circ\text{C}$  was not affected by PDI. Mannitol uptake and fermentation also persisted. Previous proteomic analysis showed that the oxidative damage caused by PDI treatment affects the expression of functional proteins involved in cell division, metabolic activities, oxidative stress responses, and sugar uptake (Alves et al., 2014, 2015a). However, the mentioned studies were performed in treated cells. Since the detection of virulence factors activity in our study was performed in the surviving cells after a period of recovery, it would be expected that the bacteria, even if injured during PDI treatments, would be able to continue producing those virulence factors and other enzymes.

Antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase can give protection against some ROS but not against singlet oxygen. So, according to the literature porphyrinic compounds (Almeida et al., 2011), namely the tetracationic porphyrin Tetra-Py<sup>+</sup>-Me used in this study, exert their photodynamic action by a type II mechanism and consequently singlet oxygen is the main ROS (Tavares et al., 2011; Costa L. et al., 2013). Moreover, has been shown that singlet oxygen is able to inactivate these enzymes, namely the catalase enzyme (Kim et al., 2001). However, no effect of the PDI process was observed for this enzyme, once that in this study, the catalase activity was only determined after a recovery period of the persistent cells, and not during the PDI treatment – condition in which could be detected some changes in the activity of catalase. Further studies are needed to clarify whether the ROS produced by the Tetra-Py<sup>+</sup>-Me during PDI treatments affect catalase enzyme and even other enzymes when they are outside the cells.

Nevertheless, some of these virulence factors are released to the extracellular compartment, becoming more exposed to the effects of the PDI process suffering a greater damage.

In this study, the presence/activity of two external virulence factors (free coagulase and enterotoxins A and C) in the supernatant of PDI treatment samples was assessed. The results show that both virulence factors are affected by PDI. This represents an advantage relatively to traditional antibiotics, which act only on bacterial cell and not on extracellular virulence factors. These results are in accordance with those observed in the previous studies by Kömerik et al. (2000) and Tubby et al. (2009).

The response of one of the most important *S. aureus* virulence factors, the SE which are the staphylococcal food poisoning causative agents, was also assessed after PDI treatment in order to confirm the effect of PDI on extracellular virulence factors. Two isolated enterotoxins, SE A and C, were treated by PDI. The porphyrin Tetra-Py<sup>+</sup>-Me at a concentration of  $5.0 \mu\text{M}$  reduced at least in 68% the amount of active SE A and C. This discovery is of great importance since it is known that this family of proteins covers very stable, resistant to heat and to degrading enzymes molecules (Schelin et al., 2011). These results demonstrate that PDI is not only effective in the inactivation of microorganisms but also in the degradation of released external virulence factors.

One of the main advantages of PDI is that because of the nature of the photoinactivation process, development of resistance is very unlikely (Tavares et al., 2010; Costa et al., 2011). The results obtained in this study do not show evidence of resistance development in the three strains subjected to ten PDI cycles, which corroborates literature conclusions. Considerable reduction in the efficiency of photosensitization of *S. aureus* strains ATCC 6538, enterotoxic 2065 MA and SA 3 MRSA after ten consecutive photosensitization sessions of 60 min with 5.0  $\mu\text{M}$  of Tetra-Py<sup>+</sup>-Me was not observed. As in this study, the viable bacterial colonies have been aseptically removed from the plate and suspended in PBS after each PDI cycle, the cellular density obtained after the colony resuspension could be different. To avoid differences in the PDI efficiency due to different bacterial densities, this parameter was controlled in all the experiments by measuring the optical density of the bacteria suspension before each assay.

## CONCLUSION

Overall, it can be concluded that (1) although the efficiency of PDI to inactivate *S. aureus* is strain-dependent, all the strains can be effectively inactivated, namely the enterotoxic strains; (2) PDI process is not only effective in the inactivation of microorganisms but also in the degradation of their external virulence factors after their release to the exterior; and (3) *S. aureus* strains do not develop resistance to PDI treatment.

The research conducted so far, allowed the development of very promising and successful *in vitro* photoinactivation protocols for enterotoxigenic and non-enterotoxigenic strains of *S. aureus*. However, their evaluation *in vivo*, along with validation of their feasibility according to different sample settings and different enterotoxigenic and non-enterotoxigenic strains of *S. aureus*, as well as on biofilms developed by these

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bacteria, are now required. Bacterial biofilms are not only more difficult to inactivate than planktonic bacteria, but bacteria in a biofilm-mode are also much more prone to become resistant to antimicrobial agents or strategies (Beirão et al., 2014). In fact, some mechanisms, such as horizontal gene transfer and quorum sensing, occur only in a biofilm-state (Madsen et al., 2012; Solano et al., 2014).

## AUTHOR CONTRIBUTIONS

MB did the experimental work and drafted the manuscript. SR participated in the experimental work. AA has been involved in the coordination, conception, design of the study and helped to draft the manuscript. AC, MF, and MN participated in the design of the study, acquisition and interpretation of data, and also helped.

## ACKNOWLEDGMENTS

The authors are thankful to the University of Aveiro, to FCT/MEC for the financial support to the QOPNA research Unit (FCT UID/QUI/00062/2013) and Centre for Environmental and Marine Studies (CESAM) unit (project Pest-C/MAR/LA0017/2013), through national funds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement, and also to the Portuguese NMR Network.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00267>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Enhanced Biocide Mitigation of Field Biofilm Consortia by a Mixture of D-Amino Acids

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## OPEN ACCESS

### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 20 January 2016

Accepted: 26 May 2016

Published: 13 June 2016

### Citation:

Li Y, Jia R, Al-Mahamedh HH, Xu D and Gu T (2016) Enhanced Biocide Mitigation of Field Biofilm Consortia by a Mixture of D-Amino Acids. *Front. Microbiol.* 7:896.  
doi: 10.3389/fmicb.2016.00896

Microbiologically influenced corrosion (MIC) is a major problem in the oil and gas industry as well as in many other industries. Current treatment methods rely mostly on pigging and biocide dosing. Biocide resistance is a growing concern. Thus, it is desirable to use biocide enhancers to improve the efficacy of existing biocides. D-Amino acids are naturally occurring. Our previous work demonstrated that some D-amino acids are biocide enhancers. Under a biocide stress of 50 ppm (w/w) hydroxymethyl phosphonium sulfate (THPS) biocide, 1 ppm D-tyrosine and 100 ppm D-methionine used separately successfully mitigated the *Desulfovibrio vulgaris* biofilm on carbon steel coupons. The data reported in this work revealed that 50 ppm of an equimolar mixture of D-methionine, D-tyrosine, D-leucine, and D-tryptophan greatly enhanced 50 ppm THPS biocide treatment of two recalcitrant biofilm consortia containing sulfate reducing bacteria (SRB), nitrate reducing bacteria (NRB), and fermentative bacteria, etc., from oil-field operations. The data also indicated that individual D-amino acids were inadequate for the biofilm consortia.

**Keywords:** biocide, biocide enhancer, D-amino acid, biofilm consortium, microbiologically influenced corrosion

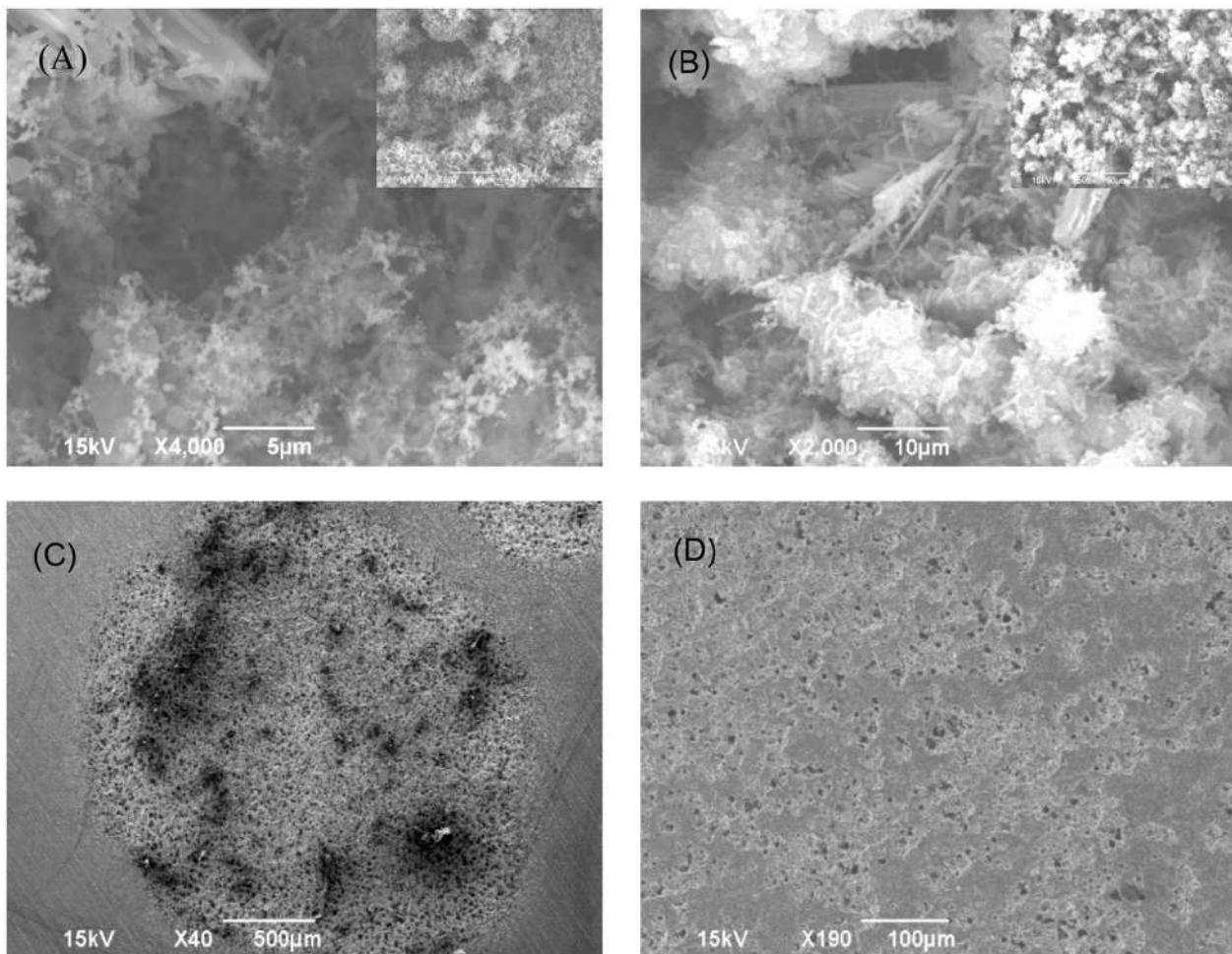
## INTRODUCTION

Microbiologically influenced corrosion (MIC) was first reported over 100 years ago (Gaines, 1910). It has become a major problem in the oil and gas industry in recent years because water injection is practiced more frequently than ever to increase well pressure. In this process, microbes and nutrients are introduced into the reservoirs and they subsequently show up in the pipelines, leading to MIC. MIC was suspected as a major contributing factor in the pipeline leak caused by a 0.25" × 0.5" hole at the bottom of a pipe in the Trans-Alaska Pipeline in 2006 (Jacobson, 2007). In a separate reported case, an 8" diameter pipeline carrying oil and produced water failed in only 8 months due to MIC (Bhat et al., 2011). A recent pipeline failure case was published suggesting that MIC was likely the culprit in the leak of a 24" CO<sub>2</sub> gathering line 2 years after commissioning, after ruling out other corrosion mechanisms (Hinkson et al., 2013). Skovhus and Eckert (2014) discussed several MIC cases and pointed out that MIC is becoming more prevalent nowadays due to aging equipment and increased awareness.

In the MIC caused by biofilms of sulfate reducing bacteria (SRB), elemental iron (Fe<sup>0</sup>) in carbon steel serves as an electron donor. Xu and Gu (2014) designed an SRB starvation experiment to support this hypothesis. They found that under carbon source starvation, MIC pitting accelerated despite the fact that the biofilm lost some cell mass due to the starvation.

SRB switched to  $\text{Fe}^0$  as electron donor for energy production to help their survival. Rajala et al. (2015) suggested that the presence of carbon steel benefited the microbial community in a nutrient-deficient anaerobic environment. In this kind of MIC, cross-cell wall electron transport is required for SRB to utilize extracellular electrons released by  $\text{Fe}^0$  oxidation in their cytoplasm for sulfate reduction, meaning that the biofilm must be electrogenic (Gu, 2012). Recent studies further suggested that electron transfer is a bottleneck in SRB MIC against carbon steel and 304 stainless steel (Li et al., 2015; Zhang et al., 2015). MIC by acid-producing bacteria (APB) belongs to a different type of MIC because the oxidant (proton) is reduced outside the cells on the steel surface rather than in the cytoplasm (Gu, 2012). Underneath an APB biofilm, the local pH is much more acidic than in the bulk-fluid pH, leading to organic acid attacks. In both types of MIC discussed above, biofilms are responsible. Thus, biofilm mitigation is a key to MIC mitigation.

In the field, microbes form synergistic biofilm consortia. It is well known that biofilm cells (sessile cells) are far more recalcitrant than planktonic cells. Through several different mechanisms, a biofilm protects inner sessile cells from harmful factors. In one way, a biofilm can slow down the diffusion of antimicrobial agents (Mah and O'Toole, 2001; Stewart and Costerton, 2001). Furthermore, it can slow down the metabolic rate to reduce the intake of antimicrobial agents. Tuomanen et al. (1986) found that the increased resistance to antibacterial agents was accompanied by a low growth rate or no growth. Biofilms also preserve persister cells when they are under attack. These persister cells quickly rebound when the environment becomes less hostile (Lewis, 2001). Sessile cells in biofilms can also use efflux pumps to prevent antimicrobial agents from entering the cells. They can upregulate resistant genes to break down antimicrobial agents (Lewis, 2001). These antimicrobial mechanisms make the mitigation of biofilm consortia difficult. It is said that as a rule of thumb, a ten times or higher biocide concentration may be required to treat biofilms



**FIGURE 1 |** Biofilm images and bare coupon surface images after biofilm removal for coupons after 10-day incubation (without treatment chemicals) at 37°C: (A) Consortium I biofilm, (B) Consortium II biofilm, (C) coupon surface after removal of Consortium I biofilm, and (D) coupon surface after removal of Consortium II biofilm.

compared with that needed for the treatment of planktonic cells (Nickel et al., 1985; Videla, 1996; Vance and Thrasher, 2005).

Pigging and biocides are two primary ways to mitigate problematic biofilms (Videla, 2002). Biocide applications can be performed during pigging by placing a biocide “plug” in a pipeline between two pigs. However, some pipelines are not piggable due to complicated elbows and other devices (Tiratsoo, 2013). Since pigging cannot completely remove biofilms, it is likely that the residual sessile cells can bounce back quickly. A biocide is needed to delay the recovery of biofilms. Tetrakis hydroxymethyl phosphonium sulfate (THPS) is one of the widely used biocides in the oil and gas industry because it is biodegradable and effective against a broad spectrum of microorganisms. THPS is designated by the US Environmental Protection Agency (EPA) as a green chemical (United States Environmental Protection Agency, 1997). It disrupts the disulphide bond in proteins and enzymes in microbes (Denyer, 1995; Russell, 2002; Ballantyne and Jordan, 2004; Greene et al., 2006). In field operations, repeated use of the same biocide will inevitably cause dosage escalation because of the selective promotion of resistant microbes over time. In some field operations, THPS dosage is so high such that the sulfate introduced by THPS precipitates with barium in the drilling fluid causing problematic scale formation downhole. High doses of biocides also raise environmental concerns in addition to increased cost. More effective uses of existing biocides are highly desirable.

Despite continued research in new biocides, it is unlikely that a blockbuster biocide will replace THPS or glutaraldehyde (another popular green biocide for large-scale applications) in oilfield applications any time soon. Thus, it is desirable to use biocide enhancers to make existing biocides such as THPS more effective. D-Amino acids were reported to enhance the efficacy of THPS in the mitigation of the *Desulfovibrio vulgaris* biofilm (Xu et al., 2012a,b, 2014). Most recently, Jia et al. (2016) demonstrated that D-amino acids enhanced two other biocides, namely alkylidimethylbenzylammonium chloride and tributyl tetradecyl phosphonium chloride against a biofilm consortium. Although previously considered rare in nature, D-amino acids are now considered ubiquitous due to the advancement of analytical techniques and the increased interests in their biological functions. They are found in microorganisms, food, plants, animals, and even in humans (Konno et al., 2009). While the biological functions of D-amino acids are not fully understood, it is believed that they could serve as a signal molecule. Lam et al. (2009) stated that the synthesis of D-amino acids might be a common way of self-adjustment of cells to the changing environment. Kolodkin-Gal et al. (2010) found that D-methionine (D-met), D-tyrosine (D-tyr), D-leucine (D-leu), and D-tryptophan (D-trp) triggered the *Bacillus subtilis* biofilm’s disassembly. They also tested an equimolar mixture of four D-amino acids. Xu and Liu (2011) confirmed that 100 ppm (w/w) D-tyr triggered the biofilm dispersal in their test using activated sludge on membrane filters. Xu et al. (2012a, 2014) found that D-tyr and D-met

were effective against the *D. vulgaris* biofilm on carbon steel coupons.

In this work, the efficacies of THPS combined with a mixture of D-amino acids against two oil-field biofilm consortia (labeled as Consortium I and Consortium II) were investigated. An equimolar mixture of four D-amino acids (D-met, D-tyr, D-leu, and D-trp) were tested with 50 ppm THPS. Individual D-met and D-tyr were also tested with THPS for comparison.

## MATERIALS AND METHODS

### Bacterium, Nutrient Medium, Biofilm Growth Substratum, and Chemicals

In this work, two biofilm consortia collected from an oil field were used to investigate the efficacy of 50 ppm THPS in combination with D-amino acids. The consortia were collected from oilfield operations in the form of planktonic cells in liquids in glass jars. The liquid samples were used to inoculate the ATCC 1249

**TABLE 1 | Metabolic assignments of dominant bacterial species (%).**

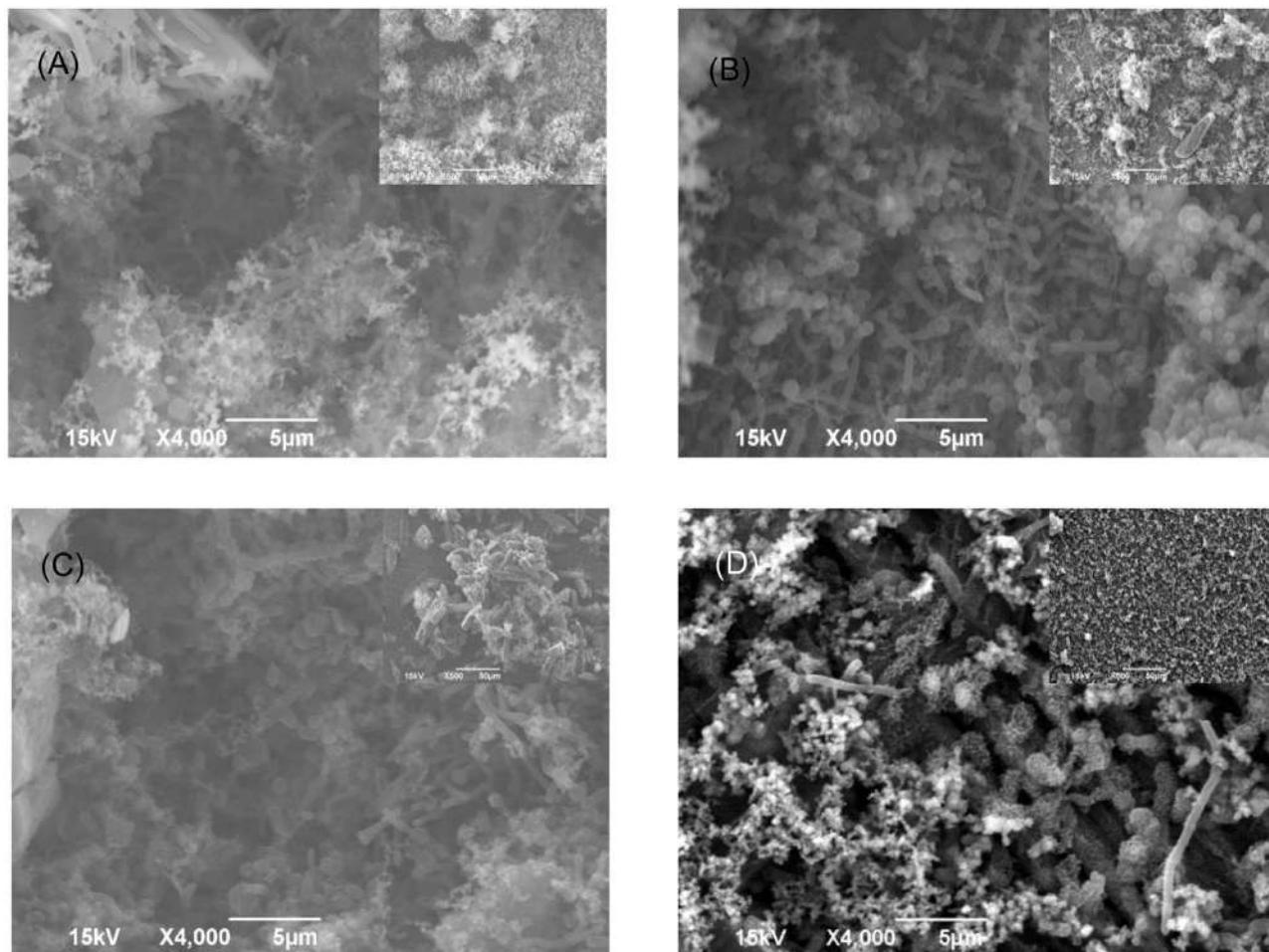
Species	Consortium I	Consortium II	Trait
<i>Bacillus</i> sp.	< 1	6.0	Biosurfactant producing; Varies
<i>Bacteroides</i> sp.	2.9	1.4	Fermenting bacteria
<i>Gardicella</i> sp.	27.7	78.8	NRB; Sulfidogen; TRB
<i>Salmonella enterica</i>	61.5	0	SRB; TRB
<i>Soehngenia</i> sp.	4.1	0	Fermenting bacteria
<i>Tepidibacter</i> sp.	2.7	13.6	Biodeg (HC)

**TABLE 2 | SRB sessile cell counts of Consortium I and II after 7-day biofilm prevention test using D-tyr and D-met.**

Biofilm	Treatment	MPN sessile cell count (cells/cm <sup>2</sup> )
Consortium I	No treatment chemical (control)	10 <sup>7</sup>
	50 ppm THPS	10 <sup>7</sup>
	50 ppm THPS + 100 ppm D-met	10 <sup>6</sup>
	50 ppm THPS + 1 ppm D-tyr	10 <sup>5</sup>
	50 ppm THPS + 10 ppm D-tyr	10 <sup>5</sup>
Consortium II	No treatment chemical (control)	10 <sup>7</sup>
	50 ppm THPS	10 <sup>7</sup>
	50 ppm THPS + 100 ppm D-met	10 <sup>6</sup>
	50 ppm THPS + 1 ppm D-tyr	10 <sup>6</sup>
	50 ppm THPS + 10 ppm D-tyr	10 <sup>6</sup>

**TABLE 3 | SRB sessile cell counts of Consortium I biofilm after 7-day biofilm prevention test using a D-amino acid mixture.**

Treatment	MPN sessile cell count (cells/cm <sup>2</sup> )
No treatment chemical (control)	10 <sup>7</sup>
50 ppm THPS	10 <sup>7</sup>
50–500 ppm D-mix	10 <sup>5</sup>
50 ppm THPS + 50 ppm D-mix	10 <sup>3</sup>



**FIGURE 2 | SEM images of Consortium I biofilm after 7-day incubation in the biofilm prevention test with: (A) no treatment chemical (control), (B) 50 ppm THPS, (C) 50 ppm D-mix, and (D) 50 ppm THPS + 50 ppm D-mix. The scale bar in the inserted small image is 50  $\mu\text{m}$ .**

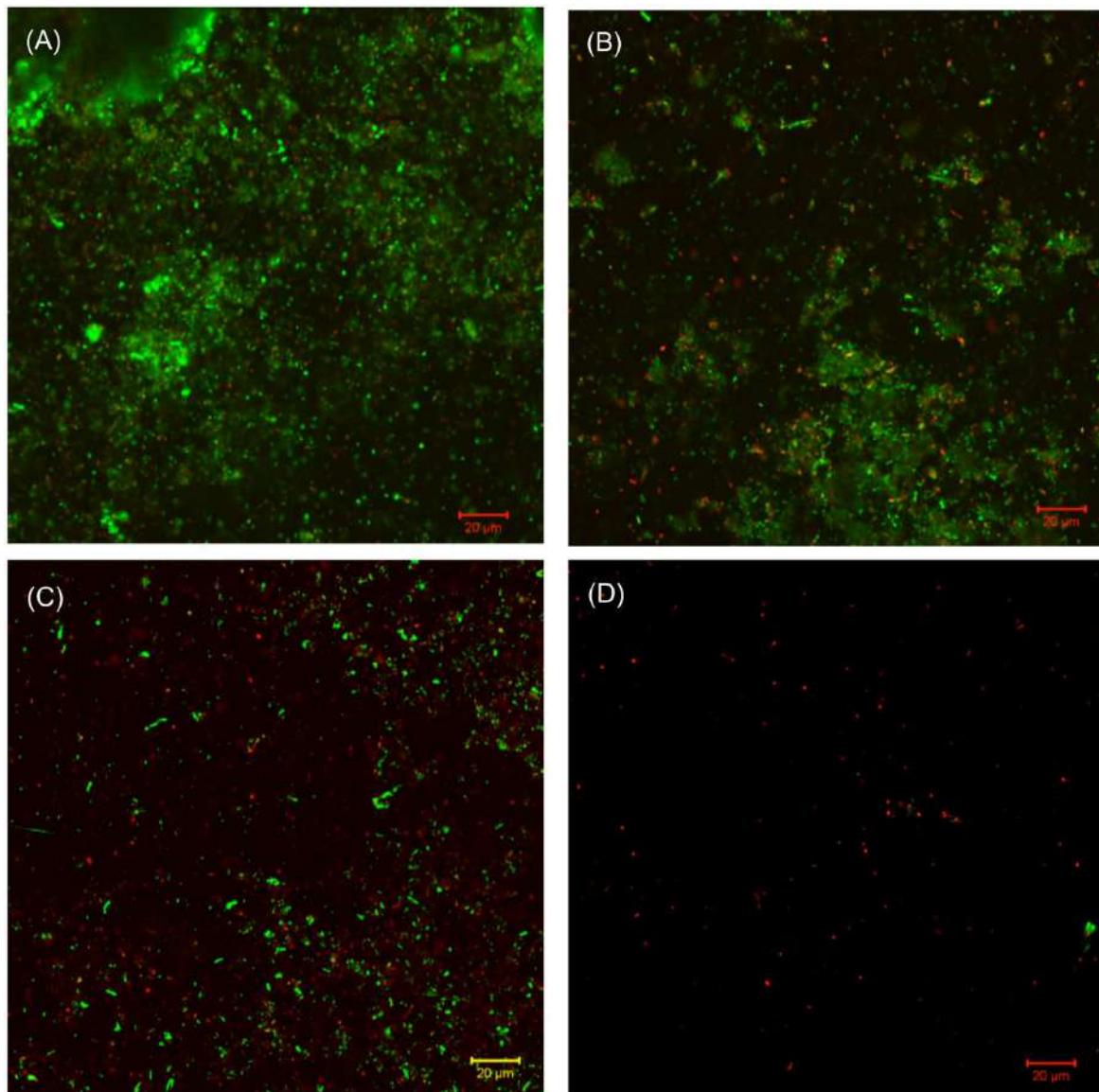
medium that is a modified Baar's medium. L-Cysteine at 100 ppm was added to the medium as an oxygen scavenger to eliminate any possible oxygen ingress.

Coin-shaped C1018 (UNS G10180) carbon steel coupons were used to grow biofilms. The composition of C1018 was (wt%): C 0.14–0.20, Mn 0.60–0.90, P 0.04, S 0.05, Si 0.15–0.30, and Fe balance. For each coupon, only the 1.2  $\text{cm}^2$  top surface was exposed to the culture medium, and the remainder was painted with Teflon. Coupons were polished with 180, 400, and 600 grit sandpapers, sequentially. They were cleaned with isopropanol and dried under UV light for 20 min.

D-Amino acids, THPS, and all chemicals used in the culture medium were purchased from Sigma-Aldrich (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA). In the 50 ppm equimolar D-amino acid mixture (D-mix), the concentrations of D-met and D-trp in the mixture were much less than the concentration of each D-amino acid which was needed to enhance 50 ppm THPS in the mitigation of the *D. vulgaris* biofilm on carbon steel (Xu et al., 2012a, 2014).

## Biofilm Prevention and Biofilm Removal Tests

In order to evaluate the efficacies of the cocktails of THPS + D-amino acid(s), both biofilm prevention and biofilm removal tests were carried out in the lab. Before the operation, the culture medium, anaerobic vials, pipettes, and tweezers were sterilized in an autoclave at 121 °C for 20 min before use. D-Amino acids were not autoclaved due to possible oxidation at a high temperature. Their stock solutions were filter-sterilized. The culture medium and solutions containing the biocide treatment chemicals were sparged with filtered N<sub>2</sub> for 45 min to remove O<sub>2</sub>. In the biofilm prevention test, two duplicate coupons, 100 ml medium, treatment chemicals, and 1 ml biofilm consortium seed culture were put into each 125 ml anaerobic vial in an anaerobic chamber. The initial planktonic cell concentration right after inoculation was 10<sup>6</sup> cells/ml. The anaerobic chamber was sparged with filtered N<sub>2</sub> for 45 min to remove oxygen before use. After the vials were sealed, they were placed in a 37°C incubator. After 7-day incubation, coupons were taken out for further measurement



**FIGURE 3 |** CLSM images of biofilms after 7-day incubation in the biofilm prevention test with: (A) no treatment chemical (control), (B) 50 ppm THPS, (C) 50 D-mix, and (D) 50 ppm THPS + 50 ppm D-mix.

and observation. In the biofilm removal test, biofilms were first grown on coupons without treatment chemicals for 3 days to achieve maturity. Coupons covered by the mature biofilm were taken out and put into a phosphate-buffered saline (PBS) solution (pH 7.4) with treatment chemicals in a Petri dish for 3 h in the anaerobic chamber at room temperature. Coupons were taken out for analysis afterwards. Each test was repeated twice and triplicate coupons were used for each test condition.

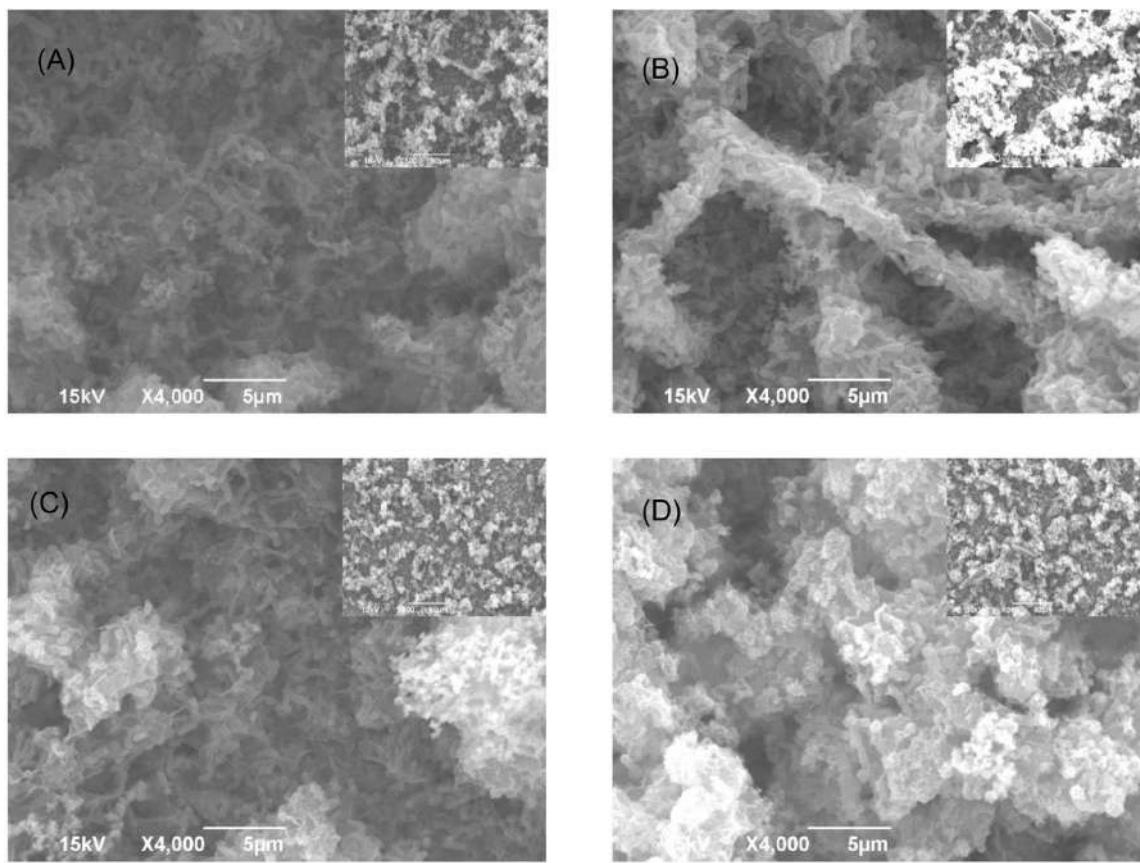
## Enumeration of Sessile Cells

The Biosan Sani-Check SRB test kit (Sani-Check® Product #100, Biosan Laboratories, Warren, MI, USA) was used for the most probable number (MPN) cell counts. The biofilm consortium on

**TABLE 4 |** SRB sessile cell counts of Consortium I after 3-h biofilm removal test.

Treatment	MPN sessile cell count (cells/cm <sup>2</sup> )
No treatment chemical (control)	10 <sup>7</sup>
50 ppm THPS	10 <sup>7</sup>
50–500 ppm D-mix	10 <sup>5</sup>
50 ppm THPS + 50 ppm D-mix	10 <sup>3</sup>

the coupon surface was removed with the small brush that was a part of the test kit. The brush was then inserted into the test kit's vial containing a solid SRB culture medium for incubation at 37°C. The time it took to show the black color (FeS) reflected the



**FIGURE 4 |** SEM images of Consortium I biofilm after 3-h treatment in PBS buffer containing: (A) no treatment chemical (control), (B) 50 ppm THPS, (C) 100 ppm D-mix, and (D) 50 ppm THPS + 50 ppm D-mix, in the biofilm removal test. The scale bar in the inserted small images is 50  $\mu\text{m}$ .

SRB cell concentration based on vendor's calibration (Xu et al., 2012a,b, 2014).

### Scanning Electron Microscope (SEM) for Biofilm Observation

Scanning electron microscope observation with a Model JSM-6390 SEM (JEOL Ltd., Tokyo, Japan) was used to observe the biofilm morphology. The coupons for SEM observation of biofilms were first submerged in 4% (w/w) glutaraldehyde for 2 h and then dehydrated in 25, 50, 75, and 100% (v/v) isopropanol sequentially for 5 min total. And then, the biofilms were dehydrated in a critical point dryer using CO<sub>2</sub>. Finally, biofilms covered coupons were coated with palladium to provide conductivity (Xu et al., 2012a,b, 2014). It should be noted that SEM images should not be used for quantitative cell counting because of uneven distribution of sessile cells.

### Confocal Laser Scanning Microscopy (CLSM) for Biofilm Observation

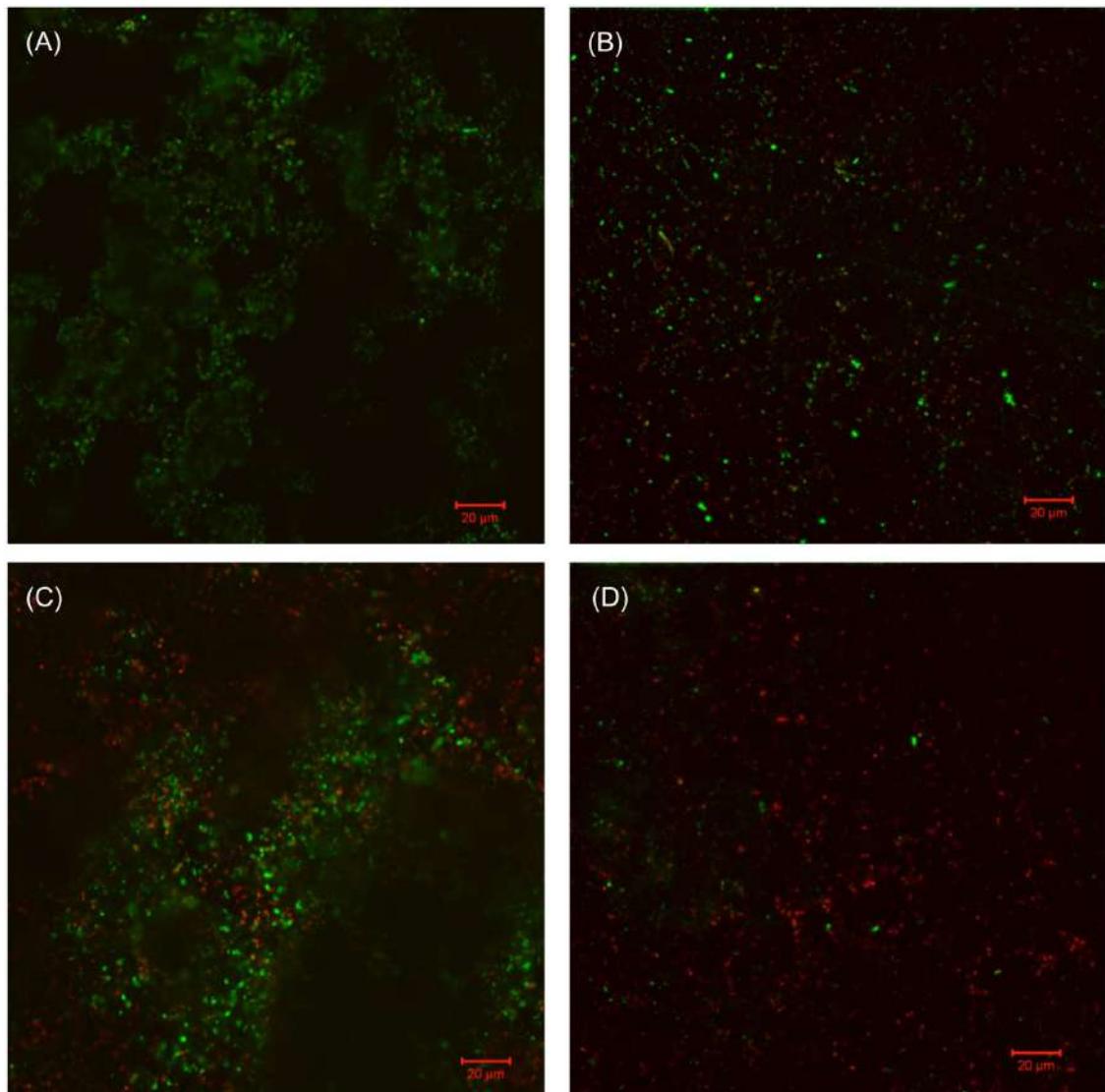
Confocal laser scanning microscopy using Model LSM 510 microscope (Carl Zeiss, Jena, Germany) was used to detect living and dead cells in biofilms. The dyes used to stain the biofilms

were in the L7012 Live/Dead<sup>®</sup> BacLight<sup>TM</sup> Bacterial Viability Kit (Life Technologies, Grand Island, NY, USA), in which SYTO 9 is a green-fluorescent stain and propidium iodide a red-fluorescent stain. Thus, living cells appear as green color at an excitation wavelength of 488 nm and dead cells red at 559 nm. Before being put into the dye solution for 15 min in a dark condition, coupons were washed with the PBS buffer for 15 s three times to wash away the culture medium, biocide residue, and planktonic cells (Mah et al., 2003; Turonova et al., 2015).

## RESULTS

### Oil-Field Biofilm Consortia

Biofilm Consortia I and II were supplied by an oil and gas company. Figure 1 shows that both biofilm consortia formed robust biofilms on coupons after incubation in the culture medium for 10 days at 37°C. The images also reveal that the sessile cells in both biofilm consortia appeared to have different morphologies, indicating that they were mixed-culture consortia. Since the ATCC 1249 culture medium is designed for SRB, the dominant sessile population observed was likely SRB. After the biofilms were removed, pits underneath the biofilms resembled



**FIGURE 5 |** CLSM images of biofilms in the 3-h treatment in PBS buffer with: (A) no treatment chemical (control), (B) 50 ppm THPS, (C) 50 ppm D-mix, and (D) 50 ppm THPS + 50 ppm D-mix.

**TABLE 5 |** SRB sessile cell counts of Consortium II after 7-day biofilm prevention test.

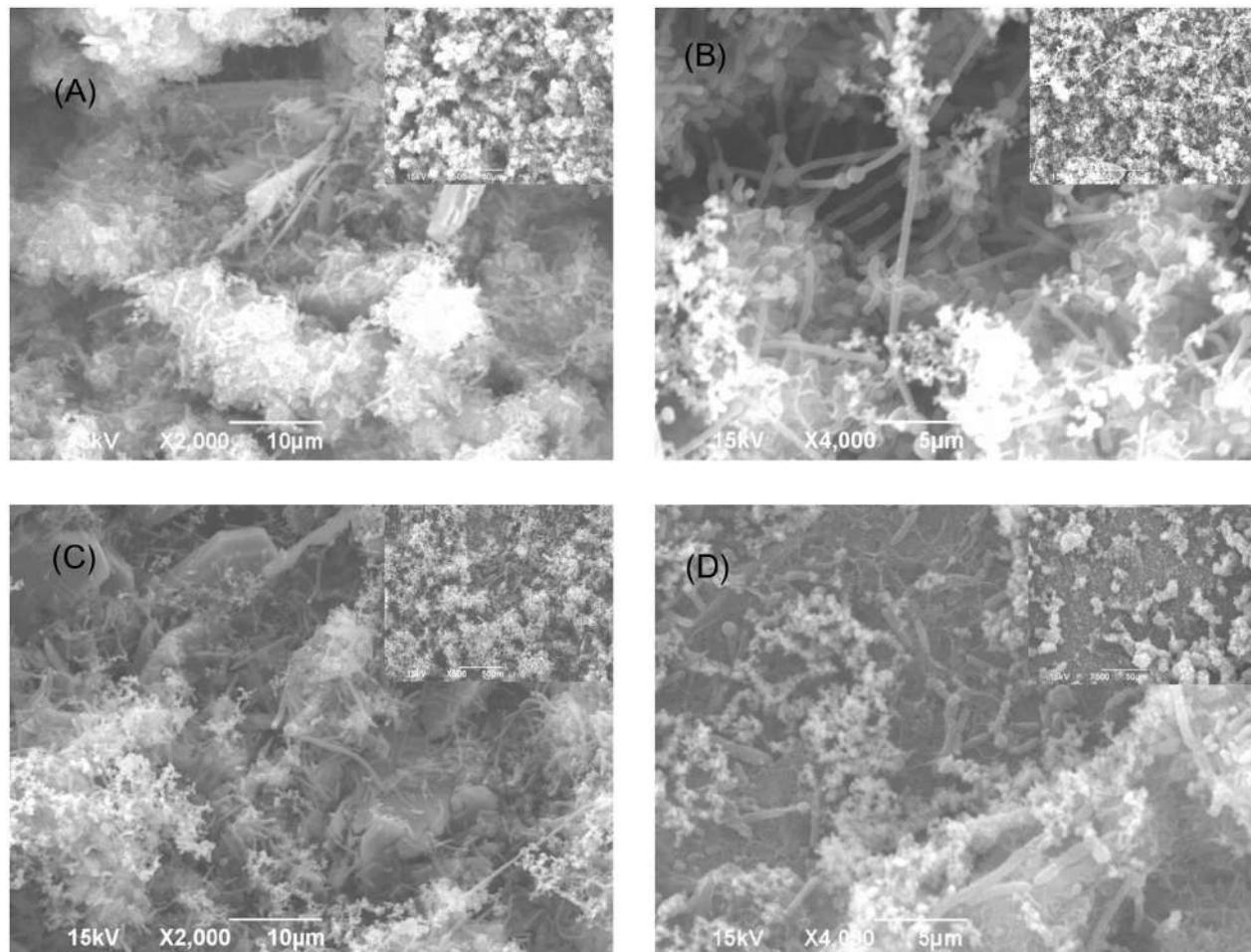
Treatment	MPN sessile cell count (cells/cm <sup>2</sup> )
No treatment chemical (control)	10 <sup>7</sup>
50 ppm THPS	10 <sup>7</sup>
50–500 ppm D-mix	10 <sup>6</sup>
50 ppm THPS + 50 ppm D-mix	10 <sup>4</sup>

characteristic MIC pits (**Figure 1**). The specific weight losses of the coupons were 0.0047 and 0.0058 g/cm<sup>2</sup> for Consortium I and Consortium II, respectively. Both values were much larger than the 0.0018 g/cm<sup>2</sup> caused by pure-strain *D. vulgaris* incubated under the same conditions.

**TABLE 6 |** SRB sessile cell counts of Consortium II after 3-h biofilm removal test.

Treatment	MPN sessile cell count (cells/cm <sup>2</sup> )
No treatment chemical (control)	10 <sup>7</sup>
50 ppm THPS	10 <sup>7</sup>
50–500 ppm D-mix	10 <sup>6</sup>
50 ppm THPS + 50 ppm D-mix	10 <sup>4</sup>

The phylogenetic identification of Consortium I and II shown in **Table 1** was carried out by Ecolyse, Inc. (College Station, TX, USA). Ecolyse used the bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) method with 515F-GTGCCAGCMGCCGCGTAA and



**FIGURE 6 | SEM images of Consortium II biofilm after 7-day incubation in the biofilm prevention test with: (A) no treatment chemical (control), (B) 50 ppm THPS, (C) 100 ppm D-mix, and (D) 50 ppm THPS + 50 ppm D-mix in the biofilm prevention test.** The scale bar in the inserted small images is 50  $\mu\text{m}$ .

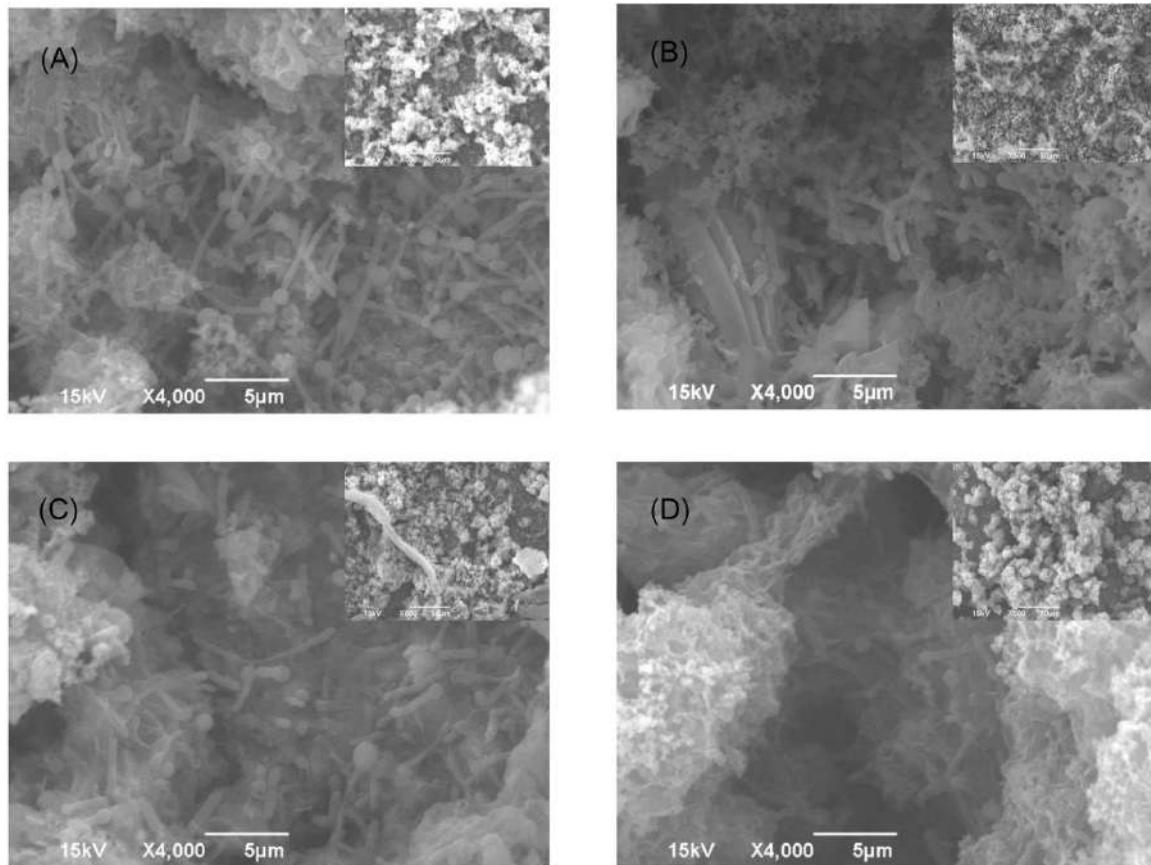
806R-TAACCTWTGGGVHCATCAGG as primers for DNA analysis. A forward primer and a reverse fusion primer were used for sample amplification. The amplified product was processed to create single-stranded DNA using the Ion PGM protocols by Life Technologies (Carlsbad, CA, USA). Sequencing was performed using the Ion PGM semiconductor sequencing method by Life Technologies.

In **Table 1**, “Sulfidogens” are bacteria that reduce sulfur and produce sulfide. “Biodeg,” abbreviated from biodegradation, refers to bacteria utilizing substrates or compounds that cannot be used by most of the other bacteria. “HC” means hydrocarbon. The metagenomics data reveal that *Bacillus* sp., *Bacteroides* sp., *Gardiella* sp., *Salmonella enterica*, *Soehngenia* sp., and *Tepidibacter* sp. were detected in Consortium I. Consortium II contained all these species, except *Salmonella enterica* and *Soehngenia* sp. The MIC caused by *Bacillus* sp. were studied since 1990s (Jack et al., 1992). *Bacteroides* sp. are normally found in mammals (Huang et al., 2011). *Bacteroides* sp. secret organic acids which are able to cause Type II MIC (Zhu et al., 2003). *Gardiella* sp. and *Soehngenia* sp. were collected from

the oil and gas field (Miranda-Tello et al., 2003; Kano et al., 2009; Naranjo-Briceño et al., 2012). *Gardiella* sp. may cause MIC by producing hydrogen sulfide from the thiosulfate reduction (Miranda-Tello et al., 2003). *Salmonella* sp. and *Soehngenia* sp. are related to MIC because they produce hydrogen sulfide in their metabolisms (Barrett and Clark, 1987; Parshina et al., 2003). *Tepidibacter* sp. were found in oil fields (Tan et al., 2012).

## Individual D-Amino Acids as Biocide Enhancers in the Mitigation of Field Biofilm Consortia

In our previous tests, the cocktails of 50 ppm THPS + 100 ppm D-met and 50 ppm THPS + 1 ppm D-tyr both achieved 5 log reduction of the SRB sessile cell count against *D. vulgaris* on carbon steel coupons (Xu et al., 2012a, 2014). However, the data in **Table 2** illustrate that in the biofilm prevention tests against Consortia I and II, the same cocktails only achieved 1 or 2 log reduction of the SRB sessile cell count, much less than 5 for *D. vulgaris*. The data presented in **Table 2**



**FIGURE 7 |** SEM images of Consortium II biofilm after 3-h treatment in PBS buffer containing: (A) no treatment chemical (control), (B) 50 ppm THPS, (C) 100 ppm D-mix, and (D) 50 ppm THPS + 50 ppm D-mix in biofilm removal test. The scale bar in the inserted small images is 50 µm.

also show that increasing D-tyr from 1 to 10 ppm did not improve the outcome. All these data suggest that the field biofilm consortia were much more recalcitrant than the pure-strain *D. vulgaris*.

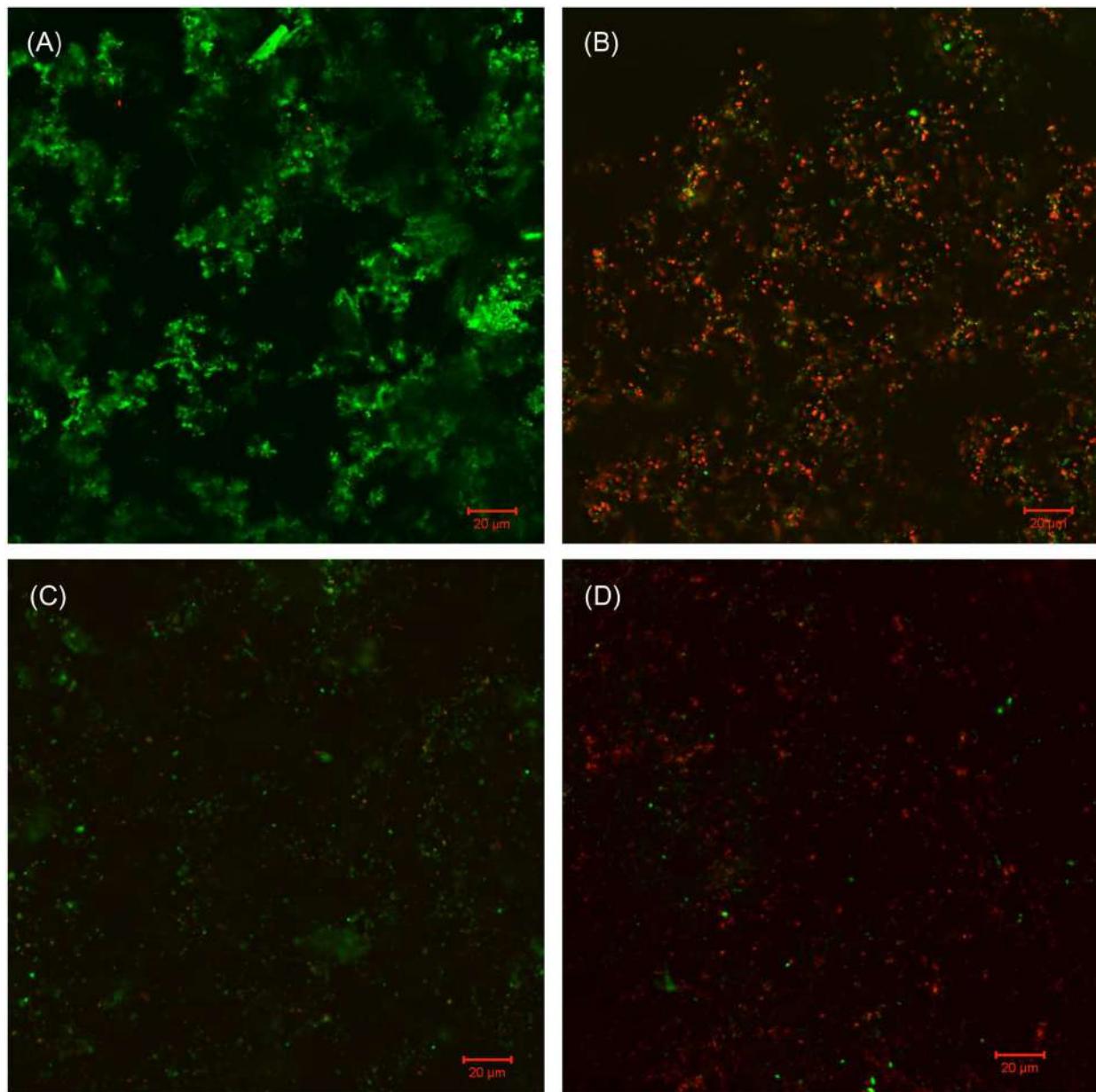
### D-Amino Acid Mixture as Biocide Enhancer Against Consortium I

For the mitigation of the biofilm Consortium I, **Table 3** data reveal that 50 ppm D-mix enhanced 50 ppm THPS in both biofilm prevention and biofilm removal tests. In the biofilm prevention test, 50 ppm THPS without enhancement did not reduce the SRB sessile cell count on the coupon surface compared with the control without treatment. While the treatment with 500 ppm D-mix without THPS achieved 2 log reduction of the SRB sessile cell count. The data in **Table 3** show that the combination of 50 ppm THPS + 50 ppm D-mix was able to achieve 4 log reduction of the SRB sessile cell count. This demonstrated that the 50 ppm D-mix enhanced 50 ppm THPS in the biofilm prevention test for biofilm Consortium I considerably.

The SEM images of the biofilm Consortium I after a 7-day biofilm prevention test in **Figure 2** are consistent with the results in **Table 3**. Different cell shapes in the biofilm image confirmed

that this was a mixed-culture biofilm. Sessile cells were easily found on coupons treated with either 50 ppm THPS or 500 ppm D-mix alone. However, with the 50 ppm THPS + 50 ppm D-mix cocktail treatment, the amount of sessile cells was much less than the amount of sessile cells after treatment with either 50 ppm THPS or 500 ppm D-mix. The CLSM images support the cell count data and are consistent with the SEM images. On the control coupon surface in **Figure 3A**, abundant living cells can be seen. In the treatments of 50 ppm THPS alone and 500 ppm D-mix alone in **Figures 3B,C**, dead cells as are seen but living cells are far more abundant. In **Figure 3D**, the amounts of both living and dead cells are much less and most cells were dead cells. It should be noted that although sessile cells were found on the SEM image in **Figure 2D**, they were dead cells that had not yet detached from the coupon according to the CLSM image in **Figure 3D**.

Similar efficacies were obtained in the biofilm removal test for biofilm Consortium I. The data provided in **Table 4** illustrate that the separate applications of 50 ppm THPS and 500 ppm D-mix had limited effects on Consortium I. It was found that 50 ppm THPS achieved no reduction of the SRB sessile cell count and 500 ppm D-mix achieved only 2 log reduction of the SRB sessile cell count. While the cocktail of 50 ppm THPS + 50 ppm D-mix

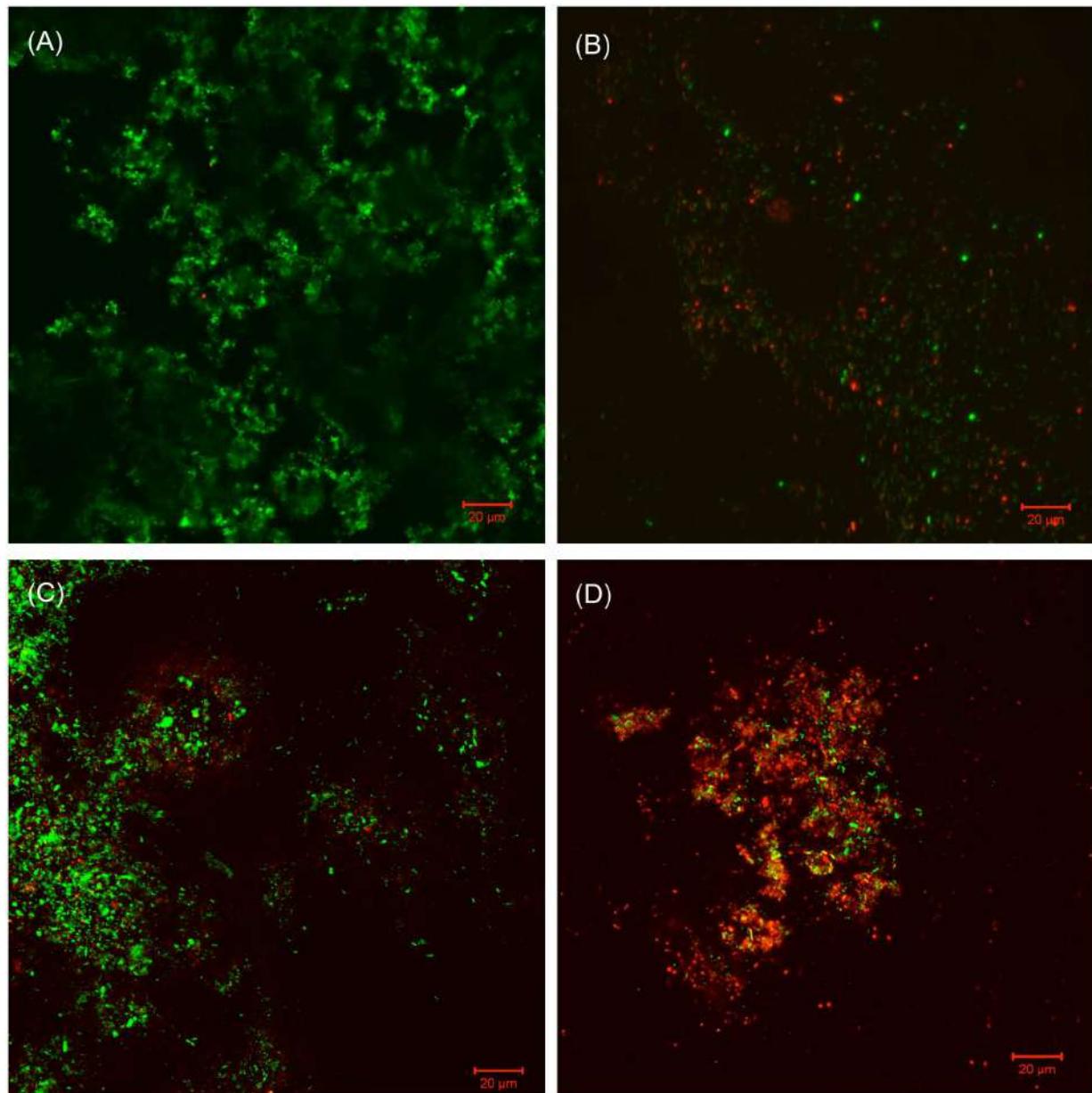


**FIGURE 8 |** CLSM images of Consortium II biofilm after 7-day incubation in the biofilm prevention test with: (A) no treatment chemical (control), (B) 50 ppm THPS, (C) 50 ppm D-mix, and (D) 50 ppm THPS + 50 ppm D-mix in the biofilm prevention test.

provided a 4 log reduction of the SRB sessile cell count after the 3-h biofilm removal test and the SEM images support the reduction of the sessile cells (**Figure 4**). **Figure 4** shows that sessile cells were present in the biofilm of Consortium I after treatment with either 50 ppm THPS or 100 ppm D-mix alone. The same trend was observed in the CLSM images (**Figure 5**) showing that more living cells were found on the control coupon surface than on the coupon surfaces treated with 50 ppm THPS and 50 ppm D-mix alone. On the coupon surface treated with 50 ppm THPS + 50 ppm D-mix, more dead cells and less living cells were found than on the coupon surface in the other three treatments.

## D-Amino Acid Mixture as Biocide Enhancer Against Consortium II

Similar treatment outcomes were obtained for Consortium II. It was found that 50 ppm D-mix enhanced 50 ppm THPS in both biofilm prevention and biofilm removal tests. Comparable to the mitigation of Consortium I, 50 ppm THPS alone had no effect on the SRB sessile cell count in both biofilm prevention and biofilm removal tests for Consortium II compared with the untreated control (**Tables 5 and 6**). The cocktail of 50 ppm THPS + 50 ppm D-mix achieved 3 log reduction of the SRB



**FIGURE 9 |** CLSM images of Consortium II biofilm after 3-h treatment in PBS buffer containing: (A) no treatment chemical (control), (B) 50 ppm THPS, (C) 50 ppm D-mix, and (D) 50 ppm THPS + 50 ppm D-mix in the biofilm removal test.

sessile cell count in both biofilm prevention and biofilm removal tests for biofilm Consortium II. In **Figures 6** and **7**, sessile cells are easily seen on the following: the control coupon (no treatment), the coupon treated with 50 ppm THPS, and the coupon treated with 500 ppm D-mix. Although sessile cells are still noticeable on the surface of coupons treated with 50 ppm THPS + 50 ppm D-mix, they are less abundant. Moreover, according to the CLSM images in **Figures 8** and **9**, on the coupon surfaces treated with 50 ppm THPS + 50 ppm D-mix (**Figures 8D** and **9D**), most cells were dead cells. The data suggest that the binary combination of 50 ppm THPS and 50 ppm D-mix

had an excellent efficacy against Consortium II owing to the enhancement of the D-mix.

## DISCUSSION

The exact mechanism of how D-amino acids enhance biofilm mitigation is not completely understood. It was hypothesized that D-amino acids trigger biofilm disassembly by replacing the D-alanine terminus in peptidoglycans that exist in all bacterial cell walls (Kolodkin-Gal et al., 2010). Kolodkin-Gal et al. (2010)

and Xu et al. (2014) found that adding a high concentration of D-alanine (D-ala) rendered D-met ineffective. It was also suggested that D-amino acids may modify the synthesis of peptidoglycans (Lam et al., 2009). Cava et al. (2011) suggested that D-amino acids were necessary in the remodeling of the cell wall structure. Leiman et al. (2013) pointed out that D-tyr, D-leu, and D-trp inhibited the formation of the *B. subtilis* biofilm because these D-amino acids interfered with protein synthesis. Their data suggested that D-tyr acted as a growth inhibitor toward *B. subtilis*. It is highly possible that specific D-amino acids are effective for specific bacteria. Sarkar and Pires (2015) tested millimolar concentrations of several D-amino acids against some bacteria and found no inhibition of biofilm formation. This is consistent with the view proposed in Xu et al.'s (2012a,b, 2014) work that a biocide stress is needed.

It is not a surprise that the combination of THPS and D-amino acid mixtures showed a synergistic effect in the mitigation of field biofilm consortia. The synergy between biocides and other agents, such as the injection of nitrate, has been demonstrated in the mitigation of reservoir souring (Xue and Voordouw, 2015). The results above proved the hypothesis that a D-amino acid mixture instead of an individual D-amino acid would be needed in the mitigation of a biofilm consortium. The cocktail of 50 ppm THPS + 50 ppm D-mix achieved a better efficacy (1 log more reduction) for Consortium I than for Consortium II, suggesting that biofilm Consortium II might be more recalcitrant. It was also possible that a different D-amino acid mixture may work better for Consortium II. Different microbial species in the biofilm consortia probably required different D-amino acids as the biofilm dispersal factor. It is reasonable that in the mitigation of other biofilm consortia, the particular D-amino acid mixture in this work may need adjustments. Optimization is likely needed to decide a cost-efficient D-amino acid mixture that offers a good efficacy.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Hospital Effluents Are One of Several Sources of Metal, Antibiotic Resistance Genes, and Bacterial Markers Disseminated in Sub-Saharan Urban Rivers

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 27 January 2016

Accepted: 06 July 2016

Published: 22 July 2016

### Citation:

Laffite A, Kilunga PI, Kayembe JM,  
Devarajan N, Mulaji CK, Giuliani G,  
Slaveykova VI and Poté J (2016)

Hospital Effluents Are One of Several  
Sources of Metal, Antibiotic  
Resistance Genes, and Bacterial  
Markers Disseminated in Sub-Saharan  
Urban Rivers.

*Front. Microbiol.* 7:1128.

doi: 10.3389/fmicb.2016.01128

Data concerning the occurrence of emerging biological contaminants such as antibiotic resistance genes (ARGs) and fecal indicator bacteria (FIB) in aquatic environments in Sub-Saharan African countries is limited. On the other hand, antibiotic resistance remains a worldwide problem which may pose serious potential risks to human and animal health. Consequently, there is a growing number of reports concerning the prevalence and dissemination of these contaminants into various environmental compartments. Sediments provide the opportunity to reconstruct the pollution history and evaluate impacts so this study investigates the abundance and distribution of toxic metals, FIB, and ARGs released from hospital effluent wastewaters and their presence in river sediments receiving systems. ARGs (*bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, and *aadA*), total bacterial load, and selected bacterial species FIB [*Escherichia coli*, *Enterococcus* (ENT)] and species (Psd) were quantified by targeting species specific genes using quantitative PCR (qPCR) in total DNA extracted from the sediments recovered from 4 hospital outlet pipes (HOP) and their river receiving systems in the City of Kinshasa in the Democratic Republic of the Congo. The results highlight the great concentration of toxic metals in HOP, reaching the values (in mg kg<sup>-1</sup>) of 47.9 (Cr), 213.6 (Cu), 1434.4 (Zn), 2.6 (Cd), 281.5 (Pb), and 13.6 (Hg). The results also highlight the highest ( $P < 0.05$ ) values of 16S rRNA, FIB, and ARGs copy numbers in all sampling sites including upstream (control site), discharge point, and downstream of receiving rivers, indicating that the hospital effluent water is not an exclusive source of the biological contaminants entering the urban rivers. Significant correlation were observed between (i) all analyzed ARGs and total bacterial load (16S rRNA) 0.51 to 0.72 ( $p < 0.001$ ,  $n = 65$ ); (ii) ARGs (except *bla*<sub>TEM</sub>) and FIB and Psd 0.57 <  $r$  < 0.82 ( $p < 0.001$ ,  $n = 65$ ); and (iii) ARGs (except

*bla<sub>TEM</sub>*) and toxic metals (Cd, Cr, Cu, and Zn) 0.44 to 0.72, ( $p < 0.001$ ,  $n = 65$ ). These findings demonstrate that several sources including hospital and urban wastewaters contribute to the spread of toxic metals and biological emerging contaminants in aquatic ecosystems.

**Keywords:** hospital and urban wastewater, water pollution, sediment receiving system, toxic metals, antibiotic resistance genes, fecal indicator bacteria, Sub-Saharan Africa, tropical conditions

## INTRODUCTION

Contamination of freshwater resources with anthropogenic pollutants is a growing concern of interest because safe and readily available water is needed for drinking, domestic use, food production, and recreational purposes (WHO, 2015). Freshwater resource pollution by various contaminants including toxic metals, persistent organic pollutants, pathogenic organisms, antibiotic resistant bacteria (ARB), and antibiotic resistant genes (ARGs) is still a major problem in many parts of the world (Poté et al., 2008; Knapp et al., 2012; Bréchet et al., 2014; Czekalski et al., 2014; Devarajan et al., 2015b). The situation is particularly alarming in developing regions such as in Sub-Saharan Africa where most rivers, lakes, and lagoons are receiving untreated hospital and industrial effluent water, mining effluents, and urban storm water runoff affected by anthropogenic pollutants due to intensive and uncontrolled urbanization (Feng et al., 2004; Chatterjee et al., 2007; Gnandi et al., 2011; Atibu et al., 2013; Mwanamoki et al., 2014, 2015).

Hospital effluents are a particular case of anthropogenic pollutants. Indeed, hospital wastewaters are complex mixtures of chemical and biological substances which are continually discharged (Barcelo and Barceló, 2003; Boillot et al., 2008; Verlicchi et al., 2010). This mixture is the result of diagnostic laboratory and research activity waste and medicine excretion which include active principles from medicinal products and their metabolites, chemicals, disinfecting agents, specific detergents, radioactive markers, iodinated contrast media, nutrients, and bacteria and their antimicrobial resistance genes (Verlicchi et al., 2010). Particularly studied among these hospital contaminants are bacteria and their antimicrobial resistance genes because of their great ability to disseminate and their clinical and financial impact (Davies et al., 1995; Cosgrove, 2006). The remarkable effectiveness of antibiotics has reduced mortality linked to bacterial diseases in only a few decades but their over- and mis-use has rapidly lead to a dramatic exponential increase in antibiotic resistance and multidrug-resistant bacteria throughout the world (Davies and Davies, 2010; Fair and Tor, 2014). The speed with which resistance has spread is explained by acquired resistance. In contrast to chromosomal resistance which is responsible for resistance against an antibiotic or an antibiotic class, acquired resistance by genetic material acquisition may be responsible for resistance against many antibiotics or antibiotic classes (INSERM, 2013). This resistance is harbored by many human and animal, pathogenic, and potentially pathogenic bacteria and can easily be spread by either conjugation, transformation, or transduction of resistance genes which are generally located on mobile elements (plasmids,

transposons, integrons; Carattoli, 2009; Cambray et al., 2010; Aminov, 2011). It is well-known that both metals and antibiotic resistance genes are located on the same mobile elements, leading to a co-selection of ARGs by metals (Baker-Austin et al., 2006; Seiler and Berendonk, 2012). Metal contaminations are widely spread in anthropogenic environment contributing on ARGs propagations (Sakan et al., 2009; Ji et al., 2012; Seiler and Berendonk, 2012).

The question of the environmental and human risks of an increasing release of bacteria carrying ARGs into the natural environment has been a subject of intense scientific and political debate in recent years. Consequently, there are a growing number of reports concerning the prevalence and dissemination of ARBs and ARGs into various environmental compartments (e.g., Kümmerer, 2004; Martínez, 2008; CDC, 2013; WHO, 2014; Devarajan et al., 2015a). Effluents from hospitals, industry, municipal organizations, and urban/agricultural runoff in many developing countries represent a significant source of emerging contaminants (metals, ARGs, ARB) in the receiving environment as the effluents are discharged into sewer systems, rivers, lakes, and seas without prior treatment which may then accumulate in sediments (Spindler et al., 2012; Mwanamoki et al., 2014; Devarajan et al., 2015a). Rivers and lakes are considered to be putative reservoirs of emerging contaminants (medicinal products, metals, ARGs) since they collect wastewaters containing various contaminants from various origins (Kümmerer, 2004; Poté et al., 2008; Allen et al., 2010). Furthermore, the sediments may accumulate 100 to 1000 times as many heavy metals, FIB and ARGs as the overlying water (e.g., Poté et al., 2008; Haller et al., 2009; Thevenon et al., 2012a,b; Mubedi et al., 2013; Mwanamoki et al., 2014, 2015; Devarajan et al., 2015a,b) and offer the opportunity for reconstructing the pollution history and evaluating the impacts.

Many studies have been performed to quantify ARB and ARGs in different environmental compartments around the world and explained the role of aquatic ecosystems as reservoirs of antibiotic resistance (e.g., Schwartz et al., 2003; Kümmerer, 2004; Levy and Marshall, 2004; Martínez, 2008; Stoll et al., 2012; Marti et al., 2014; WHO, 2014). Most of these studies formulated recommendations and hypotheses including the suggestion of further researches in different regions according to the source of drinking and recreational water, the practice of wastewater management, economic situation and sociocultural aspects of population, and climatic conditions. Nevertheless, many studies have not considered the influence of tropical conditions (e.g., in developing nations such as Sub-Saharan African countries) on the accumulation of these emerging contaminants in aquatic environment, which can vary considerably with developed

countries (under temperate conditions; e.g., Czekalski et al., 2014; Devarajan et al., 2015a). Consequently, little data are available on the assessment of heavy metals and neither there is no much information found regarding quantitative and qualitative aspects of ARB as well as ARGs in the aquatic environment under tropical conditions, which have an average daily peak temperatures reaching 30°C. Measures to reduce the potential human and environmental risks caused by hazardous substances (such as toxic metals), ARB, and ARGs include their characterizations and selection of target ARB and ARG, identification of potential sources as well as risk assessment, the development of reliable surveillance and risk assessment procedures, and finally, the implementation of technological solutions that can prevent environmental contamination with ARB and ARGs (WHO, 2014; Berendonk et al., 2015; Devarajan et al., 2015a).

The aim of the research presented in this paper is to assess the role of untreated hospital effluents discharged into freshwater receiving system under tropical conditions. This assessment was based on: (i) sediment physicochemical characterization including sediment grain size, total organic matter (OM; loss on ignition), and toxic metals including Cr, Co, Ni, Cu, Zn, As, Cd, Pb, and Hg - (ii) quantitative polymerase chain reaction (qPCR) on ARGs (*blaTEM*, *blaCTX-M*, *blaSHV*, and *aadA*), total bacterial load, and selected bacterial marker genes of fecal indicator bacteria [FIB; *E. coli* and *Enterococcus* (ENT)] and *Pseudomonas* species (Psd). To the knowledge of the authors this is first report on the accumulation of emerging microbial contaminants in the sediments of freshwater receiving systems in a central African region and specifically in the city of Kinshasa, the capital of the Democratic Republic of the Congo. Nevertheless, it should be noted that, one may argue that studies that analyze the DNA such as this study, while providing information on the presence/absence or even quantitative data but do not provide information on the expression of these ARGs (Lachmayr et al., 2009). However, the expression of genes is not the central query of this study when the purpose of this study is to address the evaluation of the tropical aquatic environment to serve as reservoirs of heavy metals and ARGs (that could be potentially transferred to other bacterial cells through horizontal gene transfer; Devarajan et al., 2016). The parameters analyzed were correlated in order to identify the potential sources of receiving system contamination.

## MATERIALS AND METHODS

### Study Site and Sampling

Kinshasa, the capital and largest city in the Democratic Republic of the Congo (DRC; **Figure 1**), is the 27th largest urban area in the world with 11,587,000 inhabitants and covering 9'965 km<sup>2</sup>. The climate is classified as humid and dry with an average temperature of 21–30°C. The city has ~20 hospitals and various medical centers and polyclinics with different intrinsic characteristics (size, type of services, medical practices...). The wastewater effluents are discharged from the big hospitals into drainage systems and ejected into the urban river receiving systems without prior treatment. For small hospitals and many

medical centers, wastewaters are directly rejected onto the soil, septic tanks, wells, or into rivers, and even using buckets. The selection of hospitals was done on the basis of the hospital size relatively to the practice of wastewater management as described above, and the hospital location in relation with the river receiving system. Three rivers receiving hospital effluent waters were selected for this study. They are affected by one very large hospital and three recent and innovative hospitals. No industry is located near the hospitals selected.

The sampling took place in December 2014. The surface sediments (0–4 cm layer) were collected from (i) outlet pipes (HOP) of the 4 hospitals selected, labeled H1, H2, H3, and H4. The collection points were adjacent to the hospital effluent outlet pipe before discharge into rivers. This sampling point is named E, (ii) at the HOP discharge points into the rivers (point named RP), (iii) in the rivers 50 m upstream from HOP discharge points (point named US), and (iv) in the rivers 50 m downstream from HOP discharge points (point named DS). Approximately 400–500 g of sediment were taken from each site in triplicate. All samples were stored in an icebox at 4°C until shipping (Mubedi et al., 2013; Mwanamoki et al., 2015; Devarajan et al., 2015b) and analyzed within 2 weeks.

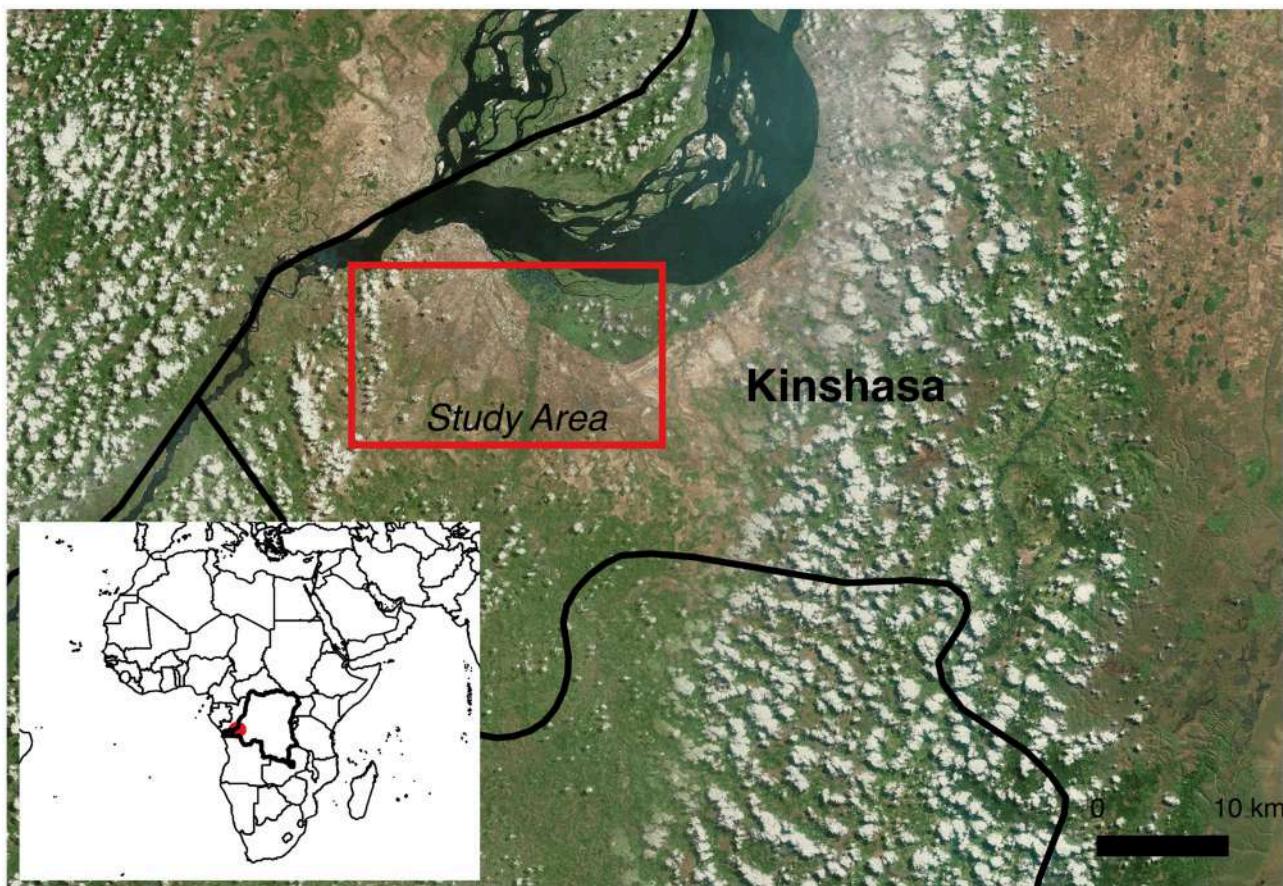
### Sediment Grain Size, Organic Matter, and Water Content

The sediment particle grain size was measured using a Laser Coulter® LS-100 diffractometer (Beckman Coulter, Fullerton, CA, USA), following 5 min ultrasonic dispersal in deionized water according to the method described by Loizeau et al. (1994). The sediment total organic matter (OM) content was estimated by loss on ignition at 550°C for 1 h in a Salvis oven (Salvis AG, Emmenbrücke, Lucerne, Switzerland). Sediment total water content was measured by drying the samples at 60°C for overnight and the weight loss was taken for the percentage of water content.

### Toxic Metal Analysis

Before being analyzed, sediment samples were lyophilized at –45°C after homogenization and air-drying at ambient room temperature. Toxic metals including Cr, Cu, Zn, Cd, and Pb were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent model 7700 series) following the digestion of sediments in Teflon bombs heated to 150°C in analytical grade 2M HNO<sub>3</sub> (Loizeau et al., 1994; Pardos et al., 2004; Poté et al., 2008). Multi-element standard solutions at different concentrations (0, 0.02, 1, 5, 20, 100, and 200 µg/L) were used for calibration. Total variation coefficients of triplicate sample measurements were under 5% and chemical blanks for the procedure were less than 2% of the sample signal. The metal concentrations of sediments were expressed in ppm (mg kg<sup>-1</sup> dry weight sediment).

Total Hg analysis was carried out using the Atomic Absorption Spectrophotometer (AAS) for mercury determination (Advanced Mercury Analyser; AMA 254, Altec s.r.l., Czech Rep.) following the method described by Hall and Pelchat (1997) and Roos-Barraclough et al. (2002). The method is based on sample combustion, gold amalgamation, and



**FIGURE 1 |** Localization of the sampling site in the province of Kinshasa, Republic Democratic of Congo (Adapted from Google Maps).

AAS. The detection limit (3 SD blank) was  $0.005 \text{ mg kg}^{-1}$  and the reproducibility better than 2%.

### Total DNA Extraction

Total DNA from sediment samples was extracted using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) according to manufacturer's instructions. DNA extraction was performed with three replicate sample (from the same sediment sample) to compensate for heterogeneity. The concentration of extracted DNA was measured using a Qubit Fluorimeter (Life Technologies Europe B.V., Zug, Switzerland). The isolated DNA was stored at  $-20^{\circ}\text{C}$  until used.

### qPCR Quantification of Selected Genes in Sediments: 16S rRNA, ARGs, and FIB

Quantification of ARGs (*blaTEM*, *blaCTX-M*, *blasHV*, and *aadA*), bacterial gene markers for *E. coli*, *Enterococcus* and *Pseudomonas* species, and 16S rRNA genes by qPCR was performed [qPCR reactions, control plasmids, calculation for absolute gene copy numbers (gene concentration) and the gene copy numbers normalized to 16S rRNA (abundance)] as previously described by Devarajan et al. (2015a). Briefly; Genes were quantified with Eco qPCR system (Illumina, Switzerland) using KAPA SYBR®

FAST qPCR Master Mix Universal Kit (KAPA Biosystems, USA). The primer sequences and reaction conditions are provided in Table 1. The following cycling parameters were applied: 10 min at  $95^{\circ}\text{C}$  for the polymerase activation; followed by 40 cycles of  $95^{\circ}\text{C}$  for 30 s, optimal Tm for 30 s and  $72^{\circ}\text{C}$  for 30 s. The temperature melting curve profile was obtained using the following conditions;  $95^{\circ}\text{C}$  for 30 s, optimal Tm for 30 s, followed by  $95^{\circ}\text{C}$  for 30 s.

All the reactions included negative (with no template DNA) and positive controls (10-fold serial dilutions of pGEM-T plasmid with respective target gene insert). All negative controls resulted either in no amplification or a threshold cycle (Ct) higher than the most diluted standard (pGEM-T plasmid). A sample was considered to be below the limit of detection (LOD) or negative for a target gene if  $\geq 2$  out of 3 technical replicates were negative or if sample Ct values were  $\geq$ Ct of negative controls. Samples above LOD were considered to be below the limit of quantification when the standard deviation of Ct values of methodological triplicates was 40.5 and their Ct value was higher than the Ct of the most diluted standard whose standard deviation of Ct values was  $\leq 0.5$ . For each reaction the efficiency of the assay was measured using the slope of the standard curve measures ( $E = 10^{[-1/\text{slope}]} - 1$ ). The absolute copy number of

**TABLE 1 |** Primers used in this study.

Target organism/gene	Primer	Oligonucleotide sequence (5'-3')	Tm (°C)	Size (bp)	References
Bacterial 16S rRNA	338F	ACTCCTACGGGAGGCAGCAG	55	197	Ovreås et al., 1997
	518R	ATTACCGGGCTGCTGG			
<i>E. coli</i> ( <i>uidA</i> )	Uida405F	CAACGAACCTGAACTGGCAGA	55	121	Chern et al., 2011
	Uida405R	CATTACGCTGCGATGGAT			
ENT (16S rRNA)	Ent376F	GGACGMAAGTCTGACCGA	55	221	Ram et al., 2004
	Ent578R	TTAAGAAACCACCTGC			
<i>Pseudomonas</i> spp.	Pse435F	ACTTTAACGGGAGGAAGGG	55	251	Bergmark et al., 2012
	Pse435R	ACACAGGAAATTCCACCA			
<i>bla</i> <sub>TEM</sub>	TEM-RT-F	GCKGCCAACTTACTTCTGACACG	55	247	Sidrach Cardona et al., 2014
	TEM-RT-R	CTTTATCCGCCTCCATCCAGTCTA			
<i>bla</i> <sub>CTX-M</sub>	<i>bla</i> CTX-M-rt-f	ATTCCRGCGAYCCGCGTGATACC	62	227	Fujita et al., 2011
	<i>bla</i> CTX-M-rt-r	ACCGCGATATCGTTGGTGGTGCAT			
<i>bla</i> <sub>SHV</sub>	<i>bla</i> SHV-rt-f	CGCTTTOCCATGATGAGCACCTT	60	110	Xi et al., 2009
	<i>bla</i> SHV-rt-r	TCCTGCTGGCGATAGTGGATCTT			
<i>aadA</i>	<i>aadA</i> -F	GCAGCGCAATGACATTCTTG	55	282	Madsen et al., 2000
	<i>aadA</i> -R	ATCCTCGCGCGATTTC			

each reaction was quantified by referring to the corresponding standard curve obtained by plotting the copy number of the constructed pGEM-T plasmid vs. threshold cycles. The serial 10-fold dilutions of plasmid DNA containing the respective target gene copies were used for the standard curve. To emphasize the relative abundance of the resistance genes the concentrations of the gene copy numbers were presented as percentage of “copy number of a gene/copy number of 16S rRNA” for each sample.

## Data Analysis

The 16S rRNA (total bacterial load), FIB and the selected marker genes and ARGs in the samples are expressed as “gene copy numbers” in per gram of dry sediment weight normalized to the DNA extraction yield. The “relative abundance” of the selected genetic marker genes (normalized to 16S rRNA) were emphasized by the ratio = (copy number of a gene) / (copy number of 16S rRNA) for each sample (Czekalski et al., 2014; Devarajan et al., 2015a). A statistical treatment of data; Correlation matrix (Pearson), Principal component analysis (PCA) and its extensions to between (BGA), and within groups (WGA) analyses (ade4 package in R) was used to analyze the grouping hospital/sampling point samples by monitoring quality variables (metal content, FIB abundance, ARGs, mean grain size and OM content). The statistical software was R version 3.2.2 (Team, 2015). Linear fixed model were fitted to the data using the functionality of the package lme4 (Bates et al., 2015). Average concentrations of gene copy numbers was modeled using the hospital and the sampling point as fixed effects and technical and biological effects as random effects. Significance of fixed effects was assessed by a *t*-test using a significance level of 5%.

Model checking was based residual plots and normal probability checking using the raw residuals. Models were reduced using the likelihood ratio test. Pairwise comparison were evaluated based on adjusted *p*-values obtained using single-step method (Hothorn et al., 2008).

## RESULTS AND DISCUSSION

### Sediment Physicochemical Parameters and Metal Content

Sediment characteristics including particle grain-size and total organic matter (OM) are presented in Table 2. The sediment grain size and the OM varied substantially internally within the sampling sites (*p* < 0.05). Surface sediments of rivers in all sites studied are generally sandy-silt. The maximum value of clay observed at sites H1, H2, and H3 was less than 3%. H4 presented the maximum value of clay (11%). The same distribution was observed for sediment OM content (*p* < 0.05). The values ranged from 15.0 to 46.2% (H1), 4.7–9.4% (H2), and 0.7–8.7%. No great difference in OM was observed in the sampling sites of H4 (8.3–8.5%). Previous studies have reported that there are large variations in the distribution of sediment OM and grain size in freshwater, lakes, rivers, and reservoirs. The OM in non-contaminated freshwater sediments varies from 0.1 to 6.0% (Poté et al., 2008; Haller et al., 2009; Mubedi et al., 2013). The sediment from all sites (US: upstream; E: exit (outlet); RP: reject point (outlet discharge) and DS: downstream) of H1 and H4 are contaminated by organic matter. These results support the hypothesis that hospital effluents are one of many sources

**TABLE 2 | Physico-chemical parameters of surface sediments from sampling points of each hospital site.**

Hospital	Sampling point <sup>b</sup>	Clay (%)	Silt (%)	Sand (%)	Mean grain size ( $\mu\text{m}$ )	OM <sup>c</sup> (%)
Hospital 1	US	3.21	27.46	69.33	46.57	15.03
	E	2.61	47.96	49.43	65.69	46.15
	RP	3.36	58.44	38.20	42.70	34.78
	DS	1.31	25.45	73.24	139.10	18.10
Hospital 2	US	0.41	14.00	85.59	157.20	6.05
	E	ND <sup>a</sup>	ND	ND	ND	ND
	RP	0.48	13.46	86.06	229.10	9.37
	DS	1.85	63.27	34.88	48.67	4.74
Hospital 3	US	0.13	10.66	89.21	226.00	0.75
	E	0.78	9.46	89.76	205.80	3.91
	RP	0.38	7.31	92.31	234.20	1.79
	DS	0.27	27.47	72.26	135.20	8.72
Hospital 4	US	5.68	38.71	55.61	52.67	8.33
	E	0.37	58.23	41.40	58.71	8.40
	RP	10.83	57.51	31.66	24.04	8.44
	DS	0.94	26.00	73.06	82.54	8.51

<sup>a</sup>ND: not determined.<sup>b</sup>US, upstream; E, exit; RP, reject point; DS, downstream.<sup>c</sup>OM, organic matter.

of contamination and that the contamination occurs at multiple points of entry along the river bank.

The results of the toxic metal analysis are reported in **Table 3**. The maximum concentration was observed in sediments at H1, reaching values (in  $\text{mg kg}^{-1}$ ) of 47.9 (Cr), 213.6 (Cu), 1434.4 (Zn), 2.6 (Cd), 274.2 (Pb), and 13.6 (Hg). Sewages and rivers, which receive the majority of drained urban wastewaters of the city of Kinshasa, are used by the local population as uncontrolled landfills for domestic solid wastes. This can explain the presence of high metal concentrations in sediment (Mavakala et al., 2016). As the studied rivers flow through the City of Kinshasa, additional pollutant sources such as domestic sewages, uncontrolled landfills and artisanal activities located in the banks of rivers can probably explain the presence of contaminants accumulation in sediment. However, the presence of other non-identified sources and untreated hospital effluent water discharge cannot be excluded. In general, the concentration of toxic metals at points E, RP, and DS are higher for all sampling sites than the upstream sampling points indicating the effect of hospital effluent waters on the contamination of rivers by toxic metals.

Metals accumulation in sediments is one of a good indicators for predicting the deterioration of a contaminated environment with inorganic pollutants. The release of heavy metals into the aquatic ecosystem can lead to the pollution of water resources and may place aquatic organisms and human health at risk. The main human and environmental risk is remobilization of the contaminants and their return to the hydrosphere either by sediment re-suspension or by infiltration into groundwater (Wildi et al., 2004; Poté et al., 2008).

The evaluation of the potential deleterious effects of the metals toward benthic fauna, which is based on sediment quality guidelines (SQG; CCME, 1999; MacDonald et al., 2000; Long et al., 2006) provides an estimate of the hazard that the sediments may represent to the local biota. The authors proposed a “Threshold effect concentration” (TEC) for specific metals which is the level above which an organism is affected (or responds) and below which it does not, and a “probable effect level” (PEL), a contaminant level that is likely to have an adverse effect on biota (Mwanamoki et al., 2014). According to the SQGs and PEL (**Table 3**), the concentration of Cr, Cu, Zn, Pb, and Hg in sediment may present a potential toxic effect on indigenous fauna and flora living in these aquatic environments. Furthermore, the high PEL values observed upstream of hospital effluent discharges indicate that hospital effluents are only one of several sources of contamination. Indeed, many anthropogenic activities (i.e., industrial, sewage, agricultural land field, mining...) are known to be responsible of heavy metal release in the environment (Poté et al., 2008; Lim et al., 2013; Devarajan et al., 2015b; Niane et al., 2015) and may be responsible of the high metal values observed upstream HOP effluents discharge.

## Abundance of Bacterial Population

The total bacterial load in sediment samples is presented in **Figure 2** (based on 16S rRNA gene copy numbers). In general, 16S rRNA gene copy numbers varied significantly between sampling sites ( $p < 0.05$ ) with values of (log copy numbers  $\text{g}^{-1}$  of dry sediment). The 16S rRNA gene copy numbers in samples from H1 were several orders of magnitude higher than those observed in others hospitals. However, these results are in conformity with those found in sediment from contaminated sediments receiving WWTP effluent waters (Devarajan et al., 2015a). Furthermore, the total bacterial load after the effluent discharge (RP) for H1 and H4 were respectively 10 and 7.85 times higher than the values observed in their respective control sites (US). In contrast, the total bacterial load in the points RP for H2 and H3 was 3.7 to 6.8 times lower than their respective control site (US), indicating the possible presence of other sources of contamination.

The average copy number of FIB bacterial marker genes including *E. coli*, ENT, and Psd in the sediment samples is presented in **Figure 3**. The bacterial density varies considerably depending on the sampling point and the type of hospital. For example, the *E. coli* range at the US site (log copy numbers  $\text{g}^{-1}$  of dry sediment) was 6.44, 6.30, 6.67, and 4.21 for H1, H2, H3, and H4, respectively; ENT range (copy numbers  $\text{g}^{-1}$  of dry sediment) of 7.28, 7.59, 7.87, and 5.41 for H1, H2, H3, and H4, respectively; and Psd range (log copy numbers  $\text{g}^{-1}$  of dry sediment) was 6.96, 6.94, 7.10, and 5.49 for H1, H2, H3, and H4, respectively. The input of hospital wastewater in the receiving system lead to a 6, 1, and 288 times greater abundance of *E. coli* for H1, H2, and H4, respectively. In H3 RP sampling point, a 6.2 times decrease in *E. coli* abundance was measured after wastewater discharge showing that H3 wastewater did not contribute more than the environmental enrichment in *E. coli*.

Raw load of selected bacterial marker genes followed the 16S rRNA trend. To avoid inconstancies between qPCR assays,

**TABLE 3 | Metal content of surface sediment samples from sampling point of each hospital site, analyzed by ICP-MS (expressed in mg. kg<sup>-1</sup>).**

Hospital	Sampling point	Cr	Co	Ni	Cu	Zn	Cd	Pb	Hg
Hospital 1	US	34.32	4.29	14.24	<b>107.45</b>	<b>884.61</b>	<b>1.79</b>	<b>189.62</b>	<b>1.43</b>
	E	<b>45.54</b>	4.55	17.42	<b>213.59</b>	<b>1004.47</b>	<b>1.88</b>	<b>137.08</b>	<b>13.60</b>
	RP	<b>47.87</b>	4.97	17.42	<b>204.13</b>	<b>1077.17</b>	<b>2.07</b>	<b>124.40</b>	<b>3.94</b>
	DS	<b>47.58</b>	7.11	21.20	<b>184.67</b>	<b>1434.78</b>	<b>2.65</b>	<b>274.19</b>	<b>3.25</b>
Hospital 2	US	13.03	1.79	6.29	<b>59.18</b>	<b>410.69</b>	0.57	<b>86.67</b>	<b>0.64</b>
	E	ND	ND	ND	ND	ND	ND	ND	ND
	RP	11.96	1.81	13.48	34.18	<b>304.92</b>	0.54	<b>281.52</b>	<b>0.44</b>
	DS	10.43	1.61	5.67	<b>51.91</b>	<b>352.41</b>	0.46	<b>81.15</b>	<b>0.74</b>
Hospital 3	US	3.20	0.37	16.73	<b>46.06</b>	87.21	0.18	<b>36.38</b>	0.15
	E	7.81	0.83	14.63	22.08	<b>147.29</b>	0.29	34.48	<b>0.68</b>
	RP	4.18	0.44	13.57	8.86	68.27	0.08	15.82	<b>0.31</b>
	DS	4.97	0.62	3.79	22.65	<b>153.77</b>	0.22	<b>40.75</b>	<b>0.37</b>
Hospital 4	US	15.36	0.99	4.85	9.38	71.99	0.10	14.96	<b>0.39</b>
	E	27.92	2.68	9.98	<b>47.54</b>	<b>365.75</b>	0.52	<b>75.98</b>	<b>0.56</b>
	RP	21.91	1.20	5.96	12.66	98.75	0.16	20.98	<b>0.51</b>
	DS	15.23	1.13	5.10	12.86	100.30	0.14	20.73	<b>0.44</b>
	SQGs <sup>a</sup>	37.30			35.70	123.00	0.60	35.00	0.17
	PEL <sup>b</sup>	90.00			197.00	315.00	3.50	91.30	0.49

<sup>a</sup>Sediment quality guidelines (mg.kg<sup>-1</sup>).<sup>b</sup>Probable effect level (mg.kg<sup>-1</sup>).

In bold: values above SQGs.

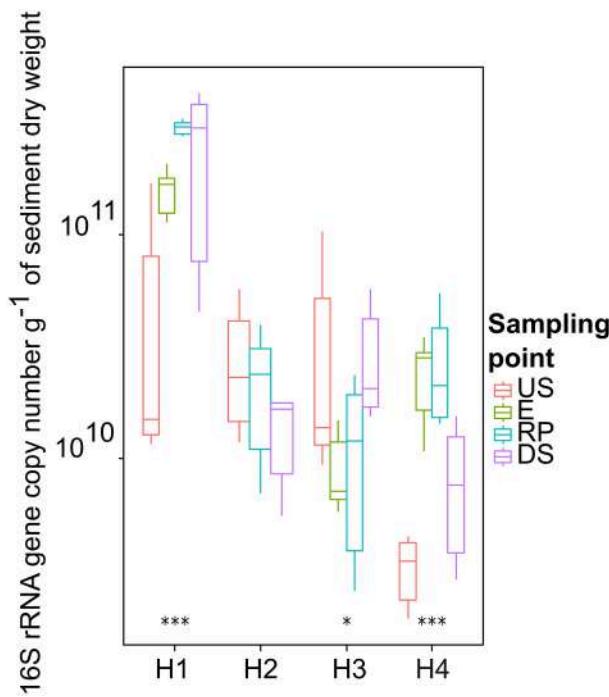
Cr, chromium; Co, cobalt; Ni, nickel; Cu, copper; Zn, zinc; Cd, cadmium; Pb, lead; Hg, mercury.

including suboptimal efficiencies, selected bacterial species marker genes and ARGs were normalized using 16S rRNA (**Figures 4, 6**). The greatest abundance of bacterial populations was recorded at H1 and H4 sampling sites (except *E. coli* for H1 and Psd for H4). H2 and H3 did not show any significant increase in bacterial marker genes abundance, which was also the case for 16S rRNA abundance ( $0.20 < p < 0.98$  for H3 and  $0.06 < p < 0.91$  for H3). The abundance of bacterial marker genes can be either accumulated or diluted depending on the river characteristics (depth, flow rate, turbulences). No uncultivable quantitative data is available for RDC aquatic systems but the high level of total bacteria and FIB in US sampling points is consistent with data from highly contaminated sediments (Devarajan et al., 2015a,b). Compared to other hospitals, the H1 sampling site had higher values for total bacterial load which could be explained by the larger size of the hospital and the shared drainage of urban and hospital wastewater. The three other hospitals are smaller than H1 which results in smaller amounts of contaminants in their effluents. In the case of H2, no canalization of hospital wastewater was used. The cumulative effect of no drainage system and the presence of wild discharge next to the US sampling point may explain why hospital effluents discharge showed no significant increase in FIB abundance in the receiving system (Orsi et al., 2007; Graham and Polizzotto, 2013). This effluent didn't contribute more to already environmental abundance and the observed decrease in FIB abundance may be due to contaminant dilution and transport downstream by river flow (Knapp et al., 2012; Chen et al., 2013).

FIB including *E. coli* and ENT are commonly used to assess microbial safety of aquatic systems. It is well-known that many *E. coli* and ENT are also responsible for numerous health care-associated infections of the bloodstream, urinary tract, and surgical incision sites (Alm et al., 2014). To develop this high level of pathogenicity, these bacteria have acquired islands of pathogenicity including antibiotic, heavy metal resistance genes, and virulence factors. Several studies in other parts of the world have revealed the presence of pathogenic micro-organisms which are multi-resistant to antibiotics in hospital effluents (Emmanuel et al., 2009). In the absence of wastewater treatment as is the case in the hospitals in the study presented in this paper, these bacteria will be discharged directly into aquatic receiving systems. It is well-known that FIB are able to survive and proliferate in sediments as sediments provide favorable conditions for proliferation and growth. (Poté et al., 2009). Furthermore, FIB have a great ability to acquire ARG (Levy and Marshall, 2004) and are able to transfer their resistance to autochthonous bacteria by HGT (Sidrach Cardona et al., 2014). Consequently, the discharge of raw hospital wastewater could lead to an environmental reservoir of clinical resistant bacteria and their associated genes developing in the sediment (Marti et al., 2014).

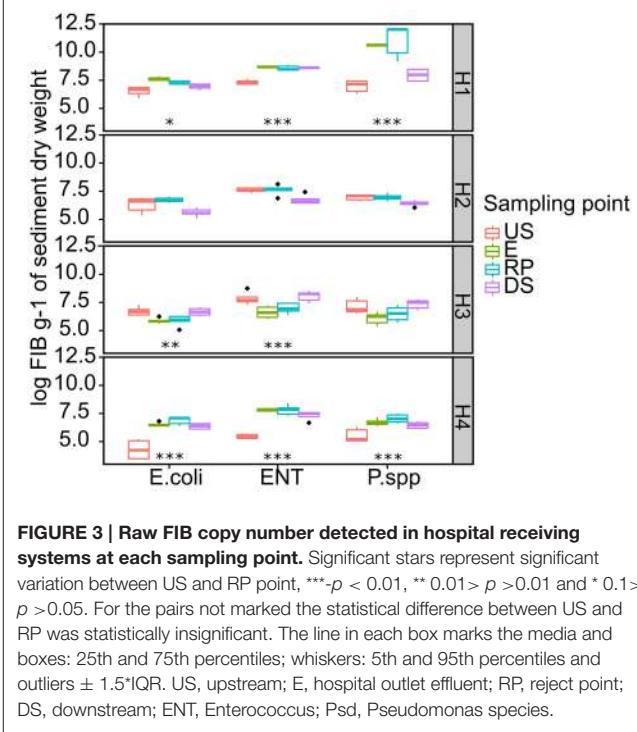
## Quantification of Antibiotic Resistance Genes

qPCR was performed to quantify the selected ARGs conferring resistance to  $\beta$ -lactam (*blaTEM*, *blaCTX-M*, and *blaSHV*) and aminoglycoside (*aadA*) in DNA extracted from the sediment

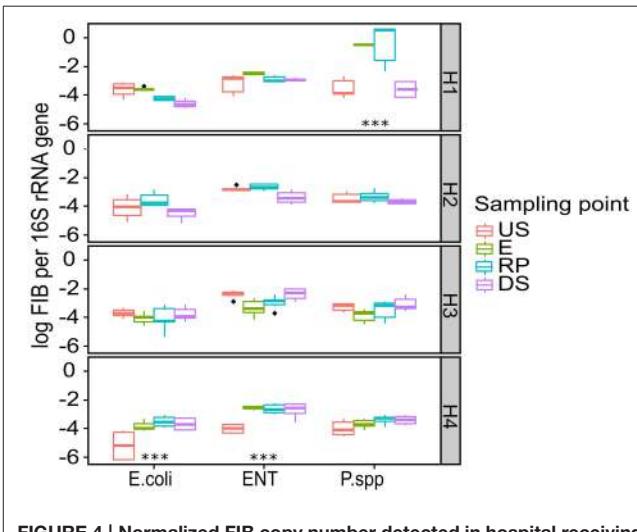


**FIGURE 2 | Raw 16S rRNA copy number detected in hospital receiving systems (16S rRNA gene copy number / g of DS) at each sampling point.** Significant stars represent significant variation between US and RP point, \*\*\*- $p < 0.01$ ; and \* $0.1 > p > 0.05$ . For the pairs not marked the statistical difference between US and RP was statistically insignificant. The line in each box marks the media and boxes: 25th and 75th percentiles; whiskers: 5th and 95th percentiles and outliers  $\pm 1.5 \times \text{IQR}$ . US, upstream; E, hospital outlet effluent; RP, reject point; DS, downstream.

samples. ARG selection was based on various criteria including (Devarajan et al., 2016): (i) clinically relevant genes (human risk); (ii) genes conferring resistance to frequently used antibiotics; (iii) ARGs previously reported in mobile genetic elements; and (iv) the antibiotics used in 6 pilot hospitals in Kinshasa (Nzolo et al., 2013). The raw gene copy number (ARGs  $\text{g}^{-1}$  of dry sediment) was used to estimate the general changes in ARGs level in receiving systems. The results are presented in Figure 5. The ARGs copy number (ARGs  $\text{g}^{-1}$  of dry sediment) for *aadA*, *bla<sub>CTX-M</sub>*, *bla<sub>SHV</sub>*, and *bla<sub>TEM</sub>* varied, respectively from 4.64 to 7.83, 4.67 to 5.01, 3.92 to 4.66, and 4.23 to 4.86 at US sites, and from 5.33 to 9.24, 4.55 to 5.61, 3.76 to 6.17, and 4.36 to 5.46 at RP/DS sampling sites. The great abundance of *bla<sub>TEM</sub>* and *aadA* genes at all sampling sites (US, E, RP, and DS) could be explained by their ubiquitous presence as housekeeping genes, which has previously been shown to occur frequently among soil bacteria as well as by their presence in sewage and effluent receiving systems (Demanèche et al., 2008; Thevenon et al., 2012a; Suzuki et al., 2015). A relevant increase in ARG level after wastewater discharge is only observed in H1 sediments ( $p < 0.05$ ) with a 25.6, 45.0, and 148 times increase observed between US and RP sampling points for *bla<sub>CTX-M</sub>*, *bla<sub>SHV</sub>*, and *aadA*, respectively. For the others hospitals, the general trend of ARGs increased after wastewater discharge was no longer observed. The abundance



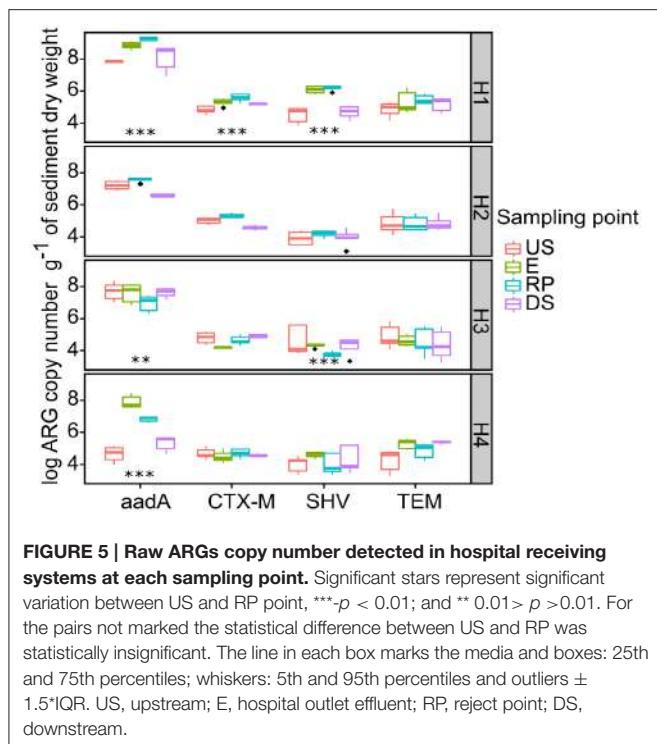
**FIGURE 3 | Raw FIB copy number detected in hospital receiving systems at each sampling point.** Significant stars represent significant variation between US and RP point, \*\*\*- $p < 0.01$ , \*\* $0.01 > p > 0.01$  and \* $0.1 > p > 0.05$ . For the pairs not marked the statistical difference between US and RP was statistically insignificant. The line in each box marks the media and boxes: 25th and 75th percentiles; whiskers: 5th and 95th percentiles and outliers  $\pm 1.5 \times \text{IQR}$ . US, upstream; E, hospital outlet effluent; RP, reject point; DS, downstream; ENT, Enterococcus; Psd, Pseudomonas species.



**FIGURE 4 | Normalized FIB copy number detected in hospital receiving systems at each sampling point.** Significant stars represent significant variation between US and RP point, \*\*\*- $p < 0.01$ . For the pairs not marked the statistical difference between US and RP was statistically insignificant. The line in each box marks the media and boxes: 25th and 75th percentiles; whiskers: 5th and 95th percentiles and outliers  $\pm 1.5 \times \text{IQR}$ . US, upstream; E, hospital outlet effluent; RP, reject point; DS, downstream; ENT, Enterococcus; Psd, Pseudomonas species.

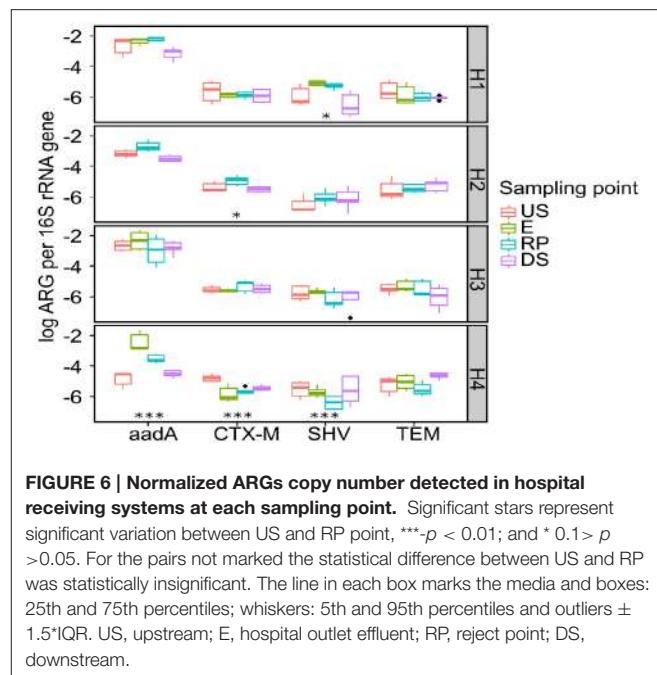
of ARGs and associated bacteria in a receiving system may vary depending on the size of the hospital and type of services.

The normalized/relative abundances of 16S rRNA were found in order to quantify relative change in ARGs abundances, that is, whether or not more or fewer ARGs appear per microbial genome (Laht et al., 2014). The relative abundance of ARGs in



the sediment samples is presented in **Figure 6**. In general, the influence of hospital effluents was not observed in the relative abundance of ARGs copy numbers in receiving systems. Some specific cases such as *aadA* in H4, *bla*<sub>CTX-M</sub> in H1, and *bla*<sub>CTX-M</sub> in H2 increased significantly after hospital effluent discharge ( $p < 0.01$ ). These data suggest that increases in ARGs abundances are directly related to wastewater discharge depending on hospital type and disposal practices, although unknown sources and/or causes also exist (Graham et al., 2011; Marti et al., 2014). The prevalence of microbial contaminants in the control sites (upstream) could be explained by, for example, input from major activities such as agricultural runoff, open defecation, urban discharge, and other anthropogenic activities along the river banks, which receives considerable amounts of wastewaters (Dekov et al., 1998; Tshibanda et al., 2014). The abundance of total bacterial load, ARGs, and FIB showed a relevant increase after wastewater discharge but the results highlight that hospital wastewater effluents are not the only source of micropollutant accumulation.

Interestingly, the relative abundances of ARGs observed in this study were greater than in other studies performed in a similar environment under tropical conditions (Graham et al., 2011; Devarajan et al., 2016) and can be compared to data obtained in industrialized countries (Devarajan et al., 2015a). However, the lack of background information and the knowledge gap in our pristine study site does not help in understanding the trends observed. ESBLs are mostly TEM, SHV, and CTX-M derivatives. The great abundance of clinically relevant ARGs such as *bla*<sub>CTX-M</sub> and *bla*<sub>SHV</sub> in the sediments studied may indicate the possible presence of ESBL in these systems (Poirel

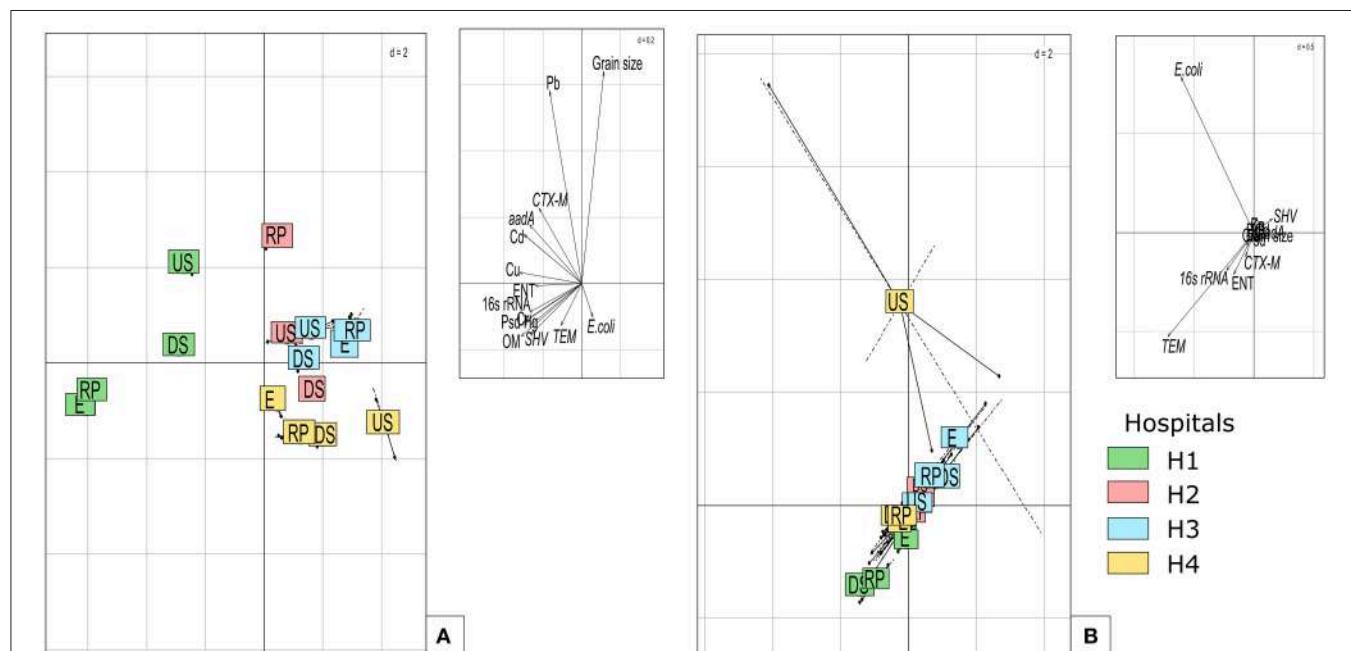


et al., 2012). The presence of ESBL in rivers (such as the sites studied) is highly alarming because the majority of ESBL are resistant to first line antibiotics but are also resistant to a large number of relevant antibiotics (Tacao et al., 2014). Furthermore, such a large abundance of ARGs represents a serious threat from resistance propagation because gene exchange can take place in sediment between both dead and living bacteria (Mao et al., 2014). It has been determined that approximately 90 bacterial species have natural transformability competences. Among them are many human pathogens, including the genera *Campylobacter*, *Haemophilus*, *Helicobacter*, *Nesseiria*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* (Mao et al., 2014). So the reservoir of ARGs in river sediments can be easily used by other bacteria to become ever more resistant.

The abundance of ARGs in the receiving systems reported in this study can be considered as alarming. Results indicate clearly that sediment receiving system under tropical condition can act as reservoirs of ARGs including *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, and *aadA*. It has been demonstrated that the intensive use of antibiotics for humans, animals, and agricultural purpose has led to the release (e.g., through the disposal of human and animal wastes) of ARB and ARGs into soil and aquatic environment (Martinez, 2008; Sommer et al., 2009). In the study region as well as in many Sub-Saharan African countries, there is no regulation for the use of antibiotics in humans, animals as well as for agricultural purpose, and data concerning the occurrence of ARGs and ARBs in aquatic environments is limited (WHO, 2014; Devarajan et al., 2016). Additionally, there is no policies and management tools to facilitate the urban wastewater treatment in study region. Thus, according to the results of this study, we strongly recommend the prudence and regulation for the use of antibiotics in humans and animals consumption, to limit spread

**TABLE 4 | Spearman's Rank-Order Correlation of selected parameters analyzed in sediment samples [n = 65, statistically significant coefficients ( $p < 0.05$ ) are in bold].**

	Cd	Cr	Cu	Grain size	Hg	16S rRNA	aadA	CTX-M	<i>E. coli</i>	ENT	Psd	SHV	TEM	OM	Pb	Zn
Cd	<b>0.90</b>	<b>0.95</b>	-0.24	0.64	0.59	0.66	<b>0.54</b>	-0.10	0.47	0.64	0.60	0.25	0.76	0.63	<b>0.99</b>	
Cr		<b>0.89</b>	-0.52	<b>0.66</b>	0.61	0.59	<b>0.44</b>	-0.05	<b>0.51</b>	0.67	0.67	0.35	0.83	0.43	<b>0.89</b>	
Cu			<b>-0.29</b>	<b>0.78</b>	<b>0.67</b>	<b>0.71</b>	<b>0.57</b>	-0.11	<b>0.54</b>	0.78	0.72	<b>0.25</b>	0.87	<b>0.49</b>	<b>0.95</b>	
Grain size				-0.26	-0.33	-0.01	0.03	-0.05	-0.20	<b>-0.29</b>	<b>-0.32</b>	<b>-0.33</b>	-0.41	0.23	-0.26	
Hg					<b>0.51</b>	<b>0.52</b>	<b>0.37</b>	-0.05	<b>0.46</b>	0.70	<b>0.63</b>	0.08	<b>0.89</b>	<b>0.25</b>	<b>0.63</b>	
16S						<b>0.72</b>	<b>0.67</b>	-0.19	<b>0.83</b>	<b>0.80</b>	<b>0.63</b>	<b>0.51</b>	<b>0.72</b>	0.24	<b>0.57</b>	
aadA							<b>0.58</b>	-0.36	<b>0.74</b>	<b>0.73</b>	<b>0.66</b>	0.18	<b>0.63</b>	<b>0.43</b>	<b>0.65</b>	
CTX-M								-0.11	<b>0.57</b>	<b>0.62</b>	<b>0.56</b>	<b>0.24</b>	<b>0.59</b>	<b>0.52</b>	<b>0.51</b>	
<i>E. coli</i>									<b>-0.34</b>	-0.17	-0.13	-0.04	-0.04	-0.12	-0.11	
ENT										<b>0.73</b>	<b>0.59</b>	<b>0.47</b>	<b>0.59</b>	<b>0.27</b>	<b>0.45</b>	
Psd											<b>0.82</b>	<b>0.26</b>	<b>0.86</b>	0.21	<b>0.61</b>	
SHV												0.15	<b>0.79</b>	0.19	<b>0.57</b>	
TEM													0.23	0.12	<b>0.25</b>	
OM															<b>0.32</b>	<b>0.74</b>
Pb																<b>0.63</b>
Zn																

**FIGURE 7 | Grouping of each sampling point according to ARGs, FIB, toxic metals, grain size, and OM. (A)** plot using between group analyses to discriminate each points in various hospital receiving system. **(B)** same samples plotted after decomposing differences in each sampling point by within group analysis. Right upper panels show correlation with variables (PCA variable scores).

of ARGs and ARB into the environment. Furthermore, the need of a strategy for hospital and urban wastewater treatment.

## Statistical Correlation

Correlation analysis between total bacterial load, FIB, ARGs, toxic metals, total organic matter, and sediment grain size was carried out to determine potential links between both parameters analyzed and possible origins of contaminants with the results

being presented in **Table 4**. Total organic matter content, metal concentrations, bacterial indicator genetic markers (except *E. coli*), and ARGs were mostly significantly, positively, and mutually correlated. Nevertheless, all ARGs studied at hospital and sampling points are significantly correlated with total bacterial load (16S rRNA): 0.51 to 0.72 ( $p < 0.001$ ,  $n = 65$ ). ARGs (except for *blaTEM*) have a positive correlation with *E. coli*, ENT, and Psd ( $0.57 < r < 0.82$ ,  $p < 0.05$ ,

$n = 65$ ) and the metals (Cd, Cr, Cu, Hg, and Zn): ( $0.37 < r < 0.71$ ,  $p < 0.001$ ,  $n = 65$ ). Strong positive and mutual correlation was observed between 16S rRNA, *E. coli*, ENT, and Psd with ARGs (except *blaTEM*). These results indicate that these biological contaminants could originate from common sources and they are carried to the receiving system by common transporters (Thevenon et al., 2012b; Devarajan et al., 2015a). In addition, there was a positive correlation between total organic content and the metals in sediments. This observation is also supported by the fact that the contaminants are attached to both large organic and small inorganic particles such as clay and they could behave in a similar way in transporting contaminants to the receiving system (Poté et al., 2008; Zhao et al., 2015).

BGA on PCA analysis (**Figure 7**) showed that sampling points varied greatly between hospital sites revealing the impact of (i) initial background level in ARGs, heavy metals, and FIB and (ii) the hospital type (**Figure 7-left**). To analyze effluent effect independently of the hospital, sampling points were decomposed before fitting (WGA on PCA using hospital as “within group” factor) (**Figure 7-right**). The results show a large increase in ARGs, FIB, toxic metals, and OM for hospital H1 and H4 linked to wastewater discharge. Lastly, these results indicate that river receiving systems could depend on hospital practices but also that river sediments are already significantly contaminated by unknown sources. As the rivers flow through the City of Kinshasa, additional pollutant sources such as domestic sewage, uncontrolled landfill and artisanal activities located in the banks of rivers can probably explain the presence of contaminants accumulation in sediment (Mubedi et al., 2013; Ngelinkoto et al., 2014; Mwanamoki et al., 2015).

## CONCLUSION

The research presented in this paper investigates the abundance and dissemination of metal, FIB and ARGs released from hospital effluents into the urban river receiving systems. It's important to note that one of the main concerns of this research consists on the evaluation of the degree to which river receiving system under tropical conditions can act as reservoir of metals, FIB and ARGs. Results demonstrate accumulation of toxic metals, *E. coli*, *Enterococcus*, and *Pseudomonas* species as well as ARGs in sediment, indicating that river receiving systems under tropical conditions (developing countries such as our study region) can act as a reservoir for metals and emerging microbial contaminants such as FIB and ARGs which can be transferred to human pathogens. Thus, the river receiving systems under tropical conditions which has average daily peak temperatures reaching  $30^{\circ}\text{C}$ , could potentially favor the transfer of mobile genetic elements carrying ARGs to susceptible bacterial pathogens. On the other hand, the presence of higher values of FIB and ARGs in sediment samples

located upstream of the hospital outlet discharges (control sites) indicates that the hospital effluent wastewaters are not the only source of deterioration of bacteriological quality of studied rivers. The pollution in the cases studied in this paper may be explained by probable multiple diffuse pollution sources including open defecation, uncontrolled landfills, unregulated effluent discharges, and inadequate sewage collection near the sites studied.

Rivers in most developing nations (such as our study region) serve as a basic network for human and animal consumption as well as irrigation for fresh urban produces. High values of metals, FIB and ARGs observed in river receiving systems indicate the human and environmental potential risks. However, further studies are required to find the pathways used by ARGs to spread, exploring their potential transfer into clinically relevant bacteria and human commensal as well as assessing the human exposure and environment potential risks. To our knowledge, this is the first study to be performed in the region regarding the quantification of ARGs in receiving systems. The quantification of FIB and ARGs as performed in this study can therefore facilitate improved risk assessments for the prudent use of antibiotics in human, animal and agriculture, and provide baseline information for developing strategies (such as hospital and urban wastewater treatment) to limit the spread of these emerging contaminants under tropical conditions.

## ETHICS STATEMENT

We confirm that the field studies and sampling did not involve misunderstanding. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## AUTHOR CONTRIBUTIONS

AL, CM, GG, VS, and JP conceived and designed research; AL, ND, PK, JK, and CM performed research: sampling and laboratory analysis; AL, GG, ND, and JP analyzed data; and AL, CM, VS, and JP wrote the paper. All authors have read, reviewed and approved the manuscript before submission.

## FUNDING

This research was supported by the Swiss National Science Foundation (grant no. 31003A\_150163/1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This study represents the tripartite collaboration between University of Geneva (Institute F.A. Forel), University of Kinshasa and Pedagogic National University of Congo (Democratic Republic of Congo).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Important Role of a Putative Lytic Transglycosylase Cj0843c in $\beta$ -Lactam Resistance in *Campylobacter jejuni*

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## OPEN ACCESS

### Edited by:

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 20 July 2015

Accepted: 04 November 2015

Published: 17 November 2015

### Citation:

Zeng X, Gillespie B and Lin J (2015)  
Important Role of a Putative Lytic  
Transglycosylase Cj0843c  
in  $\beta$ -Lactam Resistance  
in *Campylobacter jejuni*.  
*Front. Microbiol.* 6:1292.  
doi: 10.3389/fmicb.2015.01292

Beta-lactam antibiotics are an important class of antibiotics for treating bacterial infections. Despite prevalent  $\beta$ -lactam resistance in *Campylobacter jejuni*, the leading bacterial cause of human diarrhea in developed countries, molecular mechanism of  $\beta$ -lactam resistance in *C. jejuni* is still largely unknown. In this study, *C. jejuni* 81–176 was used for random transposon mutagenesis. Screening of a 2,800-mutant library identified 22 mutants with increased susceptibility to ampicillin. Of these mutants, two mutants contains mutations in *Cj0843c* (a putative lytic transglycosylase gene) and in its upstream gene *Cj0844c*, respectively. Complementation experiment demonstrated that the *Cj0843* contributes to  $\beta$ -lactam resistance. The *Cj0843c* insertional mutation was subsequently introduced to diverse *C. jejuni* clinical strains for MIC test, showing that *Cj0843c* contributes to both intrinsic and acquired  $\beta$ -lactam resistance of *C. jejuni*. Consistent with this finding, inactivation of *Cj0843c* also dramatically reduced  $\beta$ -lactamase activity. Genomic examination and PCR analysis showed *Cj0843c* is widely distributed in *C. jejuni*. High purity recombinant *Cj0843c* was produced for generation of specific antiserum. The *Cj0843* was localized in the periplasm, as demonstrated by immunoblotting using specific antibodies. Turbidimetric assay further demonstrated the capability of the purified *Cj0843c* to hydrolyze cell walls. Inactivation of *Cj0843c* also significantly reduced *C. jejuni* colonization in the intestine. Together, this study identifies a mechanism of  $\beta$ -lactam resistance in *C. jejuni* and provides insights into the role of cell wall metabolism in regulating  $\beta$ -lactamase activity.

**Keywords:** beta-lactamase, peptidoglycan, random transposon mutagenesis, lytic transglycosylase

## INTRODUCTION

Beta-lactam antibiotics, which inhibit biosynthesis of bacterial cell wall, are the most commercially available antibiotics in the market. For instance, in 2009, beta-lactam antibiotics account for more than half of the total antibiotic sales globally (Hamad, 2010). However, the efficacy of the  $\beta$ -lactam based therapy is severely threatened by a variety of  $\beta$ -lactam resistance mechanisms in bacteria, such as production of beta-lactamases (Korffmann and Wiedemann, 1988; Iaconis and Sanders, 1990; Jacoby, 2009), expression of the penicillin-binding proteins (PBPs) with reduced affinity for  $\beta$ -lactam antibiotics (Fuda et al., 2004), and extrusion of  $\beta$ -lactam using multi-drug efflux pumps (Nikaido, 1998; Vila and Martinez, 2008). Production of  $\beta$ -lactamase has been a major and

threatening  $\beta$ -lactam resistance mechanism. Recently, increasing evidence demonstrate a direct link between cell wall metabolism and regulation of  $\beta$ -lactamase activity in Gram-negative bacteria, which provides insights into the development of innovative strategies to counteract  $\beta$ -lactam resistance in pathogenic bacteria (Zeng and Lin, 2013).

*Campylobacter jejuni* is the leading bacterial cause of human gastroenteritis in the developed countries (Humphrey et al., 2007). Clinical symptoms include fever and watery diarrhea in humans. In rare case, campylobacteriosis can cause severe sequelae such as Guillain–Barré syndrome, an acute flaccid paralysis that may lead to respiratory muscle compromise and death (Nachamkin et al., 1998; Hughes and Cornblath, 2005; Nyati and Nyati, 2013). The annual incidence of campylobacteriosis in the United States is approximately 1% (Poly and Guerry, 2008), resulting in 1.7 billion dollars cost in medical practice and productivity (Hoffmann et al., 2012). In parallel to its increased prevalence, *Campylobacter* is increasingly resistant to macrolides and fluoroquinolones, the two major clinical antibiotics (Luo et al., 2005; Caldwell et al., 2008; Koningstein et al., 2011). Recently, it has been proposed that an oral  $\beta$ -lactam, such as co-amoxiclav, may provide an alternative therapy for *Campylobacter* infection (Elviss et al., 2009; Griggs et al., 2009). However,  $\beta$ -lactam resistant *C. jejuni* strains have also been observed in epidemiological studies (Lachance et al., 1991; Griggs et al., 2009). Thus, understanding the mechanisms of  $\beta$ -lactam resistance in *C. jejuni* would help us develop effective therapeutic strategy that will allow the use of existing  $\beta$ -lactams against resistant *C. jejuni*. In *Campylobacter*, two beta-lactam resistance mechanisms have been characterized, which include the presence of CmeABC multi-drug efflux pump (Lin et al., 2002) and the production  $\beta$ -lactamase OXA-61 (Alfredson and Korolik, 2005; Griggs et al., 2009). Despite these progresses, the findings from recent studies strongly suggest the existence of novel  $\beta$ -lactamases and/or regulatory system in *C. jejuni* for  $\beta$ -lactam resistance (Griggs et al., 2009; Zeng et al., 2014).

In this study, using random mutagenesis, we identified a putative lytic transglycosylase (LT) Cj0843c that is required for intrinsic and acquired  $\beta$ -lactam resistance in *C. jejuni*. This finding was further validated by a series of molecular, biochemical and genomic examination. Consistent with this finding, inactivation of Cj0843c, which is highly conserved in *C. jejuni*, dramatically reduced  $\beta$ -lactamase activity. The role of Cj0843c in peptidoglycan metabolism was validated by a standard LT assay using purified Cj0843c. This study provides compelling evidence that Cj0843c, a putative LT, plays a critical role in regulating  $\beta$ -lactamase-mediated  $\beta$ -lactam resistance in *C. jejuni*.

## MATERIALS AND METHODS

### Bacterial Strains, Plasmids, and Culture Conditions

The major bacterial strains and plasmids used in this study are listed in Table 1. The *C. jejuni* strains were grown routinely in Müller-Hinton (MH) broth or agar at 42°C in tri-gas incubator (85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% O<sub>2</sub>). *Escherichia*

**TABLE 1 | Key bacterial plasmids and strains used in this study.**

Plasmids or strains	Description	Source or reference
<b>Plasmids</b>		
pRY111	<i>Escherichia coli</i> – <i>Campylobacter</i> shuttle vector, kanamycin resistant	Yao et al., 1993
pCj843c	pRY111 derivative containing Cj0843c, Cj0844c, and Cj0845c	This study
pCj844c	pRY111 derivative containing Cj0843c and Cj0844c	This study
pET28b-Cj0843c	Expression vector for recombinant Cj0843c, Kan <sup>r</sup>	Novagen
<b>Strains</b>		
<i>Campylobacter jejuni</i>		
JL241	NCTC 11168, human isolate	Parkhill et al., 2000
JL890	JL241 derivative with transposon inserted into Cj0843c, Kan <sup>r</sup>	This study
JL990	JL890 derivative, complemented with pCj843c	This study
JL28	81–176, human isolate, for random transposon mutagenesis	Black et al., 1988
JL30	81–176 (JL28) derivative with transposon inserted into cmeB gene, Kan <sup>r</sup>	Lin et al., 2002
JL916	JL28 derivative with EZ::TN <KAN-2> transposon inserted into Cj0843c, Kan <sup>r</sup>	This study
JL926	JL916 derivative, complemented with pCj843c	This study
JL962	81–176 derivative, ampicillin resistant. The $\beta$ -lactamase gene bla <sub>OXA-61</sub> was introduced into chromosome via natural transformation using genomic DNA from ampicillin resistant <i>C. jejuni</i> 21190 (Zeng et al., 2014)	This study
JL963	JL962 derivative. The Cj0843c::kan mutation was introduced into chromosome via natural transformation using genomic DNA from JL916, Kan <sup>r</sup>	This study
JL995	81–176 (JL28) derivative, Kan <sup>r</sup> . The pgp1::kan mutation was introduced into JL28 chromosome via natural transformation using genomic DNA of the pgp1 mutant (Frirdich et al., 2012)	This study
JL996	JL962 derivative. The pgp1::kan mutation was introduced into JL962 chromosome via natural transformation using genomic DNA of the pgp1 mutant (Frirdich et al., 2012)	This study
<i>E. coli</i>		
DH5 $\alpha$	F- $\Phi$ 80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-	Invitrogen
JL985	pET28b-Cj0843c in BL21(DE3)	This study

*coli* strains were grown routinely in Luria-Bertani (LB) broth with shaking (250 rpm) or on agar at 37°C overnight. When

needed, culture media were supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml), and chloramphenicol (Cm; 20 µg/ml).

## Random Transposon Mutagenesis

We have successfully used *C. jejuni* 81–176 as a host strain for *in vivo* random transposon mutagenesis using EZ-Tn5 < KAN-2 > Tnp Transposome system (Epicentre; Lin et al., 2009; Hoang et al., 2011, 2012; Zeng et al., 2013b). In this study, the wild-type *C. jejuni* 81–176 (ampicillin Minimum inhibitory concentration (MIC) = 1 µg/ml) was subjected to *in vivo* random transposon mutagenesis; the procedure and screening strategy are detailed in previous publications (Lin et al., 2009; Hoang et al., 2011, 2012; Zeng et al., 2013b). The mutants with growth defects in MH broth containing kanamycin (50 µg/ml) and ampicillin (0.25 µg/ml) were identified. To determine transposon insertion site, genomic DNA extracted from the selected mutant was subjected to direct sequencing as described previously (Lin et al., 2009; Hoang et al., 2011; Zeng et al., 2013b).

## Antimicrobial Susceptibility Test

The susceptibilities of *C. jejuni* strains to different antimicrobials (Fisher Bioreagents) were determined by a standard microtiter broth dilution method with an inoculum of 10<sup>6</sup> CFU/ml as described previously (Lin et al., 2002; Zeng et al., 2014). MIC for specific antimicrobial was defined by the lowest concentration of the antimicrobial showing complete inhibition of bacterial growth after 2 days of incubation at 42°C under microaerophilic condition.

## Beta-lactamase Activity Assay

To measure β-lactamase activity in *C. jejuni* strains, standard assay was performed on cell lysates of different strains by following previous procedure (Griggs et al., 2009; Zeng et al., 2014) with minor modifications. Briefly, late-log phase bacterial cells were harvested from MH plates, washed with PBS, and adjusted to an optical density at 600 nm of approximately 1.2 in PBS. Bacterial suspensions were sonicated and centrifuged at 13,000 rpm for 5 min. The supernatant was subjected to protein concentration measurement using BCA Protein Assay kit (Pierce). One hundred microliter of supernatant containing 40 µg of protein was mixed with 0.8 ml of ice-chilled reaction buffer (0.1 M phosphate; 1 mM EDTA, pH 7.0). Then 100 µl of nitrocefin (500 µg/ml) was added to the above reaction mix followed by incubation at 37°C for 30 min. The activity was determined by spectrometric at 486 nm. The molar extinction coefficient of hydrolyzed nitrocefin at 486 nm is 20,500 M<sup>-1</sup> cm<sup>-1</sup>. The lysate from *C. jejuni* 81–176 was used as negative control. The β-lactamase activity was expressed as hydrolyzed nitrocefin (µM) per hr per mg of protein.

## Complementation of *C. jejuni* 81–176 Transposon Mutants

The long fragment (containing the genes *Cj0843c*, *Cj0844c*, and *Cj0845c*) and the short fragment (containing the genes *Cj0844c* and *Cj0845c*) were PCR amplified from *C. jejuni* 81–176 using

primer pairs of *Cj0843c\_F/Cj0843c\_R* and *Cj0843c\_F/Cj0844c\_R* (**Table 2**), respectively, with *Pfu*Ultra fusion II DNA polymerase (Stratagene). The resulting fragments were ligated into *Sma*I-digested shuttle vector pRY111 (Yao et al., 1993) and ligation mix was introduced into *E. coli* DH5α by heat shock transformation. The *E. coli* transformants containing the plasmid that bears the three-gene (named p*Cj0843c*) or the two-gene fragment (named p*Cj0844c*) were identified and confirmed by sequencing. Both constructs were mobilized into *C. jejuni* 81–176 transposon mutants by triparental conjugation as described previously (Zeng et al., 2013a, 2014).

## Natural Transformation

Natural transformation (biphasic method) was performed following standard procedure as described by Wang and Taylor (1990). Approximately 4 µg of *C. jejuni* genomic DNA was used for natural transformation. The insertional *Cj0843c* mutations in different strains were confirmed by PCR using primer pair *Cj0843c\_F1* and *Cj0843c\_R1* (**Table 2**).

## Production of Recombinant *Cj0843c* and Generation of Polyclonal Antiserum

N-terminal histidine-tagged recombinant *Cj0843c* was produced in *E. coli* by using pET-28b(+) vector. Briefly, approximately 1568-bp fragment of *Cj0843c* was PCR amplified from *C. jejuni* NCTC 81–176 using primer pairs *rCj0843c\_F1(NdeI)* and *rCj0843c-pET28b\_R(SalI)*; (**Table 2**). The amplified product and the vector pET-28b(+) were both digested with *NdeI* and *SalI* and ligated with each other. Cloning, expression, and purification of r*Cj0843c* were performed using the procedures described in our previous publication (Lin et al., 2005; Zeng et al., 2013a). The plasmid pET28b-Cj0843c in the *E. coli* BL21(DE3) clone (JL985) producing r*Cj0843c* was sequenced to confirm the correct insertion. The fractions containing pure r*Cj0843c* were pooled and dialyzed against 1 x PBS buffer (Fisher Bioreagents). The concentration of the purified r*Cj0843c* was determined using BCA Protein Assay Kit (Pierce™). Approximately 4 mg of highly purified r*Cj0843c* was used for the production of rabbit polyclonal antisera by Pacific Immunology Corp (Ramona, CA). Pre- and post-immune serum samples were analyzed by immunoblotting against pure r*Cj0843c* to confirm the generation of specific *Cj0843c* antibody.

## Enzymatic Activity of r*Cj0843c*

The LT activity of r*Cj0843c* was evaluated using turbidimetric assay as reported before with minor modification (Caldentey and Bamford, 1992). In brief, the isogenic *Cj0843c* mutant of *C. jejuni* 81–176 was harvested from three overnight-cultured MH agar plates and resuspended in 50 ml Tris-HCl buffer (50 mM, pH 7.5). Then 3 ml of chloroform was added in the suspension, which was kept at room temperature for 30 min with gently shaking. Subsequently, the chloroform-extracted cells (the crude cell walls) were pelleted by centrifugation at 13,000 rpm for 5 min, washed once with 50 mM Tris-HCl buffer (pH 7.5),

and resuspended in 50 ml of 50 mM Tris-HCl buffer (pH 7.5).

To perform turbidimetric assay, the crude cell walls were adjusted to final OD<sub>450 nm</sub> of approximately 0.45 using 50 mM Tris-HCl buffer. Then the purified rCj0843c was added to 1 ml of such cell wall suspension with final concentration of 24 µg/ml; the cell wall suspension containing PBS instead of rCj0843c served as control. The decrease in turbidity at 450 nm was determined after 2.5 h of incubation at 37°C. The turbidimetric assay was performed with triplicate and the statistical analysis of a Student's t test was carried out using SAS (v9.4). A P-value less than 0.05 was considered significant.

### Localization of the Cj0843c

To determine cellular localization of Cj0843c, the periplasmic and spheroplastic fractions were prepared using the PeriPreps™ Periplasting Kit (Epicentre) as detailed in our recent publication (Zeng et al., 2013a). Different cellular fractions were subsequently subjected to SDS-PAGE and immunoblotting analysis as described in our previous publications (Lin et al., 2002; Zeng et al., 2009, 2013a). Briefly, the protein samples were separated by SDS-PAGE with a 12% (wt/vol) polyacrylamide separating gel. After SDS-PAGE, proteins in the gels were then electrophoretically transferred to nitrocellulose membranes (Bio-Rad) at 60 V for 1 h at 4°C. The membranes were incubated with blocking buffer (5% Nestle skim milk powder in PBS) for 16 h at 4°C prior to incubation with primary antibodies (Rabbit anti-Cj0843c at 1:5000 dilution in the blocking buffer). To ensure the quality of periplasmic and spheroplastic fractions, antibodies directed against CmeR (cytoplasmic control, 1:2000 dilution) were also used as primary antibodies. After incubation at 25°C for 1 h, the blots were washed three times with PBS containing 0.05% Tween 20 and subsequently incubated with secondary antibodies (1:50,000 dilution of goat anti-rabbit IgG-HRP; Kirkegaard and Perry) at 25°C for 1 h. After washing, the blots were developed with the SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). Prestained molecular weight markers (Bio-Rad) were coelectrophoresed and blotted to allow estimation of the sizes of the proteins.

### Prevalence of Cj0843c in *C. jejuni*

Total 30 *C. jejuni* strains from different hosts and geographically diverse areas were grown microaerophilically to late exponential phase and harvested by centrifugation. The cell pellets were suspended in 100 µL of water and lysed by boiling for 5 min. After centrifugation, the supernatants were used as template in PCR assay with highly Cj0843c-specific primers Cj0843c\_F1 and Cj0843c\_R1 (Table 2). For bioinformatic analysis of Cj0843c prevalence among available genome sequences, total 103 *C. jejuni* genomes were extracted from Integrated Microbial Genomes<sup>1</sup> and the multi-sequence alignment was analyzed using Clustal Omega<sup>2</sup>. In addition, presence of Cj0843c homolog was also examined in the genomes of 49 *Campylobacter coli* and 298 *Helicobacter pylori* strains.

### Chicken Colonization Experiment

*Campylobacter jejuni* 81-176 wild-type strain together with its isogenic Cj0843c mutant (JL916) and the complementation strain (JL926; Table 2) were used for colonization assay in a chicken model system as described in previous studies (Lin et al., 2002; Lin and Martinez, 2006; Hoang et al., 2011; Zeng et al., 2013a). The chicken study was approved by the Institutional Animal Care and Use Committee at The University of Tennessee (Protocol 1387 under oversight of the IACUC Chair Carla Sommardahl). Briefly, 1-day-old broiler chickens were obtained from a commercial hatchery (Hubbard Hatchery, Pikesville, TN, USA). The chickens were negative for *Campylobacter* as determined by culturing cloacal swabs prior to use in this study. Three groups of 4-days-old chickens (10 birds/group) were inoculated with different bacteria via oral gavage using a dose approximately 10<sup>5</sup> CFU of fresh late log-phase *C. jejuni* per bird. Notably, the motility of parent strain and its Cj0843c mutant was confirmed to be at a comparable level prior to challenge. The isogenic Cj0843c mutant displayed reduced growth in MH broth when compared to its parent strain. However, the fresh *C. jejuni* cultures with similar growth phase, reflected by the final OD<sub>600 nm</sub> measurement, were used to challenge chickens; the bacteria in such cultures were assumed to be in the same

<sup>1</sup>[https://img.jgi.doe.gov/cgi-bin/imgm\\_hmp/main.cgi](https://img.jgi.doe.gov/cgi-bin/imgm_hmp/main.cgi)

<sup>2</sup><http://www.ebi.ac.uk/Tools/msa/clustalo/>

**TABLE 2 | Major primers used in this study.**

Primer	DNA Sequence (5'-3') <sup>a</sup>	Product size (bp)	Target gene and function
Cj0843c_F	AACAAAATCCCAAGCTAAAGTCA	3,767	Cj0843c, Cj0844c and Cj0845c operon
Cj0843c_R	TTCTAGACAAGACAAGTAAAGATGATG		
Cj0844c_R	CAAGTGAATTTCCTTCCTTTGAG	2,028	With Cj0843c_F, to amplify Cj0844c and Cj0845c
Cj0843c_F1	GCTCAAACCTTAAAAATCAAAGA	586	Cj0843c prevalence and validation of Cj0843c mutation
Cj0843c_R1	TTTGCCAAGCAAAAGGATCT		
rCj0843c_F1(NdeI)	GGAATT <u>CCATATG</u> CAATATACTATAGAAAAACTAAAAAGGAA	1,568	Recombinant Cj0843c
rCj0843c-pET28b_R(Sall)	GCGCAT <u>GTCGAC</u> CTAACAGTTGTTAGATCATTTGCC		

<sup>a</sup>Restriction sites are underlined in the primer sequence and the names are identified in parentheses.

physiological state. For each group, 5 birds were euthanized and cecal samples were collected at 6 and 10 days post-inoculation. The cecal contents from each bird were weighed and diluted in MH broth. The diluted samples were plated onto MH agar plates with *Campylobacter* specific selective supplements (Oxoid, UK). The number of CFU per gram of cecal contents was calculated for each chicken and was used as an indicator of the colonization level. The detection limit of the plating methods was 10<sup>2</sup> CFU/g of cecal contents. A bird from which no *Campylobacter* colonies were detected was assigned a conservative value of 99 CFU/g of cecal contents for the purpose of calculating means and for statistical analysis. One-way analysis of variance (ANOVA) followed by a least-significant difference test was used to analyze the significant differences in colonization level (log transformed). A P-value less than 0.05 was considered significant.

## RESULTS

### **Cj0843c, a Putative Lytic Transglycosylase, is Required for Ampicillin Resistance**

The *C. jejuni* 81-176 strain was used as a host strain to generate a library consisting of 2,800 Tn5-insertional mutants. Individual mutant grown in microtiter plates was replicated into screening plates containing MH broth with sublethal concentration of ampicillin (0.25 µg/ml). A total of 22 mutants displayed increased susceptibility to ampicillin. The insertional sites of these mutants were mapped by direct sequencing. It's not surprising that most of them (20 mutants) contains transposon insertion in *cmeABC* locus which encodes CmeABC multidrug efflux pump system which was previously shown to be involved in ampicillin resistance (Lin et al., 2002, 2003). Specifically, transposon insertional mutations were identified in *cmeA* (two different sites with four mutants), *cmeB* (two different sites with four mutants), and *cmeC* (2 different sites with 12 mutants). Interestingly, the other two mutants have transposon insertion identified in two adjacent genes, the CJJ81176\_0859 and CJJ81176\_0860 (hereinafter, the corresponding universal locus names in *C. jejuni* NCTC 11168, *Cj0843c* and *Cj0844c*, were used). The *Cj0843c* was annotated as a putative secreted LT. Specifically, *Cj0843c* contains a highly conserved domain (COG0741, E-value is 3.53e-27) represented by MltE, an endo-specific LT from *E. coli* that catalyzes hydrolysis of glycan chains to generate short chains with 1,6-anhydro-MurNAc ends (Fibriansah et al., 2012). *Cj0844c* was annotated as putative integral membrane protein. Both mutants displayed fourfold MIC reduction for ampicillin when compared to that of wild type 81-176 (**Table 3**).

Genome analysis showed that the start codon of *Cj0843c* overlapped the stop codon of *Cj0844c* by 4 bp nucleotides. In addition, there is only 2 bp nucleotide gap between the start codon of *Cj0844c* and the stop codon of its upstream gene *Cj0845c*. Therefore, the *Cj0845c*, *Cj0844c*, and *Cj0843c* may form

an operon. The transposon insertion into *Cj0844c* may cause polar effect, affecting the expression of downstream gene *Cj0843c*. To test this, two constructs with different length (pCj0843c and pCj0844c) was constructed for complementation (**Table 1**). The pCj0843c covers the whole three-gene operon while pCj0844c only contains two upstream genes *Cj0845c* and *Cj0844c*. As shown in **Table 3**, complementation with pCj0843c restored the MIC of both mutants to the level of wild type strain. However, complementation of the *Cj0844c* mutant with pCj0844c failed to restore MIC of ampicillin to wild-type level, indicating that the *Cj0843c*, not *Cj0844c*, is involved in ampicillin resistance in *C. jejuni* 81-176.

To examine the role of *Cj0843c* in ampicillin resistance in different strains, the *Cj0843c* transposon mutation was also introduced into *C. jejuni* NCTC 11168 via natural transformation. As shown in **Table 3**, the isogenic *Cj0843c* mutant of NCTC 11168 also displayed increased susceptibility to ampicillin (eightfold reduction) while the MIC of the complementation strain was the same as that of wild type strain.

### **Cj0843c is Involved in the Resistance to Various β-lactam Antibiotics in *Campylobacter***

We also examined if inactivation of *Cj0843c* affected susceptibilities of *C. jejuni* to different β-lactam antibiotics. As shown in **Table 4**, susceptibilities of *Cj0843c* mutant to various β-lactam antibiotics (penicillin G, ticarcillin, carbenicillin, and cloxacillin) significantly increased (fourfold) when compared to wild-type 81-176 strain. Complementation of the isogenic *Cj0843c* mutant with pCj0843c restored MIC of ampicillin to wild type level (**Table 4**). In terms of other types of antimicrobials, such as bile salts (cholic acid and deoxycholic acid), ciprofloxacin and ethidium bromide (**Table 4**), the susceptibilities of *Cj0843c* mutant did not change when compared to wild-type strain.

### **Cj0843c also Plays an Important Role in Acquired Ampicillin Resistance**

Although *C. jejuni* 81-176 lacks beta-lactamase gene *bla*<sub>OXA-61</sub> in the genome (Griggs et al., 2009), it could acquire high-level ampicillin resistance (Amp<sup>r</sup>) due to the acquisition of active beta-lactamase *bla*<sub>OXA-61</sub> from *C. jejuni* 21190 via natural transformation (Zeng et al., 2014). The MIC of ampicillin for the Amp<sup>r</sup> derivative of 81-176 was 128 µg/mL, which is 128-fold higher than the MIC of wild-type 81-176 (**Figure 1**), as reflected by the dramatic change of the beta-lactamase activity (**Figure 1**). However, inactivation of *Cj0843c* in the Amp<sup>r</sup> derivative of 81-176 dramatically reduced MIC (8 µg/mL) as well as the beta-lactamase activity in cell lysates (**Figure 1**). In contrast, although mutation in multidrug efflux pump gene *cmeB* (Lin et al., 2002) in the Amp<sup>r</sup> derivative led to reduced MIC of ampicillin (16 µg/mL), the beta-lactamase activity was not changed (**Figure 1**). This finding clearly indicated that *Cj0843c* also plays an important role in β-lactamase-mediated ampicillin resistance by modulating the activity of β-lactamase Bla<sub>OXA-61</sub> in *C. jejuni*.

**TABLE 3 | Susceptibilities of two representative *C. jejuni* strains and their isogenic mutants to ampicillin.**

Parent strain	MIC ( $\mu\text{g/mL}$ )						
	Wild type	<i>Cj0843c</i> <sup>-</sup>	<i>Cj0844c</i> <sup>-</sup>	<i>Cj0843c</i> <sup>-</sup> / <i>pCj0843c</i>	<i>Cj0844c</i> <sup>-</sup> / <i>pCj0843c</i>	<i>Cj0844c</i> <sup>-</sup> / <i>pCj0844c</i>	<i>CmeB</i> <sup>-</sup>
81-176	1	0.25	0.25	1	1	0.25	0.0625
NCTC 11168	16	2	ND <sup>a</sup>	16	ND	ND	4

<sup>a</sup> ND, not determined.

**TABLE 4 | *Cj0843* is only involved in *C. jejuni* resistance to various  $\beta$ -lactams.**

Chemical	81-176 WT	<i>cj0843c::Tn5</i>	<i>cj0843c::Tn5/pCj0843c</i>
Penicillin G ( $\mu\text{g/ml}$ )	16	4	16
Ticarcillin ( $\mu\text{g/ml}$ )	256	64	256
Carbenicillin ( $\mu\text{g/ml}$ )	64	16	64
Cloxacillin ( $\mu\text{g/ml}$ )	512	128	512
Cholic acid (mg/ml)	12.5	12.5	N/D <sup>a</sup>
Ciprofloxacin ( $\mu\text{g/ml}$ )	0.25	0.25	N/D
Deoxycholic acid (mg/ml)	20	20	N/D
Ethidium bromide ( $\mu\text{g/ml}$ )	1	1	N/D

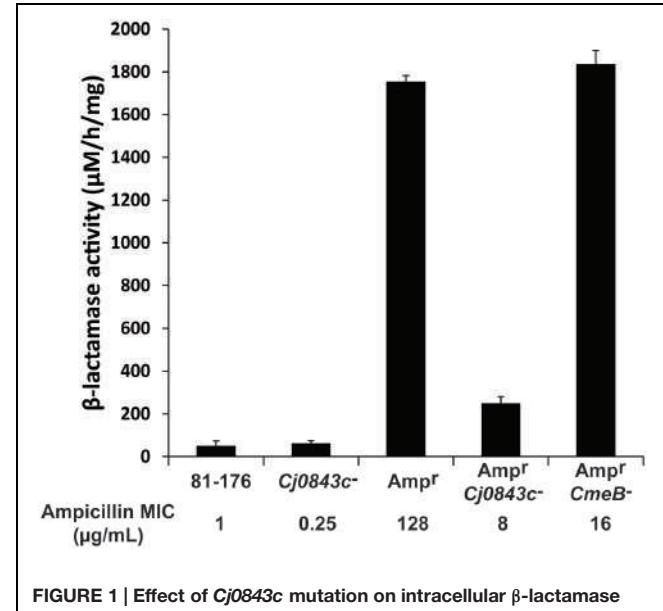
<sup>a</sup>N/D, not determined.

To determine the role of *Pgp1*, an identified gene involved in cell wall metabolism in *C. jejuni* (Frirdich et al., 2012), in ampicillin resistance, the *pgp1::kan* mutation was introduced into the wild-type 81-176 and its Amp<sup>r</sup> derivative in this study via natural transformation using genomic DNA of the *pgp1* mutant, creating isogenic *pgp1* mutant JL995 and JL996, respectively (Table 1). The MICs for JL995 and JL996 are 1 and 128  $\mu\text{g/mL}$ , respectively, which are the same as those for their corresponding parent strains. Consistent with this finding, the beta-lactamase activity was not changed due to the inactivation of *pgp1*.

## The Prevalent and Highly Conserved *Cj0843c* is Required for Ampicillin Resistance in Diverse *C. jejuni* Isolates

It's well known that the genome of *C. jejuni* species displayed considerable plasticity (de Boer et al., 2002; Poly et al., 2008; Duong and Konkel, 2009). In this study, a PCR survey was performed, demonstrating that the *Cj0843c* was present in 30 diverse *C. jejuni* isolates (data not shown). The representative isolates were listed in Table 5. Regardless of the level of  $\beta$ -lactamase activity and the level of ampicillin resistance, introduction of *Cj0843c* mutation into these isolates all resulted in the decrease of MIC of ampicillin by more than fourfold (Table 5).

In addition to this PCR survey, genome analysis also provided compelling evidence showing *Cj0843c* was highly conserved in *C. jejuni* and other closely related species. Specifically, *Cj0843c* was present in all 101 currently available *C. jejuni* genomes deposited in the database. Notably, the three-gene operon containing *Cj0843c* is also highly conserved in the genomes of different  $\epsilon$ -proteobacteria, including 101 *C. jejuni*, 49 *C. coli*,



**FIGURE 1 | Effect of *Cj0843c* mutation on intracellular  $\beta$ -lactamase activities and ampicillin resistance in *Campylobacter jejuni*.** The ampicillin MIC values were listed below each corresponding strain. Amp<sup>r</sup>, the 81-176 derivative with  $\beta$ -lactamase gene *bla<sub>OXA-61</sub>* introduced into chromosome via natural transformation using genomic DNA from ampicillin resistant *C. jejuni* 21190 (Zeng et al., 2014). The procedure for nitrocefin-based  $\beta$ -lactamase activity assay was detailed in Materials and Methods. The activity was determined by spectrometric change at 486 nm. The molar extinction coefficient of hydrolyzed nitrocefin at 486 nm is  $20,500 \text{ M}^{-1} \text{ cm}^{-1}$ . The  $\beta$ -lactamase activity was expressed as hydrolyzed nitrocefin ( $\mu\text{M}$ ) per hr per mg of protein.

and 298 *H. pylori* (Supplementary Figure S1 and detailed in Supplementary Table S1).

The multi-sequence alignment data show the percentage aa identity among *C. jejuni* strains ranges from 93 to 100%. The across-species alignment shows that the aa identity and homology between *C. jejuni* NCTC 11168 and *C. coli* RM2228 are 79 and 89%, respectively, and those between *C. jejuni* NCTC 11168 and *H. pylori* 26695 are 31 and 51%, respectively.

## The *Cj0843c* is Localized in Periplasm

Based on annotation, *Cj0843c* is predicted to interact with the glycan strand of the periplasmic peptidoglycan. To confirm this, we first cloned *Cj0843c* into expression vector pET-28b and produced N-terminal 6xHis tagged recombinant *Cj0843c* (r*Cj0843c*). Majority of r*Cj0843c* is in the soluble fraction and was successfully purified by Ni-NTA affinity column (Figure 2A).

**TABLE 5 |** Cj0843c is prevalent and involved in intrinsic and acquired ampicillin resistance in different clinical *C. jejuni* isolates.

Strain	Source/host	Cj0843c <sup>a</sup>	β-Lactamase activity <sup>b</sup>	MIC ( $\mu\text{g/ml}$ )	
				Wild type	Cj0843c::Tn5
JL241	Human	+	—	16	4
JL36	Chicken	+	±	8	1
JL78	Human	+	—	4	0.25
JL81	Human	+	—	4	0.5
JL85	Human	+	—	2	0.25
JL90	Human	+	—	2	0.5
JL95	Ovine	+	+	16	2
JL100	Human	+	+	16	0.5
JL115	Human	+	++++	256	16
49	Chicken	+	—	4	0.25
Cj41	Chicken	+	±	8	1
SO40	Bovine	+	—	16	1
JL98	Human	+	+	8	2
86	Chicken	+	—	16	2

<sup>a</sup>The prevalence of Cj0843c is detected by PCR using primer Cj0843c\_F1 and Cj0843c\_R1.

<sup>b</sup>The β-lactamase activity of each clinical isolate was measured using the cell lysate with the nitrocefin assay (Griggs et al., 2009). A yellow to red color change within 5 min at 37°C indicated a β-lactamase-positive reaction. +, positive β-lactamase activity; —, negative β-lactamase activity; ±, weak β-lactamase activity; +++, red color developed immediately upon addition of substrate nitrocefin.

The specific Cj0843c antiserum was successfully raised and was used for immunoblotting to determine localization of Cj0843c in *C. jejuni*. As shown in **Figure 2B**, Cj0843c is detected in the periplasm fraction but not in the spheroplast fraction of wild-type cells (left panel). The Cj0843c was not detected in the periplasm fraction of the isogenic Cj0843c mutant either (left panel in **Figure 2B**). As a control, CmeR, an intracellular regulator, is only detected in the spheroplast fractions in both wild type and Cj0843c mutants (right panel in **Figure 2B**). This immunoblotting analysis provides compelling evidence that that Cj0843c is localized in the periplasm, the place where peptidoglycan resides in Gram-negative bacteria.

### Lytic Transglycosylase Activity of rCj0843c

The chloroform-treated Cj0843c mutant cells was used as cell wall substrates because the rCj0843c action sites on cell walls from this mutant are possibly intact. As shown in **Figure 2C**, the decrease in OD<sub>450nm</sub> ( $\Delta\text{OD}_{450\text{nm}}$ ) in the reaction solution containing the rCj0843c is significantly larger than PBS control ( $P < 0.05$ ), indicating the capability of rCj0843c as LT to hydrolyze cell wall.

### Cj0843c is Required for *C. jejuni* Colonization

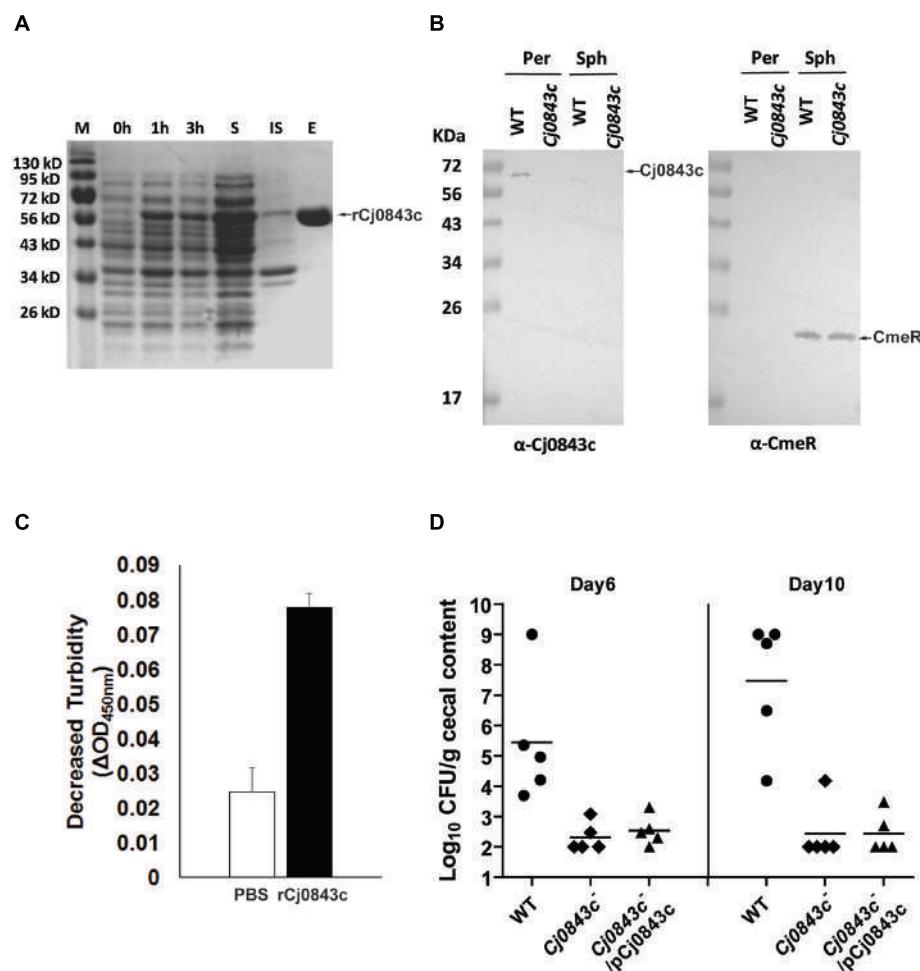
The role of Cj0843c in *in vivo* survival of *C. jejuni* was evaluated using chicken model system. As shown in **Figure 2D**, wild-type *C. jejuni* 81–176 could colonize chicken efficiently on days 6 and 10 post-challenge with colonization level as high as 10<sup>9</sup> CFU per gram of feces. In contrast, the isogenic Cj0843c mutant displayed significantly reduced colonization in the intestine ( $P < 0.05$ ); the mutant was not detected in most of chickens.

Complementation with the plasmid did not rescue the impaired colonization of the Cj0843c mutant, likely due to instability of the plasmid in the complemented strain. To test this, an *in vitro* plasmid stability assay was performed by subculturing complemented strain every 2–3 days in fresh antibiotic-free MH broth (1:400 dilution) for 10 days; by day 10, majority of the cells (85%) were observed to lose the complemented plasmid.

## DISCUSSION

In this study, random transposon mutagenesis was used to identify novel genes contributing to beta-lactam resistance in *C. jejuni*, which led to the discovery of a putative LT Cj0843c that plays an important role in the intrinsic and acquired beta-lactam resistance in *C. jejuni*. Notably, the mutation of Cj0843c significantly reduced beta-lactamase activity and MIC for ampicillin (**Table 3** and **Figure 1**). The periplasmic Cj0843c was prevalent and highly conserved in *C. jejuni* and other ε-proteobacteria, such as *C. coli* and *H. pylori* (Supplementary Figure S1 and Supplementary Table S1), indicating the critically conserved role of Cj0843c in cell wall metabolism. Cj0843c is also required for *C. jejuni* colonization in the intestine (**Figure 2D**). Mutation of another cell wall metabolism-related gene pgp1 was also observed to impair *C. jejuni* colonization (Frirdich et al., 2012). However, at this stage, it is still premature to speculate how Cj0843c is involved in colonization. Mutation of Cj0843c may affect *C. jejuni* physiology and exert pleiotropic effects that are related to *C. jejuni* *in vivo* colonization.

The LT also have been observed to be involved in beta-lactam resistance in other bacteria. For instance, single ( $\Delta\text{MltB}$ ),



**FIGURE 2 | Characterization of Cj0843c.** **(A)** Expression and purification of rCj0843c. Cj0843 fragment was cloned into pET-28b vector. Expression of rCj0843c was induced in the presence of 0.5 mM IPTG. 0, 1 and 3 h, the cell lysates from pre-induction, 1 and 3 h induced cells; S, soluble fraction; IS, insoluble fraction; E, elution fraction. **(B)** Cj0843c is localized in periplasm. The intracellular regulator CmeR was used as cytoplasm control. Per, periplasm; Sph, spheroplast; WT, wild type. **(C)** Turbidimetric assay of rCj0843c. Chloroform-treated Cj0843c<sup>-</sup> mutant cells (crude cell walls) were mixed with rCj0843c or PBS control solution. The decrease of absorbance at 450 nm was measured after 2.5 h incubation. Each bar represents the mean  $\pm$  SD from three independent measurements. **(D)** Critical role of Cj0843c in colonization of *C. jejuni* in chicken. The chickens in each group (10 birds) were inoculated with *C. jejuni* 81–176 (close circle), its isogenic Cj0843c mutant (diamond), and complementation strain (triangle) at dose of  $10^5$  CFU/chicken. At days 6 and 10 post-challenge, five chickens from each group were killed and cecal contents were collected for *C. jejuni* numeration. The detection limit is  $10^2$  CFU/gram fecal sample. Each horizontal line segment denotes the mean value of log transformed CFU of different strains at the indicated days post-inoculation. The detection limit is  $10^2$  CFU/gram fecal sample.

double ( $\Delta$ MltA/ $\Delta$ MltB; Kraft et al., 1999) or sextuple (Slt70, MltA, MltB, MltC, MltD, and EmtA; Korsak et al., 2005) LT mutants in an *E. coli* MC1061 displayed significantly decreased beta-lactamase activity. Specific inhibition of the LT Slt70 by bulgecin A suppressed the activity of beta-lactamase AmpC in *E. coli* (Kraft et al., 1999). Notably, not all PG-degrading enzymes are involved in beta-lactam resistance. For instance, the deletion of two amidases (cleaving the amide bond between N-acetylmuramic acid and L-Ala) and three DD-endopeptidases (acting on the peptide cross-links) had little effect on beta-lactam resistance (Korsak et al., 2005). In *C. jejuni*, inactivation of Pgp1, which is involved in cleaving monomeric tripeptides to dipeptides (Frirdich et al., 2012), did not affect beta-lactamase activity and the

susceptibility of *C. jejuni* to ampicillin as shown in this study.

The LT-mediated beta-lactam resistance identified in this study indicates LT is a promising target for combinational antimicrobial chemotherapy in *C. jejuni*, even in  $\epsilon$ -proteobacteria. Our genome survey suggested the three-gene operon containing Cj0843c is conserved across  $\epsilon$ -proteobacteria. The LT-mediated beta-lactam resistance mechanism might be also prevalent in  $\epsilon$ -proteobacteria. For example, the *H. pylori* homolog (Slt) shares high homology to the Cj0843c (31% aa identity and 51% aa similarity). Recently, Slt also has been found to contribute to amoxicillin resistance in *Helicobacter* (Bonis et al., 2012). Bulgecin A, the specific inhibitor of *H. pylori* Slt, could enhance the

antimicrobial effect of amoxicillin (Bonis et al., 2012). Given amoxicillin is an important  $\beta$ -lactam component of the triple therapy to eradicate *H. pylori* infection in human (Tepes et al., 2012), LT inhibitor may serve as a good combination agent (e.g., together with beta-lactam/beta-lactamase inhibitors) to maximize the clinical efficacy of beta-lactam antibiotics.

The decreased  $\beta$ -lactamase activity in LT mutants might be correlated with altered metabolism of peptidoglycan (Kraft et al., 1999). In Gram-negative bacteria, the LT-degraded PG fragments, also called muropeptides, not only serve as cell wall recycling materials during cell growth, but also serve as a signal to turn on the production of  $\beta$ -lactamase through an intracellular transcriptional regulator (Jacobs et al., 1997; Boudreau et al., 2012; Zeng and Lin, 2013). Specifically, muropeptides was either transported into cytoplasm through inner membrane transport AmpG and bound by the regulator AmpR, or directly bound by response regulators of the BlrAB-like two-component regulatory system, to activate the expression of  $\beta$ -lactamase AmpC (Boudreau et al., 2012; Zeng and Lin, 2013). Mutation of the putative LT Cj0843c likely disrupt such intracellular signaling pathway, therefore interfering the induced expression of beta-lactamase and leading to significantly reduced beta-lactamase activity as well as ampicillin MIC observed in this study (Figure 1). This speculation needs to be examined in future studies.

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## AUTHOR CONTRIBUTIONS

JL is the project leader who oversights all the experiments described in this manuscript. XZ and JL contributed with the conception and design of the study. XZ and BG were involved in the collection of data. XZ and JL were involved in the analysis and interpretation of data; drafting and revision of the article; and final approval of the article.

## ACKNOWLEDGMENTS

We thank Erin C. Gaynor (University of British Columbia, Canada) for providing genomic DNA of *pgp1* mutant, Dr. Patricia Guerry for providing *C. jejuni* BH-01-0142, and Samantha Brown for providing technical support. This work is supported by The University of Tennessee AgResearch.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01292>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Integration of PK/PD for dose optimization of Cefquinome against *Staphylococcus aureus* causing septicemia in cattle

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
Frontiers in Microbiology

Received: 20 March 2015

Accepted: 28 May 2015

Published: 17 June 2015

### Citation:

Ahmad I, Hao H, Huang L, Sanders P, Wang X, Chen D, Tao Y, Xie S, Xiuhua K, Li J, Dan W and Yuan Z (2015) Integration of PK/PD for dose optimization of Cefquinome against *Staphylococcus aureus* causing septicemia in cattle. *Front. Microbiol.* 6:588. doi: 10.3389/fmicb.2015.00588

Cefquinome is a fourth generation cephalosporin with antimicrobial activity against gram negative and gram positive bacterial species, including *Staphylococcus aureus*. The aim of our study was to observe the ex-vivo activity of cefquinome against *Staphylococcus aureus* strains by using bovine serum from intravenously treated cattle. Cefquinome kinetics were measured by liquid chromatography and UV detection. *In vitro* post antibiotic effects (PAEs) and mutant prevention concentrations were determined with *S. aureus* strain ATCC 12598. Cefquinome exhibited time-dependent killing and produced *in vitro* PAEs increasing with concentration and time of exposure. A pharmacokinetic-pharmacodynamic model was established to simulate the efficacy of cefquinome for different dosage regimens. A dosage of 2 mg/kg every 12 h for 3 days was expected to reach a bactericidal activity against *S. aureus* in case of septicemia.

**Keywords:** cefquinome, *Staphylococcus aureus*, cattle, PK/PD, septicemia

## Introduction

*Staphylococcus aureus* is a gram positive bacteria, responsible for many animal and human diseases. It can cause an extensive variety of infections from skin and soft tissue infections to septicemia. If it is not treated efficiently, the sepsis can produce inflammatory response, organ dysfunction syndrome, shock, and finally death (Fecteau et al., 2009; Fluit, 2012). During the first day of life septicemia commonly occurs in calves. Bacteremia was observed during the neonatal period in 20–30% of diarrheic calves. In bacteremia or septicemia, the bacteria isolated included *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Klebsiella* spp., and *Staphylococcus* spp (Raboisson et al., 2010). Consequently, *S. aureus* has a documented effect on mortality, with related rate of mortality at 20–40% (Uramatsu et al., 2010). Since, staphylococcal diseases are usually treated with antibiotics there is an associated risk to select antibiotic resistance (Normand et al., 2000). Without treatment, the mortality rate will be high (Gordon, 1998).

Rational Antibiotic therapy needs to ensure clinical efficacy and to reduce the risk of antibiotic resistance selection and amplification (Toutain et al., 2002; Ambrose et al., 2007; Jacobs, 2007; Tam et al., 2007). The beta-lactam antibiotic is safer in the target animals as compared to other

antibiotics (Dumka et al., 2013). The beta lactam antibiotic is time dependent and its bactericidal activity is lower as compared to other bactericidal antibiotics. Also, it has no or a minimal post antibiotic effect. Therefore to optimize the efficacy, the drug concentration should be maintained above the MIC for a longer time during the dosing interval at the site of infection (McKellar et al., 2004; Owens and Ambrose, 2007; Zonca et al., 2011; Papich, 2014). Cefquinome is the fourth generation cephalosporin, mainly used in veterinary medicine. This drug has been developed especially for use in veterinary medicine (Smiet et al., 2012; Papich, 2014), and is registered in many countries worldwide. Cefquinome has been used for the treatment of many diseases including acute mastitis, respiratory diseases, food rot in cattle, calf septicemia, metritis-mastitis-agalactia syndrome in sows, foal septicemia and respiratory diseases in horses (Uney et al., 2011; Liu et al., 2012; Dumka et al., 2013; Shan et al., 2014). The advantages of cefquinome include broad spectrum antibacterial activity, stability against  $\beta$ -lactamase, enhanced potency, and bioavailability and the ability to penetrate easily into gram negative bacteria (Dumka et al., 2013). Cefquinome pharmacokinetics (PK) have been studied in several animal species such as camels, horses, ducks, cows, wild boars, piglet, rabbits and cattle (Allan and Thomas, 2003; Ehinger et al., 2006; Li et al., 2008; Al-Taher, 2010; Hwang et al., 2011; Liguio et al., 2011; Liu et al., 2012; Shan et al., 2014).

In human medicine, international guidelines for management of severe sepsis underline the needs to base the choice of antibiotics on the knowledge of their pharmacokinetics and their pharmacodynamics (PK/PD) (Dellinger et al., 2008; Solomkin et al., 2010; Duszyńska et al., 2012). The use of PK/PD modeling has also become common in the veterinary literature. PK-PD principles are an important tool to be used by the regulatory authority when studying the application of an antimicrobial. It was also stated that the single most important factor responsible for the emergence of resistance is bacterial exposure to sub optimal concentration of an antibiotic (Papich, 2014). Therefore, some strategies have been established for dosage regimens to attain appropriate PK/PD targets in severe infection and to minimize selection of antibiotic resistance (Drusano, 2004; Olofsson and Cars, 2007; Duszyńska et al., 2012). Recently, one researcher investigated the pharmacokinetics and *ex-vivo* pharmacodynamics activity of cefquinome by using tissue cage fluid and serum obtained from pigs (Zhang et al., 2014). Efficacy of different dosage regimens was investigated in a *Staphylococcus aureus* infection in a thigh model of neutropenic mouse (Wang et al., 2014).

The objectives of the present study were to examine the PK and, establish *in vitro* PD parameters and *ex-vivo* PD characteristics of cefquinome in serum for integration in a PK-PD model for cefquinome for treatment of *Staphylococcus aureus* systemic infections in calves.

## Materials and Methods

### Animals

All the experimental procedure in this study were performed according to the guidelines of the committee on the use and

care of the laboratory animals in Hubei province China. The study was approved by the Animal Care Center, Hubei Science and Technology Agency in China (SYXK 2013-0044). All the animals were monitored throughout the study for any adverse effect signs. The study was conducted in 6 healthy cattle calves of 6–10 months of age. Body weights (BW) was  $185 \pm 10$  kg. They were housed in a  $8 \times 10$  m cattle pen, and the pen was cleaned daily. The room temperature was  $25 \pm 2^\circ\text{C}$  and a relative humidity of 45–65% was maintained in the animal house. All animals were allowed a 15 days acclimation period before the study began. The water and feed for the animals was available *ad libitum*. None of the animals had been treated with antibiotics before.

### Drug Administration and Sample Collection

Each calf received cefquinome (Cefquinome sulfate 2.5%, Shanghai Tongren Pharmaceutical Company Ltd.) at a dosage of 1 mg/kg BW. The drug was administered intravenously to 6 cattle by jugular vein at the dose rate of 1 mg/kg. The blood samples (5 mL) were collected before and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h after cefquinome administration from opposite jugular vein. Blood samples were collected into EDTA tubes and tubes without anticoagulant. Plasma and serum were obtained immediately by centrifugation at 3000 g for 20 min at  $4^\circ\text{C}$  and the supernatants (plasma or serum) were stored at  $-20^\circ\text{C}$  until analysis.

### Drug Analysis

#### Chemicals and Reagents

The cefquinome reference standard (95% purity) was kindly supplied by Dr. Ehrenstorfer (Augsburg, Germany). The reagents used were of analytical grade. Formic acid, methanol (MeOH), and acetonitrile (ACN) were provided by TEDIA (USA). Solid phase extraction (SPE) cartridges (Waters Oasis<sup>TM</sup> HLB, waters) were used in the analytical method. De-ionized water (Milli-Q Millipore Corp.) was used during the study.

### Development of HPLC Method

Plasma samples were analyzed using a Waters 2695 series high performance liquid chromatography (HPLC) and a Waters 2587 UV detector set at a wavelength of 268 nm. The chromatographic separation was achieved with an analytical ZORBAX SB-C<sub>18</sub> column (250 × 4.6 mm i.d., 5  $\mu\text{m}$ ; Agilent Technology, USA) at  $30 \pm 5^\circ\text{C}$ . The separation was on isocratic mode with mobile phase A containing 0.1% formic acid and B acetonitrile (90/10, v/v) at 0.9 mL/min flow rate. The injection volume was 50  $\mu\text{L}$ .

After thawing at room temperature, aliquots of 250  $\mu\text{L}$  cattle plasma were collected in 1.5 mL tubes. Then, 500  $\mu\text{L}$  methanol was added, tubes were shaken for 20 s, and were centrifuged (8000 g) for 10 min at  $4^\circ\text{C}$ . After centrifugation, the supernatant was pipetted into a tube and 10 mL water was added. The mixture was then cleaned up on a HLB SPE cartridge (3 mL, Waters Corp., Milford, MA, USA), preconditioned with 3 mL methanol and 3 mL water. After transfer, the cartridge was washed with 3 mL water and 3 mL (10% methanol). The analytes were eluted with 3 mL acetonitrile. The samples were dried with a stream of nitrogen at  $50^\circ\text{C}$ . The residue remaining after evaporation with nitrogen was reconstituted with 500  $\mu\text{L}$  of 15% acetonitrile.

After Vortex mixing for 20 s, the samples were filtered through 0.22 µm nylon Millipore chromatographic syringe filter into an autosampler vial.

For calibration, 250 µL blank plasma were spiked with 0.01–5 µL of a series of diluted cefquinome working standard solutions and analyzed as above. The cefquinome concentrations in the prepared standard samples were 0.01, 0.02, 0.04, 0.08, 0.16, 1, 2, and 5 µg/mL. Spiked quality control samples were prepared at the concentrations of 0.04, 0.08, and 0.16 µg/mL. Retention time for cefquinome in plasma was 13.40 min. The limit of detection (LOD) and limit of quantification (LOQ) were 0.01 and 0.04 µg/mL. Cefquinome quantification is linear within a range of 0.01–5 µg/mL ( $r^2 > 0.999$ ). The recovery of cefquinome from plasma was  $76.24 \pm 2.55\%$  (mean ± SD). The coefficient of variability (CV %) was all <15% for intra- and inter-day variation.

### Pharmacokinetic Analysis

Pharmacokinetic analysis was performed using Win-Nonlin (version 5.2.1, Pharsight Corporation, Mountain View, CA, USA). The data obtained after plasma concentration determination were analyzed by compartmental methods. The compartmental analysis was evaluated based on Akaike Information Criteria estimates and coefficient of determination application for the best fit model (Liu et al., 2012). The data were analyzed by non-compartment modeling. To find the values of the area under the concentration-time curve (AUC) and area under the first moment curve (AUMC), we used the linear trapezoidal route. The apparent volume of distribution at steady state (VSS) and volume of distribution (VDarea) were calculated according the following equation.

$$\begin{aligned} VD_{area} &= \frac{\text{Dose}}{\text{AUC} \times \beta} \\ VSS &= \frac{\text{Dose}/\text{AUC}}{\text{AUMC}/\text{AUC}} \end{aligned}$$

The pharmacokinetic parameters are presented as mean ± SD.

### Pharmacodynamics

#### Bacterial Strain and Animals

*Staphylococcus aureus* Rosenbach (ATCC BAA-934), subsp. Aureus (ATCC-12598), and ATCC-29213 strains were purchased from the American Type Culture Collection (ATCC). *Staphylococcus aureus* subsp. Aureus Rosenbach (ATCC-12598) was isolated from septic arthritis ([www.atcc.org](http://www.atcc.org)). Thirty *S. aureus* strains isolated from healthy cattle in Huazhong Agriculture University Wuhan, China were also evaluated. The strains were stored at –80°C. Prior to each experiment, the bacteria were grown freshly on Chrom agar and MH agar and incubated at 37°C.

#### Determination of Minimal Inhibitory Concentration, Minimal Bactericidal Concentration, Mutant Prevention Concentration, and Post Antibiotic Effect

The minimal inhibitory concentration (MIC) of cefquinome against *S. aureus* strains were determined in both broth and serum by micro dilution method according to the CLSI (Clinical

and Laboratory standards Institute, 2008), at concentrations between 8 and 0.015 µg/mL. Microplates were incubated at 37°C for 24 h. MIC was determined as the lowest cefquinome concentration where at the end of the incubation period for 24 h, growth of visible bacteria was inhibited. For the minimal bactericidal concentration (MBC) of cefquinome against *S. aureus* strains, 100 µL from each well were successively diluted in 0.85% sodium chloride solution by 1:10 steps and 10 µL were spread on MH agar plates for colony forming unit (cfu) counting and incubated at 37°C for 24 h. MBC was defined as the lowest drug concentration which resulted in a 99.9% reduction in the bacterial density. MBC is the result of five independent experiments. The mean was expressed as the final result.

The mutant prevention concentration (MPC) of cefquinome is determined by agar method. The inoculum of *Staphylococcus aureus* was concentrated to  $10^{10}$  CFU/mL according (Balaje et al., 2013). Bacterial suspensions were inoculated on the agar plates containing serial dilutions of cefquinome and cultured for 96 h. MPC was the lowest drug concentration on agar plates without bacterial growth under anaerobic conditions. Drug range tested for MPC was 1 MIC, 2 MIC, 4 MIC, 8 MIC, 16 MIC, and 32 MIC.

Post-antibiotic effect (PAE) of cefquinome against *Staphylococcus aureus* is estimated with removal of drug methods. The strain of *S. aureus* was incubated with 1 MIC, 2 MIC, 4 MIC of drug. After one and two hours' incubation, the drug was eliminated by several times of centrifuge and wash with fresh medium. The colony forming units (CFU) per milliliter were determined at different time points. The recovery growth kinetic curves of bacteria were established in order to calculate the PAE.

#### In vitro and Ex-vivo Bacterial Killing Curves

After MIC and MBC determinations, different concentration of cefquinome were prepared in MHB ranging from 1/8 to  $16 \times$  MIC before bacterial inoculation ( $10^6$  cfu/mL). Growth was checked with a control. The tubes containing bacteria and different concentration of cefquinome were incubated at 37°C and the viable counts of bacteria were determined at 2, 5, 8, and 24 h. At each time, 100 µL obtained were sampled, gradient diluted by saline and then colony forming units were counted. The limit of detection was 10 cfu/mL.

Serum samples obtained from calves that had received cefquinome intravenously were used. Controls were prepared from serum samples collected from the same calves before administration. A 10 µL volume of bacterial culture in stationary phase was added to 1 mL of serum to give a final suspension of approximately  $10^6$  cfu/mL. The tubes containing bacterial culture and serum were than incubated at 37°C, and viable counts were determined at 0, 2, 5, 8, and 24 h (Shan et al., 2014).

The time-kill curves obtained with serum were analyzed with a pharmacodynamic model described by the following equation

$$\frac{dB}{dt} = k_{net} \times \left(1 - \frac{B}{B_{max}}\right) \times B - \left(\frac{E_{max} + C^\gamma}{EC_{50}^\gamma + C^\gamma}\right) \times B$$

Where  $B$ , is the number of bacterial cell expressed as cfu/mL,  $k_{net}$  the net growth rate,  $B_{max}$  the maximum number of bacteria,  $E_{max}$

the maximum killing rate,  $EC_{50}$ , the concentration to reach half of maximal killing rate and  $\gamma$ , the steepness.

The *ex-vivo* time kill curve was fitted with this model with the hypothesis of a decrease in cefquinome concentration according the incubation time using the proc lsqnonlin (Matlab).

### Pharmacodynamic Analysis, PK-PD Integration and PK-PD Modeling Analysis

For cefquinome, the surrogate markers of antimicrobial activity,  $AUC_{24h}/MIC$ ,  $C_{max}/MIC$ , and  $T > MIC$ , were determined by linking *in vitro* data and *in vivo* pharmacokinetic parameters for serum.

$$\frac{dB}{dt} = k_{net} \times \left(1 - \frac{B}{B_{max}}\right) \times B - k_{death} \times B \\ - \left(\frac{E_{max} + C^\gamma}{EC_{50}^\gamma + C^\gamma}\right) \times B$$

The relationship between the *ex-vivo*  $AUC_{24h}/MIC$  ratio and the variation between the initial bacterial count and the bacterial count after 24 h of incubation (cfu/mL) in serum content was established by using the inhibitory sigmoid  $E_{max}$  model (FS and Lees, 2001). This model is described by the following equation

$$E = E_{max} - \frac{(E_{max} - E_0) \cdot C^N}{C^N + EC_{50}^N}$$

where  $E$  is the effect of antibacterial agent measured as the change in  $\log_{10}$  difference of bacterial count after 24 h incubation compared with the initial  $\log_{10}$  cfu/mL in the serum sample;  $E_0$  is the change in  $\log_{10}$  difference in bacterial count of control sample between 0 and 24 h;  $E_{max}$  is the change in  $\log_{10}$  difference in bacterial count between 0 and 24 h in the cefquinome containing samples;  $EC_{50}$  is the  $AUC_{24h}/MIC$  value producing 50% of the  $E_{max}$ ;  $C$  is the  $AUC_{24h}/MIC$  ratio being examined; and  $N$  is the Hill coefficient that describes the steepness of the ( $AUC_{24h}/MIC$ ) effect curve. These PD parameters were calculated using the GraphPad Prism software (version 5.01, USA).

The *ex-vivo* antibacterial effect of cefquinome after intravenous administration were quantified MIC from the sigmoid  $E_{max}$  equation by determining  $AUC_{24h}/MIC$  for four levels of effect: for bacteriostatic action (no change in bacterial count, that is  $E = 0$ ), for 50% reduction in the bacterial count, for bactericidal action (a 99.9% reduction in bacterial count) and for bacterial elimination (a 99.99% reduction) (FS and Lees, 2001). The dose was calculated by the using the following formula.

$$Dose = \frac{(AUC_{24}/MIC) \cdot MIC \cdot CL}{fu \cdot F}$$

Cefquinome is time dependent drug and the PK/PD index responsible for the efficacy is  $T > MIC$ . To find  $T > MIC\%$  we used the following formula

$$T > MIC = \ln \left( \frac{D}{Vd \cdot MIC} \right) \cdot \frac{T1/2\beta}{\ln 2} \cdot \frac{100}{t}$$

Where  $T > MIC$  (in percent) is the time interval during which the drug plasma concentration is above or equal to the minimal inhibitory concentration (MIC) values;  $D$  is the planned dose;  $t_{1/2}$  the terminal elimination half-life; and  $t$  is the dose interval (Smiet et al., 2012).

To investigate the effect of different dosage regimens, the pharmacodynamic model describing bacterial growth rate in function of cefquinome concentration was combined with the pharmacokinetic model and simulations were performed with mlxplor software (version-1.1.0, Lixoft, Orsay, France).

## Results

### Pharmacokinetics of Cefquinome

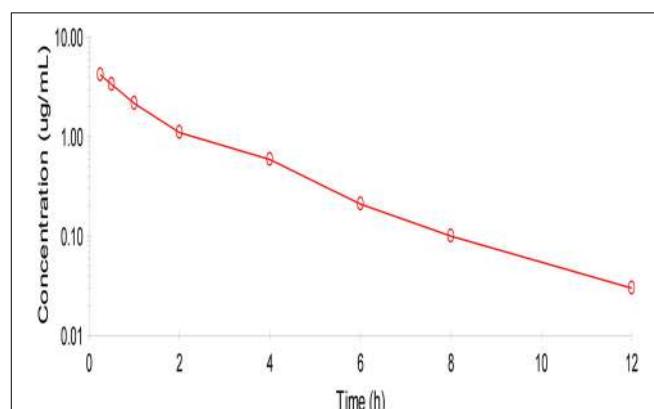
No adverse reactions were observed after intravenous drug administration. The plasma concentration-time profiles are illustrated in (Figure 1). After intravenous administration, plasma concentration of cefquinome was best fitted with a two-compartment model (Table 1). The elimination half-life was  $2.1 \pm 0.45$  h showing rapid elimination after intravenous administration. The area under the concentration time curve  $AUC_{0-\infty}$  was  $8.04 \pm 0.34 \mu\text{g.h/mL}$ . The volume of distribution at steady state ( $V_{ss}$ :  $0.28 \pm 0.02 \text{ L/kg}$ ) was low. The mean residence time up to last was  $2.3 \pm 0.3$  h.

### Pharmacodynamics

#### MIC, MBC, MPC, and PAE of Cefquinome against *Staphylococcus aureus* Strains

The MIC and MBC of cefquinome against *S. aureus* were for the three tested strains at 0.25 and 0.5  $\mu\text{g/mL}$  in culture medium MHB and in serum (Table 2).  $MIC_{90}$  of 30 strains isolated from healthy cattle in our laboratory was 0.25  $\mu\text{g/mL}$ . The mutant prevention concentration (MPC) of cefquinome against the 3 strains in MHB and serum were 2  $\mu\text{g/mL}$ .

Post-antibiotic effect (PAE) of cefquinome for different concentrations (1x, 2x, 4x MIC) and time exposure (1, 2 h)



**FIGURE 1 |** Semi-logarithmic plot of mean serum concentration after intra venous administration of cefquinome (1 mg/kg) in cattle ( $n = 6$ ).

Two compartment method (Winonlin software) were used to observe the concentration-time curve.

is lower than 1 h and increases with time and concentration (**Table 3**).

### In vitro and Ex-vivo Antimicrobial Activity

Time kill curves obtained against *S. aureus* ATCC-12598 for different concentrations of cefquinome expressed as multiple of MIC were reported (**Figure 2**). The curves were characteristics of time-dependent antibiotic activity. The net growth rate is lower for concentration below the MIC. Meanwhile the bactericidal activity increased with increasing concentration of the cefquinome up to 4 MIC. A further increase in concentration resulted in the same death rate.

Serum samples from six cattle that had been administered cefquinome intravenously collected at different time points were used to determine *ex-vivo* killing rate. The results show that at the highest concentration, the number of bacteria decreased slightly (**Figure 3**). A net killing rate is obtained with samples collected before 6 h and growth is observed for serum samples collected after 8 h. In the data set with serum, a phenomenon of decrease

of bacteria number followed by a regrowth was observed on the time kill curves obtained with serum sampled at 8 and 12 h. After fitting, the net killing rate was  $0.95 \text{ h}^{-1}$ , the net growth rate was  $k_{net} = 0.6915 \text{ h}^{-1}$ , the maximum number of bacteria  $B_{max}$  was  $(10E9.25)$ , the maximum killing rate was  $E_{max} = 1.67 \text{ h}^{-1}$ ,  $EC_{50} = 0.06 \mu\text{g/mL}$ ,  $\gamma = 0.70$  and cefquinome decrease rate =  $0.15 \text{ h}^{-1}$ .

### PK/PD Integration

The mean values of the PK/PD indices  $C_{max}/\text{MIC}$ ,  $AUC_{24}/\text{MIC}$ ,  $C_{max}/\text{MBC}$ ,  $AUC_{24}/\text{MBC}$ , and  $C_{max}/\text{MPC}$ ,  $AUC_{24}/\text{MPC}$  of cefquinome against *staphylococcus aureus* are shown in (**Table 4**). The values determined for  $C_{max}/\text{MIC}$  and  $AUC_{24}/\text{MIC}$  were 16.72 and 32.16 h. The mean values for  $C_{max}/\text{MPC}$  and  $AUC_{24}/\text{MPC}$  were 2.09 and 4.02.

### PK/PD Modeling

The association between  $AUC_{0-24}/\text{MIC}$  ratio and antibiotic efficacy was best described by using inhibitory sigmoid  $E_{max}$  model. The parameters obtained  $N$ ,  $E_0$ ,  $E_{max}$  and  $AUC_{0-24}/\text{MIC}$  values required for various degrees of antibacterial activity are shown in **Table 5**. The values of  $AUC_{0-24}/\text{MIC}$  ratios for bacteriostasis activity, bactericidal action and virtual eradication were 29.71, 51.97, and 67.51, respectively.

### Estimation of Dose

The dose of cefquinome in cattle calves after a single intravenous administration, based on the observed  $AUC_{24h}/\text{MIC}$  values by modeling PK/PD data, MIC values and PAE values of our study and  $MIC_{90}$  required for bacteriostatic, bactericidal and bacterial eradication responses are 3.6, 6.4, and 8.3 mg/kg for bacteriostatic, bactericidal action and virtual eradication activity for 24 h dosage interval. The  $t > \text{MIC}$  for the above mentioned dose are 50, 57, and 60%.

**TABLE 1 | Pharmacokinetics parameters after intravenous administration of Cefquinome in cattle at the dose (1 mg/kg) body weight.**

PK parameters	Mean	SD
AUC ( $\mu\text{g.h/mL}$ )	8.04	0.34
$K_{10}$ (1/h)	0.67	0.03
$K_{12}$ (1/h)	0.39	0.09
$K_{21}$ (1/h)	0.69	0.24
$T \frac{1}{2}\alpha$ (h)	0.48	0.09
$T \frac{1}{2}\beta$ (h)	2.10	0.45
$V_1$ (L/kg)	0.18	0.00
CL (L/h.kg)	0.12	0.00
$V_{ss}$ (L/kg)	0.28	0.02
MRT (h)	2.30	0.30
AUMC ( $\mu\text{g.h}^2/\text{Ml}$ )	18.56	2.19

AUC, Area under the concentration-time curve;  $K_{10}$ , First order elimination rate constant;  $K_{12}$ , First order transfer rate constant describing distribution between central and peripheral compartment;  $K_{21}$ , First order transfer rate constant describing distribution between peripheral and central compartment;  $T \frac{1}{2}\alpha$ , Half-life of distribution;  $T \frac{1}{2}\beta$ , Half-life of elimination; CL, Systemic clearance;  $V_{ss}$ , Volume of distribution at steady state; MRT, Mean residence time; AUMC, Area under the first moment-time curve.

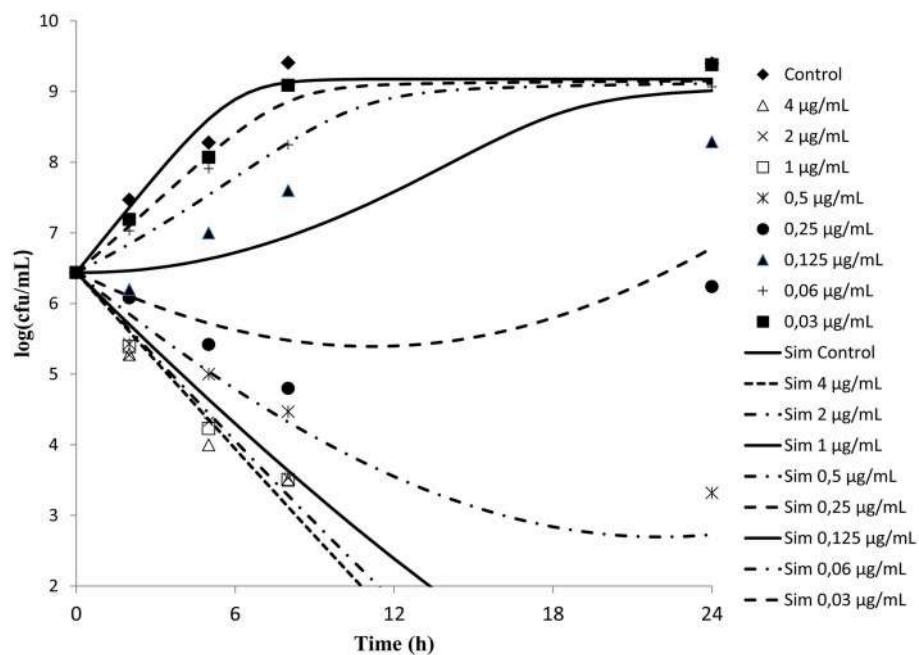
**TABLE 3 | Post antibiotic effect (PAE) after 1 and 2 h. (ATCC-12598).**

Antibacterial concentration	PAE after 1 h (h)	PAE after 2 h (h)
1MIC	0.10	0.10
2MIC	0.20	0.20
4MIC	0.30	0.60

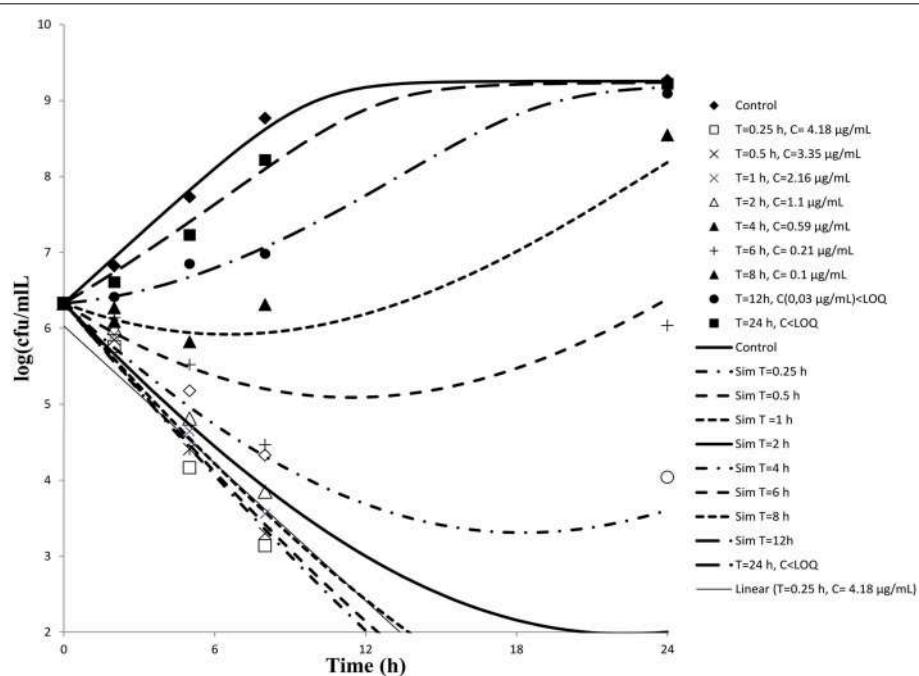
**TABLE 2 | MIC, MBC and MPC ( $\mu\text{g/ml}$ ) of Cefquinome against *staphylococcus aureus*.**

Parameters	Matrix	Strain 1 (ATCC-29213)	Strain 2 (ATCC-BAA-934)	Strain 3 (ATCC-12598)	Mean $\pm$ SD
MIC	MHB	0.25	0.25	0.25	$0.25 \pm 0.00$
	Serum	0.25	0.25	0.25	$0.25 \pm 0.00$
MBC	MHB	0.5	0.5	0.5	$0.50 \pm 0.00$
	Serum	0.5	0.5	0.5	$0.50 \pm 0.00$
MBC/MIC	MHB	2	2	2	$2 \pm 0.00$
	Serum	2	2	2	$2 \pm 0.00$
MPC	MHB	4	2	2	$2.66 \pm 1.15$
MPC/MIC	MHB	16	8	8	$10.66 \pm 4.61$

MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration; MPC, Mutant prevention concentration.



**FIGURE 2 |** *In vitro* antibacterial activity of cefquinome against *Staphylococcus aureus* in MH broth. The figures show that cefquinome exhibit time dependent activity when the concentration increases.



**FIGURE 3 |** *Ex-vivo* antibacterial activity of cefquinome in serum of cattle against *staphylococcus aureus* after intravenous administration ( $n = 6$ ). The concentration of cefquinome in serum at different time interval shows its effect on *staphylococcus aureus*.

Using a PK/PD model with the PD parameters derived from *ex-vivo* analysis, actions of different doses (0.5, 1, 2.5, 5, 7.5 mg/kg) were simulated (Figure 4). According these figures, a dose of 1 mg/kg is not sufficient to reduce the bacterial load

while a dose of 2 mg/kg lead to a net reduction of a factor of 10 after 12 h.

Different dosage regimen for 3 days of treatment (1 mg/kg every 12 h, 1 mg/kg every 24 h and 2 mg/kg every 24 h, 2 mg/kg

**TABLE 4 | Integration of PK/PD data obtained after intra venous administration of Cefquinome (1 mg/kg) in cattle ( $n = 6$ ).**

Parameter	Unit	Mean
$C_{MAX}$	$\mu\text{g/ml}$	4.18
$AUC_{0-24\text{h}}$	$\mu\text{g.h/ml}$	8.04
MIC	$\mu\text{g/ml}$	0.25
MBC	$\mu\text{g/ml}$	0.50
MPC	$\mu\text{g/ml}$	2.00
$C_{MAX}/\text{MIC}$	—	16.72
$AUC_{0-24\text{h}}/\text{MIC}$	h	32.16
$C_{MAX}/\text{MBC}$	—	8.36
$AUC_{0-24\text{h}}/\text{MBC}$	h	16.08
$C_{MAX}/\text{MPC}$	—	2.09
$AUC_{0-24\text{h}}/\text{MPC}$	h	4.02

every 12 h, and 5 mg/kg every 24 h) (Figure 5) were simulated. A dosage regimen of 2 mg/kg every 12 h should be efficient to reach a bactericidal activity in serum (Figure 5).

## Discussion

Pharmacokinetic properties of cefquinome have been studied previously in the serum of cattle after intravenous administration at 1 mg/kg (Shan et al., 2014). The reported terminal half life, clearance and Vss were respectively  $2.4 \pm 0.21$  h,  $0.11 \pm 0.03$  L/h.kg,  $0.3 \pm 0.5$  L/kg and were very close to our values. The terminal elimination half-life of cefquinome is similar to values that were observed in piglet (Zhang et al., 2014), ducks (Liguo et al., 2011), rabbit (Hwang et al., 2011), and horses (Winther et al., 2011) in the range of (0.9–2.77 h) following IV administration. The volume of distribution at steady state was low which means that cefquinome was not as widely distributed as previously reported for piglet (Zhang et al., 2014), sheep (Unay et al., 2011), rabbits (Hwang et al., 2011), and horses (Winther et al., 2011) in the range (0.19–0.36 L/kg).

The MIC of cefquinome against *S. aureus* strains tested ( $0.25 \mu\text{g/mL}$ ) were in the same range as those previously reported (Limbert et al., 1991; Wang et al., 2014). The MBC was determined to be two fold higher.

Cefquinome is considered to be a time-dependent agent and its activity is a function of the time remaining in excess of MIC. There was a regrowth observed in the inoculated bacteria on the *ex-vivo* time kill curves obtained for low concentration. A model with a decreasing of cefquinome concentration in serum was used to fit the data. This mathematical model is the simplest to describe a bacterial regrowth. As antimicrobial concentration in serum after 24 h of incubation was not measured, it was not supported by any data and must be considered as theoretical. A theoretical MIC value for the strain tested was calculated as  $0.064 \mu\text{g/mL}$ . This value means a better susceptibility of *S. aureus* in serum than those observed after 24 h of culture in Mueller Hinton.

The PAE observed *in vitro* below 1 h is short in comparison of 2.9 h obtained *in vivo* in neutropenic mice thigh model of

**TABLE 5 | PK/PD Modeling of *ex-vivo* data after administration of Cefquinome in cattle ( $n = 6$ ).**

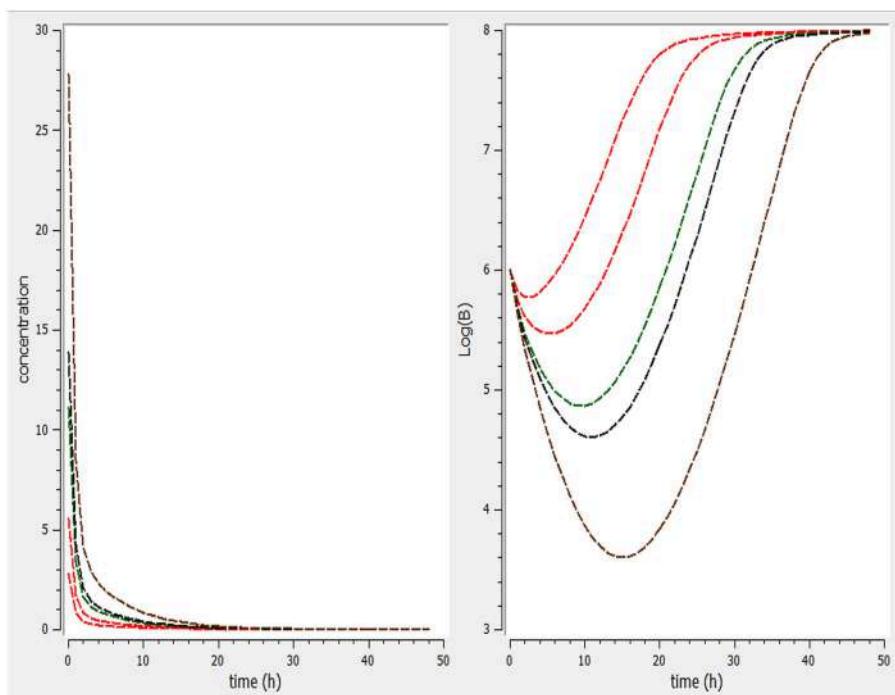
Parameters	Unit	Mean
$E_0$	$\log_{10} \text{CFU/ml}$	2.93
$E_{MAX}$	$\log_{10} \text{CFU/ml}$	-5.33
$E_{MAX}-E_0$	$\log_{10} \text{CFU/ml}$	8.26
Slope (N)	—	2.74
$AUC_{24\text{h}}/\text{MIC EC}_{50}$	h	36.96
$AUC_{24\text{h}}/\text{MIC}$ for bacteriostatic action	h	29.71
$AUC_{24\text{h}}/\text{MIC}$ for bactericidal action (99.9% reduction)	h	51.97
$AUC_{24\text{h}}/\text{MIC}$ for bacterial eradication (99.99% reduction)	h	67.51

$E_0$ , difference in bacterial count in control sample (without drug) between 0 and 24 h;  $E_{MAX}$ , difference in bacterial count in sample incubated with Cefquinome between 0 and 24 h;  $AUC_{24\text{h}}/\text{MIC EC}_{50}$ ;  $AUC_{24\text{h}}/\text{MIC}$  of drug producing 50% of maximal antibacterial effect; N, slope of the  $AUC_{24\text{h}}/\text{MIC}$ —response curve.

*staphylococcus aureus* (Wang et al., 2014). The *in vivo* PAE of cephalosporin were between 2 and 6 h in the former model. Then, PAE determined *in vitro* is a poor predictor of those observed *in vivo*. For our simulation, PAE will not be considered as our data are not enough to build a model to predict *in vivo* scenario.

The bactericidal action of a time dependent drug is relatively slow as compared to concentration dependent drugs (Levison, 2004), and little increase in bactericidal activity is seen when the concentration is increased more than maximal killing concentration, which is approximately equal to 4 times the MIC. These drugs have short or no PAE for gram negative bacilli and have short PAE for gram positive bacilli; the drug concentration above the MIC relative to dosing interval is important, consequently the dosing administration frequency is an important factor for the efficacy. Shorter dosing intervals will increase the duration of time at which the drug concentration is above the MIC of the causative agent (McKellar et al., 2004). For time dependent drugs, AUC/MIC is a poor predictor of efficacy while  $T > \text{MIC}$  is the best surrogate. To estimate this surrogate from PK and *in vitro* and *ex-vivo* data, it is necessary to estimate the maximum kill rate,  $\text{EC}_{50}$  and steepness from the time kill curve data using a differential model (Nielsen and Friberg, 2013). So use of mathematical model describing simultaneously the pharmacokinetics of a drug and the effect on bacterial growth is the best approach to investigate time dependent drugs.

The mutant selection window hypothesis was initially proposed using agar plate assays and then explored in several *in vitro* and *in vivo* models mainly for fluoroquinolones for which resistance appears by mutation. For time-dependent drugs such as cephalosporin, it was rarely reported and as far as we know, only one recent paper investigated *in vivo* the mutant selection window for cefquinome against *E. coli* in an *in vitro* model (Zhang et al., 2014). For *S. aureus*, the observed *in vitro* MPC ( $2 \mu\text{g/mL}$ ) was 8 times higher than the MIC ( $0.25 \mu\text{g/mL}$ ). Compared with the MIC definition, the mutant prevention concentration (MPC) is defined as the lowest drug concentration that prevents the growth of the least susceptible first-step



**FIGURE 4 | Simulate the effect of different doses (0.5, 1, 2.5, 5, 7.5 mg/kg).** The effect of different doses were observed on bacteria and its elimination.

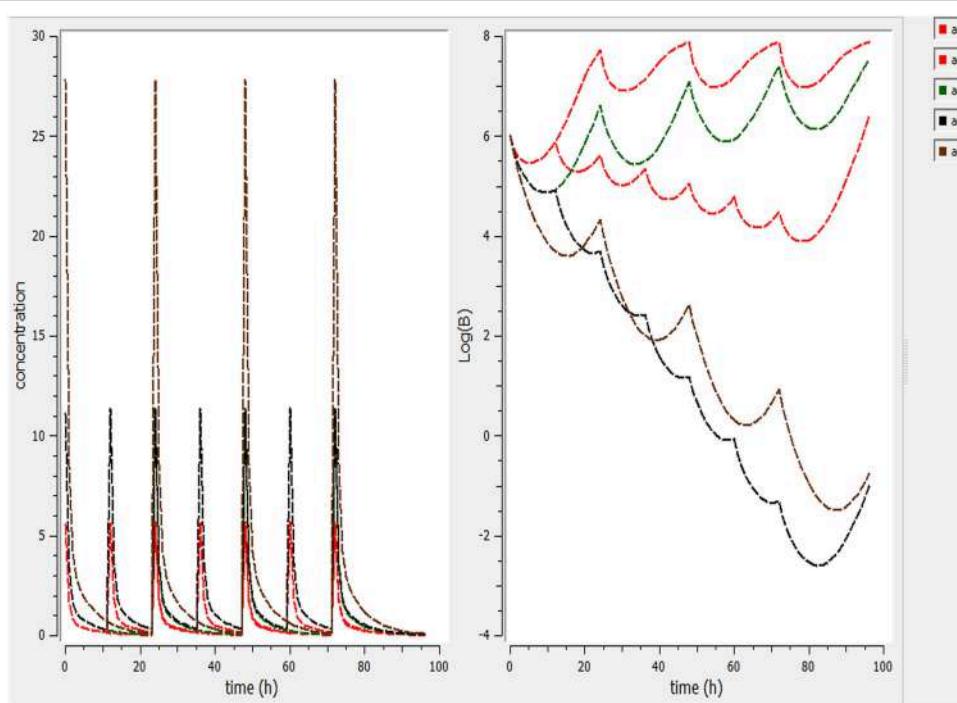
resistant mutants. It has been proposed that the  $AUC_{24\text{ h}}/\text{MPC}$  ratio could help as an indicator of drug exposure that stops the selection of drug-resistant mutants (Zhao and Drlica, 2001; Olofsson et al., 2006). Further investigations are needed to determine the mechanisms of resistance (induced or acquired) observed in our study. For this reason, mutant prevention concentration was not taken into account in our dosage regimen optimization.

PK/PD model helps as a bridge between *in vitro* and *in vivo* study and permits predicting the suitable dose for bacteriological bend and emergence. According our pharmacokinetic and pharmacodynamic parameters, single dose to reach bacteriostatic, bactericidal and eradication activity corresponding to 3.6, 6.4, and 8.3 mg/kg doses to maintain a concentration higher than the observed MIC (0.25  $\mu\text{g/mL}$ ) for a fraction of 50, 57, or 60% of a 24 h interval (Figure 4). For the treatment, we simulate different dosage regimen and show that a 3 day treatment of 2 mg/kg every 12 h should be efficient against *S. aureus* ATCC 12598 in cattle. These PK/PD interrelationships expect that cefquinome treatment is likely to be effective clinically against *S. aureus* strains with the same or lower MIC. Our model is based upon a single strain and with pharmacokinetic parameters derived from healthy animals. Rather it would be better to base our dose determination on the  $MIC_{90}$  values and to obtain more information about the population pharmacokinetics of cefquinome to take into account all the variability associate to animals and bacteria.

The therapeutic effect of an antimicrobial agent depends on several factors such as disease severity, animal immune response,

pathogen load and strain virulence. The objective of the approach described here is to clear an organism of a pathogen, which was expected to be present in the central compartment during acute infection. Our model for *S. aureus* with a MIC of 0.25  $\mu\text{g/mL}$  lead to  $AUC_{24\text{ h}}/\text{MIC}$  for bacteriostasis and bactericidal action close 30 and 52 h which are higher than those (21 and 35 h, respectively) reported in piglet cage tissue fluid for a strain of *E. coli* with an MIC of 0.03  $\mu\text{g/mL}$  (Zhang et al., 2014). The PK-PD surrogates have been widely used to provide dosages that aim to ensure clinical cures. These surrogates are less appropriate to prevent emergence of resistance strains and it has been suggested that the  $AUC/\text{MPC}$  ratio could serve as an indicator of drug exposure that prevents the selection of drug resistant mutant. However, this ratio should be a surrogate of the prevention of resistance selection at the target site. But, the same approaches must be applied to other body compartments such as the intestinal lumen where the microbiota can be exposed to selective concentration of the drug (Zhang et al., 2013).

Resistance to antimicrobials is a major threat for human health, the overuse and misuse is considered to be the main factor for increasing bacterial resistance in both humans and animals. The gut microbiota constitutes one of the key reservoirs of resistance genes between commensal bacterial ecosystems (Andremont, 2003; Phillips et al., 2004; Baquero et al., 2011; De Lastours et al., 2012; Vasseur et al., 2014). Antibiotic dosages presently used in humans and animals have not been developed to prevent the collateral choice on the gut microbiota and the selection and amplification of resistant strains (Fantin et al., 2009; De Lastours et al., 2012; Vasseur et al., 2014). An



**FIGURE 5 | Simulate different dosage regimen (1 mg/kg every 12 h, 1 mg/kg every 24 h, and 2 mg/kg every 24 h, 2 mg/kg every 12 h, and 5 mg/kg every 24 h).** The different doses were simulated for different intervals of time to find the efficient dose and dose intervals

investigation of the interactions between microbial populations and antibiotics, as well as better understanding of the significant factors governing antimicrobial action and resistance range, might lead to the development of strategies combining maximum efficacy with minimum impact on the commensal bacterial ecosystems (Baquero et al., 2011; Cantón and Morosini, 2011; Martinez et al., 2012; Vasseur et al., 2014). For example, current studies confirmed that the degree of increasing of antimicrobial resistance in the gut microbiota was directly associated with the magnitude of the antibiotic dosage, irrespective of the route of administration (Nguyen et al., 2012; Zhang et al., 2013; Vasseur et al., 2014). Therefore there is an essential need to improve the antibiotic dose by taking this effect in the process of drug optimization.

## Conclusion

The purpose of our study was to estimate a dosage regimen of cefquinome after intravenous administration that would be efficient for treatment of *S. aureus* septicemia in cattle. The dosage estimate was based on a pharmacokinetic analysis and some pharmacodynamic studies. As previously described we observed that cefquinome is a time dependent drug with a low *in vitro* PAE for *S. aureus*. Pharmacodynamic parameters were

derived from the analysis of static time kill curves obtained *ex-vivo*. The dosage regimen was simulated using a mathematical model. For a time dependent drug, this approach for dose determination is better than the analysis of the relationship between AUC/MIC value and the number of viable bacteria after 24 h. A dosage regimen of 2 mg/kg every 12 h during 3 days should be efficient in the treatment of *S. aureus* septicemia.

## Author Contributions

Design of the work: HH, LH, ZY. Analysis and interpretation of the work: PS, IA. Drafting the work or revising it critically for important intellectual content: DC, YT, SX, XW. Experimental procedure performed: IA. Paper writing: IA, JL, WD, KX.

## Acknowledgments

This article is financially supported by National Natural Science Foundation of China (31101856/31302143), Grants from National Basic Research program of China (2013CB127200), National Key Technology R&D Program (2012BAK01B00), Fundamental Research Funds for the Central Universities (2662015PY035) and project supported by the morning program of Wuhan in China (2015070404010191).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Salmonella Enteritidis Isolate Harboring Multiple Efflux Pumps and Pathogenicity Factors, Shows Absence of O Antigen Polymerase Gene

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## OPEN ACCESS

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equally to this work.

### Specialty section:

This article was submitted to  
Antimicrobials, Resistance and  
Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 29 January 2016

Accepted: 06 July 2016

Published: 03 August 2016

### Citation:

Jones-Dias D, Clemente L, Egas C, Froufe H, Sampaio DA, Vieira L, Fookes M, Thomson NR, Manageiro V and Caniça M (2016) Salmonella Enteritidis Isolate Harboring Multiple Efflux Pumps and Pathogenicity Factors, Shows Absence of O Antigen Polymerase Gene. *Front. Microbiol.* 7:1130.  
doi: 10.3389/fmicb.2016.01130

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**Keywords:** *Salmonella Enteritidis*, omphalitis, *wzy* deletion, epidemiology, pathogenicity factors, MGE, metal tolerance

## BACKGROUND

*Salmonella enterica* is one of the most important causes of gastrointestinal infection in humans, being the great majority of infections related to the consumption of poultry meat and eggs (Foley and Lynne, 2008; EFSA/ECDC, 2015).

In animals, infections caused by serotype Enteritidis are rarely responsible for severe disease with animals frequently becoming asymptomatic carriers, except in the case of young chicks and pouls, where outbreaks exhibiting clinical disease are often accompanied by high mortality rates (Foley et al., 2008, 2013). Indeed, *S. enterica* subsp. *enterica* serovar Enteritidis (*S. Enteritidis*) has been responsible for severe disease in industrial poultry farming facilities worldwide, posing a potential hazard for public health (Lutful Kabir, 2010).

In order to be infectious, *Salmonella* needs to adapt to different niches and conditions, where virulence and heavy-metal-tolerance factors play an important role, through co-selection events and the formation of pathogenicity islands, respectively (Hensel, 2004; Medardus et al., 2014). Furthermore, antibiotic resistance determinants can also facilitate their survival, with ubiquitous chromosomally encoded efflux mechanisms, playing an important role in both intrinsic, and acquired multidrug resistance. Other resistance mechanisms, such as changes in the membrane permeability, enzymatic modification, and target alterations may increase the levels of bacterial resistance, contributing to the success of the infection (Poole, 2004; Delmar et al., 2014; Li et al., 2015).

Both antibiotic susceptibility determination and serotyping constitute very useful tools for the epidemiologic classification of *S. enterica* isolates. Indeed, in *S. enterica*, the resistance rates fluctuate according to the serotype and with the antibiotic (Clemente et al., 2015). Classically, serotyping is based on the antigenic reactivity of lipopolysaccharide (O antigen) and flagellar proteins (H antigen), followed by a designation using names or formulas (Grimont and Weill, 2007). In this study, we aimed to analyze the genome of a *S. Enteritidis* isolate responsible for omphalitis in chicks,

exploring the molecular features associated with antibiotic resistance and pathogenicity, as well as the ability to spread the respective determinants.

## METHODS

### Bacterial Isolate, Antibiotic Susceptibility Testing, and Serotyping

The isolate (LV60) was recovered from a sample collected from the yolk sac of a chick with omphalitis, under the scope of the “*Salmonella* National Control Programme in food-producing animals and food of animal origin for bacteriological diagnosis, serotype identification and antibiotic susceptibility testing.” The guidelines of the Commission Decision (CD),

2007/407/EC were followed. LV60 was tested for its antimicrobial resistance through the determination of minimum inhibitory concentrations (MICs) using the agar dilution method, as previously described (Clemente et al., 2013) and according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (<http://www.eucast.org/>). Briefly, a panel of 11 antibiotic compounds was tested in a 2-fold concentration series over the following ranges: ampicillin and tetracycline (0.5–64 µg/mL), gentamicin and trimethoprim (0.25–32 µg/mL), ciprofloxacin (0.008–8 µg/mL), cefotaxime (0.06–8 µg/mL), nalidixic acid and streptomycin (2–512 µg/mL), chloramphenicol (2–256 µg/mL), florfenicol (1–128 µg/mL) and sulphamethoxazole (8–1024 µg/mL). The epidemiological cut-off values recommended by EUCAST to *Salmonella* spp. were

**TABLE 1 | Single nucleotide variants that represent amino acid substitutions in *S. Enteritidis* LV60 using *S. Enteritidis* strain p125109 as the reference genome.**

Reference Position	Reference	Allele	Gene (Product)	Amino acid change	Coverage
40158	C	T	SEN_RS00180 (arylsulfatase)	Pro92Ser	155
55278	C	A	<i>ileS</i> (isoleucine-tRNA ligase)	Ala557Glu	144
93979	G	A	SEN_RS00415 (hypothetical protein)	Ala96Thr	127
156264	G	A	SEN_RS00685 (peptidase M23)	Gly299Asp	123
353437	T	C	SEN_RS01600 (isopropylmalate isomerase)	Val454Ala	119
357149	A	T	SEN_RS01625 (hypothetical protein)	Leu1Met	177
401018	C	A	<i>prpE</i> (acetyl-CoA synthetase)	Arg9Ser	132
411602	T	G	SEN_RS01845 (hypothetical protein)	Trp209Gly	58
561577	T	C	SEN_RS02560 (MFS transporter)	Ser333Pro	68
659902	T	G	<i>dpiB</i> (sensor histidine kinase)	Tyr3Asp	52
988620	G	C	SEN_RS04610 (hypothetical protein)	Ala89Pro	130
1044895	G	T	<i>helD</i> (DNA helicase IV)/Mobile element	Ala204Ser	75
1156702	G	C	<i>sirA</i> (virulence gene transcriptional regulator)	Val181Leu	112
1325689	A	G	SEN_RS06450 (hydrogenase-1 operon protein HydF)	Tyr209His	93
1427037	T	A	SEN_RS06930 (diguanylate phosphodiesterase)	Asp16Glu	92
1787654	A	G	SEN_RS08735 (transporter)	Arg348Gly	79
1807289	G	A	SEN_RS08820 (lipoprotein)	Ala14Val	79
1931818	C	T	SEN_RS09505 (NAD-dependent deacetylase)	Met37Ile	82
2115337	C	T	SEN_RS10585 (cobalamin biosynthesis protein CbiB)	Gly167Ser	104
2419980	G	A	SEN_RS11950 (NADH:ubiquinone oxidoreductase subunit M)	Leu474Phe	130
2426844	A	G	SEN_RS11980 (NADH dehydrogenase subunit G)	Val610Ala	125
2463887	T	C	SEN_RS12170 (amino acid transporter)	Ile452Val	34
2647060	G	A	SEN_RS12985 (outer membrane protein RatA)	Pro459Ser	108
2647626	G	T	SEN_RS12985 (outer membrane protein RatA)	Ala270Glu	111
2672592	A	C	SEN_RS13070 (hypothetical protein)	Ile313Ser	61
2956057	C	A	SEN_RS14420 (2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase)	Arg53Leu	123
3185834	C	A	SEN_RS15495 (D-mannonate oxidoreductase)	Asn151Lys	81
3659470	G	T	SEN_RS17815 (membrane protein)	Gln71Lys	122
3802073	G	A	<i>coaD</i> (phosphopantetheine adenyllyltransferase)	Val116Ile	127
4051393	T	C	SEN_RS19620 (DNase TatD)	Ser141Pro	150
4059155	G	A	<i>fadB</i> (3-ketoacyl-CoA thiolase)	Ala395Val	84
4348398	A	G	SEN_RS20980 (membrane protein)/ Salmonella Pathogenicity Island 4	Asn2902Asp	158
4402123	C	T	SEN_RS21190 (sugar:sodium symporter)	Ala350Val	77
4476625	T	C	SEN_RS21580 (hypothetical protein)	Lys76Glu	170
4555382	C	T	SEN_RS21985 (DNA polymerase III subunit chi)	Asp10Asn	110

used for the interpretation of susceptibility testing results. Quality control was performed using the *Escherichia coli* ATCC 25922 strain. LV60 isolate was then serotyped by the slide agglutination method for its O and H antigens using the method of Kauffmann-White scheme (Grimont and Weill, 2007).

## Whole Genome Sequencing (WGS), Assembly, and Annotation

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen), and DNA quantification was performed by Qubit Fluorometric Quantitation (Life Technologies), according to with the manufacturer's instructions. The genome was sequenced using a double strategy of 454 (Roche) and MiSeq (Illumina) sequencing.

Five hundred nanograms of bacterial DNA were fragmented by nebulization, followed by adaptor ligation to create double stranded DNA libraries and sequenced on a 454 GS FLX Titanium according to the standard manufacturer's instructions (Roche-454 Life Sciences). The second genome library was prepared from 1 ng of genomic DNA using the Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced on the Illumina MiSeq sequencer (Illumina) using paired-end 2 × 150 bp reads.

First quality evaluation of raw read sequences and their corresponding quality values were assigned by the FastQC software. Reads were then trimmed and filtered according to quality criteria, and *de novo* assembled with Ray, version 2.3.1 (Boisvert et al., 2010). Contigs were searched for identity through blastn (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the nr/nt NCBI database to identify the closest bacterial genome and/or plasmid. Therefore, LV60 genome was mapped against the bacterial genome of *S. Enteritidis* strain p125109 and its plasmid (NC\_011294 and HG970000, respectively) using GS Mapper version 2.9 (Roche). Additionally SNV (single nucleotide variants) and structural variants were also detected with the GS Mapper (Roche, version 2.9).

Structural and functional annotation was performed using PGP (Prokaryotic Genome Prediction) (Egas et al., 2014), an in-house developed pipeline. Taxonomy identification was performed by BLASTP search against the NCBI GenBank non-redundant (nr) database of the 16 s rRNA sequence gene, identified in the previous step and confirmed using RNAmmer v1.2 (Lagesen et al., 2007).

The final data was submitted in the DDBJ/EMBL/GenBank databases, using the Sequin software tool (<http://www.ncbi.nlm.nih.gov/Sequin/>). This dataset, which includes files in Genbank (LIHI01.1.gbff.gz), Fasta (LIHI01.1.fsa\_nt.gz), and ASN.1 (LIHI01.1.bbs.gz) formats, can be accessed and/or reused at <http://www.ncbi.nlm.nih.gov/nuccore/LIHI00000000>.

## In silico Analyses

CLC genomics workbench 8.0 (QIAGEN, Aarhus), PathogenFinder 1.1, ResFinder 2.1, PlasmidFinder 1.3, and MLST 1.8 (MultiLocus Sequence Typing) were used to estimate the number of pathogenicity determinants, acquired antibiotic resistance genes, plasmids and the MLST using the *S. Enteritidis* genome (Larsen et al., 2012; Zankari et al., 2012; Cosentino et al.,

2013; Carattoli et al., 2014). SeqSero tool was used for *Salmonella* serotyping by whole genome sequencing (Zhang et al., 2015).

PHAST search web tool was applied to detect, identify and annotate prophage sequences (Zhou et al., 2011). ISsaga was used for the high throughput identification and semiautomatic annotation of insertion sequences in the genome (Varani et al., 2011). The presence of molecular determinants of antimicrobial resistance was predicted based on homology and SNP models using the Comprehensive Antibiotic Resistance Database (CARD; <https://card.mcmaster.ca/analyze/rgi>), through Resistance Gene Identifier software (RGI; McArthur et al., 2013).

## RESULTS

LV60 isolate was serotyped as *S. Enteritidis*, using the method of Kauffmann-White scheme, and found to be wild-type to all the antibiotics tested, except tetracycline.

The *de novo* assembly yielded 4.977 Mbp distributed in 83 contigs (largest contig with 970,921 bp) with a N50 of 491,005 bp. Overall, the structural and functional annotation with PGP detected 97 tRNA genes, 7 rRNA genes and identified 4656 mRNA genes.

From mapping against the bacterial genome of *S. Enteritidis* strain p125109, the main difference between the two genomes was the absence of the O-antigen polymerase gene *wzy* in the LV60 isolate, which in *S. Enteritidis* is located outside the O antigen gene cluster (Liu et al., 2014). The coding sequence of *wzy* gene was searched against the assembled genome using blastn, confirming its absence. The flanking regions of *wzy* gene, which coded for a disrupted membrane and a hypothetical protein, were also absent. The *wzy* gene is involved in the Wzx/Wzy-dependent pathway, which constitutes the predominant pathway for O-antigen production in Gram-negative bacteria, specifically in *Salmonella* (Hong et al., 2015).

However, in this study, the absence of the *wzy* gene did not compromised the use of a high-throughput genome sequencing serotype determination method (Zhang et al., 2015), which corroborated the result obtained by the gold standard method. Indeed, this method, based on the detection of O and H antigens encoding genes, predicted an antigenic profile 9:g,m:- based on the O-9,46 *wbaV* gene, which encodes to the O-antigen tyvelosyl transferase. Furthermore, the *S. Enteritidis* serotype was confirmed by the presence of *sdf* gene (*Salmonella* difference fragment virulence gene), a characteristic marker of commonly circulating *S. enterica* serovar Enteritidis (Agron et al., 2001).

Sixty-one SNVs were detected between LV60 and the *S. Enteritidis* strain p125109. The SNVs that resulted in amino acid substitutions are represented in Table 1. *In silico* analysis with ResFinder tool did not reveal the presence of any acquired antibiotic resistance genes (90% identity and 40% minimum length) or plasmids (95% identity). However, the RGI analysis, using the *perfect algorithm*, showed the presence of a *Salmonella*-specific MerR-like gold (Au) sensor- GolS—involved in Au resistance (Pontel et al., 2007). This constitutes a matter of concern since antibacterial biocides and metals can contribute

**TABLE 2 | Perfect and strict best hit results, by predicted gene, obtained using the Resistance Gene Identifier (RGI).**

Predicted gene	e-value	Identity (%)	Contig	Average coverage	Start	Stop	RGI Cut-off	RGI Protein Model_type	Antibiotic Resistance Ontology (ARO) category
<i>golS</i>	1.41E-108	100	4	147.97	80575	81039	Perfect	homolog	efflux pump conferring AR; chloramphenicol RG; beta-lactam RG; gene modulating antibiotic efflux
<i>acrF</i>	0	99	4	147.97	73608	76775	Strict	homolog	efflux pump conferring AR; beta-lactam RG; fluoroquinolone RG
<i>sdiA</i>	0	99	2	127.7	1179091	1179813	Strict	homolog	chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
<i>cpr</i>	1.30E-151	99	7	160.37	388833	389465	Strict	homolog	efflux pump conferring AR; macrolide RG; beta-lactam RG; gene modulating antibiotic efflux; fluoroquinolone RG
<i>mdsA</i>	0	98	4	147.97	76772	77977	Strict	homolog	efflux pump conferring AR; chloramphenicol RG; beta-lactam RG
<i>mdsC</i>	0	98	4	147.97	72134	73624	Strict	homolog	efflux pump conferring AR; chloramphenicol RG; beta-lactam RG
<i>aac(6')-ly</i>	2.36E-101	97	2	127.7	808040	808477	Strict	homolog	antibiotic inactivation enzyme; aminoglycoside RG
<i>cpxR</i>	1.24E-160	97	3	152.34	67603	68301	Strict	homolog	efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux
<i>bacA</i>	0	97	14	155.64	142061	142882	Strict	homolog	peptide AR gene; gene conferring AR via molecular bypass
<i>cpxA</i>	0	96	3	152.34	66233	67606	Strict	homolog	efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux
<i>baeR</i>	5.11E-165	96	2	127.7	107261	107983	Strict	homolog	efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux
<i>emrY</i>	0	95	8	158.13	93935	95473	Strict	homolog	efflux pump conferring AR; tetracycline RG
<i>marA</i>	1.35E-82	95	2	127.7	702301	702690	Strict	homolog	chloramphenicol RG; gene modulating antibiotic efflux; gene modulating permeability to antibiotic; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
<i>H-NS</i>	9.89E-75	94	2	127.7	965098	965511	Strict	homolog	gene modulating antibiotic efflux; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; beta-lactam RG
<i>mexD</i>	0	94	5	135.43	37513	40626	Strict	homolog	chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG
<i>phoP</i>	6.18E-151	93	2	127.7	417112	417786	Strict	homolog	efflux pump conferring AR; polymyxin RG; macrolide RG; gene modulating antibiotic efflux; gene altering cell wall charge conferring AR
<i>emrR</i>	7.58E-115	93	8	158.13	92089	92619	Strict	homolog	efflux pump conferring AR; gene modulating antibiotic efflux; fluoroquinolone RG
<i>mexD</i>	0	93	4	147.97	209028	212177	Strict	homolog	chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG
<i>mdtH</i>	0	92	2	127.7	349496	350704	Strict	homolog	efflux pump conferring AR
<i>mdtK</i>	0	92	2	127.7	607306	608679	Strict	homolog	efflux pump conferring AR; fluoroquinolone RG
<i>mexN</i>	0	92	2	127.7	113873	116995	Strict	homolog	efflux pump conferring AR; chloramphenicol RG

(Continued)

**TABLE 2 | Continued**

Predicted gene	e-value	Identity (%)	Contig	Average coverage	Start	Stop	RGI Cut-off	RGI Protein Model_type	Antibiotic Resistance Ontology (ARO) category
<i>mexN</i>	0	91	2	127.7	110792	113872	Strict	homolog	efflux pump conferring AR; chloramphenicol RG
<i>emrD</i>	0	90	7	160.37	11534	12718	Strict	homolog	efflux pump conferring AR
<i>mdtG</i>	0	90	2	127.7	339682	340896	Strict	homolog	efflux pump conferring AR
<i>pmrA</i>	1.77E-143	90	9	160.96	119082	119750	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
<i>emrA</i>	0	89	8	158.13	92719	93918	Strict	homolog	efflux pump conferring AR; fluoroquinolone RG
<i>pmrE</i>	0	89	2	127.7	174573	175739	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
<i>baeS</i>	0	89	2	127.7	107980	109383	Strict	homolog	efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux
<i>tolC</i>	0	89	14	155.64	163404	164879	Strict	homolog	chloramphenicol RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; aminocoumarin RG; tetracycline RG; rifampin RG; beta-lactam RG
<i>acrE</i>	0	88	1	155.02	4223	5380	Strict	homolog	efflux pump conferring AR; beta-lactam RG; fluoroquinolone RG
<i>mexD</i>	0	88	1	155.02	1098	4211	Strict	homolog	chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG
<i>mdfA</i>	0	87	13	131.07	105101	106333	Strict	homolog	efflux pump conferring AR
<i>pmrF</i>	0	87	5	135.43	231615	232598	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
<i>mdtM</i>	0	86	11	163.1	148308	149549	Strict	homolog	efflux pump conferring AR
<i>ramA</i>	1.93E-71	86	4	147.97	311233	311622	Strict	homolog	chloramphenicol RG; gene modulating antibiotic efflux; gene modulating permeability to antibiotic; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
<i>mdtD</i>	0	86	2	127.7	109383	110795	Strict	homolog	efflux pump conferring AR
<i>acrA</i>	0	85	4	147.97	212200	213393	Strict	homolog	chloramphenicol RG; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
<i>phoQ</i>	0	85	2	127.7	415649	417112	Strict	homolog	efflux pump conferring AR; polymyxin RG; macrolide RG; gene modulating antibiotic efflux; gene altering cell wall charge conferring AR
<i>pmrB</i>	0	85	9	160.96	118002	119081	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
<i>mdtA</i>	0	82	2	127.7	116995	118332	Strict	homolog	efflux pump conferring AR; aminocoumarin RG
<i>pmrC</i>	0	82	9	160.96	119747	121390	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
<i>acrR</i>	1.83E-124	82	4	147.97	213535	214188	Strict	variant	chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; antibiotic resistant gene variant or mutant; tetracycline RG; rifampin RG; beta-lactam RG
<i>robA</i>	0	81	11	163.1	77518	78387	Strict	homolog	chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
<i>arnA</i>	0	79	5	135.43	229636	231618	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR

(Continued)

**TABLE 2 | Continued**

Predicted gene	e-value	Identity (%)	Contig	Average coverage	Start	Stop	RGI Cut-off	RGI Protein Model_type	Antibiotic Resistance Ontology (ARO) category
<i>mdtL</i>	0	77	16	156.65	44691	45878	Strict	homolog	efflux pump conferring AR
<i>rosB</i>	0	74	4	147.97	230248	231924	Strict	homolog	polymyxin RG
<i>rosA</i>	0	71	4	147.97	232128	233348	Strict	homolog	efflux pump conferring AR; polymyxin RG
<i>rpoB</i>	0	58	19	154.2	4220	8248	Strict	variant	rifampin RG; antibiotic resistant gene variant or mutant
<i>katG</i>	0	56	3	152.34	121560	123740	Strict	variant	antibiotic resistant gene variant or mutant; isoniazid RG
<i>gyrB</i>	0	55	16	156.65	54369	56783	Strict	homolog	aminocoumarin RG; antibiotic resistant gene variant or mutant
<i>macB</i>	0	50	13	131.07	143618	145564	Strict	homolog	efflux pump conferring AR; macrolide RG
<i>vanG</i>	8.15E-81	38	4	147.97	113335	114447	Strict	homolog	glycopeptide RG; AR gene cluster, cassette, or operon; gene conferring AR via molecular bypass
<i>macA</i>	2.30E-51	35	13	131.07	142503	143621	Strict	homolog	efflux pump conferring AR; macrolide RG

RG, resistance gene; AR, antibiotic resistance.

to the development and maintenance of antibiotic resistance in bacterial communities through mechanisms of cross- or co-resistance (Baker-Austin et al., 2006; Lemire et al., 2013; Pal et al., 2015).

Furthermore, the RGI strict algorithm, which detects previously unknown variants of known antimicrobial resistance genes, identified 52 genes involved in efflux, transport, and permeability, which might justify the low-level tetracycline resistance identified by phenotypic methods (Table 2). Resistance to additional classes of antibiotics such as fluoroquinolones, aminoglycosides, and chloramphenicol were bioinformatically predicted. Indeed, efflux pumps are often associated with discrete decreases in antibiotic susceptibility that may not necessarily reflect an alteration in interpretation categories (Fernández and Hancock, 2012). Genes responsible for the intrinsic resistance to benzylpenicillin, glycopeptides, macrolides, and rifampicin were also detected.

The total number of pathogenicity determinants present in the genome of *S. Enteritidis* LV60, matching 1164 pathogenic families, showed a 94.1% certainty of the isolate being a human pathogen. Here we highlight the presence of *Salmonella* Pathogenicity Island 4, which usually encodes a non-fimbrial adhesion and the cognate type 1 secretion system (Gerlach et al., 2007).

The use of complementary web tools assigned this isolate to ST11, which according with MLST data (<http://mlst.warwick.ac.uk/>) is commonly found among CTX-M-14 and CTX-M-15-producing *S. Enteritidis* human isolates (Kim et al., 2011; Bado et al., 2012). In this study, the identification of ST11 in an isolate of animal origin, together with other pathogenicity determinants may suggest its zoonotic potential.

We also identified 6 prophage regions, among which three were incomplete and three were intact. The last included prophage regions reaching the lengths of 64.3, 49.2, and 31.7 Kb, and encoding 42, 78, and 66 DNA coding sequences, respectively.

Overall, 33 different IS were detected within the genome, which were distributed as follows: 27.03% of IS3 family,

18.92% of IS256 family, 13.51% of IS unclassified elements, 10.81% of IS200/IS605 complex, and of ISL3 family, 8.11% of IS481 family, 5.41% of IS630 family, and 2.7% of IS1 and IS110 families. All identified structures (pathogenicity island, prophages, ISs) constitute a multiplicity of pathogenicity factors in LV60 *S. Enteritidis* isolate and contribute for the fitness of the isolate in different environments; its presence may also suggest the possibility of acquisition of other factors by different mechanisms, including resistance genes e.g., by horizontal gene transfer, contributing to its biological diversity and genetic evolution.

## CONCLUSION

The detection of an avian *S. Enteritidis* isolate harboring multiple efflux pumps, pathogenicity factors, a variety of mobile genetic elements and heavy-metal-tolerance genes raises concerns regarding the dissemination of infection in birds and potential risk of zoonotic transmission.

This study demonstrated the added value of WGS as a routine tool for surveillance programs directed to food-producing animals, which might complement sanitary measures, essential to prevent the spread of *Salmonella* infections among animals. It also proved to have an added value as a complementary typing method. Moreover, the simultaneous detection of putative Au resistance, intrinsic antibiotic resistant genes, and mobile genetic elements, underline this method as a helpful resource to follow the spread and evolution of antibiotic resistance in this species by genomic comparison studies.

## DATA ACCESS

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LIHI00000000. The version described in this paper is version LIHI01000000.

## AUTHOR CONTRIBUTIONS

DJ designed the study, performed molecular experiments, analyzed the data and wrote the manuscript. LC performed the microbiological experiments and reviewed the manuscript. CE, HF performed 454 Roche genome sequencing experiments and analyze the data; DS, LV performed Illumina genome sequencing experiments. MF, NT analyzed the data. VM designed the study, analyzed the data and reviewed the manuscript. MC designed the study, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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## FUNDING

DJ has received research funding from Fundação para a Ciência e a Tecnologia (FCT, grant number SFRH/BD/80001/2011). VM was supported by FCT fellowship (grant SFRH/BPD/77486/2011), financed by the European Social Funds (COMPETE-FEDER) and national funds of the Portuguese Ministry of Education and Science (POPH-QREN). We thank the support of FCT grant number PEst-OE/AGR/UI0211/2011-2014 and UID/MULTI/00211/2013.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The IncP-6 Plasmid p10265-KPC from *Pseudomonas aeruginosa* Carries a Novel $\Delta$ ISEc33-Associated bla<sub>KPC-2</sub> Gene Cluster

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
*Frontiers in Microbiology*

Received: 10 November 2015

Accepted: 25 February 2016

Published: 10 March 2016

### Citation:

Dai X, Zhou D, Xiong W, Feng J, Luo W, Luo G, Wang H, Sun F and Zhou X (2016) The IncP-6 Plasmid p10265-KPC from *Pseudomonas aeruginosa* Carries a Novel  $\Delta$ ISEc33-Associated bla<sub>KPC-2</sub> Gene Cluster. *Front. Microbiol.* 7:310.  
doi: 10.3389/fmicb.2016.00310

*Pseudomonas aeruginosa* strain 10265 was recovered from a patient with pneumonia in a Chinese public hospital, and it displays the carbapenem resistance phenotype due to the acquisition of a non-conjugative but mobilizable IncP-6-type plasmid p10265-KPC. p10265-KPC carries a Tn5563-borne defective *mer* locus, and a novel  $\Delta$ ISEc33-associated bla<sub>KPC-2</sub> gene cluster without paired inverted repeats and paired direct repeats at both ends. Mobilization of this  $\Delta$ ISEc33-associated element in p10265-KPC would be attributed to homologous recombination-based insertion of a foreign structure Tn3-ISAp1-orf7-ISAp2-ISKpn27- $\Delta$ bla<sub>TEM-1</sub>-bla<sub>KPC-2</sub>- $\Delta$ ISKpn6-korC-orf6-kICa- $\Delta$ repB into a pre-existent intact ISEc33, making ISEc33 truncated at the 3' end. The previously reported pCOL-1 represents the first sequenced KPC-producing IncP-6 plasmid, while p10265-KPC is the second one. These two plasmids carry two distinct bla<sub>KPC-2</sub> gene clusters, which are inserted into the different sites of the IncP-6 backbone and have different evolutionary histories of assembly and mobilization. This is the first report of identification of the IncP-6-type resistance plasmid in China.

**Keywords:** *Pseudomonas aeruginosa*, KPC-2, p10265-KPC, IncP-6

## INTRODUCTION

*Klebsiella pneumoniae* carbapenamases (KPCs) were initially discovered in USA in 1996, and they have disseminated worldwide among Enterobacteriaceae, *Pseudomonas* and *Acinetobacter* with *K. pneumoniae* being the most predominate species (Munoz-Price et al., 2013; Chen et al., 2014b). At least 23 KPC protein variants (KPC-2 to KPC-24; KPC-1 is essentially identical to KPC-2) have been identified<sup>1</sup>. The bla<sub>KPC</sub> genes are typically present on plasmids, varying in size, genetic structure and incompatibility group (e.g., IncFII, FIA, I2, A/C, N, X, R, P, U, W, L/M, and Cole; Munoz-Price et al., 2013; Chen et al., 2014b). Chromosomal location of bla<sub>KPC</sub> has also been evidenced in *Pseudomonas aeruginosa*, indicating that the bla<sub>KPC</sub> genes can be integrated into host genome (Villegas et al., 2007; Cuzon et al., 2011). KPC-producing bacteria are resistant to almost all  $\beta$ -lactams and often to other classes of antibiotics (such as aminoglycosides, quinolones, and tetracyclines), resulting from the co-selection of additional resistance determinants encoded by

<sup>1</sup><http://www.lahey.org/studies/>

insertion sequence-, integron- and transposon-associated mobile gene clusters and, thereby, leaves few or no antimicrobial treatment options (Muñoz-Price et al., 2013; Chen et al., 2014b).

At least thirteen incompatibility groups, IncP-1 to IncP-7 and IncP-9 to IncP-14, have been recognized for the plasmids found in the natural isolates of *Pseudomonas*, and about one third of these plasmids characterized belong to the IncP-2 group which typically have a size > 300 kb (Sagai et al., 1976; Boronin, 1992). Plasmids belonging to IncP-2, IncP-5, IncP-7, IncP-10, IncP-12, and IncP-13 incompatibility groups have a narrow host range and cannot be transferred from *Pseudomonas* to *Escherichia coli*, while the other IncP types especially including IncP-1, IncP-4, and IncP-6 seem to have a broad host range (Sagai et al., 1976; Boronin, 1992; Xiong et al., 2013). IncP-1 corresponds to IncP in the *E. coli* plasmid classification system, and plasmids of this group can transfer and replicate virtually in all Gram-negative bacteria, contributing to the spread of antibiotic and heavy metal resistance (Popowska and Krawczyk-Balska, 2013).

The IncP-6 plasmids are capable of replicating in both *E. coli* (where there are assigned into the IncG group) and *Pseudomonas*. Only a few IncP-6 plasmids, such as Rms149 from *P. aeruginosa* (Haines et al., 2005), pCOL-1 from *P. aeruginosa* (Naas et al., 2013) and pRIO-5 from *Serratia marcescens* (Bonnin et al., 2012), have been fully sequenced, and all these plasmid have acquired various mobile genetic structures harboring resistance markers. In addition, pRSB105 from an uncultured eubacterium represents a mosaic plasmid that carries the IncP-6 backbone as well as the Rep1 replicon module, most likely contributing to the extension of plasmid's host range (Schluter et al., 2007).

Data presented here reveal that *P. aeruginosa* strain 10265 harbors a novel IncP-6 resistance plasmid p10265-KPC. The complete sequence of p10265-KPC was determined and compared with other sequenced IncP-6 plasmids. p10265-KPC carries a novel  $\Delta$ ISEc33-associated *bla<sub>KPC-2</sub>* gene cluster as well as a Tn5563-borne defective mercury resistance (*mer*) gene locus, providing further insights into drug resistance mechanism of the KPC-encoding IncP-6 plasmids.

## MATERIALS AND METHODS

Bacterial species was identified using Bruker MALDI Biotyper (Bruker Daltonics, Bremen, Germany) and 16S rRNA gene sequencing (Frank et al., 2008). The major acquired carbapenemase and extended-spectrum  $\beta$ -lactamase genes were detected by PCR, followed by amplicon sequencing on ABI 3730 Sequencer (Applied Biosystems, Foster City, CA, USA; Chen et al., 2015). The experimental protocols were approved by the Ethics Committee of the Third Military Medical University.

Plasmid electroporation or conjugal transfer was performed with *E. coli* TOP10 (LacZ<sup>-</sup>, resistant to streptomycin and tetracycline) and EC600 (LacZ<sup>-</sup>, resistant to nalidixic acid and rifampicin) being used as recipient for selection of *bla<sub>KPC</sub>*-positive electroporants or transconjugants, respectively (Chen et al., 2015). Transfer of the *bla<sub>KPC</sub>* gene on the plasmid was determined by S1-PFGE and Southern blot hybridization (Lee et al., 2012; Chen et al., 2015).

Activity of Ambler class A/B/D carbapenemases in bacterial cell extracts was determined by CarbaNP test (Dortet et al., 2012) with modifications (Chen et al., 2015). Bacterial antimicrobial susceptibility was tested by VITEK 2 (BioMérieux Vitek, Hazelwood, MO, USA) and interpreted as per Clinical and Laboratory Standards Institute guidelines (Twenty-Fourth Informational Supplement M10-S24, 2014).

Plasmid DNA was isolated from *E. coli* electroporant using Qiagen large construct kit (Qiagen, Hilden, Germany), and sequenced by whole-genome shotgun strategy in combination with Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) sequencing technology. The contigs were assembled with Velvet, and the gaps were filled through combinatorial PCR and Sanger sequencing on ABI 3730 Sequencer. The genes were predicted with GeneMarkS<sup>TM</sup> and further annotated by BLASTP and BLASTN against UniProt and NR databases. Gene organization diagrams were drawn with Inkscape<sup>2</sup>. The complete sequence of p10265-KPC was submitted to GenBank under accession number KU578314.

## RESULTS AND DISCUSSION

### Case Report

In September 2010, an 81-year-old male with hemafecia visited a public hospital in Beijing of China, and the progressive symptoms of fever, cough, and pulmonary infection were observed after hospitalization. The patient had the underlying diseases hypertension, diabetes, multiple cerebral infarction, and chronic renal insufficiency. The patient received long-term hospital care under the hospital and was bed-ridden with indwelling catheter. His symptoms of hemafecia were generally well controlled during hospitalization, but he started to suffer from the recurrent urinary tract infections since August 2013. About 2 weeks later, bacterial colonies were observed after cultivation of the urine specimens on the Mueller-Hinton agar, and the bacterial isolate designated 10265 was identified as *P. aeruginosa*. Based on the antimicrobial susceptibility test results, the patient received the intravenous administration with amikacin, and his symptoms associated with urinary tract infections progressively disappeared.

### Overview of Plasmid p10265-KPC

Screening for the *bla<sub>GES</sub>*, *bla<sub>KPC</sub>*, *bla<sub>SME</sub>*, *bla<sub>IMI</sub>*, *bla<sub>BIC</sub>*, *bla<sub>IMP</sub>*, *bla<sub>VIM</sub>*, *bla<sub>NDM</sub>*, *bla<sub>TMB</sub>*, *bla<sub>FIM</sub>*, *bla<sub>SPM</sub>*, *bla<sub>DIM</sub>*, *bla<sub>GIM</sub>*, *bla<sub>SIM</sub>*, *bla<sub>AIM</sub>*, *bla<sub>SMB</sub>*, *bla<sub>OXA</sub>*, *bla<sub>CTX-M</sub>*, *bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, *bla<sub>GES</sub>*, *bla<sub>PER</sub>*, *bla<sub>VEB</sub>*, and *bla<sub>OXA</sub>* genes by PCR (Chen et al., 2015) indicated the presence of only *bla<sub>KPC-2</sub>* (but not any of the other *bla* genes tested) in strain 10265. Electroporation of the plasmid DNA of strain 10265 into *E. coli* TOP10 generated a *bla<sub>KPC</sub>*-positive electroporant 10265-KPC-TOP10, but repeated attempts of plasmid conjugal transfer with *E. coli* EC600 being used as recipient and strain 10265 as donor failed to obtain a *bla<sub>KPC</sub>*-positive *E. coli* transconjugant. S1-PFGE followed by Southern hybridization (Chen et al., 2015) indicated the presence

<sup>2</sup><https://inkscape.org/>

of a ~40 kb plasmid, being able to hybridize with a *bla*<sub>KPC</sub>-specific probe (Lee et al., 2012), in both 10265 and 10265-KPC-TOP10 (data not shown). 10265 and 10265-KPC-TOP10 but not TOP10 have the class A carbapenemase activity (data not shown). Strain 10265 is resistant to all the  $\beta$ -lactam,  $\beta$ -lactamase inhibitor, fluoroquinolone, and sulfonamide drugs tested but remains susceptible to aminoglycosides, while 10265-KPC-TOP10 is resistant to  $\beta$ -lactams and  $\beta$ -lactamase inhibitors but remains susceptible to all the other drugs (Table 1). Taken together, strain 10265 harbors a non-conjugative plasmid p10265-KPC, which carries the *bla*<sub>KPC-2</sub> gene to mediate the resistance to  $\beta$ -lactams including monobactam and carbapenems.

The complete sequence of p10265-KPC, recovered from the 10265-KPC-TOP10 strain, was determined with a mean coverage of 124, resulting in a circular plasmid sequence of 38,939 bp with an average G + C content of 58.2% (Figure 1). Sequence annotation generated a total of 41 predicted open reading frames. The modular structure of p10265-KPC is divided into the backbone [especially including the regions for plasmid replication (*repA*) and stability (*parABC* and *mob*)], and three separate accessory modules (a novel *bla*<sub>KPC-2</sub> gene cluster, Tn5563, and ISPa19) inserted at the different sites of the backbone.

## Accessory Modules of p10265-KPC

The rapid spread of *bla*<sub>KPC</sub> genes in European and American countries is linked to their location in a Tn3-family unit transposon Tn4401 with at least eight designated isoforms *a* to *g* and a separate *d* (Chen et al., 2012; Bryant et al., 2013; Chmelnitsky et al., 2014). Tn4401b is the prototype one and has a modular structure *tnpRA* (transposition core module)-ISKpn7-*bla*<sub>KPC</sub>-ISKpn6, which is delimited by two 39 bp inverted repeats (IRs), and the other isoforms result from distinct deletion or insertion events occurring at the different sites of Tn4401b (Chen et al., 2012; Bryant et al., 2013; Chmelnitsky et al., 2014).

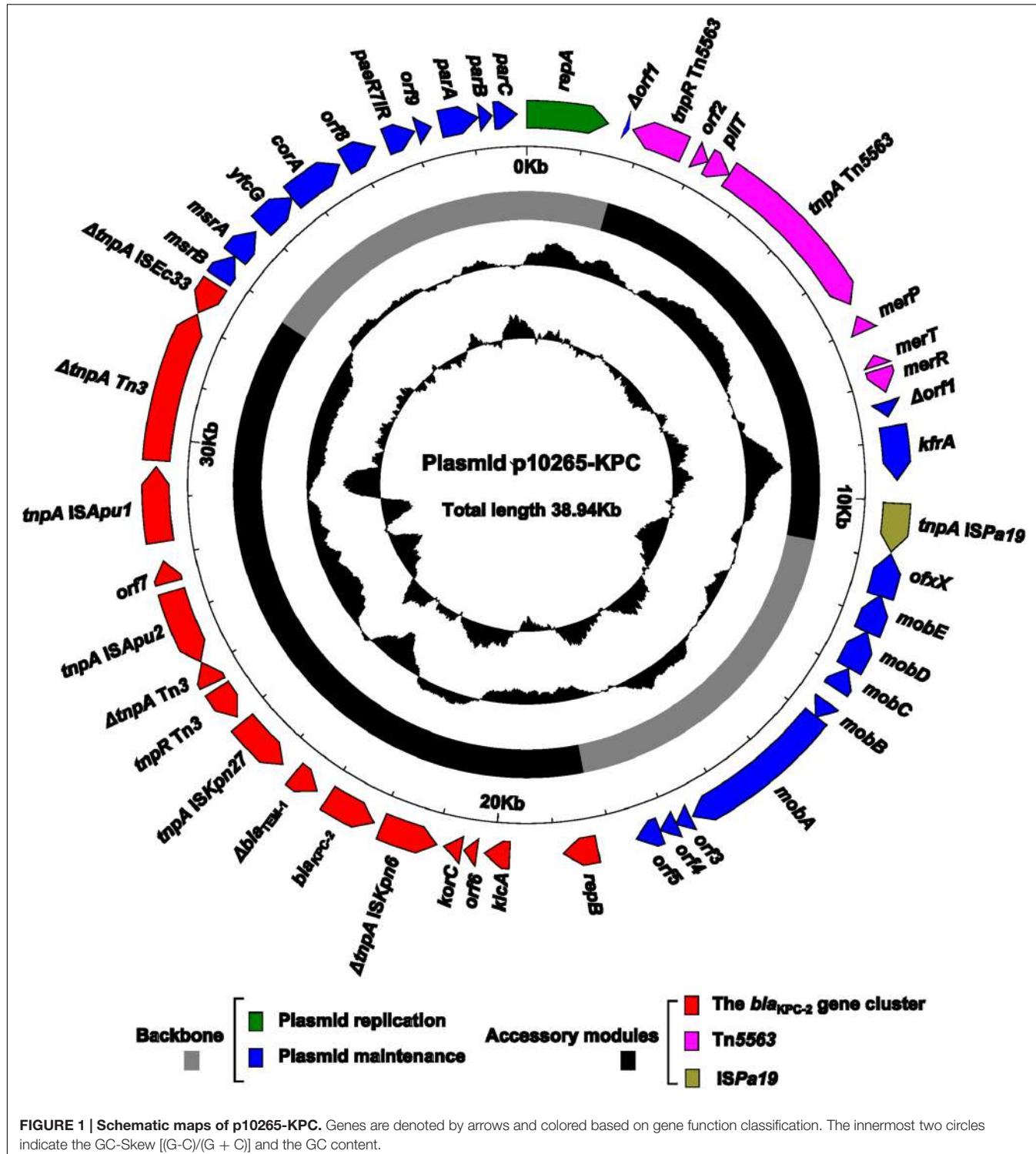
In China, Tn4401 is rarely found (Ho et al., 2013) and, instead, a core module Tn3-ISKpn27-*bla*<sub>KPC</sub>- $\Delta$ ISKpn6 is frequently identified as the *bla*<sub>KPC</sub> platform (Shen et al., 2009; Chen et al., 2014a,c; Li et al., 2015). For generation of the above core module, an intact ISKpn27 is inserted into the 3'-end of Tn3, and then the resulting Tn3-ISKpn27 is connected with *bla*<sub>KPC-2</sub> and  $\Delta$ ISKpn6 (Figure 2). This core module is imbedded in two major classes of transposons (Wang et al., 2015), the Tn1722-based unit transposons [e.g., pKP048 (Shen et al., 2009)] and the IS26-based composite transposons [e.g., pKPC-LKEc (Chen et al., 2014c)].

The Tn1722-based transposon in pKP048 (Shen et al., 2009) looks like a prototype one, and it is generated from the

**TABLE 1 |** Antimicrobial resistance phenotypes of *Pseudomonas aeruginosa* and *Escherichia coli* with plasmid p10265-KPC.

Antibiotics	MIC (mg/L)/antimicrobial susceptibility		
	<i>P. aeruginosa</i> 10265	<i>E. coli</i> 10265-KPC-TOP10	<i>E. coli</i> TOP10
<b>Penicillins</b>			
Ampicillin	$\geq 32/R$	$\geq 32/R$	4/S
Ampicillin/sulbactam	$\geq 32/R$	$\geq 32/R$	4/S
Piperacillin	$\geq 128/R$	$\geq 128/R$	$\leq 4/S$
Piperacillin/tazobactam	$\geq 128/R$	$\geq 128/R$	$\leq 4/S$
<b>Cephalosporins</b>			
Cefazolin	$\geq 64/R$	$\geq 64/R$	$\leq 4/S$
Cefuroxime sodium	$\geq 64/R$	$\geq 64/R$	4/S
Cefuroxime axetil	$\geq 64/R$	$\geq 64/R$	4/S
Cefotetan	$\geq 64/R$	32/R	$\leq 4/S$
Ceftriaxone	$\geq 64/R$	$\geq 64/R$	$\leq 1/S$
Ceftazidime	$\geq 64/R$	$\geq 64/R$	$\leq 1/S$
<b>Carbapenems</b>			
Imipenem	$\geq 16/R$	$\geq 16/R$	$\leq 1/S$
Meropenem	$\geq 16/R$	8/R	$\leq 0.25/S$
<b>Monobactam</b>			
Aztreonam	$\geq 64/R$	$\geq 64/R$	$\leq 1/S$
<b>Fluoroquinolones</b>			
Ciprofloxacin	$\geq 4/R$	$\leq 0.25/S$	$\leq 0.25/S$
Levofloxacin	$\geq 8/R$	$\leq 0.25/S$	$\leq 0.25/S$
<b>Sulfonamide</b>			
Trimethoprim/sulfamethoxazole	$\geq 320/R$	$\leq 20/S$	$\leq 20/S$
<b>Aminoglycosides</b>			
Amikacin	$\leq 2/S$	$\leq 2/S$	$\leq 2/S$
Gentamicin	$\leq 1/S$	$\leq 1/S$	$\leq 1/S$
Tobramycin	2/S	$\leq 1/S$	$\leq 1/S$

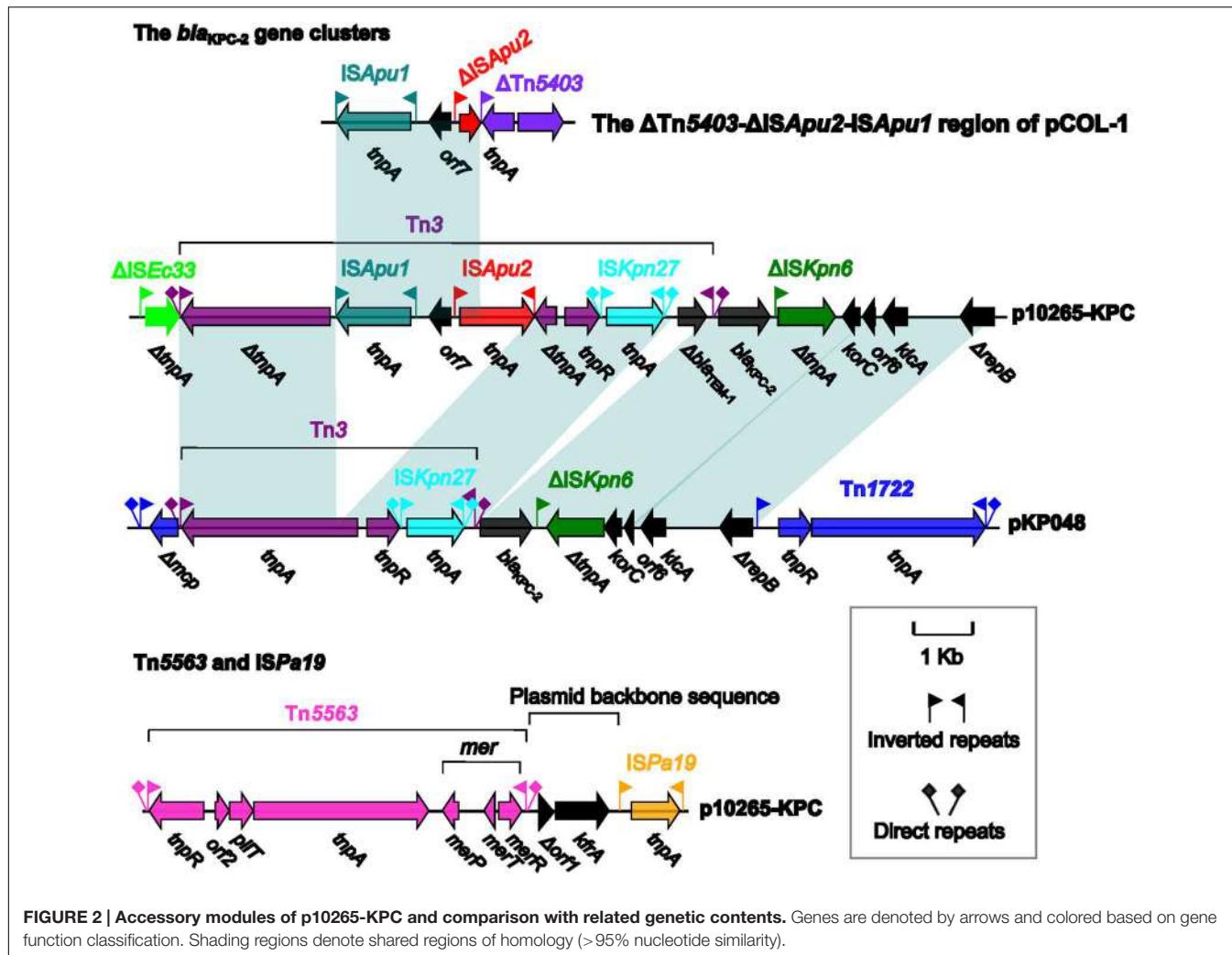
S, susceptible; R, resistant.



insertion of IS $Kpn$ 27-*bla*<sub>KPC-2</sub>-ΔIS $Kpn$ 6-*korC*-*orf*6- *kIC*A-Δ*repB* into the *mcp* gene of the cryptic transposon Tn1722, making *mcp* to be truncated; moreover, it is bounded by 39 bp IRs and further flanked by 5 bp direct repeats (DRs: target site duplications which are usually the signature of a transposition event) at both ends (**Figure 2**). Before being captured by

Tn1722, the above mentioned core module  $\text{ISKpn}27\text{-bla}_{KPC-2}$ - $\Delta\text{ISKpn}6$  is connected with a gene cluster  $\text{kocC}\text{-orf}6\text{-klcA}\text{-}\Delta\text{repB}$ .

In p10265-KPC, the primary genetic content Tn3-*ISKpn27*-*bla<sub>KPC-2</sub>*-Δ*ISKpn6*-*korC*-*orf6*-*kIC*A-Δ*repB* is also found but it has undergone two major evolutionary events: (i) the



**FIGURE 2 | Accessory modules of p10265-KPC and comparison with related genetic contents.** Genes are denoted by arrows and colored based on gene function classification. Shading regions denote shared regions of homology (>95% nucleotide similarity).

insertion of a  $\Delta$ *bla*<sub>TEM-1</sub> gene between ISKpn27 and the Tn3 IRR (IR right), and (ii) the insertion of ISApnu1-*orf7*-ISApnu2 into the Tn3 *tnpA* gene, disrupting it into two separate parts (Figure 2). The resulting structure is then connected with  $\Delta$ ISEc33 to finally constitute a 15.1 kb  $\Delta$ ISEc33-associated element  $\Delta$ ISEc33-Tn3-ISApnu1-*orf7*-ISApnu2-ISKpn27- $\Delta$ *bla*<sub>TEM-1</sub>-*bla*<sub>KPC-2</sub>- $\Delta$ ISKpn6-*korC*-*orf6*-*klcA*- $\Delta$ *repB (Figure 2). In contrast to the Tn1722-based unit transposons, the  $\Delta$ ISEc33-associated element is not bracketed by IRs and DRs. In addition, the Tn3 in this  $\Delta$ ISEc33-associated element is heavily fragmented due to insertion of various elements and most likely defective in the activity of transposition. The mobilization of this  $\Delta$ ISEc33-associated element in p10265-KPC would attribute to homologous recombination-based insertion of a foreign element Tn3-ISApnu1-*orf7*-ISApnu2-ISKpn27- $\Delta$ *bla*<sub>TEM-1</sub>-*bla*<sub>KPC-2</sub>- $\Delta$ ISKpn6-*korC*-*orf6*-*klcA*- $\Delta$ *repB* into a pre-existent intact ISEc33 element (making it truncated at 3' end), rather than resulting from a transposition event of the whole  $\Delta$ ISEc33-associated element followed by the deletion of its adjacent extremities removing IR and DR sequences.*

In p10265-KPC, the 6.5 kb transposon Tn5563 is located upstream of ISPa19, with two consecutive backbone genes *orf6* and *kfrA* as the interval between Tn5563 and ISPa19. ISPa19 contains the single transposase gene *tnpA*, and this insertion sequence is bordered by 18 bp IRs; this insertion sequence was initially described in plasmid Rms149 (Haines et al., 2005). Tn5563, belonging to the Tn3 subgroup of the Tn3 family transposons, was initially identified in plasmid pRA2 from *P. alcaligenes* and organized sequentially as *tnpR* (resolvase), *orf2* (hypothetical protein), *pliT* (PilT domain-containing protein), *tnpA*, *merP* (mercuric transport protein periplasmic component), *merT* (mercuric transport protein), and *merR* (mercuric resistance operon regulatory protein); this gene cluster is bounded by 39 bp IRs and further flanked by 5 bp (this study) or 7 bp DRs at both ends (Yeo et al., 1998). Various truncated versions of Tn5563 are also found in pOZ176 (Xiong et al., 2013) and pUM505 (Ramirez-Diaz et al., 2011) from *P. aeruginosa*. The Tn5563 element of p10265-KPC differs from the prototype Tn5563 of pRA2 with a 286 bp insertion (nucleotide position 7270 to 7555) between *merP* and *merT*. The above observations indicate the frequent

inter-plasmid transmission of Tn5563 among *Pseudomonas* species.

## Replication and Maintenance Determinants of p10265-KPC

Plasmid p10265-KPC belongs to the IncP-6 incompatibility group because it carries three partition genes *parABC* and a downstream replicase gene *repA*, which constitute an IncP6-type consecutive *par-rep* gene cluster. The *parA* gene encodes an ATPase, whereas *parB* and *parC* encode auxiliary partition proteins. The *repA* gene and the *parABC* locus of p10265-KPC show >97% and >99%, respectively, nucleotide sequence identity with the three IncP-6 plasmids Rms149, pRIO-5, and pCOL-1. The Rms149 RepA has been shown to confer the plasmid's replication ability in *E. coli*, *P. aeruginosa*, and *P. putida* (Haines et al., 2005), while the pRIO-5 RepA is able to replicate in *S. marcescens* and *Acinetobacter baumannii* but not in *P. aeruginosa* (Bonnin et al., 2012). The *parABC* locus of Rms149 is known to promote the plasmid mobilization in *E. coli* (Haines et al., 2005).

All of p10265-KPC, Rms149, pRIO-5, and pCOL-1 contain a 5.6 kb MOB<sub>p</sub> family mobilization module (Francia et al., 2004), which is composed of six genes *mobA* (relaxase/primase fusion protein), *mobB* (*oriT* recognition-like protein), *mobC* (relaxosome protein), and *mobD* and *mobE* (auxiliary proteins). The study with Rms149 denotes that the *mob* gene cluster is functional for the plasmid mobilization in *E. coli* (Haines et al., 2005). In addition, the above *mob* gene clusters are similar to those of the IncQ plasmids pTF-FC2 and pTC-F14; as shown by the genetic analysis using these two plasmids, the minimal region essential for mobilization are *mobA*, *mobB*, and *mobC*, while *mobD* and *mobE* are non-essential but together they greatly enhance the mobilization frequency (van Zyl et al., 2003).

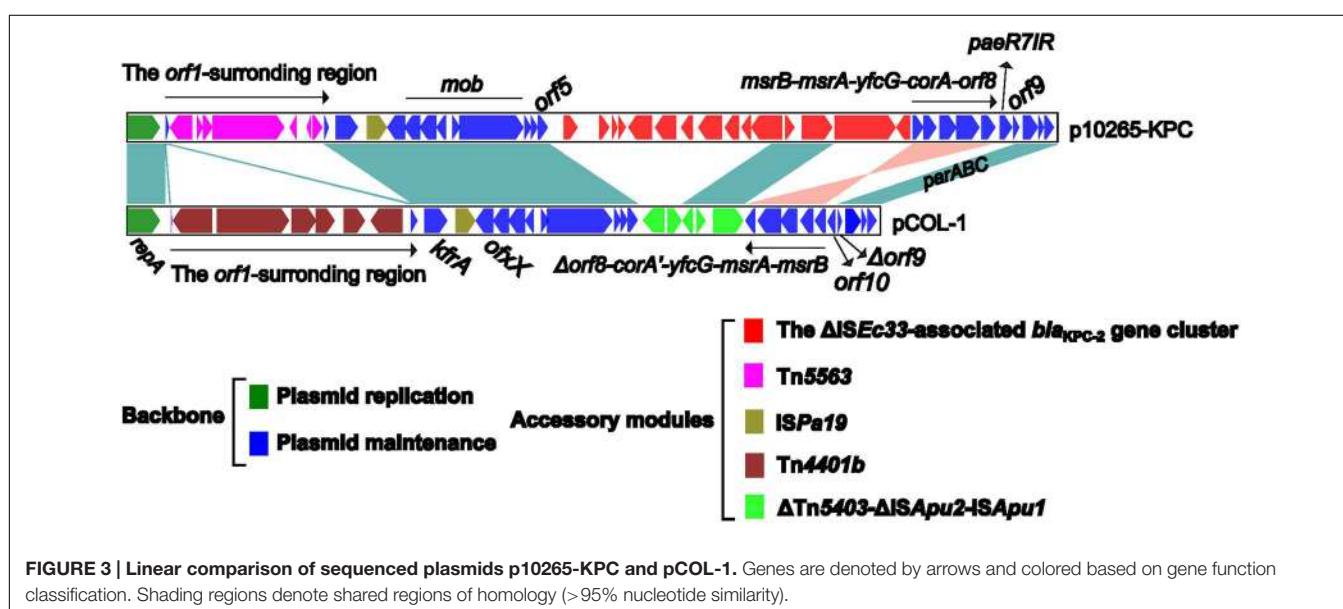
## Genomic Comparison of p10265-KPC with pCOL-1

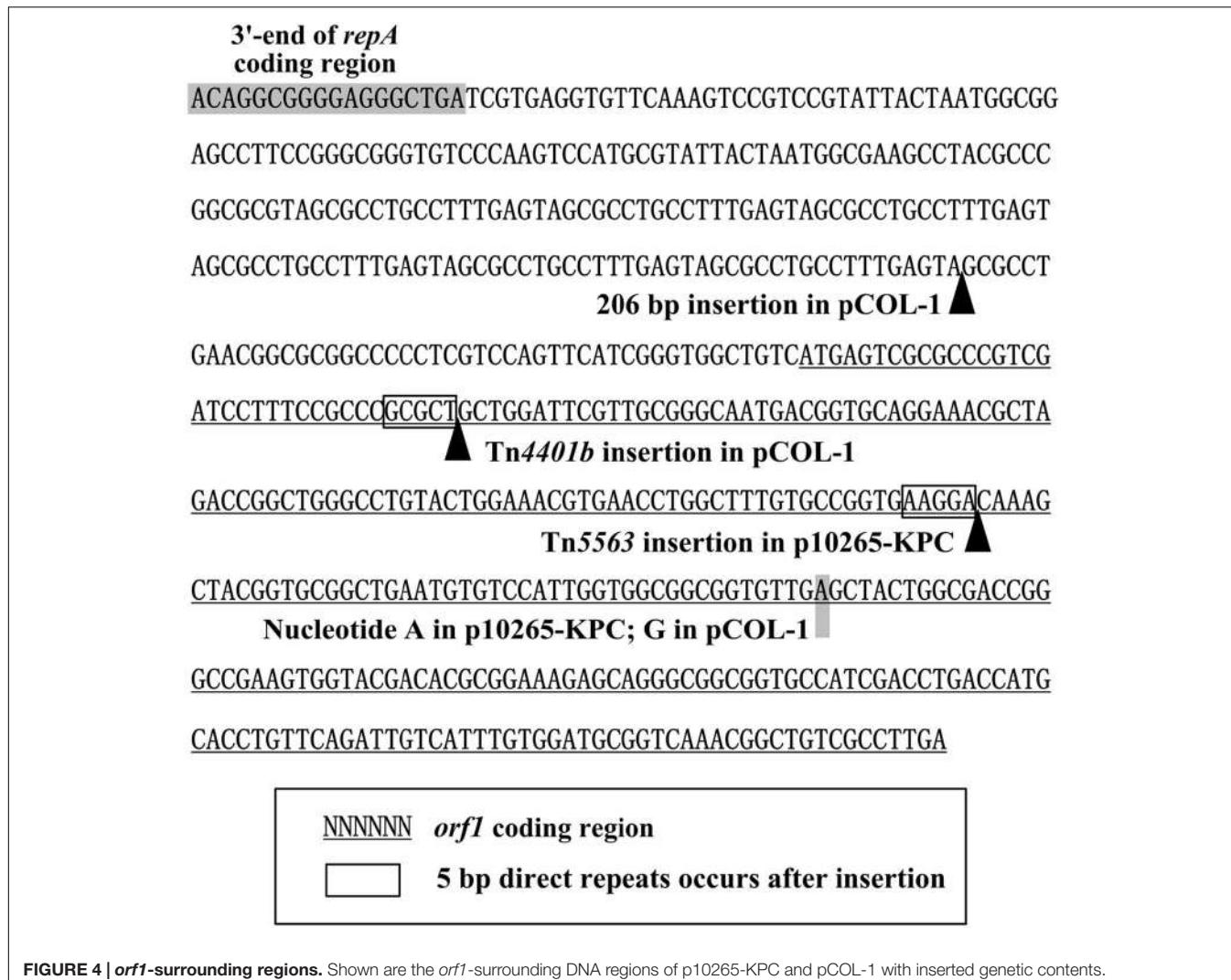
pCOL-1 represents the first sequenced KPC-producing IncP-6 plasmid (Naas et al., 2013), while p10265-KPC is the second one. The *par-rep* regions for partition-replication and the *mob* gene modules for mobilization are conserved in the p10265-KPC and pCOL-1 backbones (Figure 3), allowing these two plasmids to transfer if a conjugative plasmid is also present in the cell. The p10265-KPC and pCOL-1 backbones lack the conjugal transfer gene regions, which is in accordance with the fact that these two plasmids are unable to self-transfer their drug resistance markers via conjugation. An inversion of the backbone gene cluster *msrB-msrA-yfcG-corA-orf8* in p10265-KPC turns it into *Δorf8-corA'-yfcG-msrA-msrB* in pCOL-1 with a truncation of *orf8* and, moreover, *corA* becomes a pseudo gene with the accumulation of multiple indels.

A 206 bp fragment comprising 12 copies of tandem repeat of GCGCCTGCCTTGAGTA is inserted into the *repA-orf1* intergenic region of pCOL-1 relative to p10265-KPC (Figures 3 and 4). The open reading frame *orf1* is disrupted at two distinct sites, respectively, by the insertion of two different accessory elements, namely a 10 kb *bla<sub>KPC-2</sub>*-carrying Tn4401b in pCOL-1 and a *mer* locus-carrying Tn5563 in p10265-KPC (Figures 3 and 4).

pCOL-1 and p10265-KPC share the accessory element ISPa19, which is inserted between the backbone genes *kfrA* and *ofxX* (Figure 3). Downstream of the backbone gene *orf5* of p10265-KPC and pCOL-1 are two distinct inserted accessory regions, namely the ΔISEc33-associated *bla<sub>KPC-2</sub>* gene cluster and a 4.3 kb ΔTn5403-ΔISApU2-ISApU1 region (showing sequence similarity to the ΔISEc33-associated element), respectively (Figures 2 and 3).

Remarkably, pCOL-1 and p10265-KPC carry two distinct *bla<sub>KPC-2</sub>* gene clusters, Tn4401b and the ΔISEc33-associated





**FIGURE 4 |** **orf1-surrounding regions.** Shown are the *orf1*-surrounding DNA regions of p10265-KPC and pCOL-1 with inserted genetic contents.

element, respectively; these two gene clusters are inserted at two different sites of the IncP-6 backbone and seem to have completely different evolutionary histories of genetic assembly and transposition.

## CONCLUSION

Plasmid p10265-KPC is a novel IncP-6 resistance plasmid from *P. aeruginosa*, and it carries the IncP-6-type replication, partition and mobilization systems and a novel  $\Delta$ ISEc33-associated *bla*<sub>KPC-2</sub> gene cluster accounting for carbapenem resistance. The  $\Delta$ ISEc33-associated element has a complex mosaic structure, which is genetically related to the previously characterized Tn1722-based *bla*<sub>KPC-2</sub>-carrying unit transposons (Wang et al., 2015). Besides *bla*<sub>KPC-2</sub>, p10265-KPC still carries a truncated *bla*<sub>TEM-1</sub> gene in the *bla*<sub>KPC-2</sub> gene cluster and an incomplete *mer* locus in Tn5563, both of which are thought defective to mediate the corresponding resistance phenotypes. KPC-2 appears to be the sole determinant of

antimicrobial resistance for p10265-KPC. Epidemiological study and routine surveillance of p10265-KPC-like plasmids in China is needed.

The IncP-6 resistance plasmids are not commonly found in the natural isolates of *P. aeruginosa*. The accumulating complete sequences of the IncP-6 plasmids would make it possible to chart their evolutionary history and to draw the inferences about the processes that lead to these complex plasmid genomes. As seen from p10265-KPC, Rms149, pRIO-5, and pCOL-1, the IncP-6 plasmid backbones are able to integrate a wide variety of foreign genetic contents through transposition or homologous recombination mediated by the transposable elements such as insertion sequences, transposons and integrons. The IncP-6 plasmids are quite unusual in having a relatively small backbone but carrying a large amount of accessory modules which are mainly composed of mobile genetic elements and resistance determinants. It is worth elucidating whether there are specific mechanisms associated with the IncP-6 plasmids to promote the involvement of themselves in the complex processes of acquisition of foreign genetic contents.

## AUTHOR CONTRIBUTIONS

DZ, FS, and XZ designed the study. XD, WX, JF, WL, GL, HW, and FS performed experiments. DZ, XD, and FS analyzed data. XD, WX, JF, WL, GL, HW, and FS contributed reagents, materials and analysis tools. DZ, XD, FS, and XZ wrote this manuscript.

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## ACKNOWLEDGMENTS

This work was funded by the Chongqing Application and Development Program (cstc2014yykfA110021), the National Basic Research Program of China (2015CB554202), and the National High-Tech Research and Development Program of China (2014AA021402).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Bacteriophages as Potential Treatment for Urinary Tract Infections

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**Background:** Urinary tract infections (UTIs) are among the most prevalent microbial diseases and their financial burden on society is substantial. The continuing increase of antibiotic resistance worldwide is alarming so that well-tolerated, highly effective therapeutic alternatives are urgently needed.

**Objective:** To investigate the effect of bacteriophages on *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from the urine of patients suffering from UTIs.

**Material and methods:** Forty-one *E. coli* and 9 *K. pneumoniae* strains, isolated from the urine of patients suffering from UTIs, were tested *in vitro* for their susceptibility toward bacteriophages. The bacteriophages originated from either commercially available bacteriophage cocktails registered in Georgia or from the bacteriophage collection of the George Eliava Institute of Bacteriophage, Microbiology and Virology. *In vitro* screening of bacterial strains was performed by use of the spot-test method. The experiments were implemented three times by different groups of scientists.

**Results:** The lytic activity of the commercial bacteriophage cocktails on the 41 *E. coli* strains varied between 66% (Pyo bacteriophage) and 93% (Enko bacteriophage). After bacteriophage adaptation of the Pyo bacteriophage cocktail, its lytic activity was increased from 66 to 93% and only one *E. coli* strain remained resistant. One bacteriophage of the Eliava collection could lyse all 9 *K. pneumoniae* strains.

**Conclusions:** Based on the high lytic activity and the potential of resistance optimization by direct adaption of bacteriophages as reported in this study, and in view of the continuing increase of antibiotic resistance worldwide, bacteriophage therapy is a promising treatment option for UTIs highly warranting randomized controlled trials.

**Keywords:** bacteriophages, antibiotics, urinary tract infection, bacteriophage adaptation, antibiotic resistance

**Citation:**

Sybesma W, Zbinden R, Chanishvili N, Kutateladze M, Chkhota A, Ujmajuridze A, Mehnert U and Kessler TM (2016)

Bacteriophages as Potential Treatment for Urinary Tract Infections.  
*Front. Microbiol.* 7:465.

doi: 10.3389/fmicb.2016.00465

**Abbreviation:** AM, ampicillin; AMC, amoxicillin/clavulanic acid; CIP, ciprofloxacin; CL, confluent lysis; CXM, cefuroxime; *E. coli*, *Escherichia coli*; ESBL, extended spectrum beta lactamase; EUCAST, European Committee on Antimicrobial Susceptibility Testing; F, female; I, intermediate; IP, individual clear or opaque plaques; *K. oxytoca*, *Klebsiella oxytoca*; *K. pneumonia*, *Klebsiella pneumoniae*; M, male; n, no (absent); ND, not done; NF, nitrofurantoin; NOR, norfloxacin; OL, overgrown lysis; *P. aeruginosa*, *Pseudomonas aeruginosa*; R, resistant; S, sensitive; SCL, semi-confluent lysis; SXT, sulfamethoxazole(trimethoprim or co-trimoxazole; tv, taches vierges; UTIs, urinary tract infections; y, yes (present).

## INTRODUCTION

Urinary tract infections (UTIs) are among the most prevalent bacterial infections and their financial burden for the society is substantial. In the USA alone, UTIs are responsible for over 7 million physician visits annually (Foxman, 2002) and approximately 15% of all community-prescribed antibiotics are used for UTIs resulting in an annual cost in excess of 1 billion US dollars (Mazzulli, 2002). UTIs account for more than 100'000 hospital admissions annually in the USA (Foxman, 2002), most often for pyelonephritis. These infections are held responsible for at least 40% of all hospital-acquired infections, and the majority of cases are catheter-associated (Rüden et al., 1997). The wide and often uncritical use of antibiotics contributes to the global increase in resistant strains and the present state of microbial resistance is alarming (Carlet et al., 2011; Verbeken et al., 2014). Thus, there is an urgent need for effective, well-tolerated therapeutic alternatives.

After the discovery of bacteriophages by d'Hérelle in 1917, the use of bacteriophage therapy was proposed in the 1920s and has a long and colorful history. At present, bacteriophage therapy is a registered medicine in East European countries like Georgia, Armenia, Ukraine, and Russia, and well accepted by physicians and patients. Several researchers (Abedon et al., 2011; Chanishvili, 2012) have reviewed numerous examples of successfully applied bacteriophage therapy for a diverse area of medical specializations, including dermatology, surgery, wound treatment, intestinal infections, ophthalmology, gynecology and urology. In the Western world bacteriophages have been approved as food processing aid for the decontamination of food (e.g., ListShield<sup>TM</sup>). However, the use of bacteriophages as treatment against selected bacterial infectious diseases has not yet been approved by regulatory authorities and a regulatory framework is still missing (Pirnay et al., 2011, 2015). Nevertheless, the interest from Western clinicians to collaborate with specialists in bacteriophage therapy is rising, as is illustrated by the growing number of scientific papers in peer reviewed journals, for instance regarding the identification of bacteriophages against the O104:H4 *E. coli* outbreak in Germany, (Merabishvili et al., 2012) and the treatment of *P. aeruginosa* and *Staphylococcus aureus* infections in burn wound patients (Merabishvili et al., 2013). In addition, a number of recent reviews have addressed the safety of bacteriophage therapy (Kutter et al., 2010; Pirnay et al., 2015).

Technically, bacteriophage therapy involves the application of bacteriophages that, upon encounter with specific pathogenic bacteria, can infect and kill the pathogenic bacteria. Bacteriophages can specifically dock on bacteria (host species), introduce their DNA, multiply by using the host cell machinery bacteria, and subsequently lyse the bacteria with release of virion progeny that can re-initiate the cycle. In this way, bacteriophages are unique among antibacterial agents in their ability to increase their numbers in the presence of specific bacterial targets.

Bacteriophages might be rather appropriate means to treat patients suffering from UTIs: compared to last generation of antibiotics, bacteriophages are relatively cheap and the treatment can be done under the control of medical personnel by using

a catheter for intravesical instillation several times a day. In addition, bacteriophage therapy for UTI treatment will be applied locally and does not involve a systemic treatment. It is also likely that the absence of physical and metabolic barriers in this kind of treatment increases the potential efficacy and reduces the occurrence of potential adverse events. Thus, in view of the high prevalence of UTIs, the continuing increase of antibiotic resistance worldwide, and the renewed interest to apply bacteriophage therapy in the Western world, we investigated the effect of bacteriophages on *E. coli* and *K. pneumoniae* strains isolated from the urine of patients suffering from UTIs.

## MATERIALS AND METHODS

### Information on Bacterial Strains and Patients

Between February 2012 and January 2013, 41 *E. coli* and 9 *K. pneumoniae* strains were isolated from urinary cultures of 50 patients (22 women and 28 men) suffering from UTIs defined according to the European Association of Urology Guidelines on Neuro-Urology (Groen et al., 2016). Urine samples were collected by sterile catheterization at the Balgrist University Hospital Zürich and sent within 16 h to the microbiological laboratory of the University of Zürich, Switzerland. None of the patients received antibiotic treatment within 4 weeks before urinary culture. The mean age of the 50 patients was  $55 \pm 18$  years. The causes of lower urinary tract dysfunction were spinal cord injury in 38 (76%), multiple sclerosis in 8 (16%) and spinal stenosis in 4 (4%) patients. Of the 50 patients, 34 (68%) relied on aseptic intermittent self-catheterization, 12 (24%) on an indwelling (transurethral  $n = 4$ , suprapubic  $n = 8$ ) catheter and 4 (16%) voided spontaneously.

The urine samples were cultivated on sheep blood agar (COS, bioMérieux, Marcy l'Etoile, France), on sheep blood agar with colistin and nalidixic acid (CNA, bioMérieux) and on the chromogenic agar (Uriselect 4, Bio-Rad, Marnes – la Coquette, France). The isolated bacteria were identified according to standard procedures. The susceptibility testing was performed according to the EUCAST Version 3.1, 2013 ([www.eucast.org](http://www.eucast.org)). The strains were stored at  $-70^{\circ}\text{C}$  in skimmed milk. Before shipping to Georgia, the isolated strains were sub-cultured and transferred to Amies transport agar (Copan, Brescia, Italy).

### Bacteriophages

Four commercial bacteriophage preparations registered in Georgia, Pyo, Intesti, Ses, and Enko bacteriophages, along with 29 *E. coli* and 10 *K. pneumoniae* bacteriophages from the bacteriophage collection of the George Eliava Institute of Bacteriophage, Microbiology and Virology, were used in a bacterial cell lysis screening assay. In order to eliminate effects of batch to batch variations, the individual bacteriophage preparations used for the screening came from the same production batch. The bacteriophages composing the four commercial bacteriophage preparations were grown on a set of bacterial strains, and underwent titration by Appelmans method

(Appelmans, 1921) on each individual strain previously used for multiplication of the bacteriophages. In agreement with current regulation the average titer of the mixture is  $10^{-4}$ – $10^{-5}$  for at least 48 h (Appelmans method), which corresponds to titers of  $10^7$ – $10^9$  pfu/mL.

The individual bacteriophages have been selected on basis of their capability to eliminate currently prevailing pathogenic microorganisms in Georgia, including *E. coli*, *P. aeruginosa*, *Staphylococcus* spp., *Shigella* spp., *Salmonella* spp., *Enterococcus* spp., and *Proteus* spp. More information of the composition and origin of the four bacteriophage preparations can be found in earlier work on Pyo and Intesti (Abedon et al., 2011) and Ses and Enko (Fitzgerald-Hughes et al., 2014).

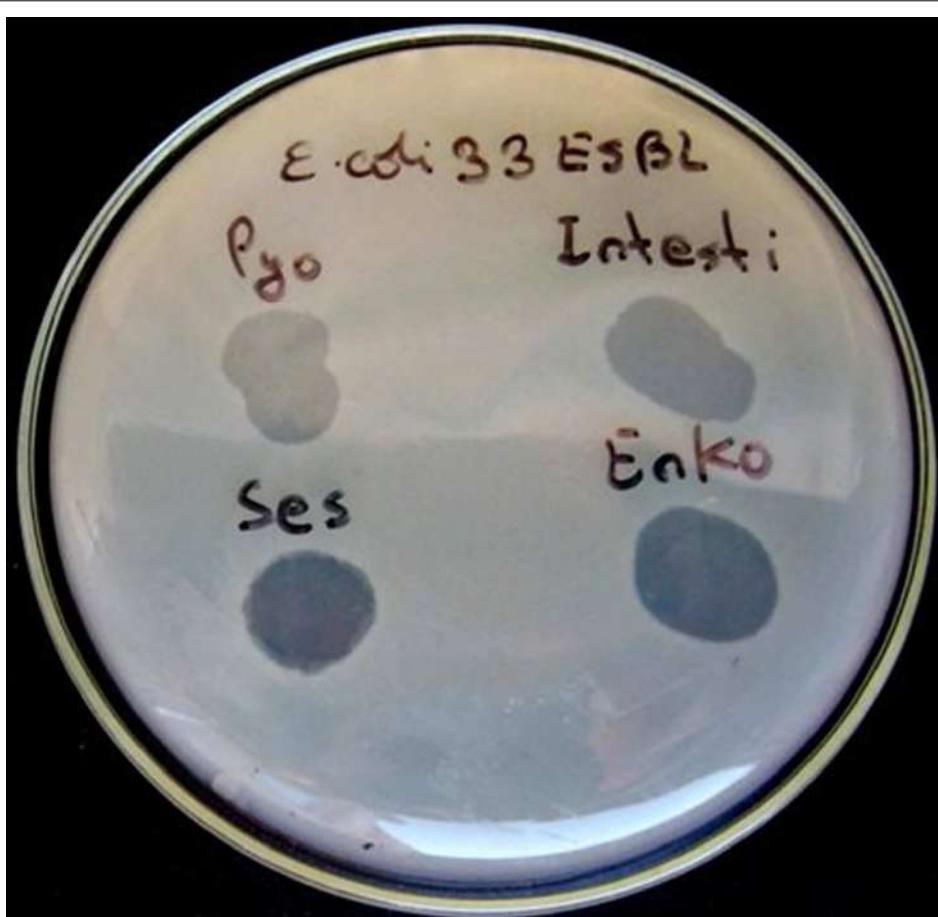
The 29 *E. coli* bacteriophages were freshly prepared and originally isolated for their potential as antimicrobial agents for therapeutical applications from a broad range of different sources during the period 2000–2012, like sewage waters, agricultural farm isolates (Khurtsia et al., 2001) and clinical isolates (Ghudumidze et al., 2012).

The 10 *K. pneumoniae* bacteriophages (Table 3) were isolated between 2002 and 2012 from sewage samples (Karumidze et al., 2013) with the aim to treat patients suffering from UTIs and

prostatitis using clinical *K. pneumoniae* strains or *K. oxytoca* strains as hosts. All bacteriophages used in this study were lytic bacteriophages, as was concluded on basis of a combination of analyses and observations, including plaque morphology and clarity of lytic zones on the lawn of bacterial agar, host range, time to infection, time of latent period, and concentration of progeny (Clokie and Kropinski, 2009).

## Screening

Screening of bacterial strains for susceptibility to bacteriophages was performed by using a spot test method (Craigie and Yen, 1938) and then modified by different authors and adjusted for different species (Felix and Callow, 1943; Felix, 1956). Bacterial cultures were grown overnight (18–24 h at 37°C) to receive a concentration of  $10^9$  colony forming units (cfu) mL<sup>-1</sup> and diluted 10 times up to a final concentration of  $10^8$  cfu mL<sup>-1</sup>. 100 µL of the diluted culture was transferred into 2–3 mL of previously melted and cooled soft agar (0.7% Brain-Heart agar), vortexed for 30–60 s and poured on the top of solidified and previously dried Petri dishes containing 2% of Brain-Heart agar. After solidification of the top agar (30–60 min) at room temperature, 10 µL of bacteriophage suspension with a titer of



**FIGURE 1 | Plaque morphology of *Escherichia coli* strain #33.** The figure shows overgrown (partial) lysis (OL) in case of Pyo and Intesti bacteriophages and confluent (complete) lysis (CL) in case of Ses and Enko bacteriophages. All these results are positive.

$10^7$  plaques forming units (pfu) mL<sup>-1</sup> was placed on top of the soft agar using an automatic pipette. The plates were let to dry for 20–30 min and then incubated overnight (18–24 h) at 37°C. Next, the results were visually evaluated.

### Primary Outcome Measure

The primary outcome measure was the assessment of lytic activity of the bacteriophages on the bacteria, as could be identified by the plaque morphology in a double-layered plaque assay using *E. coli* or *K. pneumoniae* strains. A bacterial lawn inoculated with a range of bacteriophages gives different visual patterns, similar to a bacteriophage typing pattern and depending on the effect of the bacteriophage-host strain interaction. The clear zones usually differ by their intensity and structure (**Figures 1 and 2**). The positive reactions were classified as “CL” – confluent lysis, i.e., a full clear zone on the well-developed lawn of bacterial culture; “SCL” – semi-confluent lysis, i.e., a not fully cleared bacterial lawn; “OL” – overgrown lysis, i.e., single bacterial colonies that overgrew a complete clear zone; “IP” – individual clear or opaque plaques, which look like multiple small clear zones on the bacterial lawn within a drop contour, previously assigned by d’Hérelle as “tv” from “taches vierges” (Clokie and Kropinski, 2009; Chanishvili, 2012).

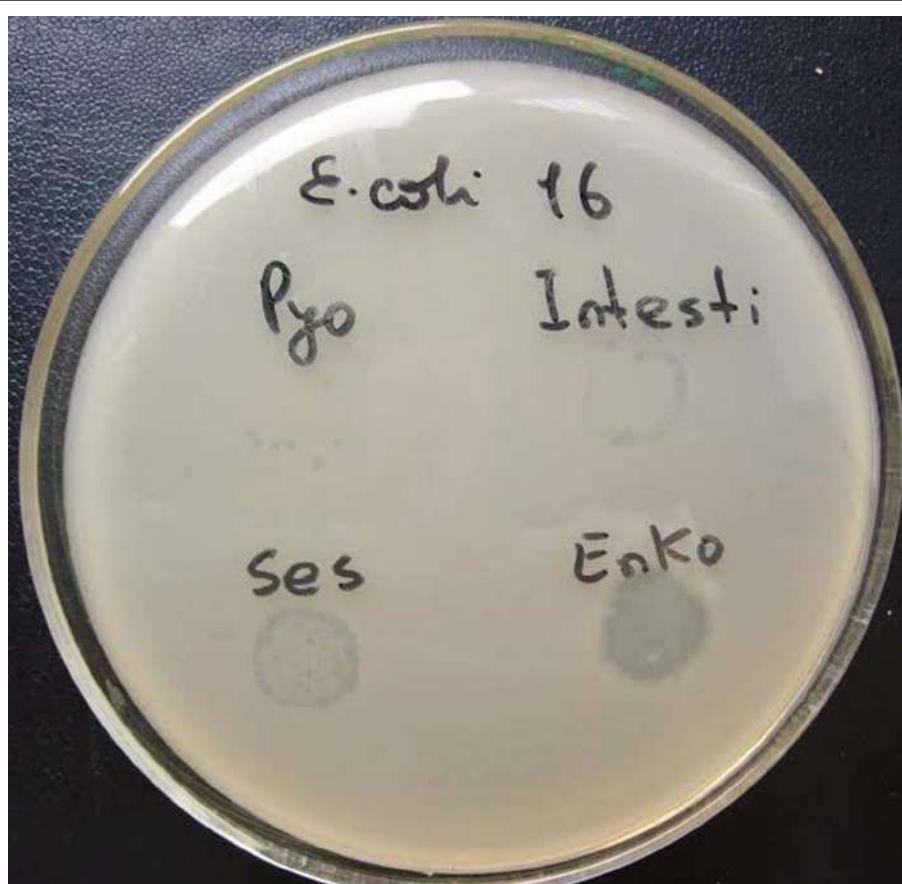
The negative reactions were classified as “R” – resistant, i.e., no lysis.

### Extension and Optimisation of the Bacteriophage Preparation Host-Range (Adaptation)

Expansion of the range of lytic activity of the Pyo bacteriophages cocktail was achieved by selection of mutant bacteriophages with extended host-range (h-mutants) and by increasing their titers in the bacteriophage preparation. For this purpose the bacteriophages were propagated in a suspension containing the bacterial cultures of choice with low or no susceptibility to these bacteriophages. The initial steps of adaptation technique are similar to Appelmans’ method for titration in liquid media (Appelmans, 1921). The adapted bacteriophages were used for the repeated screening.

## RESULTS

The antibiogram of the 41 *E. coli* and 9 *K. pneumoniae* strains shows the ESBL production capability of the strains and their sensitivity or resistance for each of seven tested



**FIGURE 2 | Plaque morphology of *E. coli* strain #16.** The figure shows individual plaques (IP) in case of Pyo and Intesti bacteriophages and two degrees of overgrown (partial) lysis (OL) in case of Ses and Enko bacteriophages. All these results are positive.

antibiotics, **Table 1**. In summary, the strain susceptibility varies between sensitive for all antibiotics to resistant for at least six of the seven tested antibiotics. The lytic (killing) activity of the commercial bacteriophage cocktails on the 41 *E. coli* strains varied between 66% (27 strains sensitive for Pyo bacteriophages) and 93% (38 strains sensitive for Enko bacteriophages), **Table 2**. From the 29 *E. coli* bacteriophages from the Eliava collection, the highest lytic activity came from the bacteriophages vB\_E. coli\_4t, vB\_E. coli\_4s, and vB\_E. coli\_Pic with 42, 39, and 24% coverage, respectively. Three of the *E. coli* strains (*E. coli* 5, *E. coli* 29, and *E. coli* 35 ESBL) showed complete resistance to all bacteriophage from any of the 4 commercial bacteriophage cocktails and 29 *E. coli* bacteriophages from the Eliava collection.

However, after adaptation of the Pyo bacteriophages the lytic activity could be increased from 27 to 38 sensitive strains (66 to 93%, **Table 2**), including two of the three previously mentioned resistance strains. In total, all but one *E. coli* strain could be lysed by one of the four commercial bacteriophage preparations and

only strain *E. coli* 35 ESBL remained resistant, even after the adaptation of the Pyo bacteriophage preparation.

Regarding *K. pneumoniae* strains (**Table 3**), the best result was shown by bacteriophage v\_B-KpS10 that could lyse all nine strains.

Bacteriophage and antibiotic susceptibility/resistance did not correlate. For example, *E. coli* strain #33 was resistant to all tested antibiotics except nitrofurantoin (NF; **Table 1**), while the same strain was susceptible to all tested bacteriophages (**Table 2**, **Figure 1**). On the contrary, *E. coli* strain #35 was resistant to all tested bacteriophages (**Table 2**), but showed susceptibility to two antibiotics, i.e., amoxicillin (AMC) and sulfamethoxazole (SXT; **Table 1**).

## DISCUSSION

The *in vitro* lytic activity of bacteriophages has already been demonstrated in several studies (Chanishvili, 2012) but data

**TABLE 1 |** Antibiogram of 41 *Escherichia coli* and 9 *Klebsiella pneumoniae* strains isolated from the urine of patients suffering from UTIs.

	Antibiotic resistance profile							
	AM	AMC	CXM	SXT	NOR	CIP	NF	ESBL
<b><i>E. coli</i> strain number</b>								
3,9,10,13,17,19,21,22, 23,25,27,28,38,39,40	S	S	S	S	S	S	S	N
12	S	S	S	S	S	S	R	N
11,30	S	S	S	S	R	R	S	N
29,36	R	S	S	S	S	S	S	N
14,24	R	S	S	S	R	R	S	N
8	R	S	S	S	I	S	S	N
37	R	S	S	R	S	S	S	N
7,16,18,20	R	S	S	R	R	R	S	N
1,4	R	S	R	S	S	S	S	Y
5	R	S	R	S	S	S	S	N
2,31	R	S	R	S	R	R	S	Y
15	R	S	R	S	R	R	S	N
35	R	S	R	S	R	R	R	Y
41	R	S	R	R	R	R	S	Y
6	R	R	S	R	R	R	S	N
26	R	R	S	I	R	R	S	N
32	R	R	R	R	S	S	S	Y
33,34	R	R	R	R	R	R	S	Y
<b><i>K. pneumoniae</i> strain number</b>								
43	R	S	S	S	S	S	S	Y
46,50	R	S	S	S	S	S	S	N
42	R	S	S	S	S	S	ND	N
48	R	S	S	R	S	S	S	N
49	R	S	R	R	S	S	ND	Y
47	R	S	R	R	R	R	S	Y
44	R	S	R	R	I	I	R	N
45	R	R	R	R	R	R	ND	Y

Strains were isolated from 22 different women and 28 different men with age between 25 and 80. Numbers 1–41 are *E. coli* strains with at least 18 different antibiotic resistance profiles. Numbers 42–50 are *K. pneumoniae* strains with at least seven different antibiotic resistance profiles. AM, ampicillin; AMC, amoxicillin/clavulanic acid; CXM, cefuroxime; SXT, sulfamethoxazole/trimethoprim or co-trimoxazole; NOR, norfloxacin; CIP, ciprofloxacin; NF, nitrofurantoin; ESBL, extended-spectrum-beta-lactamase; Y, yes (present); N, no (absent); R, resistant; S, sensitive; I, intermediate; ND, not done. Notwithstanding the occurrence of several identical antibiograms, all strains have different bacteriophage resistance/sensitivity profiles, except strains 14 and 24 (compare with **Tables 2** and **3**).

**TABLE 2 | Results of spot tests on 41 *E. coli* strains using commercial bacteriophage cocktails.**

Strain number	Commercial bacteriophage cocktails				
	Pyo				
	Before adaptation	After adaptation	Intesti	Ses	Enko
39	CL	CL	CL	OL	OL
7	IP	IP	IP	OL	OL
20	IP	OL	IP	OL	OL
6,16	IP	SCL	IP	OL	OL
9	OL	CL	OL	OL	OL
1	OL	CL	OL	SCL	OL
23	OL	OL	CL	OL	OL
12,21,33	OL	OL	OL	CL	CL
31	OL	OL	OL	IP	SCL
4,8,14,24,26,30,34	OL	OL	OL	OL	OL
40	OL	OL	OL	SCL	OL
28	OL	OL	SCL	CL	CL
27,32	OL	OL	SCL	SCL	SCL
17	OL	OL	SCL	OL	OL
38	OL	SCL	CL	SCL	SCL
19	OL	SCL	OL	OL	OL
3	R	CL	OL	IP	OL
18,36,37	R	CL	OL	OL	OL
25	R	CL	R	R	OL
15	R	CL	R	SCL	OL
22	R	CL	R	SCL	SCL
2	R	CL	SCL	SCL	CL
5	R	IP	R	R	R
10	R	IP	SCL	SCL	SCL
11	R	R	R	OL	SCL
35	R	R	R	R	R
41	R	R	SCL	SCL	CL
29	R	SCL	R	R	R
13	SCL	SCL	SCL	CL	CL
Lytic activity	65.9%	92.7%	82.9%	90.2%	92.7%

CL, confluent lysis; SCL, semi-confluent lysis; OL, overgrown lysis; IP, individual plaques; R, resistant. Twenty-nine unique bacteriophage resistance/sensitivity profiles can be distinguished.

on the application of bacteriophages for treating UTIs are scarce (Perepanova et al., 1957; Arshba and Bagdoeva, 1965; Kolomeitsev et al., 1966; Danilova, 1996; Letkiewicz et al., 2010; Khawaldeh et al., 2011; Chanishvili, 2012). In the present study we showed for the first time the lytic activity of commercially existing bacteriophage cocktails on 41 *E. coli* strains, isolated from urinary cultures of patients suffering from UTIs. The applied spot test demonstrated the results of the bacteriophage–bacteria interactions: All reactions assigned as CL, SCL, OL, and IP were positive, which means that the bacteriophages lysed the bacteria, although with different degrees of effectiveness. In case of CL the bacteria could not develop bacteriophage resistance during the incubation period on the plate (18 h), indicating that this kind of bacteriophage–bacterium interaction can be considered as the most effective one. The other spot morphologies reflected less lytic activity, which could partially be caused by the different concentrations of specific bacteriophages in the commercial

cocktails. Interestingly, our results show a similar order of lytic activity for the four commercial bacteriophages (before adaptation) as reported in an earlier bacteriophage sensitivity test on *E. coli* strains (Fitzgerald-Hughes et al., 2014). Furthermore, in the present study, we have shown that, as is common in the development of bacteriophages, the impact and activity of the bacteriophage cocktails can be increased in adaptation experiments, as illustrated by the change from IP into OL, or even CL (Table 2). The commercial bacteriophage preparations, after the adaptation experiments of the Pyo bacteriophage cocktail in successive series of adaptation, showed lytic activity on 40 of the 41 *E. coli* strains. *K. pneumoniae* bacteriophages from the Eliava collection could lyse all of the 9 *K. pneumoniae* strains tested. Besides adaptation, the efficacy of bacteriophage cocktails can also be increased by addition of new bacteriophages that show lytic activity toward the targeted clinical bacterial isolate.

**TABLE 3 | Results of spot tests on 9 *K. pneumoniae* strains using laboratory bacteriophages from the Eliava collection.**

Strain number	Bacteriophages									
	vB_KlpR1	vB_KloxR2	vB_KlpR3	vB_KlpR4	vB_KlpR5	vB_KlpR6	vB_KlpR7	vB_KlpR8	v_BR KpM 9	v_BRKpS 10
45,47	R	R	OL	OL	R	R	R	R	OL	OL
42	R	R	R	R	R	CL	R	CL	OL	OL
48	R	R	R	R	R	R	OL	R	SCL	
50	R	R	R	R	R	R	R	OL	OL	
43,44,46	R	R	R	R	R	R	R	R	OL	OL
49	R	R	R	R	R	R	R	SCL	CL	
Lytic activity	0%	0%	22%	22%	0%	11%	0%	22%	56%	100%

CL, confluent lysis; SCL, semi-confluent lysis; OL, overgrown lysis; IP, individual plaques; R, resistant. Six unique bacteriophage resistance/sensitivity profiles can be distinguished.

Since the mode of action of antimicrobial drugs like antibiotics differs considerably from bacteriophages, resistance mechanisms are consequently different, as can be concluded by comparing **Table 1** with **Tables 2** and **3**. Furthermore, these data also indicate that all but two bacterial strains with a similar antibiotic resistance profile have different bacteriophage resistance profiles, which indicates a broader (genetic) variability among the isolates than could have been expected on the basis of the antibiotic resistance profiles only. Although beyond the scope of this study, detailed genetic characterisation could indicate the effect of bacteriophages on specific serotypes or genotypes of *E. coli* and *K. pneumonia* and determine the spectrum of the (adapted) bacteriophage cocktails vs. the diversity of the strains. Full genome sequencing could further reveal which defense mechanisms by the bacteria and which counter-strategies by the bacteriophages may have been evolved (Samson et al., 2013).

The applied bacteriophage cocktails Pyo, Intesti, Ses, and Enko are allowed by the Georgian medicinal products legislation. In terms of safety, these bacteriophage preparations are already commercially available for more than 50 years and have been used millions of times without relevant incidence of adverse events. In addition, the bacteriophages in these cocktails are all lytic and do not have the ability to integrate and remain in the bacterial genome, contrarily to the temperate bacteriophages that after infection include their DNA into the host genome without making much harm to the host (see Materials and Methods). For a complete overview on quality and safety requirements for sustainable bacteriophage therapy products see (Pirnay et al., 2015).

Notwithstanding the potential of application of bacteriophage therapies in treating patients suffering from UTIs, and its broad acceptance in some East European countries, one major limitation today is the regulatory framework in the Western world (Verbeken et al., 2014; Pirnay et al., 2015), which requires a long and expensive road of assessments and randomized, placebo-controlled, double-blind trials. These studies should also investigate the best routes of administration, duration and dosage of treatment, adverse events, stability and definition of formulations and applicability for acute infections. Furthermore, and in order to accelerate potential regulatory approval for bacteriophage therapy, bacteriophages should not be considered

in a similar way as chemical drugs, but, because of their typical mode of action, more like the components of the influenza vaccine, which is re-evaluated and changed yearly and does not require clinical trials for each revision (Pirnay et al., 2011). In addition, it may be appropriate to classify bacteriophages in different ways for different applications. For example as a drug for systemic applications, but as a medical device for more topical applications, as proposed for the treatment of UTIs (Pirnay et al., 2011). Recently, a set-up has been proposed where bacteriophage therapy is coordinated and standardized (in a first instance) by national bacteriophage therapy centers, which operate under the supervision of relevant public health authorities and in interaction with private stakeholders (Pirnay et al., 2015).

## CONCLUSION

The results of this study have clearly shown the lytic activity of commercial bacteriophage cocktails on *E. coli* and *K. pneumonia* strains isolated from patients suffering from UTIs. In addition, the presented data has shown the potential of bacteriophage adaptation experiments with the aim to increase the lytic activity of the bacteriophage cocktails.

In times of increasing antibiotic resistance worldwide and in combination with the long history of safe use of bacteriophages in East European countries, our findings can be seen as a stimulus to invest in the set-up of randomized, placebo-controlled trials and investigate the efficacy of bacteriophage therapies in the Western world as a common alternative to strictly chemical-based treatment of bacterial infections.

In view of the high prevalence of UTIs, bacteriophages, as natural and self-amplifying antibacterial “drugs,” might offer a non-systemic effective therapeutic option urgently warranting randomized controlled trials.

## AUTHOR CONTRIBUTIONS

All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of data analysis. Study concept and design: WS, TMK. Acquisition, analysis, or interpretation of data: WS, RZ, MK, NC, AC, AU,

UM, TMK. Drafting of the manuscript: WS and TMK. Critical revision of the manuscript for important intellectual content: RZ, MK, NC, AC, AU, UM. Data analysis: WS, TMK. Obtained funding: UM, TMK. Study supervision: TMK. All authors read and approved the final version to be published.

## FUNDING

This study was supported by the Swiss Continence Foundation ([www.swisscontinencefoundation.ch](http://www.swisscontinencefoundation.ch)), the Swiss National Science Foundation ([www.snsf.ch](http://www.snsf.ch)) and the Swiss Agency for Development and Cooperation in the framework of the

programme SCOPES (Scientific co-operation between Eastern Europe and Switzerland).

## ACKNOWLEDGMENTS

Marina Goderdzishvili, Nana Balarjishvili, Nino Ghudumidze, Darejan Zhgenti, Darejan Bolkvadze, Natia Skhirtladze, Natia Karumidze, and Khatuna Makalatia are acknowledged for technical assistance with the preparation of bacteriophages and related optimization protocols. Martina Marchesi is acknowledged for technical support in preparing all the strains for shipping. Harald Bruessow is acknowledged for providing valuable comments on the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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