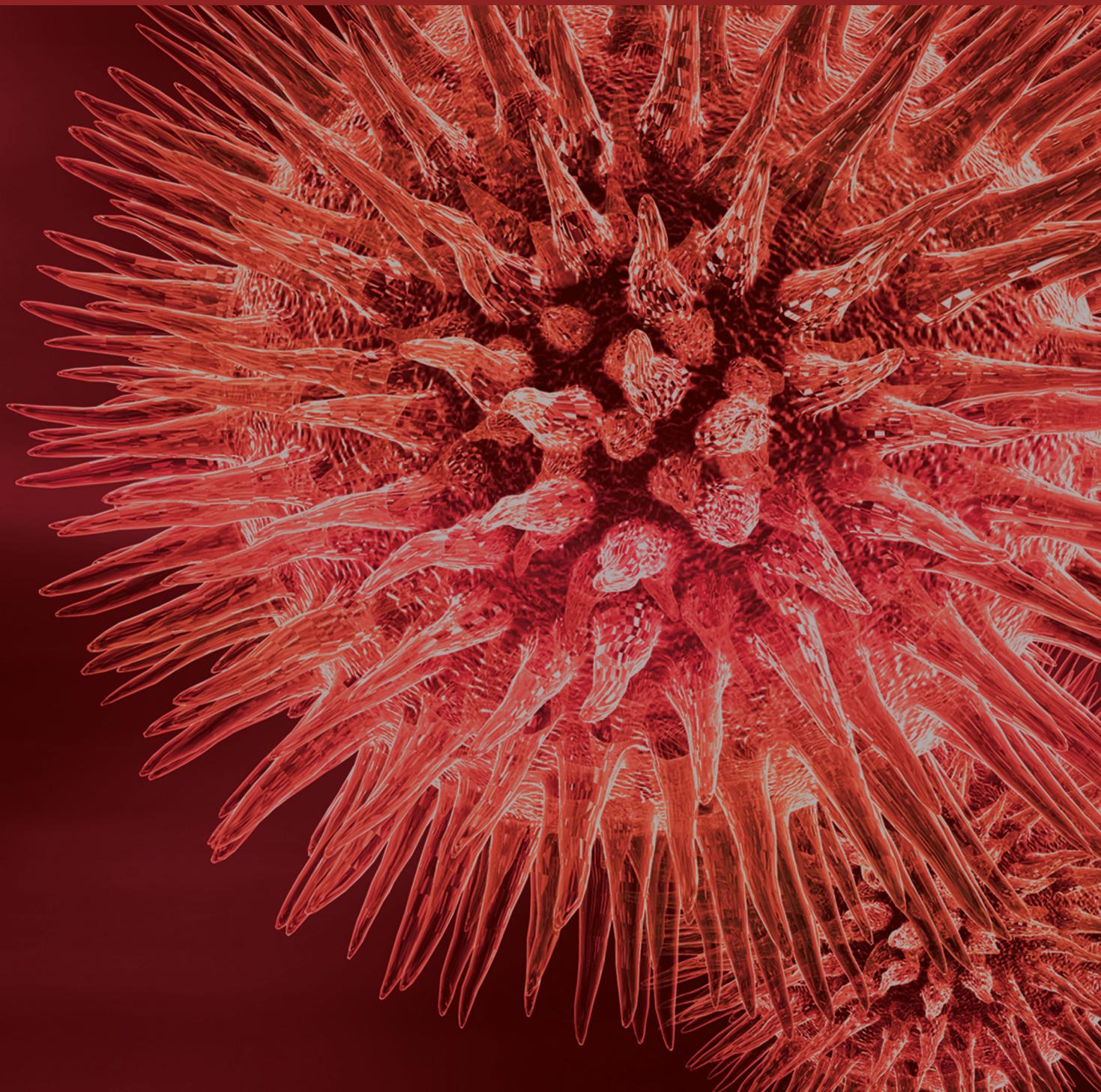


Antibiotic Resistance of Bacteria

Guest Editors: Madhab K. Chattopadhyay, Ranadhir Chakraborty,
Hans-Peter Grossart, Gundlapally S. Reddy, and Medicharla V. Jagannadham



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Editorial

Antibiotic Resistance of Bacteria

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Antibiotic resistance of bacteria and other microorganisms is one of the most serious and grievous challenges of the twenty-first century. The life-saving drugs, which held a great deal of promises during the 1940s to eradicate all the infectious life-threatening diseases in the world, have ceased to work, because of the increasing emergence of microbial strains invulnerable to them. Many of the previously efficacious antibiotics are no longer usable because of widespread occurrence of multiresistant microbial strains. Lately, discovery of new antibiotics is failing to keep pace with the emergence of (multi)resistance of pathogenic and also environmental bacterial strains. Consequently, the prospect of chemotherapy looks bleak. The trepidation that we might be pushed back to a situation analogous to the preantibiotic era, when no chemotherapeutic agent was available to contain and combat deadly bacterial infections, does not appear to be an overblown imagination.

Based on this backdrop, this special issue appears to be an aptly undertaken and well-timed endeavour to address this global problem. The articles contributed by investigators from various research laboratories with different scientific backgrounds have not only portrayed the width of the problem but also displayed some silver lining in the management of the looming crisis. Rapid detection of the profile of resistance is essential for timely application of the right antibiotic to a patient. H. Frickmann et al. summarize the efficacy and limitations of various molecular and mass spectrometric

methods for the detection of resistance. The omnipresent nature of the resistant organisms is revealed in a number of articles. F. B. Atique and Md. M. R. Khalil report on the occurrence of antibiotic resistance among bacteria (predominantly skin commensal coagulase-negative staphylococci) isolated from allogenic bone samples for grafting, collected from different hospitals of Bangladesh. Food materials are believed to serve as a vehicle for transmission of resistance. This issue is addressed by F. S. Dehkordi et al. who report on the genotype and resistance-profile of *Helicobacter pylori* isolated from vegetables and salad samples, picked up from groceries and supermarket in a province of Iran. The high similarity in the genotype pattern of the isolates obtained from vegetables and humans indicates transmission. A. B. Flórez et al. reveal tetracycline and erythromycin-resistant bacteria and genes conferring resistance to these antibiotics in 10 Spanish and 10 Italian samples of commercial cheese. P. Krupa et al. report on the population structure (based on *spa* typing) of oxacillin-resistant *Staphylococcus aureus* isolated from nasal swabs of pigs, collected from two slaughter houses of Poland. Some meat samples bought from the shops were also included into their studies. D. De Vito et al. characterize multidrug-resistant clinical isolates of *Salmonella typhimurium* for resistance genes in an area of southern Italy by pulsotyping and phage typing. C. Zhang et al. report on the resistant phenotype and genotype of *Streptococcus suis* serotype 2, isolated from 62 clinically healthy sows and 34

diseased pigs reared in different farms of China. Antibiotic resistance in the nosocomial isolates is a matter of serious concern. F. Lombardi et al. look into the molecular epidemiology of carbapenemase-producing strains of *Klebsiella pneumoniae* isolated from the surgery unit at a cardiovascular centre of Italy. D. Ojdana et al. demonstrate the ability of an *E. coli* strain obtained from a hospital of Poland to produce carbapenemase enzymes and also the presence of genes responsible for the production of carbapenemases and other β -lactamases. Extended-spectrum- β -lactamase (ESBL) is a bacterial enzyme having the ability to hydrolyse even the third-generation cephalosporins and aztreonam. Besides *Klebsiella pneumoniae* some strains of *Escherichia coli* are also known to produce this enzyme. This is indicated by M. S. Rezai et al. who performed genotyping of ESBL-producing strains of *E. coli*, obtained from a paediatric hospital of north Iran. The authors also show the association of ESBL-positive *E. coli* strains with resistance to various other antimicrobials. Occurrence of ESBL-producing Enterobacteriaceae in iceberg lettuce obtained from the retail market of Rochester (US) is described by N. Bhutani et al. A wide spectrum of diseases is caused by the virulent strains of ESBL-positive isolates of *E. coli*. Regional difference in the prevalence of virulence genes in 432 phenotypically ESBL-positive patient-isolates of *E. coli* (obtained from the Baltic Sea region) is shown by J. Lillo et al. Keeping in mind the tremendous challenge posed by drug-resistant tuberculosis, a number of relevant articles are included in this collection. The susceptibility profile of *M. tuberculosis* isolates to various antitubercular antibiotics varies significantly depending on the test system as revealed by Z. Mei et al. They have also shown that changes in bacterial susceptibility are further caused by mixed infection with particular genotypes of *M. tuberculosis* strains. Resistance-profile of 100 strains of *M. tuberculosis*, isolated from patients in northeast Iran, is reported by A. T. Sani et al. Occurrence of nontuberculosis *Mycobacterium*, in 25 out of 125 patients (20%) surveyed, underscores the need of proper diagnosis before the onset of chemotherapy.

Discovery of new drugs and strategies to circumvent antibiotic resistance is the need of the hour to contain the problem. N. Jafari et al. report on the isolation of an antibiotic-producing strain of a soil Actinomycetes belonging to the genus *Pseudonocardia*. The antibacterial compound produced by it is effective against *Staphylococcus aureus*. They have also purified and partially characterized this compound. R. D. Wojtyczka et al. demonstrate high antibacterial activity of two new quinoline derivatives of a structure of 3-thioacyl 1-methyl 4-arylaminoquinolinium salts against some nosocomial strains of staphylococci in both planktonic and biofilm form. In view of the widespread nature of the problem caused by inefficacy of the antibiotics produced by fermentation and chemical synthesis, it is necessary to tap alternative sources (e.g., plant kingdom) for novel antibiotics. P. Del Serrone et al. demonstrate antibacterial activity of Neem seed oil (*Azadirachta indica* A. Juss.) against enteropathogenic strains of *E. coli* and indicate that some of the ciprofloxacin-resistant isolates lost their virulence following treatment with Neem seed oil. Antimicrobial peptides are considered potential candidates for the management of multidrug-resistant infections.

M. Singh and K. Mukhopadhyay evaluate the antimicrobial potential of an anti-inflammatory neuropeptide whereas C. Chen et al. report on the efficacy of recombinant lysostaphin against methicillin-resistant *S. aureus* (MRSA) in a mouse model. Widespread use of carbapenems is associated with emergence of resistance. The polymyxin antibiotic colistin is not used at present because of its nephrotoxicity. H.-J. Tang et al., however, demonstrate the efficacy of a combination of colistin and imipenem against carbapenem-resistant *Klebsiella pneumoniae*. Bacteriophages could be suitable alternatives for antibiotics, which currently have lost efficacy because of the emergence of resistant strains. N. Shivshetty et al. demonstrate the potential of a bacteriophage isolated from sewage to protect diabetic mice against *Pseudomonas aeruginosa*-induced bacteremia. Reversal of bacterial resistance to antibiotics is essential to restore the efficacy of the existing antimicrobials. C. Santiago et al. claim to achieve an increase in susceptibility of a MRSA strain to ampicillin when it was combined with a plant extract. A number of computerized models have been developed during the recent past to assist the physicians with the necessary information to enable prescription of the right antibiotic in the right moment. M. Rodriguez-Maresca et al. report on the efficacy of a new electronic device based on laboratory data on the most probable susceptibility profile of pathogens responsible for infections and also on local epidemiology.

Acknowledgment

We are hopeful that the compendium will be highly useful for researchers in deciding on the future course of investigation. We feel highly appreciative of all contributions made by the investigators.

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Ranadhir Chakraborty
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Research Article

First Report of *Klebsiella pneumoniae*-Carbapenemase-3-Producing *Escherichia coli* ST479 in Poland

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An increase in the antibiotic resistance among members of the *Enterobacteriaceae* family has been observed worldwide. Multidrug-resistant Gram-negative rods are increasingly reported. The treatment of infections caused by *Escherichia coli* and other *Enterobacteriaceae* has become an important clinical problem associated with reduced therapeutic possibilities. Antimicrobial carbapenems are considered the last line of defense against multidrug-resistant Gram-negative bacteria. Unfortunately, an increase of carbapenem resistance due to the production of *Klebsiella pneumoniae* carbapenemase (KPC) enzymes has been observed. In this study we describe the ability of *E. coli* to produce carbapenemase enzymes based on the results of the combination disc assay with boronic acid performed according to guidelines established by the European Community on Antimicrobial Susceptibility Testing (EUCAST) and the biochemical Carba NP test. Moreover, we evaluated the presence of genes responsible for the production of carbapenemases (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{OXA-48}) and genes encoding other β-lactamases (*bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}) among *E. coli* isolate. The tested isolate of *E. coli* that possessed the *bla*_{KPC-3} and *bla*_{TEM-34} genes was identified. The tested strain exhibited susceptibility to colistin (0.38 μg/mL) and tigecycline (1 μg/mL). This is the first detection of *bla*_{KPC-3} in an *E. coli* ST479 in Poland.

1. Introduction

E. coli is a common etiological factor of urinary tract infection, gastroenteritis, neonatal meningitis, and many nosocomial infections such as pneumonia, bloodstream infections, and surgical site infections [1]. The treatment of infections caused by *E. coli* is challenging, because of the increasing resistance of bacteria to antibiotics. The phenomenon of multidrug resistance has been reported worldwide and results in reduction of therapeutic possibilities [2].

The aim of this study was to evaluate the presence of *bla* genes responsible for carbapenemases production (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{OXA-48}) and genes encoding other β-lactamases (*bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}). Additionally, we sought to determine the sequence type (ST) of a tested *E. coli* strain.

2. Materials and Methods

The tested *E. coli* strain was isolated in February 2014 from the swab of an intestinal fistula obtained from a patient hospitalized in the intensive care unit at the University Hospital of Białystok (Poland).

Biochemical identification (GN cards) and the preliminary susceptibility test (AST-N259 cards) were performed using the VITEK 2 automated system (bioMérieux, France). Additionally, the susceptibility to antibiotics of the tested strain was performed using E-tests (bioMérieux, France). The results of the susceptibility tests were interpreted according to EUCAST recommendations [3]. The screening detection of carbapenemases was performed according to EUCAST. Moreover, the biochemical Carba NP test was

performed according to the Nordmann and Poirel protocol [4]. Further, molecular analysis was performed with the use of polymerase chain reactions (PCRs). Plasmid DNA was extracted with the use of Plasmid Mini (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. PCR amplifications for *bla* genes responsible for carbapenemases production (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{OXA-48}) and genes encoding other β -lactamases (*bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}) were performed using appropriate primers and conditions as described previously [5–8]. PCR amplicons were separated electrophoretically according to a previously described protocol [8]. Moreover, sequencing of *bla* amplicons was performed at Genomed (Warsaw, Poland). Multilocus sequence typing (MLST) was performed according to Institut Pasteur's MLST scheme (http://www.pasteur.fr/recherche/genopole/PF8/mlst/primer_Ecoli.html).

3. Results

The combination disc assay showed that the difference in the size of the inhibition zone between meropenem and meropenem with boronic acid was higher than 7 mm. The biochemical Carba NP test was positive after 1 minute. The obtained results indicated carbapenem resistance mediated by KPC among the tested strains of *E. coli*.

The tested strain was analyzed for the presence of resistance mechanisms against β -lactam antibiotics using PCR amplifications for *bla* genes responsible for carbapenemases production (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{OXA-48}) and genes encoding other β -lactamases (*bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}). The *bla*_{KPC} and *bla*_{TEM} genes were found in *E. coli*. The obtained sequence of the *bla*_{KPC} gene showed identity with the sequence of the *bla*_{KPC-3} gene (GeneBank accession no. AF395881.1). The obtained sequence of the *bla*_{TEM} gene showed identity with the sequence of the *bla*_{TEM-34} gene (GeneBank accession no. KC844056.1) responsible for production of broad-spectrum β -lactamase type TEM-34. Results of PCRs and minimum inhibitory concentration (MIC) values of tested antibiotics are presented in Table 1.

The analysis of allelic profile (*dinB-5*, *icdA-37*, *pabB-4*, *polB-10*, *putP-78*, *trpA-8*, *trpB-2*, *uidA-30*) with use of the *E. coli* MLST sequence type database (http://www.pasteur.fr/cgi-bin/genopole/PF8/mlstdbnet.pl?page=profile-query&file=Eco_profiles.xml) showed that the tested *E. coli* strain belonged to the ST479 type.

4. Discussion

A significant increase of *E. coli* isolates resistant to third-generation cephalosporins has been observed in Europe [9]. Studies have shown a high percentage (65%–100%) of extended-spectrum β -lactamase (ESBL) production among *E. coli* isolates resistant to third-generation cephalosporins [10]. One of the therapeutic options for treatment of infections due to ESBL-producing *E. coli* may be carbapenems. Resistance against carbapenems among *E. coli* rods is uncommon, which may be a result of AmpC β -lactamase production and loss of porins. Unfortunately, strains resistant to

TABLE 1: MIC values of antimicrobial agents tested for *E. coli* 140 2594-2 and results of PCRs for *bla* genes.

Antimicrobial agents	MIC [μ g/mL]	
	Diffusion test with use of E-tests	VITEK 2 automated system and AST-N259 card
Amikacin	R 96	R \geq 64
Amoxicillin/clavulanic acid	N	R \geq 32
Cefepime	I 4	I 2
Piperacillin/tazobactam	R > 256	R \geq 128
Cefuroxime	N	R \geq 64
Cefotaxime	N	R 2
Ceftazidime	R > 256	R 32
Colistin	S 0.38	S \leq 0.5
Ertapenem	R 8	R 4
Gentamicin	R 16	I 4
Tobramycin	N	R \geq 16
Aztreonam	R 192	N
Imipenem	I 3	I 8
Meropenem	S 0.75	I 1
Doripenem	I 1.5	N
Tigecycline	S 1	S \leq 0.5
Ciprofloxacin	N	R \geq 4
Trimethoprim/sulfamethoxazole	N	R \geq 320

Results of PCRs	
Genes encoding carbapenemases	Genes encoding other β -lactamases
<i>bla</i> _{KPC} -positive*	<i>bla</i> _{TEM} -positive**
<i>bla</i> _{VIM} -negative	<i>bla</i> _{SHV} -negative
<i>bla</i> _{OXA-48} -negative	<i>bla</i> _{CTX-M} -negative
<i>bla</i> _{IMP} -negative	

R: resistant; S: susceptible; I: intermediate; * genes encoding β -lactamase type KPC-3, ** genes encoding β -lactamase type TEM-34; N: not tested.

carbapenems due to the production of KPCs have recently been observed [11].

KPC producers have previously been reported in distinct geographic locations: European countries (Greece, Israel, Spain, Italy, Portugal, France, Poland, Germany, UK, and the Czech Republic), the United States, China, and South America [12]. KPC production is mainly prevalent among *Enterobacteriaceae* species. The significant majority of reports describe identification and the prevalence of *bla*_{KPC} genes among nosocomial *K. pneumoniae* strains. Moreover, the occurrence of *bla*_{KPC} genes among other *Enterobacteriaceae* species, for example, *E. coli*, *Enterobacter*, and *Citrobacter freundii* was observed [13]. The most commonly reported variant is KPC-2. Single reports describe the occurrence of KPC-3 among *E. coli* in Europe. In Spain, a multiresistant *E. coli* strain producing both KPC-3 and VIM-1 carbapenemases was described. In Italy, a KPC-3-producing *E. coli* isolate was found in abdominal drainage. Both cases were reported in

2014 [14]. Our study is first report of *bla_{KPC-3}* genes in *E. coli* ST479, in Poland.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Antimicrobial Resistance Profile and Genotypic Characteristics of *Streptococcus suis* Capsular Type 2 Isolated from Clinical Carrier Sows and Diseased Pigs in China

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Streptococcus suis serotype 2 is an important zoonotic pathogen. Antimicrobial resistance phenotypes and genotypic characterizations of *S. suis* 2 from carrier sows and diseased pigs remain largely unknown. In this study, 96 swine *S. suis* type 2, 62 from healthy sows and 34 from diseased pigs, were analyzed. High frequency of tetracycline resistance was observed, followed by sulfonamides. The lowest resistance of *S. suis* 2 for β -lactams supports their use as the primary antibiotics to treat the infection of serotype 2. In contrast, 35 of 37 *S. suis* 2 with MLS_B phenotypes were isolated from healthy sows, mostly encoded by the *ermB* and/or the *mefA* genes. Significantly lower frequency of *mrp*+/*epf*+/*sly*+ was observed among serotype 2 from healthy sows compared to those from diseased pigs. Furthermore, isolates from diseased pigs showed more homogeneously genetic patterns, with most of them clustered in pulsotypes A and E. The data indicate the genetic complexity of *S. suis* 2 between herds and a close linkage among isolates from healthy sows and diseased pigs. Moreover, many factors, such as extensive use of tetracycline or diffusion of Tn916 with *tetM*, might have favored for the pathogenicity and widespread dissemination of *S. suis* serotype 2.

1. Introduction

Streptococcus suis is an important swine pathogen leading to big loss in pig production worldwide [1]. Among 35 serotypes currently identified, serotype 2 has gained more attention for the high prevalence and mortality rates in swine and human and is considered as an emerging zoonotic agent [2]. Furthermore, epidemiological surveillance has confirmed that *S. suis* type 2 can transmit from carrier pigs to humans [3, 4]. Clinically healthy carrier sows, harboring *S. suis* type 2, are considered as the major source of infection for their offspring [5]. Since the infection of *S. suis* type 2 commonly occurred among suckling and weaned piglets, it is essential to investigate the association of antimicrobial resistance profile

and genotypic characteristics of isolates from carrier sows and diseased pigs.

In the absence of effective vaccines to fight against *S. suis*, antimicrobial agents have become increasingly important in treating and controlling the infection of *S. suis* type 2. Of these, β -lactams, tetracyclines, sulphonamides, and macrolides are the most common antimicrobials used for the prevention and treatment of streptococcal infection in pig production. But inappropriate use of antibiotic has led to the development of resistance of *S. suis* to these drugs worldwide [6–9]. Furthermore, coresistance to tetracyclines and macrolides/lincosamides in human *S. suis* isolates was observed, and the relevance of Tn916-like conjugative transposon in coresistant mechanisms and clone diffusion have

been studied [9, 10]. However, to date, few reports about coresistance to these three classes of antibiotics and the Tn916 family were found in swine *S. suis* serotype 2.

In recent years, research on *S. suis* type 2 has mainly concentrated on its potential virulence factors and pathogenic mechanisms. Many factors, including polysaccharide capsule (*cps*), muramidase-released protein (*mrp*), the extracellular protein factor (*epf*), the suilysin (*sly*), glyceraldehyde-3-phosphate dehydrogenase (*gdh*), and a fibronectin/fibrinogen-binding protein (*fbp*), were found to be associated with virulence of *S. suis* 2 [11–14]. Of these, *mrp*, *epf*, and *sly* were considered as the most relevant factors to the pathogenesis of *S. suis* by many researchers [15–17]. Recently, other new putative virulence factors, such as surface-associated subtilisin-like serine protease (*SspA*), factor H-binding protein (*fhb*), have also been identified [18, 19]. However, our knowledge about pathogenesis of serotype 2 is still limited despite the increasing number of studies.

To deeply understand *S. suis* type 2 infection, molecular typing methods are usually applied to identify individual isolates and establish genotypic characterization. Currently, many typing methods, such as randomly amplified polymorphic DNA (RAPD) [20], ribotyping [21], multilocus sequences typing (MLST) [22, 23], and genome sequencing [24], have been used to define the diversity of *S. suis* and to distinguish virulent from nonvirulent isolates. Of these, pulsed-field gel electrophoresis (PFGE) is one of the most powerful molecular typing methods. Many researches had been done to compare differences between *S. suis* from different animals, and genetic diversity was found on the basis of isolates of the different pathogenic serotypes [25–27]. However, a thorough characterization of serotype 2 isolates from healthy sows and diseased pigs has not thus far been reported.

The aim of the study is to investigate antimicrobial resistance phenotypes, genotypes, and genetic patterns of *S. suis* type 2 from clinical carrier sows and diseased pigs. Furthermore, the phenotypic and genotypic characterizations of these isolates were compared. To the best of our knowledge, this is the first integrative report about resistance profile and genetic diversity of *S. suis* serotype 2 from clinically healthy sows and diseased pigs.

2. Materials and Methods

2.1. *S. suis* Type 2 Isolates. A total of 96 swine *S. suis* serotype 2 were included in this study. 62 isolates were recovered from tonsils of clinically healthy sows of 15 epidemiologically unrelated farms in 10 regions (Jiangsu, Sichuan, Guangdong, Guangxi, Anhui, Henan, Hebei, Jiangxi, Shandong, and Beijing) from March 2005 to November 2012. Of the 34 *S. suis* serotype 2 obtained from diseased pigs, 31 outbreak isolates were from 6 epidemiologically unrelated farms in Sichuan, Jiangsu, Anhui, Henan, and Beijing during 2005–2008, and three historical isolates (C55604, C55609, and C55612) were provided by China Veterinary Culture Collection Center (CVCC). All isolates were determined as *S. suis* types 2 by biochemical characteristics (API 20 strep, bioMérieux SA,

France) and sera agglutination reaction (special antisera provided by the Statens Serum Institut, Copenhagen, Denmark) and further confirmed as serotype 2 by positive PCR for the genes coding for the 16S rRNA of *S. suis* and for the capsule of *S. suis* serotype 2 (*cps2J*) [23].

2.2. Antimicrobial Susceptibility. Antimicrobial susceptibility testing was performed using the standard broth microdilution method [28]. The following antimicrobial agents, the representatives of commonly used drug classes in China, were tested (with dilution ranges in parentheses): penicillin G (0.06–8 µg/mL), ampicillin (0.12–16 µg/mL), ceftiofur sodium (0.12–16 µg/mL), enrofloxacin (0.015–4 µg/mL), clindamycin (0.03–4 µg/mL), erythromycin (0.06–8 µg/mL), tilimicosin (0.5–64 µg/mL), chloramphenicol (1–128 µg/mL), tiamulin fumarate (0.25–32 µg/mL), tetracycline (0.06–8 µg/mL), sulfisoxazole (16–512 µg/mL), and trimethoprim/sulfamethoxazole (0.5/9.5–16/304 µg/mL). *Streptococcus pneumoniae* ATCC 49619 was used as the quality control strain. The isolate was defined as an MLS_B-phenotype that was found to be resistant to erythromycin, lincomycin, and tilimicosin.

2.3. Genotyping. PCR virulence genotyping of all isolates was performed for the *mrp*, *epf*, *sly*, *orf2*, *fbp*, and *gdh* genes. Moreover, 85 tetracycline-resistant (*tet*^r) isolates were also analyzed for the presence of tetracycline-resistant genes (*tetK*, *tetL*, *tetM*, and *tetO*), macrolide-resistant genes (*ermB*, *ermA*, and *mefA*), lincosamide-resistant gene (*lnuB*), and Tn916-like transposon family (*intTn* and *xis*), respectively. Positive and negative controls were included with each PCR assay. Target genes and the corresponding primer sequences were listed in Table 1. Confirmation of the amplicons was determined by DNA sequencing and the results were analyzed using BLAST software (<http://www.ncbi.nlm.nih.gov>).

2.4. PFGE Analysis. All *S. suis* serotype 2 isolates were typed using pulsed-field gel electrophoresis (PFGE) as described previously [31, 32] with minor modifications. Briefly, cell culture was suspended in cell suspension buffer (100 mM Tris : 100 mM EDTA, pH8.0) to 10 McFarland standards. Proteinase K was then added at final concentration of 0.5 mg/mL and mixed with equal volume of molten 1% Seakem Gold Agarose before loading into the plug mold. After solidification, the agarose plugs were submerged in cell lysis buffer (CLB, 50 mM Tris : 500 mM EDTA, pH8.0 + 1% sarkosyl) with lysozyme (1 mg/mL) before incubation at 37°C for 12 h and then CLB with proteinase K (0.5 mg/mL) was added and cultured at 54°C for 2 h with vigorous shaking. After washing, the plugs were sliced and then digested in fresh restriction buffer with the enzyme *Sma*I (50 U/µL) at 25°C for 12 h. The electrophoresis was performed with CHEF-DR III system (Bio-Rad) at 14°C under the electric field strength 6 v/cm for 19 h with pulse time ramping from 2.2 s to 63.8 s.

Salmonella enterica serovar Braenderup H9812 restricted with *Xba*I was used for molecular weight and size determinations. Similarities between restriction endonuclease digestion profiles were analyzed using BioNumerics software (Applied

TABLE 1: Target genes and PCR primers used in this study.

Gene target(s)	Primer sequence (5'-3')	Amplicon size (bp)	Reference
Macrolide/Lincosamide resistance genes			
<i>ermB</i>	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639	[29]
<i>ermA</i>	GAAGTTTAGCTTCCTAA GCTTCAGCACCTGTCTTAATTGAT	395	[29]
<i>mefA</i>	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAGTGG	346	[29]
<i>lnuB</i>	CCTACCTATTGTTGTGGAA ATAACGTTACTCTCCTATTTC	944	[29]
Tetracycline resistance genes			
<i>tetK</i>	TATTTGGCTTGTATTCTTCAT GCTATACCTGTTCCCTCTGATAA	1159	[26]
<i>tetL</i>	ATAAATTGTTCGGGTCGGAAT AACCAAGCCAACTAATGACAATGAT	1077	[26]
<i>tetO</i>	AACTTAGGCATTCTGGCTCAC TCCCACGTCCATATCGTCA	519	[26]
<i>tetM</i>	GAACTCGAACAAGAGGAAAGC ATGGAAGGCCAGAAAGGAT	740	[26]
<i>IntTn</i> and <i>xis</i> genes			
<i>intTn</i>	GGTCTTCGTATTCAGAGTTGG GTTGCATGTGCGTAATAGTTAG	473	[30]
<i>xis</i>	AAGCAGACTGACATTCTCA GCGTCCAATGTATCTATAA	193	[30]
Virulence-associated factors			
<i>mrp</i>	ATTGCTCCACAAGAGGATGG TGAGCTTACCTGAAGCGGT	188	[15]
<i>epf</i>	CGCAGACAACGAAAGATTGA AAGAATGTCTTGGCGATGG	744	[15]
<i>sly</i>	GCTTGACTTACGAGCCACAA CCGCGCAAACTGATAAGC	248	[15]
<i>fbp</i>	GACGGATCCTTTTACATCACATGACGG CCGTCGACGTATTCGCAGAACAT	247	this study
<i>orf2</i>	CAAGTGTATGTGGATGGG ATCCAGTTGACACGTGCA	860	this study
<i>gdh</i>	GGCGCCGAATTGTCGACATTAGCAATTGGCG CGCCGGATCCGTAGTTAAAGTTGGTATTAAC	1039	this study

Maths, Kortrijk, Belgium) with Dice coefficients and clustering by an unweighted paired group with arithmetic averaging. The dendrogram of PFGE patterns of isolates tested was drawn with a 1.5% position tolerance and 1% optimization. And the cluster cutoff was set at an 85% similarity level. The different PFGE fingerprints were assigned as different uppercase letters.

2.5. Statistical Analysis. SPSS for Windows, version 16.0, was used for statistical analysis. The frequencies of antibiotic resistance, resistant genes, and virulence-associated factors were compared between the isolates from healthy carrier

sows and from diseased pigs. The chi-square (or Pearson chi-square) and Fisher exact tests were used when appropriate. Differences were considered significant when two-sided *P* value was less than 0.05.

3. Results

3.1. Antimicrobial Susceptibility Testing and Detection of Resistance Genes. The collections of 96 *S. suis* 2 were tested for susceptibility to 12 antimicrobials (Table 2). High frequency of resistance was observed for tetracycline, followed by sulfonamides. 57 and 46 isolates from healthy sows and 28

TABLE 2: Antimicrobial resistance profile of *S. suis* serotype 2 from clinically healthy carrier sows and diseased pigs.

Antimicrobials	MIC breakpoint* ($\mu\text{g/mL}$)			Healthy carrier sows (n = 62)		Diseased pigs (n = 34)		Total (n = 96)	
	S	I	R	MIC range	n**	MIC range	n**	MIC range	n**
Penicillin	0.12	0.25–2	4	≤ 0.06 –>8	2	≤ 0.06 –1	0	≤ 0.06 –>8	2
Ampicillin	0.25	0.5–4	8	≤ 0.12 –2	0	≤ 0.12 –0.5	0	≤ 0.12 –2	0
Erythromycin	0.25	0.5	1	0.12–>8	35	≤ 0.06 –4	2	≤ 0.06 –>8	37
Clindamycin	0.5	1–2	4	0.06–>4	35	0.06–>4	2	0.06–>4	37
Enrofloxacin	0.25	0.5–1	2	0.12–>4	6	0.06–1	0	0.06–>4	6
Tetracycline	2	4	8	0.5–>8	57	0.25–>8	28	0.25–>8	85
Ceftiofur	2	4	8	≤ 0.12 –>16	3	≤ 0.12 –2	0	≤ 0.12 –>16	3
Tiamulin	16	—	32	≤ 0.25 –>32	7	≤ 0.25 –16	0	≤ 0.25 –>32	7
Tilmicosin	16	—	32	1–>64	35	≤ 0.5 –64	2	≤ 0.5 –>64	37
Chloramphenicol	4	8	16	≤ 1 –32	3	≤ 1 –8	0	≤ 1 –32	3
Sulfisoxazole	256	—	512	32–>512	46	32–>512	18	32–>512	64
Trimethoprim/ sulfamethoxazole	2/38	—	4/76	≤ 0.5 /9.5– >16/304	13	≤ 0.5 /9.5– 16/304	2	≤ 0.5 /9.5– >16/304	15

* MIC breakpoints were taken from Clinical and Laboratory Standards Institute standards (CLSI).

S: susceptible; I: intermediate; R: resistant.

** Number of resistance isolates.

and 18 from diseased pigs were resistant to tetracycline and sulfisoxazole, respectively. These two antimicrobial agents had MIC₅₀ values (>8, 512 $\mu\text{g/mL}$, resp.) equal to or higher than the highest concentration tested. The lowest resistant rates of *S. suis* 2 for β -lactams were found, and all isolates were susceptible to ampicillin. Data also suggested the high incidence rates of resistance for macrolides and lincosamides in the isolates from healthy carrier sows. Among 85 tet^r isolates, 37 had MLS_B resistance phenotypes, 35 from carrier sows and the remaining two from diseased pigs. No inducible resistance pattern was discovered. A significantly higher occurrence of MLS_B resistance was observed in *S. suis* 2 from carrier sows than those from diseased pigs ($P < 0.005$).

Antimicrobial resistance patterns and resistant determinants for tetracyclines, macrolides/lincosamides were analyzed in Table 3. No detection of *tetK* and *tetL* genes was observed among tet^r *S. suis* 2. The *tetM* gene was found among 77 and the *tetO* gene among 51 of 85 tet^r *S. suis* type 2. None of erythromycin- and clindamycin-resistant isolates carried *ermA* or *lnuB*. 35 *S. suis* 2 with MLS_B-phenotype were shown to be *ermB* and *tetM* positive and 18 *mefA* positive. Neither *ermB* nor *mefA* was detected in the non-MLS_B tet^r isolates. Presence of the *ermB* gene was strongly associated with MLS_B-phenotype of *S. suis* 2. Significant carrier difference of the *tetO* gene, but not *tetM*, was observed between erythromycin-resistant (32/37) and erythromycin-susceptible (19/48) isolates.

None of 85 tet^r *S. suis* serotype 2 was positive for the *xis* gene. 38 tet^r isolates (26 from diseased pigs and the remaining 12 from healthy sows) carried the *intTn* gene. Unexpectedly, all isolates (38/96) with *intTn* gene were resistant to tetracycline only, while they were susceptible to macrolides/lincosamides. And higher frequency of *intTn*

with *tetM* was observed in isolates from diseased pigs (22/34) compared to those from healthy carrier sows (12/62).

3.2. Virulence-Associated Genes Analysis. 96 serotype 2 isolates were cloned and screened for the presence of the *mrp*, *epf*, *sly*, *orf2*, *fbp*, and *gdh* genes. The distribution of virulence-associated genes was reported in Table 3. All isolates were positive for the virulence genes coding for *fbp* and *orf2* and negative for *gdh*. 41, 58, and 47 of 62 healthy sows isolates harbored *mrp*, *epf*, and *sly*, respectively. In contrast, the *mrp*, *epf*, and *sly* genes were detected in all *S. suis* 2 recovered from diseased pigs but two *epf*-negative isolates.

Six kinds of virulence genotypes were obtained in *S. suis* capsular type 2 from carrier sows, with high frequency of *mrp+/epf+/sly+* (30/62), *mrp-/epf+/sly+* (15/62), and *mrp+/epf+/sly-* (9/62) (Table 4). All *S. suis* type 2 from diseased pigs had the virulence genotype of *mrp+/epf+/sly+* with two exceptions of *mrp+/epf-/sly+*. Significantly lower carrier rate of *mrp+/epf+/sly+* genotype was observed among isolates from healthy sows compared to those from diseased pigs ($P < 0.005$).

3.3. PFGE Typing. On the basis of an investigation of 96 *S. suis* type 2, PFGE typing produced 15 different fingerprints, which were grouped into types A to H (Figure 1). Pulsotypes C1–C3, E1–E4, G1–G2, and H1–H2 were considered to be respectively related, with more than 85% similarity.

In contrast, significant difference was observed between the isolates from diseased pigs and healthy sows. All isolates from diseased pigs were classified as pulsotypes A, E, and G (Tables 3 and 5). Pulsotypes A and E predominated in diseased pigs and were detected in 32 of 34 *S. suis* 2 isolates. For isolates from clinically healthy sows, pulsotype G (21/64)

TABLE 3: Distribution of antimicrobial resistance patterns, resistant determinants, virulence factors, and pulsotypes of *S. suis* type 2 isolates.

Origin of isolates	Resistant pattern ^a	Number of isolates	Number of isolates with resistant determinants ^b						Number of isolates with virulence genes ^c (%)	PFGE subtypes (number of isolates)
			<i>intI1n</i>	<i>ermB</i>	<i>mefA</i>	<i>tetM</i>	<i>tetO</i>	<i>mrp</i>		
Healthy sows (<i>n</i> = 62)	Ery ^r Til ^r Cli ^r Tet ^r	35	0	33	16	33	30	16	33	C (5), D (8), F (4), G (10), H (8)
	Ery ^s Til ^s Cli ^s Tet ^r	22	12	0	0	20	12	20	20	B (2), C (8), G (8), E (4)
	Ery ^s Til ^s Cli ^s Tet ^s	5	/	/	/	/	/	5	5	G (3), H (2)
	Total	62	12	33	16	53	42	41	58	47
Diseased pigs (<i>n</i> = 34)	Ery ^r Til ^r Cli ^r Tet ^r	2	0	2	2	2	2	2	2	A (2)
	Ery ^s Til ^s Cli ^s Tet ^r	26	26	0	0	22	7	26	24	A (10), E (14), G (2)
	Ery ^s Til ^s Cli ^s Tet ^s	6	/	/	/	/	/	6	6	A (2), E (4)
	Total	34	26	2	2	24	9	34	32	34

^aEry^r: erythromycin resistant; Til^r: tilimicosin resistant; Cli^r: clindamycin resistant; Tet^r: tetracycline resistant; Ery^s: erythromycin susceptible; Til^s: tilimicosin susceptible; Cli^s: clindamycin susceptible; Tet^s: tetracycline susceptible.

^bThe *ermA*, *lmuB*, *tetK*, *tetL*, and *xis* genes were not detected in the isolates included in this study.

^cAll isolates carried the *fbp* and *orf2* genes and none harbored the *gadH* gene.

/: Not detected.

TABLE 4: Virulence genotypes of 96 *S. suis* capsular type 2.

Virulence genotypes*	Number of <i>S. suis</i> capsular type 2		
	Healthy sows	Diseased pigs	Total
<i>mrp+/epf+/sly+</i>	30	32	62
<i>mrp+/epf-/sly+</i>	2	2	4
<i>mrp-/epf+/sly+</i>	15	0	15
<i>mrp-/epf+/sly-</i>	4	0	4
<i>mrp+/epf+/sly-</i>	9	0	9
<i>mrp-/epf-/sly-</i>	2	0	2
Total	62	34	96

* All isolates had the genotype of *fbp*+/orf2+/gdh-.

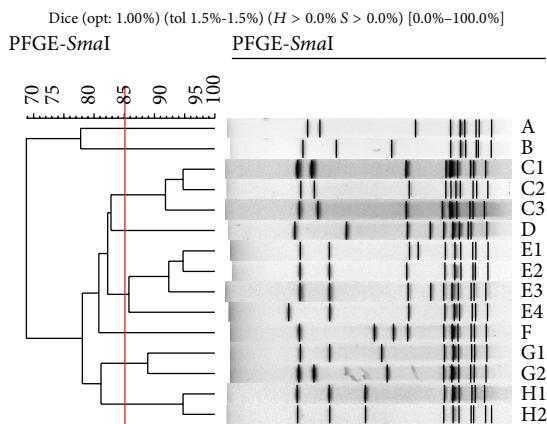


FIGURE 1: Genetic relationship of representative bands of *S. suis* type 2 isolated from clinically healthy sows and diseased pigs. Dendrogram showed the genetic relatedness of *S. suis* type 2 pulsotypes A to H2. The cluster cutoff (red line) was set at an 85% similarity level. Numbers at the upper left indicate percent similarity.

was the most frequently observed one, followed by types C (13/64), H (10/64), D (8/64), E (4/64), F (4/64), and B (2/64). Interestingly, the same pulsotype, E or G, was found in *S. suis* 2 both from healthy sows and from diseased pigs. For example, isolates C55604 (from historically diseased pigs) and one isolate (farm GD-2, from healthy sows) with 100% homogeneity were present in pulsotype E2 (Table 5).

All *S. suis* serotype 2 from the same farm, including those isolated in different years, shared the pulsotype with more than 85% similarity; most of them had the identical PFGE patterns. 6 isolates from farm GD-1 in 2006 and 2009 were assigned to PFGE type C, and the similar results were observed for pulsotypes D, G2, H1, and E3. Complex relationship among *S. suis* 2 isolates from different farms was discovered. Three isolates from Beijing had the pulsotype (E4) different from Sichuan and Jiangsu isolates (pulsotype A) even if these *S. suis* serotype 2 were isolated during the largest outbreak of human *S. suis* 2 infection occurring in 2005. However, some pulsotypes were more frequently isolated and exhibited a wide distribution over herds compared to others. For instance, 13 isolates from different farms in Henan, Jiangsu, and Shandong provinces were classified as PFGE subtype G1, and similar results were

TABLE 5: PFGE patterns of 96 *S. suis* serotype 2 isolates included in this study.

Origin	PFGE pattern (n [#])	Number of isolates	Farm number ^{##}	Isolated years
	B (2)	2	AH-2	2011
	C1 (4)	2	GD-1	2009
		2	GX-1	2007
	C2 (5)	3	SD-1	2006
		2	HB-1	2007
	C3 (4)	4	GD-1	2006, 2009
Healthy sows	D (8)	2	BJ-2	2008
		6	HN-3	2009, 2010
	E1 (3)	3	GD-2	2009
	E2 (1)	1	GD-2	2007
	F (4)	4	HB-2	2007
		7	HN-1	2006
	G1 (13)	1	JS-2	2009
		5	SD-2	2011
	G2 (8)	8	GX-2	2010, 2012
	H1 (8)	8	JX-1	2007, 2009
	H2 (2)	2	SC-3	2007
		8	SC-1	2005
	A (14)	2	SC-2	2005
		4	JS-1	2005
Diseased pigs	E1 (2)	2	C55609, C55612	/
	E2 (1)	1	C55604	/
	E3 (12)	12	AH-1	2007, 2008
	E4 (3)	3	BJ-1	2005
	G1 (2)	2	HN-2	2006

[#] Number of isolates with the same PFGE pattern.

^{##} Farm number was named as capital letters (abbreviation of the province/region)—serial number. AH: Anhui; GD: Guangdong; GX: Guangxi; SD: Shandong; HB: Hebei; BJ: Beijing; HN: Henan; SC: Sichuan; JS: Jiangsu; JX: Jiangxi. C55604, C55609, and C55612 were provided by CVCC. /: isolated time was not provided.

obtained for patterns C1, C2, and D. Furthermore, *S. suis* serotype 2 with types G1, C1, C2, and D were resistant to

tetracycline and positive for the *tetM* gene, although four different virulence genotypes, *mrp+/epf+/sly+*, *mrp-/epf+/sly+*, *mrp+/epf-/sly+*, and *mrp-/epf+/sly-*, were involved (not shown in tables).

4. Discussion

4.1. Resistant Phenotypes and Genotypes of *S. suis* 2. The lowest resistance of *S. suis* serotype 2 for β -lactams was in accordance with other discoveries [7, 8], supporting their use as the primary drugs to treat the infection of swine *S. suis* serotype 2. The resistance to tetracyclines in *S. suis* has become a major worldwide problem, closely related to the widespread use of tetracycline in swine production. And tetracycline-resistance has been considered to be an important cofactor in the selection of resistance to macrolides/lincosamides [9]. In this study, 85 of the 96 isolates were resistant to tetracycline, 37 of which were coresistant to macrolides and lincosamides antibiotics. This indicated less frequent coresistance of *S. suis* 2 to tetracyclines and macrolides/lincosamides. And the similar results were also observed by other researchers [6, 10, 33]. Furthermore, 35 of 37 *S. suis* 2 with MLS_B-phenotype were isolated from healthy sows, indicating the presence of selective pressure of antimicrobial agents since tilmicosin and tylosin were widely used as swine feed additives in China. In contrast, most of tet^r isolates from diseased pigs in backyard without feed additives were susceptible to macrolides/lincosamides.

Tetracyclines resistance in streptococci is mediated by ribosomal protection proteins or efflux proteins, encoded mainly by the *tet* genes [34]. Neither *tetK* nor *tetL* was detected in this study, which was consistent with other recent analyses [10]. The *tetM* and *tetO* genes, both coding for ribosomal protection protein, were widespread in tet^r *S. suis*, and higher carrier rate of the *tetM* gene than the *tetO* gene was also observed in other studies [9, 35].

Resistance to macrolide of streptococcal clinical isolates is commonly encoded by ribosomal methylase (*erm*) genes and efflux (*mef*) genes [29]. The *ermB* gene was found in all but two erythromycin-resistant isolates, confirming its frequency in *S. suis* type 2 in China [9]. High number of MLS_B-resistant isolates with the *ermB* gene is in agreement with other research findings [30, 36]. To our knowledge, no large scale survey concerning the distribution of the *mefA* gene in *S. suis* type 2 has been described in the literature. Wierzbowski et al. found that the *mefA* gene conferred low-level resistance of *Streptococcus pneumoniae* to macrolides only (M phenotype) [36]. In this study, about half of *S. suis* 2 with MLS_B resistance harbored the *mefA* gene. In the view of the fact that all *mefA*-positive isolates showed MLS_B-phenotype and harbored the *ermB* gene, *mef*-mediated resistance can be obscured by the effects of the *erm* gene in phenotypic tests.

4.2. Tn916-Like Transposon Family. Tn916 is one of the most extensively studied conjugative transposons in gram-positive bacteria. The integrase *intTn* gene is responsible for transposition, and the excisase *xis* gene may increase the frequency of excision but is not required [37]. In this present study, it is interesting that the *intTn* gene was detected only in

erythromycin- and clindamycin-susceptible isolates and no *xis* genes were detected, indicating the absence of relatedness between presence of Tn916-like conjugative transposon and macrolides/lincosamides resistance phenotypes of *S. suis* type 2.

It is worth noting that the *tet* genes are often carried by Tn916-like conjugative transposon and erythromycin resistance genes are also carried on the same element, which contributes to the coresistance of streptococci to tetracyclines and macrolides. Previous studies have investigated the association between *tetM/tetO* and *ermB/mefA* in *S. pneumoniae* or *S. pyogenes*, and conjugal transfer experiments demonstrated that *tetM/tetO* and *ermB/mefA* were consistently cotransferred by Tn916-Tn1545-like transposons [38, 39]. However, the *intTn* gene was not detected among 35 MLS_B isolates with *tetM* and *ermB* in this study, and therefore, elements other than the Tn916 family might be associated with coexistence of these two genes in *S. suis* serotype 2 with MLS_B phenotype.

The *tetM* and *intTn* genes, the markers for the Tn916 family of elements [40], were harbored by 22 of 36 isolates from diseased pigs in the study. Ye et al. also found that *tetM* was associated with Tn916 in *S. suis* type 2 from human outbreak [9]. Thus, the presence in *S. suis* 2 of elements related to Tn916 with *tetM* could play an important role in the pathogenicity of this bacterial pathogen. Further studies are necessary to monitor the spread of these elements in *S. suis* serotype 2 circulating in environments.

4.3. Virulence-Associated Factors. To further characterize the molecular features of the isolates from the diseased pigs and healthy sows, six virulence-associated genes were detected. The *gdh* gene was not detected in any isolates, revealing that presence of this gene may be not necessary for these isolates included in this study. Different results from other studies indicated that carriage of the *gdh* gene was associated with multilocus sequence type (ST) or origins of isolates [41]. Moreover, the *fbp* gene was present in all *S. suis* type 2, and similar results were also observed by de Greeff et al. [12], who suggested that the *fbp* gene is present among most serotypes except for serotypes 32 and 34. Thus, virulence difference between the isolates from clinically healthy sows and those from diseased pigs may lie in the frequency of the *mfp*, *epf*, and *sly* genes, since all *S. suis* serotype 2 analyzed in the present study also carried the *orf2* gene. Interestingly, more than 60% isolates from clinically healthy sows harbored the *mfp*, *epf*, and *sly* genes. The higher carrier rate of virulent genotype of *mfp+/epf+/sly+* in *S. suis* type 2 from diseased pigs compared to healthy sows showed that these three genes together may contribute to differentiating the virulence of serotype 2, which is in accordance with results of other epidemiological reports [9, 17]. However, since 30 of 62 isolates from clinical carrier sows were also genotyped *mfp+/epf+/sly+* in this study and similar result from healthy pigs was obtained by other researchers [15], it is necessary to perform further studies to specify the virulence of serotype 2 *mfp+/epf+/sly+* isolates.

4.4. PFGE Subtyping. Many studies showed that PFGE could effectively detect relationship between genetic background, virulence traits, and epidemiologic implication of many bacterial pathogens [27, 31]. In this study, all isolates were typed by PFGE and clustered in 8 pulsotypes. *S. suis* serotype 2 from diseased pigs showed more homogeneously genetic patterns than those from healthy sows, with most of them clustering in pulsotypes A and E. In contrast, the majority of isolates from healthy sows clustered in the patterns G, C, and H, presenting a high level of divergence.

Despite the genetic diversity observed, four PFGE profiles, G, E, A, and C (accounted for 72 of 96 isolates), were more frequently observed than other patterns. These four prevalent PFGE profiles were isolated from diseased pigs and healthy sows from 9 provinces in different years (Table 5), indicating their widespread distribution in Chinese swine population. Of these, PFGE pattern G1 was unique and predominant among *S. suis* serotype 2 isolates from four epidemiologically unrelated herds, suggesting the existence of a prevalent clone. In consideration of the characteristic of their *tetM*-positive tetracycline resistance, diffusion has probably provided considerable advantages by the use of antimicrobial agents in different farms or horizontal acquiring of genetic elements, such as Tn916 with *tetM* [9, 42]. In addition, *S. suis* serotype 2 with pulsotype G1 showed dissimilarity at virulence genotypes, including *mrp+/epf+/sly+*, *mrp+/epf-/sly+*, and *mrp-/epf+/sly+*, which demonstrates that factors other than antimicrobial susceptibility might have favored for its diffusion.

The identical PFGE pattern (C3, D, G2, H1, or E3) was detected for the isolates from the same farm in different years (Table 5), and the persistent dissemination of *S. suis* serotype 2 clone within the herd of swine could be confirmed [26]. Furthermore, pulsotype E1 (E2 or G1) was found among the isolates from historical diseased pigs and from healthy sows. In our opinion, this can be explained with two main reasons. Firstly, after a long-term adaption to the healthy sows, *S. suis* 2 gradually lost virulence and finally became avirulent. Secondly, as a kind of conditional pathogenic bacteria, the possibility that healthy carrier sows harbor *S. suis* 2 is capable of causing disease under specific circumstances cannot be ruled out [5], suggesting a close linkage of *S. suis* serotype 2 from healthy sows and diseased pigs.

Taken together, the present study is the first systematic description of resistant phenotypes and genetic genotypes of *S. suis* type 2 isolated from clinically healthy sows and diseased pigs in China. Significant differences of MLS_B resistance phenotype, virulence-associated genotypes, and PFGE pulsotypes were observed between isolates from clinical carrier sows and those from diseased pigs. The results indicate that β -lactams are still the primary drugs to treat the infection of swine *S. suis* serotype 2. The unique and predominant PFGE types within and between herds show persistent dissemination of *S. suis* 2 and a close linkage among isolates from healthy sows and diseased pigs. Moreover, our data also support the contention that extensive use of tetracycline and horizontal acquiring of genetic element, Tn916 with *tetM*, could act as a selective factor for the pathogenicity and widespread diffusion of serotype 2 [9, 42].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Emergence of Carbapenem-Resistant *Klebsiella pneumoniae*: Progressive Spread and Four-Year Period of Observation in a Cardiac Surgery Division

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Frequent use of carbapenems has contributed to the increase to *K. pneumoniae* strains resistant to this class of antibiotics (CRKP), causing a problem in the clinical treatment of patients. This investigation reports the epidemiology, genetic diversity, and clinical implication of the resistance to drugs mediated by CRKP in our hospital. A total of 280 *K. pneumoniae* strains were collected; in particular 98/280 (35%) were CRKP. Sequencing analysis of CRKP isolated strains showed that 9/98 of MBL-producing strains carried the *bla_{VIM-1}* gene and 89/98 of the isolates were positive for *bla_{KPC-2}*. Antimicrobial susceptibility tests revealed a complete resistance to third-generation cephalosporins and a moderate resistance to tigecycline, gentamicin, and fluoroquinolones with percentages of resistance of 61%, 64%, and 98%, respectively. A resistance of 31% was shown towards trimethoprim-sulfamethoxazole. Colistin was the most active agent against CRKP with 99% of susceptibility. Clonality was evaluated by PFGE and MLST: MLST showed the same clonal type, ST258, while PFGE analysis indicated the presence of a major clone, namely, pulsotype A. This finding indicates that the prevalent resistant isolates were genetically related, suggesting that the spread of these genes could be due to clonal dissemination as well as to genetic exchange between different clones.

1. Introduction

In patients undergoing cardiac surgery, healthcare-associated infection often represents a dramatic event, with a consequent prolonged hospitalization and increased mortality [1]. The most common microorganisms causing infection are gram positive bacteria, with *Staphylococcus* spp. being the most frequent, followed by *Enterococcus* spp., *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii* [2]. During the last decade, various multidrug-resistant organisms (MDRO) emerged, constituting a new challenge for pharmacological treatment and the implementation of infection control practices.

In the class of Enterobacteriaceae, *Klebsiella pneumoniae* was widespread in hospital environments and their diffusion was being facilitated by their being a normal colonizer of the gastrointestinal tract and by their having a high efficiency of resistant strains selection. This resistance was due to chromosomal mutations and to the presence of many transmissible plasmids. During outbreaks, a high number of carriers have been reported among patients and personnel in these wards, due to the colonization of hands and nasopharynx [3].

Klebsiella pneumoniae has become progressively resistant to penicillin, aminoglycosides, extended-spectrum β -lactamase, and fluoroquinolones. In the 2000s, when carbapenems represented the last resort for the treatment

of infection caused by extended-spectrum β -lactamase (ESBL) producing bacteria, strains producing carbapenemases encoded by mobile elements arose, with a variety of enzymes produced.

The clinically most important *Klebsiella pneumoniae* carbapenemase (KPC) belongs to the class A enzymes encoded by *bla*_{KPC} genes and the class B metallo- β -lactamases (MBL), mainly NDM (New Delhi metallo-beta-lactamase) and VIM (Verona integron-encoded metallo-beta-lactamase). Both of these classes of A and B enzymes have been implicated in the rapid dissemination of the MDRO carbapenem-resistant *Klebsiella pneumoniae* (CRKP); this species seems to represent a “reservoir” of resistance, transmittable to other enterobacteria, including *Escherichia coli* and *Enterobacter* spp. [4, 5]. CRKP strains are implicated in nosocomial outbreaks and cause serious infections in intensive care units (ICUs); the respiratory tract was the most common site of infection [6], but also catheter related infection, surgical site, and urinary infection are reported [7].

Heart surgery patients are frequently affected by important comorbidity (diabetes mellitus, renal disease, respiratory disease, etc.) and are exposed to several healthcare-associated risk factors, such as mechanical ventilation, parenteral nutrition, arterial and central lines, and urinary catheter [8]. Some of these patients have additional risk factors for postoperative infection, like hemodialysis, blood transfusion, and readmission to the ICU [9].

Diagnosis and hospital management of CRKP infection represent a major problem in hospitals worldwide. For these patients, time is a critical parameter for the successful implementation of treatment and this should be considered in relation to the time required by the microbiology laboratory to confirm the bacteriological identification and the antimicrobial pattern, to support the decision on antibiotic therapy. This information is important for designing and implementing interventions aiming at reducing the spread of antimicrobial resistance [10].

The emergence of *K. pneumoniae* resistance to carbapenems is well documented in several studies [11–17] but more information about the molecular characterization of CRKP and their antimicrobial resistance patterns and molecular typing could be keys for the epidemiological investigation of hospital-onset CRKP infection. The Policlinico San Donato is an IRCCS (Scientific Institute for Research, Hospitalization and Health Care), with a particular attention on cardiovascular diseases. In the cardiovascular centre “E. Malan,” five operating rooms and 102 beds for patients of Heart Surgery (41 beds), Pediatric Heart Surgery (33 beds), and Postoperative Intensive Care (28 beds) Divisions are functional. Between 2010 and 2013, 1450 heart surgical interventions have been performed, representing more than 20 percent of all cardio-surgery activities in the Italian region of Lombardy. Therefore, the principal objective of the current work was to study the molecular epidemiology of *K. pneumoniae* strains circulating in this institution between 2010 and 2013. Secondly, all *K. pneumoniae* strains were characterized phenotypically by antimicrobial susceptibility detection and genotypically by multilocus sequence typing (MLST) and by pulsed-field gel

electrophoresis (PFGE) analysis. The respective allelic variants were determined to understand the clonal relationship and to better control their dissemination.

2. Materials and Methods

2.1. Laboratory Data

2.1.1. Microbiologic Analysis. Between February 2010 and December 2013, a total of 280 nonduplicate *K. pneumoniae* strains were isolated from patients hospitalized in the Cardiac Surgery ward of the IRCCS Hospital of San Donato, including Postoperative Intensive Care, Cardiac Surgery for Adults, and Pediatric Cardiac Surgery. All strains were identified by Clinical Microbiology Laboratory and extracted from several biological sources such as blood, bronchoalveolar lavage (BAL), uroculture, wound swab, and other sources. Species identification and susceptibility testing were performed by the semiautomated systems VITEK 2 (bioMerieux, France). Minimum inhibitory concentrations (MIC) of meropenem and imipenem were confirmed by Etest (AB Biodisk, Sweden) on Mueller-Hinton agar. Susceptibility results were interpreted in accordance with clinical guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). All strains with a meropenem level greater than 0.5 μ g/mL were subject to genotypic determination; carbapenemases detection was confirmed by a modified Hodge test [11] and by a Synergy test that combined disk for meropenem (10 μ g), tested with meropenem + dipicolinic acid (100 mg/mL in DMSO) (MRPDP) and meropenem + 3-aminophenylboronic acid (60 mg/mL in DMSO) (MR+BO) (Rosco Diagnostica, Denmark).

2.1.2. Molecular Analysis. For chromosomal DNA extraction, several colonies were suspended in about 50 μ L of sterile distilled water and heated to 95°C for ten minutes. Each sample was then centrifuged for five minutes at 2500 $\times g$. After centrifugation, DNA concentration was assessed by spectrophotometry and stored at -20°C.

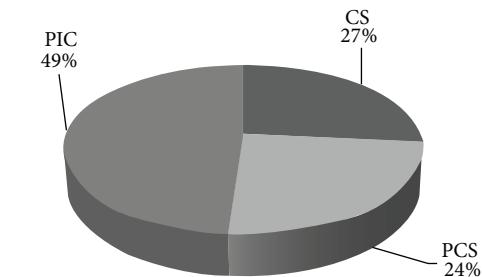
Primer specific for carbapenemase genes (*bla*_{KPC}, *bla*_{VIM}, and *bla*_{IMP}) and polymerase chain reaction (PCR) amplification conditions were performed as previously described [18].

Sequencing reactions were performed by the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and by an automated ABI PRISM 3100 genetic analyzer sequencer (Applied Biosystems). The nucleotide sequences were analyzed using software by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

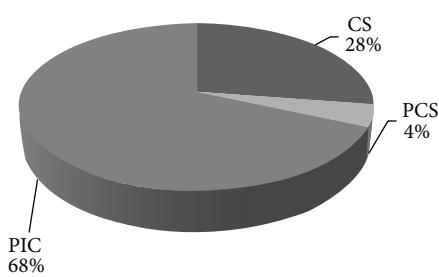
We investigated clonal relatedness for epidemiological comparison using MLST and PFGE.

The genotype of carbapenem-resistant strains was determined by MLST analysis, performed as described by Diancourt et al. [19]. Fragments of seven housekeeping genes *rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB* were obtained from chromosomal DNA and directly sequenced. Allelic profiles and sequence types (STs) were designated at the website <http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>.

Total strains



Resistant strains



PIC: postoperative Intensive Care

CS: Cardiac Surgery

PCS: Pediatric Cardiac Surgery

FIGURE 1: Distribution of *K. pneumoniae* isolates by Cardiac Surgery department. The above pie chart shows the total distribution of *K. pneumoniae* isolates by department. The pie chart below shows the distribution of *K. pneumoniae* resistant isolates by department.

We performed a second genotype analysis using PFGE of *Xba*I-digested total DNA with gene path system (Bio-Rad, Hercules, CA, USA). Band profiles were inspected by Fingerprinting II software (Bio-Rad). The clonal relationship was interpreted according to Tenover criteria [20].

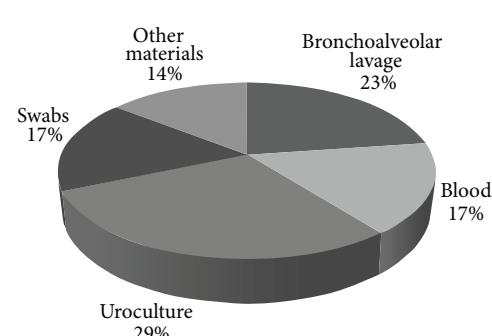
2.1.3. Statistical Analysis. We used the Student's *t*-test for statistical analysis. *P* values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. Department and Material Distribution of *K. pneumoniae* Strains. Between February 2010 and December 2013, a total of 280 consecutive nonreplicate clinical isolates of *K. pneumoniae* were isolated from patients hospitalized in the Cardiac Surgery ward of the IRCCS Hospital of San Donato Milanese, including Postoperative Intensive Care ($n = 137$; 49%), Cardiac Surgery for Adults ($n = 75$; 27%), and Pediatric Cardiac Surgery ($n = 68$; 24%) (Figure 1, above).

K. pneumoniae isolates were extracted from several clinical samples: uroculture 29% ($n = 82$), BAL 23% ($n = 64$), blood 17% ($n = 46$), swabs 17% ($n = 48$), and other sources 14% ($n = 40$) (Figure 2, above). Uroculture and bronchoalveolar lavage were the most commonly infected by *K. pneumoniae* strains. Of the total *K. pneumoniae* strains, 98/280

Total strains



Resistant strains

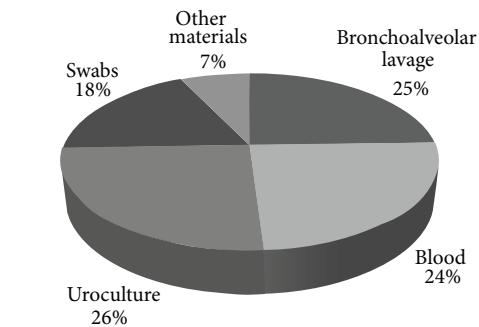


FIGURE 2: Distribution of *K. pneumoniae* isolates by type of biological materials. The above pie chart shows the total distribution of *K. pneumoniae* isolates by several biological materials. The pie chart below shows the distribution of *K. pneumoniae* resistant isolates by several biological materials.

(35%) isolates were resistant to carbapenems (CRKP) and, of the remaining 182 isolates, 36/280 (13%) were susceptible to all drugs while 146/280 (52%) were ESBL (resistant to third-generation cephalosporins).

67 of 137 strains derived from Postoperative Intensive Care (49%) were resistant to carbapenems. For what concerns Cardiac Surgery for Adults, a similar incidence of resistance was observed (27/75; 41%) whereas only 4 out of 68 strains derived from Pediatric Cardiac Surgery (5%) were found to be CRKP.

In Figure 1 (below) is reported the distribution of CRKP by ward: Postoperative Intensive Care ($n = 67$; 68%), Cardiac Surgery for Adults ($n = 27$; 28%), and Pediatric Cardiac Surgery ($n = 4$; 4%). In Figure 2 (below) is reported the distribution by biological source: urine, bronchoalveolar lavage, and blood were the most frequent sources of CRKP strains with an incidence of 26% ($n = 25$), 25% ($n = 24$), and 24% ($n = 24$), respectively. These resistant isolates that exhibited reduced carbapenems susceptibility were selected as the object of this study.

3.2. Molecular Characterization of CRKP Strains. To gather information on the molecular epidemiology of the CRKP strains diffused in Northern Italy, the 98 isolates were characterized by MLST and PFGE genotyping and their allelic variants were determined.

PCR assays performed for the 98 isolates collected during the four years exhibited reduced susceptibility to carbapenems. The resulting amplifications demonstrated that 9/98 MBL-producing isolates carried a *bla_{VIM}* gene and 89/98 isolates were positive with the primer specific for *bla_{KPC}*. None of the total isolates contained *bla_{IMP}* or produced either VIM or KPC carbapenemase. Sequencing analysis allowed identifying VIM-positive isolates as type 1 and KPC-positive as type 2.

Genetic relationship between all the resistant isolates was investigated using MLST. The most prevalent profile of CRKP was ST258 with the allelic profile of 3-3-1-1-1-79.

In addition, PFGE detected 3 pulsotypes when a similarity cut-off value of 80% was implemented. PFGE profiles indicated the presence of a major pulsotype, namely, pulsotype A, followed by pulsotype B and pulsotype C.

The A and C clones include VIM-producing and KPC strains, while the clone B includes only VIM-producing carbapenem-resistant *Klebsiella pneumoniae*. The clones A and C harbored both resistance determinants (*bla_{VIM}* and *bla_{KPC}*), while the B clone harbored only the gene *bla_{VIM}*.

The pulsotype A was found for the first time in January 2010 in the Postoperative Intensive Care and it was observed that it still persists in this care unit and in Cardiac Surgery for Adults. The pulsotype B was identified in the month of February 2010 in only Postoperative Intensive Care, while the spread of the clone C derived from Pediatric Cardiac Surgery and Postoperative Intensive Care.

3.3. Temporal and Age Group Distribution of CRKP Strains. The epidemic curve revealed three phases: period 1 (February 2010 to December 2010) during which the first cases of resistant strains began to emerge: 5 isolates were MBL-KP and 10 strains were KPC-KP; period 2 (January 2011 to December 2012) during which we observed a dramatic increase in KPC-KP strains which persisted until the end of 2012. In particular, we identified 35 KPC-KP strains in 2011 and 32 KPC-KP strains in 2012. Period 3, during which the spreading subsided, presented 4 cases of MBL-KP and 12 isolates of KPC-KP strains (Figure 3).

The age of the patients contributing the CRKP isolates varied between 2 months and 86 years and the gender ratio male : female was 2 : 1. Table 1 shows the distribution of resistant isolates based on the age of the patients. Most of the MBL-KP strains (6/9) were found in patients under 5 years of age while 56/89 were KPC-KP isolates from patients with an age ranging between 66 and 80 years. The male group constituted the majority of the patients except in the case children.

3.4. Antimicrobial Susceptibility Profile in CRKP Strains. Susceptibility testing of the 182 carbapenems susceptible strains revealed that 20% ($n = 36$) were susceptible to all drugs tested, while 80% ($n = 146$) were nonsusceptible to third-generation cephalosporin (data not shown). In February 2010, we had the first case of MBL-KP isolate resistant to meropenem (MIC > 8 mg/L), isolated from the respiratory tract of a child who was admitted to the Pediatric Cardiac

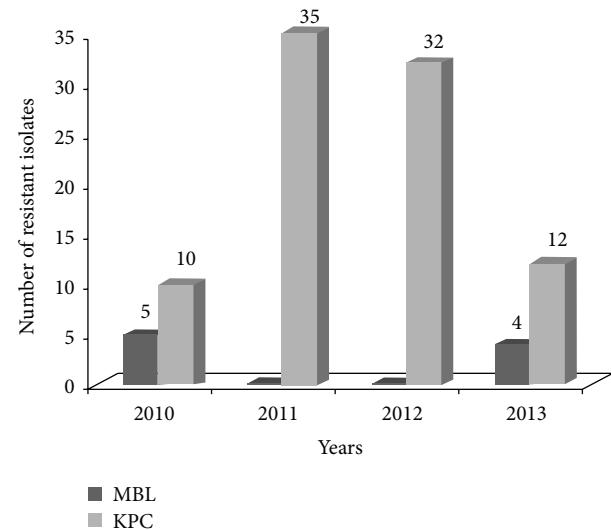


FIGURE 3: Temporal distribution of carbapenem-resistant *K. pneumoniae* isolates during the study period (February 2010–December 2013).

TABLE 1: Age groups and sex-wise details of patients from whom carbapenem-resistant *K. pneumoniae* was isolated.

Age years	Resistant strains		MBL		KPC	
	M	F	M	F	M	F
<5 y	3	5	2	4	1	1
6–25 y	2	1	1	0	1	1
26–45 y	2	0	0	0	2	0
46–65 y	9	5	0	0	9	5
66–80 y	38	19	1	0	37	19
>80 y	6	8	0	1	6	7

Surgery ward. After one month, KPC-KP spread rapidly becoming increasingly prevalent, while only a few cases of MBL-KP isolates were recorded between 2010 and 2013. A wide spectrum of resistance patterns to most classes of antibiotics was shown in the CRKP strains (Table 2).

Resistance to imipenem, meropenem, and ertapenem in all 98 isolates was variable (MICs ranging from 1 to >8 mg/L). Antimicrobial susceptibility tests revealed a complete resistance (100%) to third-generation cephalosporins (cefotaxime, cefepime, and ceftazidime MICs > 4 mg/L). A moderate resistance, between 60% and 65%, was shown to tigecycline (MICs > 2 mg/L) and gentamicin (MIC > 4 mg/L), while a higher resistance, of 96%, was shown to fluoroquinolones: ciprofloxacin and levofloxacin (MICs > 1 mg/L and MICs > 2 mg/L, resp.). Resistance of 31% was present toward trimethoprim-sulfamethoxazole. Colistin was the most effective agent against CRKP with 99% of susceptibility (MICs < 2 mg/L).

In that order, among CRKP isolates, tigecycline resistance increased from 60% (9/15) in 2010 to 91% (29/32) in 2012 and this percentage has remained unchanged throughout 2013.

TABLE 2: Antimicrobial resistance profile of clinical isolates of carbapenem-resistant *K. pneumoniae* from our hospital (February 2010–December 2013).

Antibiotic/MIC ($\mu\text{g/mL}$)	Total no (%)	Carbapenemase MBL/no	KPC/no
IMP			
>4	98 (100)	9	89
MER			
>8	98 (100)	9	89
EPT			
>8	98 (100)	9	89
GEN			
>4	63 (64)	6	57
SXT			
>40	30 (31)	4	26
CS			
>2	1 (1)	—	1
TGC			
>2	60 (61)	6	54
FEP			
>4	98 (100)	9	89
CTX			
>4	98 (100)	9	89
CAZ			
>4	98 (100)	9	89
CIP			
>1	96 (98)	7	89
LVX			
>2	96 (98)	7	89

IMP: imipenem; MER: meropenem; EPT: ertapenem; GEN: gentamicin; SXT: trimethoprim-sulfamethoxazole; CS: colistin; TGC: tigecycline; FEP: cefepime; CTX: cefotaxime; CAZ: ceftazidime; CIP: ciprofloxacin; LVX: levofloxacin.

On the other hand, we observed a decrease in CRKP strains resistant to gentamicin and trimethoprim-sulfamethoxazole going from a rate of about 69% (22/32) in 2012 to 43% (7/16) in 2013 and from a rate of 41% (13/32) in 2012 to 25% (4/16) in 2013, respectively. Notably, only one KPC-KP strain was resistant to colistin and it appeared in 2013.

The susceptibility to fluoroquinolones was observed only in MBL-KP strains and it was of 4%. Most of the MBL-KP isolates were found in the bronchoalveolar lavage in the Pediatric Cardiac Surgery ward (4/9) while KPC-KP strains were more prevalent in urocultures (26/89) of which 62% (16/26) were isolated in the Postoperative Intensive Surgery ward.

In addition, the predominant resistant antibiotyping was the same: in particular, 44% (4/9) of the MBL-KP and 21% (19/89) KPC-KP strains were characterized by a resistance to gentamicin, trimethoprim-sulfamethoxazole, tigecycline, fluoroquinolones, and third-generation cephalosporins and were susceptible to colistin.

4. Discussion

KPC spread rapidly in our hospital, becoming increasingly prevalent between 2011 and 2012, while in this period VIM-producing isolates disappeared. Since mid-2013, the incidence of KPC isolates has been gradually declining, whereas VIM producers reappeared in a few cases, at a much lower rate. We PCR-amplified and sequenced the DNA of carbapenemase genes and used MLST or PFGE to examine the molecular epidemiology to better characterize the dissemination of these isolates. The MLST and PFGE data aided in identifying the possible route of dissemination of the isolates; MLST showed that all CRKP strains belonged to the same clonal type, ST258, while PFGE revealed that there were three different strains and most of the isolates were owing to the same clone A. The other sample was genetically unrelated among the common clone. Though all the strains were phenotypically the same, PFGE revealed genetical discrepancy between the strains.

Microbiological investigation showed multidrug-resistant profiles between MBL-KP and KPC-KP strains, even if the prevalent resistant antibiotyping was the same. Fortunately, a high proportion of CRKP isolates still showed *in vitro* susceptibility to aminoglycosides (37%), tigecycline (39%), and trimethoprim-sulfamethoxazole (69%). In addition, they presented complete coresistance to the fluoroquinolones and to third-generation cephalosporins. A colistin-resistant CRKP isolate was also identified from a patient with a history of colistin therapy. This strain was resistant to all drugs tested. A double carbapenem regimen was employed to treat this patient.

The infections caused by multidrug resistant *K. pneumoniae* have been reported with an increasing frequency in the Intensive Care Unit and in the Cardiac Surgery Unit. CRKP strains constitute an emergent public health hazard and they are associated with a significant morbidity and mortality [21]. Many studies have focused on the risk factors for acquiring this pathogen and the impact of bacteraemia on the outcome. Our results are concordant with the previous studies: KPC type 2 is present in the majority of clinical specimens and accounts for most of the epidemic outbreaks. KPC-2 appears to be more predominant worldwide, with outbreaks arising in Europe and especially in Italy [22]. Starting from 2010, KPC-2 was detected in different regions of Italy. Interestingly, we found that the predominant type of *K. pneumoniae* associated with KPC and MBL production was ST258. It is increasingly recognized that ST258 is the predominant clone of resistant isolates all over the world and has caused outbreaks in many countries [23, 24].

The fact that CRKP isolates belonged to ST258 and were acquired in our hospital indicates the possibility that the spread of these genes could be due to clonal dissemination as well as to genetic exchange between different clones. However, the failure to find a common environmental reservoir indicated that patient-to-patient transmission may be the main mechanism of CRKP spread in our hospital. Frequent bed transfers of patients, particularly after isolation of CRKP, combined with the lack of adequate preventive measures, might have facilitated this process.

As of today, few safe and practical therapeutic options remain for patients infected with KPC producers. Many clinicians have resorted to the use of tigecycline, polymyxins, and the few remaining aminoglycosides [25]. Tigecycline consistently shows *in vitro* activity against most isolates of KPC-producing organisms. In any case, some shortcomings limit its use in monotherapy regimens: due to its high volume of distribution, for example, tigecycline does not always achieve high serum or urinary concentrations after infusion at standard doses [26]. Furthermore emergent resistance to tigecycline has been reported; thus, clinicians should remain vigilant for clinically refractory infections when tigecycline is being used. Despite these limitations, tigecycline has been successfully used to treat and cure patients infected with KPC-producers. Another class of antibiotics that has been successfully used to treat CRKP strains is polymyxins, such as colistimethate and colistin. These drugs cause very little toxicity (namely, mild dose-dependent renal toxicity) and have shown good efficacy in several studies. According to these, a significant finding of this report is that we observed only one strain resistant to colistin in all CRKP isolates. This may be due to the restricted use of the drug in our hospital. Indeed it is employed almost exclusively to treat MDR infections caused by CRKP or *Acinetobacter Baumannii*. It is suggested to utilize polymyxins in combination with other antibiotics [27–30].

Combination therapy improves the chances of cure in highly resistant infections, due to a synergistic effect and to the minimization of prolonged therapy which could abet resistance spreading [31]. It is noteworthy that in some cases colistin represents the “last-line” therapeutic drug against CRKP pathogens. Flowcharts for selecting mainstream and adjuvant therapy against gram-negative bacteria are described by Zavascki et al. [32]; these guidelines are recently adopted also in our departments.

With regards to other antibiotics tested in the study (Table 2), we found high resistance rates for cephalosporins, chinolons, and gentamicin. This was not surprising taking into account the selective pressure due to the frequent use of these drugs.

On the contrary, a discrete trimethoprim-sulfamethoxazole (SXT) sensibility characterized our strains. But, due to its high restitution volumes and sodium content, SXT represents an inconvenient drug for intravenous administration. For this reason, SXT is infrequently used in surgery and intensive care units like those considered in this work. Further studies are advocated in order to evaluate the real consistency of this finding.

Finally, we think that the significant reduction in CRKP isolates observed from 2011 onwards is important. This coincided with a strong enhancement, in our institution, of the recommendations for prevention of healthcare-associated infections that include performance of contact precautions in addition to standard precautions (hand hygiene, use of gloves, and protective coating), isolation of the infected patients in separate rooms with designated nursing staff; promotion of more targeted antibiotic therapy, minimization of the invasive medical devices use, and rectal swab screening for

all new admitted patients with risk factors for CRKP in the cardiosurgery area.

These results and the decline in the discovery of new effective drugs suggest that the prudent and conservative use of available active agents combined with good control practices still represent the keys to curtailing the spread of this dramatic antimicrobial resistance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

In Vitro Activity of Imipenem and Colistin against a Carbapenem-Resistant *Klebsiella pneumoniae* Isolate Coproducing SHV-31, CMY-2, and DHA-1

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We investigated the synergism of colistin and imipenem against a multidrug-resistant *K. pneumoniae* isolate which was recovered from a severe hip infection. PCR and DNA sequencing were used to characterize the outer membrane porin genes and the resistance genes mediating the common β -lactamases and carbapenemases. Synergism was evaluated by time-kill studies. The *bla*_{SHV-31}, *bla*_{CMY-2}, and *bla*_{DHA-1} were detected. Outer membrane porin genes analysis revealed loss of *ompK36* and frame-shift mutation of *ompK35*. The common carbapenemase genes were not found. Time-kill studies demonstrated that a combination of 1x MIC of colistin (2 mg/L) and 1x MIC of imipenem (8 mg/L) was synergistic and bactericidal but with inoculum effect. Bactericidal activity without inoculum effect was observed by concentration of 2x MIC of colistin alone or plus 2x MIC of imipenem. In conclusion, colistin plus imipenem could be an alternative option to treat carbapenem-resistant *K. pneumoniae* infections.

1. Introduction

The widespread multidrug resistant Enterobacteriaceae is challenging physicians to effectively treat nosocomial infections. Followed by the extensive use of broad-spectrum antibiotics, the plasmid-mediated extended-spectrum β -lactamases (ESBLs) and/or AmpC β -lactamases have limited the choice of antibiotics to carbapenems for therapy against serious infections [1, 2]. However, increased use of carbapenems may contribute to the rising occurrence of carbapenem-resistant Enterobacteriaceae [3, 4]. In an era with limited antimicrobial agents available to us, old potential candidate (such as colistin) has therefore commonly been used or recommended to combine with a carbapenem to treat serious

carbapenem-resistant Enterobacteriaceae infections [5, 6]. We have reported the synergistic effect by a combination of colistin and tigecycline against an ESBL-producing *Klebsiella pneumoniae* urine isolate with resistance to ertapenem, imipenem, and meropenem. However, there was a concern of inoculum effect because colistin plus tigecycline achieved less bactericidal effect against the strain with higher inoculum density in the bacterial suspensions [7]. Similarly, we experienced a multidrug resistant *K. pneumoniae* designed Kp830 isolated from a patient with severe hip infection. The isolate was resistant to imipenem according to the breakpoints of 2012 CLSI [8]. The goals of the study were to investigate the mechanisms of resistance to carbapenems and to evaluate the potential synergism between colistin and imipenem.

2. Material and Methods

2.1. Bacterial Isolates. A 79-year-old diabetic man was referred to the hospital due to persistent wound discharge from the left hip for one month. The computed tomography showed left hip and thigh necrotizing fasciitis and left iliac muscle abscess. Both of the deep-seated abscess and blood cultures yielded multidrug resistant *K. pneumoniae* isolates with the same antibiogram, which was resistant to amikacin, ampicillin, cefazolin, cefuroxime, ceftazidime, ciprofloxacin, ertapenem, flomoxef, gentamicin, and piperacillin-tazobactam but was susceptible to imipenem, using standard disc diffusion test [8]. The organism was not eradicated and the patient died in acute renal failure, acute respiratory failure, and septic shock after 9-week hospitalization, despite of three times of surgical debridement and 7-week imipenem therapy. The last wound pus *K. pneumoniae* isolate became resistant to imipenem and was designated strain Kp830, which was subcultured and frozen at -70°C until being used in the study.

2.2. Antimicrobial Susceptibility Testing. Minimal inhibitory concentrations (MICs) for imipenem (Merk, Sharp & Dohme, West Point, PA, USA) and colistin sulphate (Sigma Chemical Company, St. Louis, MO, USA) were determined by standard agar dilution method according to CLSI [9].

There is no CLSI recommendation for colistin susceptible breakpoints against Enterobacteriaceae [8]. According to the British Society for Antimicrobial Chemotherapy Working Party on Susceptibility Testing, the susceptible MIC breakpoint for colistin against Enterobacteriaceae is $\leq 4 \mu\text{g/mL}$ and should be considered resistant if MIC $> 4 \mu\text{g/mL}$ [10]. We applied the British Society MIC breakpoints to our results.

2.3. Phenotypic Methods for Detection of β -Lactamases. The ESBL production was interpreted by the phenotypic confirmatory test according to CLSI disk diffusion method [8]. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as the negative and positive control, respectively. An increase of ≥ 5 mm between the growth-inhibitory zone diameter of either cefotaxime or ceftazidime tested in combination with clavulanate and its zone diameter when tested alone is interpreted as positive ESBL phenotype.

In the present study, 3-aminophenylboronic acid (APB) (Sigma-Aldrich, Steinheim, Germany) was used in the disk potentiation test and double-disk synergy test for the identification of class C β -lactamase production. The enlargement and discernible expansion of diameter of the growth-inhibitory zone were observed in plasmid-mediated class C β -lactamase producing bacteria [11]. The modified Hodge test (MHT) was used to detect carbapenemase production when the test isolate produced the enzyme and allowed growth of a carbapenem susceptible strain (*E. coli* ATCC 25922) towards a carbapenem disk [8]. The modified MHT is limited by unknown sensitivity and specificity for detecting low-level metallo- β -lactamase production [8].

2.4. Detection of β -Lactamase Genes. Plasmid DNA was extracted as templates and polymerase chain reaction (PCR)

was used to amplify *bla_{CTX-M}*, *bla_{TEM}*, *bla_{IMI}*, *bla_{GES}*, *bla_{IMP}*, *bla_{VIM}*, *bla_{KPC}*, *bla_{OXA}* (OXA-23, OXA-24, OXA-48, and OXA-58), and *bla_{NDM}* using specific primers as previously published [3, 12, 13]. For AmpC genes, the following primers were used: (a) CMY-2-forward (TTT TCA AGA ATG CGC CAG GC), CMY-2-reverse (CTG CTG CTG ACA GCC TCT TT); (b) DHA-1-forward (CTG ATG AAA AAA TCG TTA TC) and DHA-1-reverse (ATT CCA GTG CAC TCA AAA TA). For SHV genes, the following primers were used: (a) SHV-forward (GAT CCA CTA TCG CCA GCA GG) and SHV-reverse (ACC ACA ATG CGC TCT GC TTT G); (b) SHV-12-forward (ATG CGT TAT ATT CGC CTG TG) and SHV-12-reverse (TTA GCG TTG CCA GTG CTC G). Amplicons were purified with PCR clean-up kits (Roche Diagnostics, GmbH, Penzberg, Germany) and sequenced on an ABI PRISM 3730 sequencer analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. Detection of Outer Membrane Porin Genes. Outer membrane porin-associated genes (*ompK35* and *ompK36*) were screened by using PCR assay as previously described [14]. The PCR amplicons were sequenced and analyzed with the BLAST program.

2.6. Time-Kill Assay. Model time-kill curves were determined by plotting mean colony counts (\log_{10} CFU per milliliter) from each model versus time. All model simulations were conducted over 48 h and were performed in duplicate to ensure reproducibility. The 1x MIC and 2x MIC for drugs concentration of imipenem and colistin alone or combination of both drugs were investigated in time-kill studies. Approximately 2×10^5 CFU/mL (standard inoculum) and 1.6×10^6 CFU/mL (higher inoculum) were used at baseline. Serial samples (baseline, 2, 4, 6, 8, 12, 24, 28, 32, and 48 h) were obtained for 48 hours. Total bacterial populations were quantified by serial dilution. Bactericidal activity was defined as a $\geq 3\log_{10}$ CFU/mL decrease in the viable cell counts within 24 hours with respect to the original inoculum. Synergistic effect was defined as a $\geq 2\log_{10}$ CFU/mL decrease in the viable cell counts compared to the most active drug. Inoculum effect was defined as a decreasing antibiotic efficacy with high inoculum. The lower limit of detection was $1\log_{10}$ CFU/mL.

3. Results

The MICs of imipenem and colistin for the strain Kp830 were 8 mg/L and 2 mg/L, respectively. The 2012 CLSI recommended imipenem MIC breakpoints as susceptible, ≤ 1 mg/L, and resistant, ≥ 4 mg/L [8].

The carbapenemase phenotype of the strain Kp830 was negative, whereas the class C β -lactamase phenotype was positive (Figure 1). Further detection of the β -lactamase genes for the plasmid DNA extract from the strain Kp830 confirmed the presence of plasmid-mediated *bla_{SHV-31}* (GenBank accession number, KC880337), *bla_{CMY-2}*, and *bla_{DHA-1}*. The amino acid sequence of SHV-31 differs from SHV-1 by two mutations, namely, L35Q and E240K, and differs from

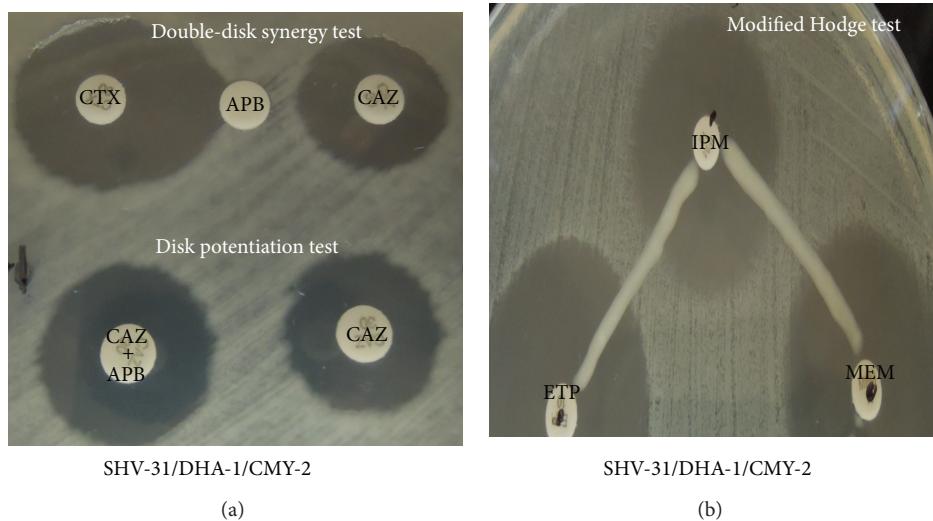


FIGURE 1: Phenotypic disc confirmatory tests. (a) Double-disk synergy test and disk potentiation test using cefotaxime (CTX) disk and ceftazidime (CAZ) disk are confirming the production of class C β -lactamases which were inhibited by APB. (b) The modified Hodge test is showing negative results for carbapenemase production, when the test isolate (Kp830) grew towards three carbapenem disks, including imipenem (IPM), meropenem (MEM), and ertapenem (ETP).

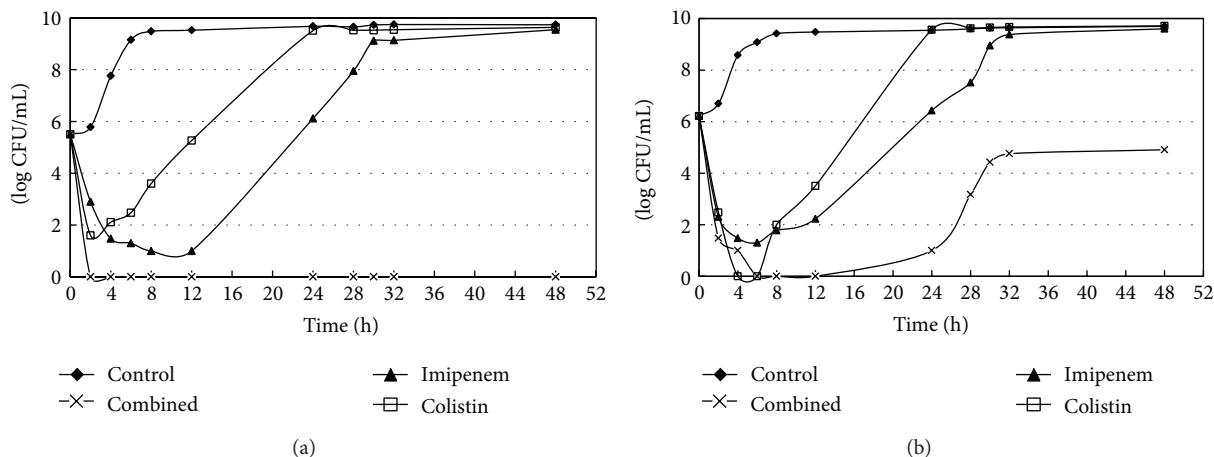


FIGURE 2: Survival curves of Kp830 (a) 1x MIC, in standard inoculum, and (b) 1x MIC, in high inoculum. All models with duplicate performance demonstrated similar bactericidal kill (duplicate data not shown).

SHV-12 by one mutation (G134S). PCR results for other β -lactamase genes were all negative. PCR for the outer membrane porin genes and DNA sequencing analysis revealed loss of porin *ompK36* gene and frame-shift mutation in *ompK35* gene (100% identity to GenBank accession number GU945370), similar to previous reports [15, 16].

Data from the time-kill studies in different inocula and multiples of MIC are shown in Figure 2(a) (standard inoculum, 1x MIC), Figure 2(b) (high inoculum, 1x MIC), Figure 3(a) (standard inoculum, 2x MIC), and Figure 3(b) (high inoculum, 2x MIC). The initial bactericidal activities of colistin and imipenem were attenuated by regrowth after 6–8 hours of antibiotic exposure at 1x MIC with different inocula (Figures 2(a) and 2(b)). Colistin showed excellent bactericidal activity against Kp830 strain at 2x MICs with different inocula

(no inoculum effect), which began at 2 h after inoculations and has sustained for 48 h (Figures 3(a) and 3(b)). For 2x MICs of imipenem, inoculum effect and delayed regrowth after 12-hour exposure were also observed, even the initial bactericidal activity producing a decrease $>3\log_{10}$ CFU/mL after a 6-hour exposure.

Synergism was observed in the combination of colistin and imipenem each at 1x MIC with standard inoculum (Figure 2(a)). However, combination of colistin with imipenem each at 1x MIC for high-inoculum bacterial densities demonstrated early bactericidal effect with delayed regrowth after 24-hour exposure (Figure 2(b)). Due to excellent bactericidal activity of colistin, synergism of colistin and imipenem was not observed at 2x MICs with different inocula (Figures 3(a) and 3(b)).

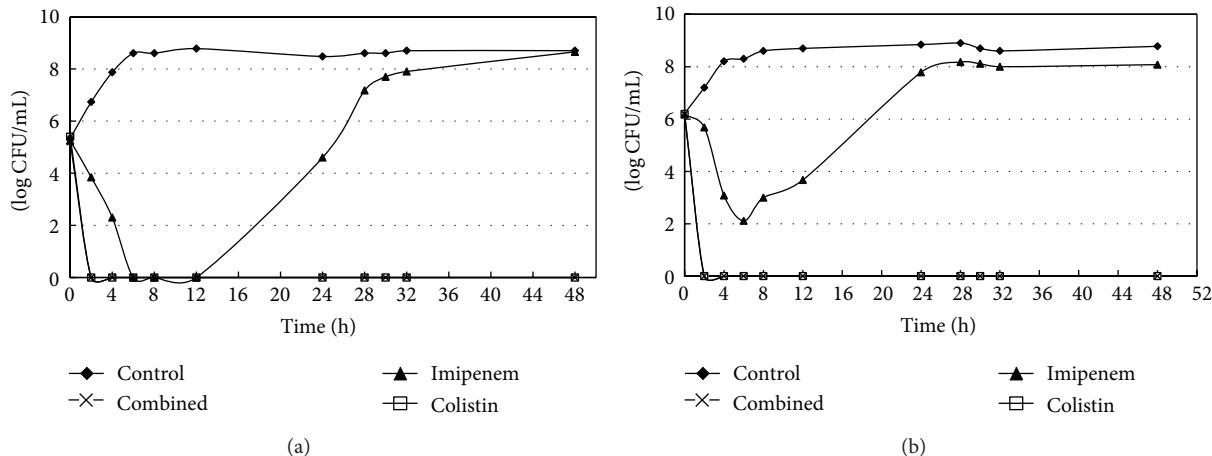


FIGURE 3: Survival curves of Kp830 (a) 2x MIC, in standard inoculum, and (b) 2x MIC, in high inoculum. All models with duplicate performance demonstrated similar bactericidal kill (duplicate data not shown).

4. Discussion

The coproduction of ESBL (SHV-31) and AmpC β -lactamases (CMY-2 and DHA-1) may confer resistance of strain Kp830 to multiple drugs, such as ceftazidime, flomoxef, and piperacillin-tazobactam. The SHV-31 differs from SHV-1 by the two mutations, namely, L35Q and E240K, which was first reported in The Netherlands [15]. Although there might be a chance of false-negative result for modified MHT, we extensively surveyed the common carbapenemase genes and all the results were negative. The negative modified MHT in Kp830 may correspond to the bactericidal activity of 1x MIC and 2x MIC imipenem, even though the strain was resistant to imipenem according MIC breakpoints. The reasons of carbapenem resistance could be explained by the OmpK36 and/or OmpK35 defects, which have represented the major mechanism for the development of carbapenem resistance in the ESBL- and/or AmpC-producing *K. pneumoniae* isolates in Taiwan [16–18]. Our case highlighted that prolonged imipenem use may be associated with *ompK35* gene mutation and *ompK36* gene loss on ESBL-producing *K. pneumoniae* and led to emergence of carbapenem resistance.

Lai et al. reported an increasing trend with a prevalence of about 2.5% of carbapenem-nonsusceptible Enterobacteriaceae in Taiwan [19]. In that study, carbapenem-based therapy (most commonly combined with amikacin) still had a good outcome with a 90% clinical success rate. Because our strain Kp830 was resistant to amikacin but susceptible to colistin, there is a need to document the in vitro efficacy of carbapenem plus colistin. The time-killing data might suggest high-dosage colistin alone or in combination with imipenem to treat serious carbapenem-resistant *K. pneumoniae* infections. Souli et al. reported that synergy of colistin and imipenem was observed only against isolates exhibiting susceptibility or low-level resistance to colistin [20]. The current study revealed that synergy of colistin and imipenem was observed only with 1x MICs of both drugs against the isolate with normal inoculum but there was an inoculum effect. The inoculum phenomenon of declining

antibiotic efficacy may lead to treatment failure for serious infections with high bacterial population in the lesions, such as endocarditis, meningitis, septic arthritis, osteomyelitis, abscesses, and other deep-seated infections [21].

In conclusion, an imipenem-resistant *K. pneumoniae* with outer membrane porin defect as well as coproducing SHV-31, CMY-2, and DHA-1 emerged from a prolonged course of imipenem therapy. A high-dosage colistin alone or in combination with imipenem may be considered an alternative option to treat the carbapenem-resistant *K. pneumoniae* infections.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Epidemiological Characterization of Drug Resistance among *Mycobacterium tuberculosis* Isolated from Patients in Northeast of Iran during 2012-2013

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Introduction. Tuberculosis is still one of the most important health problems in developing countries and increasing drug resistance is the main concern for its treatment. This study was designed to characterize the drug resistant *Mycobacterium tuberculosis* isolated from patients suffering from pulmonary tuberculosis in northeast of Iran. **Method.** In this cross-sectional study during 2012-2013, drug susceptibility testing was performed on *Mycobacterium tuberculosis* isolated in northeast of Iran using proportional method. Epidemiological data concerning these strains were also analyzed. **Results.** Among 125 studied isolates, 25 mycobacteria (20%) were diagnosed as nontuberculosis mycobacteria. Among the remaining 100 *Mycobacterium tuberculosis* isolates, the resistance rates were 7%, 7%, 3%, and 9% against isoniazid, rifampin, ethambutol, and streptomycin, respectively. Four isolates were resistant against both isoniazid and rifampin (MDR tuberculosis). The highest resistance rate was observed among 15–45-year-old patients. The MDR tuberculosis was much more prevalent among those who had previous history of treatment. **Conclusion.** Considering these findings, DOTS strategy should be emphasized and promptly used in order to prevent further resistance. Regarding the high rate of nontuberculosis mycobacteria, it is recommended that confirmatory tests were performed before any therapeutic decision.

1. Introduction

One-third of the world population is contaminated by *Mycobacterium tuberculosis*. Every year, about 9 million people suffer from active tuberculosis, and about 2 million die because of this disease [1]. In 2009, about 9.4 million new cases of tuberculosis were reported around the world. In the same year in Iran, 10,099 cases of tuberculosis were reported; among those 5,100 cases had positive sputum which were highly contagious [1]. Tuberculosis has been reported in all the provinces in Iran, but this rate was higher in eastern part, as neighboring to Afghanistan and Pakistan which have the highest rate of tuberculosis in the world.

Drug resistant tuberculosis is the main problem in controlling tuberculosis. In contrast to drug susceptible cases, patients who have been infected with drug resistant strains need two years of treatment instead of 6 months and the treatments are 70 times more costly in addition to much higher mortality rate (about 40–60%) [2]. According to World Health Organization, there were 9.2 million new cases of tuberculosis and 500 thousand cases of drug resistance in 2006. Also, World Health Organization and other international studies stated that the resistance against antituberculosis antibiotics exists all around the world, and the global prevalence of primary resistance is almost 10.7% [1, 3]. *Mycobacterium tuberculosis* strains which are resistant against

TABLE 1: Pattern of drug resistance among *Mycobacterium tuberculosis* isolates in northeast of Iran during 2012-2013.

Pattern of resistance against each antibiotic for those 14 resistant isolates									
Isoniazid	Resistant isolate 1	Resistant isolate 2	Resistant isolate 3	Resistant isolate 4	Resistant isolate 5	Resistant isolate 6	Resistant isolate 7	Resistant isolate 8	Resistant isolate 9
*	*	*	*	*	*	*	*	*	*
Rifampin	*	*	*	*	*	*	*	*	*
Ethambutol	*	*	*	*					
Streptomycin	*	*			*	*	*		
History of previous treatment for TB	*	*			*	*	*	*	*

several drugs (MDR-TB) are also prevalent and cause high mortality rate in spite of treatment [4, 5]. In the past decade, MDR-TB has become the main threat for tuberculosis control strategies [6]. More attention was given to this issue when tuberculosis with extensive drug resistance (XDR) has been reported in some locations [7]. The latest reports indicate that, in Iran, there is a high rate of MDR and XDR and even resistance against all drugs (TDR) [8]. This study was designed to characterize the epidemiology of drug resistance among *Mycobacterium tuberculosis* isolates in northeast of Iran during 2012-2013 to plan better controlling strategies.

2. Method

In this study, all culture positive samples from patients with pulmonary tuberculosis in northeast of Iran during 2012 and 2013 were referred to Regional Reference Laboratory of Tuberculosis, Mashhad, Iran. All isolates were examined by Ziehl Neelsen staining and biochemical and phenotypic methods. Biochemical and phenotypic methods for identification of mycobacteria include observation of rate of growth, colony morphology, pigmentation, and biochemical profiles. Biochemical testing (i.e., niacin, nitrate reduction, and 68°C labile catalase tests) was used to definitively identify the isolated mycobacteria once they are categorized into a preliminary subgroup based on their growth characteristics [9, 10]. Also a multiplex polymerase chain reaction was carried out for confirmation as prescribed previously [11-14]. For this PCR assay, two different pairs of oligonucleotide primers targeting the gene encoding for 85 KDa protein (common to all mycobacteria) and the insertion sequence IS6110 (specific for *M. tuberculosis* complex) were used [11-14].

The susceptibilities of the isolates which were identified as *Mycobacterium tuberculosis* were determined against isoniazid, rifampicin, ethambutol, and streptomycin by standard proportional method using Lowenstein-Jensen medium as recommended by World Health Organization (WHO) [15]. For this purpose, several dilutions of inoculums (10^{-2} and 10^{-4}) were planted onto both control and drug containing media. The proportion of resistant bacilli against given drug is then determined by comparing these numbers and expressing the resistant portion as a percentage. Susceptibility was defined as no or less than 1% growth on media containing the critical concentration of drug, and the resistance was defined as growth of 1% or more of the bacterial population. To ensure that results of drug susceptibility testing are reliable and accurate, the standard strain H37Rv and two strains of drug resistant *M. tuberculosis* were included in each series of testing [15]. Epidemiological data about the patients were gathered. The data was then reviewed and analyzed using SPSS.

3. Results

Among 125 patients in this study, 25 samples were diagnosed as nontuberculosis mycobacteria by biochemical and molecular methods (Figure 1).

From 100 patients whose culture resulted in *Mycobacterium tuberculosis*, 86 isolates were susceptible to antituberculosis antibiotics, and 14 isolates were resistant. Among

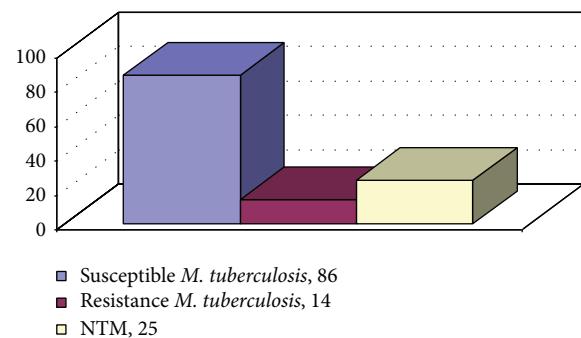


FIGURE 1: The distribution of mycobacteria studied in this research project.

TABLE 2: The distribution of drug resistance by age.

Age range	Number	Sensitive cases	Resistant cases	Percentage
<15	0	0	0	0
45–15	36	28	8	58%
65–45	15	13	2	14%
>65	31	27	4	28%
Total	82	68	14	100%

resistant isolates, 7 isolates (7%) were resistant against isoniazid, 7 isolates (7%) were resistant against rifampin, and 3 and 9 isolates were resistant against ethambutol and streptomycin, respectively. Four isolates were resistant against both isoniazid and rifampin. Three isolates (3%) were resistant against isoniazid and streptomycin and 4 isolates (4%) were resistant against rifampin and streptomycin. In this study, only two isolates (2%) were resistant against all 4 antibiotics (Table 1).

As it has been shown in Table 2, no *Mycobacterium tuberculosis* was isolated from people younger than 15 years old, and the most resistance cases were among 15–45-year-old groups.

As the epidemiological data of 18 patients were unclear, we performed all the analysis on the remaining 82 patients. Among these 82 patients who have been infected with *Mycobacterium tuberculosis*, there were 45 male patients and 37 female patients. The age range was between 16 and 94 years old, with the average 53 years old. Seventy-three patients were Iranian, and 9 patients were non-Iranian. Seventy-one patients live in urban areas and 11 in rural area. There were 56 new cases and 26 cases with previous history of treatment. The relations between demographic and epidemiological data with resistance and multiple drug resistance were analyzed and there was not any significant relation between these parameters and resistance (Table 3).

Interestingly, out of these 4 multiple drug resistant cases, 3 patients had a history of previous tuberculosis and treatment, so the rate of MDR tuberculosis among patients with relapse history was 11.53%. And only one patient diagnosed with MDR tuberculosis was a new case (1.78%) (Table 4).

TABLE 3: The demographic data of patients.

Demographics status	Condition	Total	Sensitive	Resistant	MDR-TB	P value	NTM
Sex	Male	45	37	8	2	0.749	5
	Female	37	31	6	2		13
Nationality	Iranian	73	61	12	3	0.301	18
	Non-Iranian	9	7	2	1		0
Living area	Urban	71	59	12	3	1	15
	Rural	11	9	2	1		3
History of previous treatment	Yes	26	19	7	3	0.066	6
	No	56	49	7	1		12
Diabetes	Yes	11	11	0	0	0.344	5
	No	71	57	14	4		13
Smoking	Yes	9	7	2	0	0.301	1
	No	73	61	12	4		17
Family history of TB	Yes	15	12	3	1	1	3
	No	67	56	11	3		15
Opium addiction	Yes	12	8	4	0	1	0
	No	70	60	10	4		18
History of imprisonment (jail)	Yes	25	22	3	1	0.635	0
	NO	57	46	11	3		18

NTM: nontuberculosis mycobacterium, MDR TB: multiple drug resistant tuberculosis.

TABLE 4: Percentage of MDR tuberculosis in new cases and patients with relapse during 2012-2013.

	Total number of cases	MDR cases
History of previous treatment	26	3 (11.53%)
New case	56	1 (1.78%)

TABLE 5: Grading scale of smear positivity in sensitive and resistant isolates.

Grading scale of smear positivity	Sensitive	Resistance	P value
1+	22 (32%)	4 (28%)	
2+	11 (16%)	1 (7%)	0.056
3+	35 (52%)	9 (65%)	
Total	68 (100%)	14 (100%)	

Although the grading scale of smear positivity was highly associated with multiple drug resistant, the relation was not statistically significant (Table 5).

4. Discussion

Nowadays, drug resistance is the main problem in controlling tuberculosis in the world. During the last years, the resistance rate has been steadily increased. As the effective drugs are very limited, resistance is the main threat for STOP-TB programs. Traditionally, patients with drug resistant tuberculosis have been assumed that acquired drug resistance due to their previous treatment history. The term “acquired drug resistance” in patients with tuberculosis implies that resistance has developed during treatment [16].

In this study, the resistance rates of *Mycobacterium tuberculosis* against isoniazid, rifampin, ethambutol, and streptomycin were 7%, 7%, 3%, and 9%, respectively. The rate of MDR tuberculosis was 4% which is similar to the rate obtained in another study in Mashhad during 2008 in which the rate of MDR tuberculosis was 4.65%. Our resistance rate seems very close to the rate reported by Dr. Velayati and colleagues. They reported 5.6% MDR tuberculosis among 146 patients suffering from tuberculosis in Iranian National Reference TB Laboratory [17]. As they received sample from all over the country, this rate sounds highly reliable.

However, some researchers reported lower rate for resistance in Iran. In Shamaei's study on 546 patients in Tehran, 2.8% people were reported with MDR [18]. And also in Hadizadeh and colleagues' study in Tehran between 2006 and 2009, the resistance against isoniazid and rifampin was 11% and 10%, respectively, and 2.5% of cases were MDR tuberculosis [19]. This lower rate of resistance might be due to obtaining sample from restricted regions with low rate of resistance. On the contrary, the reported rate for resistance from southeast of Iran is much higher than our results. For example, Metanat and colleagues reported 27%, 33%, 39%, and 55% resistance rate against rifampin, isoniazid, ethambutol, and streptomycin, respectively, as they conducted a study on 84 *Mycobacterium tuberculosis* isolates in Buali Hospital in Zahedan (southeast of Iran). The rate of MDR was 16% in their study [20]. In another study, during 2012 by Metanat and colleagues, the multidrug resistance rate of 12% was reported for tuberculosis in Zahedan (southeast of Iran) among 88 tuberculosis cases [21]. This high rate of resistance can be interpreted by considering neighboring of that region with Afghanistan and Pakistan as the most prevalent area for tuberculosis.

In one study in Uzbekistan and Kazakhstan, drug resistance was reported as 13%, and in China, it was reported as 10% [22]. A study in Pakistan during 2009–2011 showed that the resistance rate against isoniazid was 15.5% and simultaneous resistance against isoniazid, pyrazinamide, and ethambutol was 1% [23]. In another study, in Saudi Arabia during 2012, the resistance rate against isoniazid was 33%, and the resistance against rifampin, streptomycin, and ethambutol was 23%, 13%, and 3%, respectively. In this study, the resistance rate against two drugs was 20% [24]. Considering these data, our resistance rate is much lower which could be due to good tuberculosis controlling measures. For example, tuberculosis treatment is free of charge in Iran and paid completely by government. Our results confirmed that prompt implementation of DOTS strategy in our region has slowed down the increasing incidence of resistant tuberculosis and has prevented a widespread resistance in this community.

Around the world, the prevalence of MDR among pulmonary tuberculoses is 1.4% in new cases and 13% in recurrence of tuberculosis [25]. Our findings show that in northeast of Iran the prevalence of MDR in new cases of pulmonary tuberculosis was 1.78 and among patients with history of relapse was 11.53%. Our rates are very similar to the global rate of resistance in new and recurrent cases.

Another notable finding of this study is the high prevalence of nontuberculosis mycobacterium (NTM) consisting of 20% of the cases. Such patients often were treated as tuberculosis which did not respond and categorized as treatment failure. This means that, in addition to receiving inappropriate treatment, improper antituberculosis antibiotics were also used. This could cause an increased resistance against antituberculosis antibiotics, especially isoniazid and rifampin. Also in our study, the resistance against streptomycin was much higher than other drugs which is expected because of using this drug for other diseases.

5. Conclusions

As northeast of Iran is in neighborhood of Afghanistan and Turkmenistan which are countries with a very high burden of disease and also high rate of resistant tuberculosis, prompt preventive policies and complete DOTS strategy should be implemented in order to prevent more incidences of resistance.

Given the high rate of nontuberculosis mycobacterium, it is recommended that treatment starts after accurate diagnostic procedure in order to prevent improper drug usage and also to prescribe proper treatment from the beginning.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Population Structure and Oxacillin Resistance of *Staphylococcus aureus* from Pigs and Pork Meat in South-West of Poland

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The genotypes and oxacillin resistance of 420 *S. aureus* isolates from pigs ($n = 203$) and pork ($n = 217$) were analyzed. Among 18 *spa* types detected in *S. aureus* from pig t011, t021, t034, t091, t318, t337, and t1334 were the most frequent. Among 30 *spa* types found in *S. aureus* isolates from pork t084, t091, t499, t4309, t12954, and t13074 were dominant. The animal *S. aureus* isolates were clustered into MLST clonal complexes CC7, CC9, CC15, CC30, and CC398 and meat-derived isolates to CC1, CC7, and CC15. Thirty-six MRSA were isolated exclusively from pigs. All MRSA were classified to *spa* t011 SCCmecV. BORSA phenotype was found in 14% *S. aureus* isolates from pigs and 10% isolates from pork meat. *spa* t034 dominated among BORSA from pigs and t091 among meat-derived BORSA. This is the first report on *spa* types and oxacillin resistance of *S. aureus* strains from pigs and pork meat in Poland. Besides *S. aureus* CC9, CC30, and CC398 known to be distributed in pigs, the occurrence of genotype belonging to CC7 in this species has been reported for the first time. To our knowledge it is also the first report concerning CC398 BORSA isolates from pigs and pork meat.

1. Introduction

Staphylococcus aureus is one of the most serious pathogens of humans and important animal pathogen. *S. aureus* infections can easily turn into life-threatening diseases if they are not antibiotically treated. The ability of this microorganism to survive in the presence of β -lactam antibiotics remains the main problem in the therapy [1]. Several phenotypes of resistance to β -lactams have been described in *S. aureus* so far. These phenotypes reflect different mechanisms of resistance and include acquisition of β -lactamase, modification of normal penicillin-binding proteins (PBPs), and acquisition of genes coding for low-drug-affinity PBPs. Methicillin-resistant *S. aureus* (MRSA), representing the latest of these aforementioned mechanisms, show resistance to both cefoxitin and oxacillin, which is conferred by acquisition of the *mecA* or recently discovered *mecC* gene [2, 3]. Another relatively frequently described phenotype amongst *S. aureus*

strains is borderline oxacillin-resistant *S. aureus* (BORSA). These strains are cefoxitin susceptible and do not carry the *mecA* or *mecC* genes but are characterized by oxacillin resistance with MIC between 1 and 8 $\mu\text{g}/\text{mL}$ [2, 3]. Hyperproduction of β -lactamase has been proposed to explain BORSA phenotype [4–6].

Increasing number of community-acquired MRSA (CA-MRSA) infections led to investigation of new sources of their origin. Current knowledge on population structure of cefoxitin-susceptible *S. aureus* from animals is still scarce. Some *S. aureus* genotypes are thought to be predominantly associated with particular animal species. The others can be isolated from both human and animals [7, 8]. Most of available data concern prevalence of animal MRSA [9–12]. Evidence for multiple, independent acquisition of the methicillin resistance determinant of methicillin-susceptible *S. aureus* (MSSA) strains, associated with animal breeding, enforces the research on revealing the structure of this

population. Some of *S. aureus* lineages observed in animals are thought to arise from relatively recent transmission from humans. They may represent important reservoir of strains affecting global health systems. Research on genetic diversity of animal MSSA may allow identification of new clones potentially contributing to emergence of community-acquired staphylococcal infections [7].

Until recently MRSA were rarely isolated from livestock animals. However, in recent years, livestock-associated MRSA have been repeatedly isolated from pigs initially in Netherlands [13] and later in various countries in Europe [10], Canada [14], and USA [11]. Lineage ST398 was found to predominate among MRSA in pigs in Europe. It was observed that MRSA CC398 can be readily transferred from animal to animal and animal to human. Studies in humans showed that rapid transmission of MRSA CC398 is possible even after short-term occupational contact with colonized pigs [15].

spa genotyping scheme, first introduced by Harmsen et al. [16], enables interlaboratory comparison of genotypic data. Its association to the results of MLST typing allows for reliable genotypic characterization of *S. aureus* populations [17].

The aim of this work was to determine the population structure of *S. aureus* isolates derived from pig carriage and porcine meat, based on *spa* typing, with emphasis on detection of oxacillin-resistant isolates, that is, BORSA and MRSA.

2. Materials and Methods

2.1. Isolation and Identification of *S. aureus*. One thousand and seventy-four nasal swabs were taken between 2011 and 2012 from pigs in two slaughterhouses (S1 and S2) possessing their own meat processing plants located in south-west of Poland. Eight hundred and four nasal swabs were taken from the slaughterhouse S1 and 270 from the slaughterhouse S2. Average slaughter capacity was 200 pigs per day in both plants. The swabs were taken (ca. one hundred swabs per sampling) four times from slaughterhouse S1 and two times from S2 in 2011, as well as four times from S1 and once from S2 in 2012. Animals at each sampling session originated from different farms (11 objects in total). Samples were collected from the nasal cavity by introducing a cotton swab for approximately 10 cm into the nares. The swabs were taken after electric stunning, before steaming of the pigs. Slaughtered animals originated from local breeding farms.

Additionally, 396 and 140 samples of pork meat from S1 and S2 company shops were examined, respectively. Meat samples (ca. 20) were purchased from the company shops everyday successively during maximum of 4 days following the slaughter.

One-gram food samples and whole nasal cotton swabs were cultured in a final volume of 10 mL of Giolitti-Cantoni enrichment broth and subcultured on Baird-Parker agar. The isolates were identified as *S. aureus* based on their ability to coagulate rabbit plasma and clumping factor production. All isolates were screened by PCR using *S. aureus*-specific primers for *nuc* gene, encoding thermonuclease [18]. Reference *S. aureus* strain ATCC 29213 served as a control.

One *S. aureus* isolate per sample/swab was taken for further characterization.

2.2. Preparation of Bacterial DNA. Two millilitres of bacterial cell suspension from an overnight culture grown in brain-heart infusion (BHI) broth was centrifuged for 5 min at 12,000 × g and suspended in 100 μL of 100 mM Tris-HCl buffer, pH 7.4, containing 10 μg of lysostaphin (A&A Biotechnology, Gdańsk, Poland). After 30-minute incubation at 37°C, 10 μL of 10% SDS was added and the sample was incubated for another 30 min at 37°C. Two hundred μL of 5 M guanidine hydrochloride was added and the sample was mixed by vortexing and incubated at room temperature for 10 min. The DNA was extracted by phenol and chloroform, precipitated with ethanol, and dissolved in water.

2.3. Detection of *mecA* and Determination of SCCmec Cassette Type. All *S. aureus* isolates were tested for the presence of *mecA* gene using the primers described by Milheirço et al. [19]. Each PCR contained *mecA*-positive (*S. aureus* ATCC 43300) and -negative (*S. aureus* ATCC 29213) strains as controls. SCCmec cassette type was determined according to Milheirço et al. [19]. The PCR products were electrophoretically resolved in 1.5% agarose containing 0.5 μg/mL ethidium bromide and photographed with the GelDocXR System (Bio-Rad, Hercules, CA).

2.4. Antibiotic Resistance and Oxacillin MIC Determination in *mecA*-Positive *S. aureus*. Susceptibility of *mecA*-positive *S. aureus* isolates to penicillin G (10 units/disc), cefoxitin (30 μg/disc), tetracycline (30 μg/disc), clindamycin (2 μg/disc), gentamicin (10 μg/disc), erythromycin (15 μg/disc), ciprofloxacin (5 μg/disc), norfloxacin (10 μg/disc), and vancomycin (30 μg/disc) (all substances from Oxoid Ltd., UK) was tested by the disk-diffusion method and interpreted according to CLSI document M100-S22 [20]. The MIC for oxacillin was determined with the E-test and interpreted according to the manufacturer's instructions (bio-Mérieux, Inc.). Reference *S. aureus* strains ATCC 25923, ATCC 43300, and ATCC 29213 served as controls.

2.5. Detection of Borderline Oxacillin-Resistant *S. aureus* (BORSA). All *mecA*-negative *S. aureus* isolates were plated on oxacillin resistance screening agar (ORSA, Oxoid) plates containing 2, 3, and 4 μg/mL oxacillin, respectively. The results were recorded after 24- and 48-hour incubation at 35°C and interpreted according to the manufacturer's instructions. For isolates able to grow in medium containing 4 μg/mL oxacillin MIC for oxacillin was determined using E-test (bio-Mérieux, Inc.). Reference MRSA (ATCC 43300) and MSSA (ATCC 29213) strains served as controls. All phenotypically oxacillin-resistant isolates were analyzed for susceptibility to amoxicillin with clavulanic acid (20/10 μg/disc) by the disk-diffusion method and interpreted according to CLSI document M100-S22 [20]. Reference *E. coli* ATCC 35218 strain served as control. All BORSA isolates were screened for *blaZ* gene according to Rizzotti et al. [21] and for

TABLE 1: *spa* types of *S. aureus* isolates from pig nasal swabs and pork meat derived from slaughterhouses S1 and S2 in 2011.

Sampling	Nasal swabs			Pork meat		
	Number of samples	Number of isolates	<i>spa</i> types (n)	Number of samples	Number of isolates	<i>spa</i> types (n)
<i>S. aureus</i> isolates from S1						
Sampling I	101	44	t318 (29), t034 (7), t1430 (3), t091 (2), t7568 (1), t4309 (1), t8588 (1)	95	32	t091 (12), t084 (12), t4309 (3), t273 (1), t499 (2), t015 (1), t118 (1)
Sampling II	101	19	t021 (15), t318 (3), t034 (1)	65	11	t091 (7), t084 (2), t273 (2)
Sampling III	100	0	—	Not tested	0	—
Sampling IV	100	2	t1334 (2)	36	0	—
Total	402	65		196	43	
<i>S. aureus</i> isolates from S2						
Sampling I	100	1	t252 (1)	50	21	t091 (6), t084 (4), t034 (2), t499 (2), t156 (1), t289 (1), t304 (1), t346 (1), t519 (1), t1491 (1), t3358 (1)
Sampling II	100	3	t12950 (2), t1430 (1)	50	22	t084 (6), t1491 (5), t091 (4), t3478 (2), t034 (1), t127 (1), t267 (1), t337 (1), t499 (1)
Total	200	4		100	43	

hyperproduction of beta-lactamase using Cefinase test (bio-Mérieux, Inc.).

2.6. Detection of *mecC*. All *mecA*-negative *S. aureus* isolates able to grow on ORSA plates containing 2 µg/mL oxacillin were tested for *mecC* gene using the primers described by Cuny et al. [22]. DNA from *mecC*-positive *S. aureus* strain 1140/12, from the National Medicines Institute, Warsaw, Poland, served as a control.

2.7. Determination of *spa* Type and ST. The *spa* types of all *S. aureus* isolates were determined according to Harmsen et al. [16]. The nucleotide sequencing of the repeat-containing region of the *spa* gene was performed from both DNA strands of the PCR product by Genomed (Warsaw, Poland), using BigDye Terminator Ready Reaction Cycle Sequencing kit. The analysis of repeats and the assignment of *spa* types were performed with the resources of the Ridom SpaServer (<http://spa.ridom.de>). Grouping of *spa* types was done using BURP, Ridom StaphType Software. *Spa* types were clustered if cost between members of the group was less than or equal to 4. *Spa* types shorter than 5 repeats were excluded from analysis [23].

Sequence types (STs) of selected *S. aureus* isolates (one t091 isolate from pig and 9 isolates from meat, i.e., t091, t118, t289, t519, t3358, t9031, t12953, t12954, and t12955) were determined according to Enright et al. [24]. The sequences obtained from both strands of the PCR product were analyzed using BioEdit software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) and further assignment of the sequence type (ST) was performed using the <http://www.mlst.net/> platform.

3. Results

3.1. Frequency of *S. aureus* Isolates. In total, 420 *S. aureus* isolates were obtained, including 203 isolates from 1074 nasal swabs and 217 isolates from 536 meat samples. The prevalence of *S. aureus* was different in the two slaughterhouses. The bacterium was found in 197 (25%) from a total of 804 nasal swabs in S1, but only in 6 (2%) from 270 swabs in S2. Screening of meat from S1 and S2 company shops revealed comparable prevalence of samples contaminated with *S. aureus*. The pathogen was isolated from 157 (40%) of a total of 396 meat samples derived from S1 and 60 (43%) from 140 samples originating from S2.

3.2. Genotypes of *S. aureus* Isolates. Forty-three *spa* types were determined in studied *S. aureus* population. *S. aureus* isolates obtained from pig nasal swabs were classified into 18 *spa* types. Among them t318 (28.7%), t011 (18.3%), t034 (13.4%), t337 (11.9%), t021 (8.9%), t091 (8.4%), and t1334 (3.0%) were most frequent (Table 3), with genotypes t318, t034, and t091 isolated during 5 out of a total of 11 sampling sessions (Tables 1 and 2).

S. aureus isolates from pork meat were assigned to 30 *spa* types. Among them isolates belonging to t091 (41.7%), t4309 (14.2%), t084 (11.5%), t499 (5.5%), t12954 (5.5%), and t13074 (3.7%) were dominating (Table 3). *S. aureus* genotype t091 was isolated during 9 out of 11 sampling sessions, while t4309 was found in samples from 5 sessions (Tables 1 and 2).

The most abundant genotype amongst isolates from animals, t318 and t011, were not found in food (Tables 1 and 2). *spa* genotypes t034 and t337 were frequently isolated from animals (13.3% and 11.8%, resp.), but sporadically from meat (1.8% and 0.9%, resp.). In contrast, genotypes t4309 and t084

TABLE 2: *spa* types of *S. aureus* isolates from pig nasal swabs and pork meat derived from slaughterhouses S1 and S2 in 2012.

Sampling	Number of samples	Number of isolates	Nasal swabs		Pork meat		
			<i>spa</i> types (n)	Number of samples	Number of isolates	<i>spa</i> types (n)	
<i>S. aureus</i> isolates from S1							
Sampling I	101	39	t318 (24), t034 (6), t091 (4), t1334 (4), t1939 (1)	50	26	t4309 (11), t091 (10), t13074 (2), t034 (1), t1187 (1), t9031 (1)	
Sampling II	101	49	t011 (37), t091 (4), t021 (3), t034 (2), t026 (1), t084 (1), t1334 (1)	50	31	t091 (9), t4309 (7), t12954 (7), t499 (3), t084 (1), t337 (1), t12955 (1), t13074 (1)	
Sampling III	100	32	t337 (21), t034 (9), t091 (1), t318 (1)	50	27	t091 (15), t4309 (7), t13074 (2), t078 (1), t499 (1), t12954 (1)	
Sampling IV	100	12	t091 (6), t337 (3), t034 (2), t8893 (1)	50	30	t091 (19), t12954 (4), t4309 (3), t13074 (2), t499 (1), t774 (1)	
Total	402	132		200	114		
<i>S. aureus</i> isolates from S2							
Sampling I	70	2	t318 (1), t12952 (1)	40	17	t091 (9), t499 (2), t3380 (2), t008 (1), t1333 (1), t3358 (1), t12953 (1)	
Total	70	2		40	17		

occurred sporadically in pigs (0.5% each) but were frequent in pork meat (14.3% and 11.5%, resp.) (Tables 1 and 2). Thirteen out of 18 *S. aureus* genotypes identified in animals were not detected in meat, whereas among 30 *spa* types found in meat 25 were not detected in animals. Only 5 *S. aureus* genotypes, that is, t034, t084, t091, t337, and t4309, were identified in both nasal swabs and meat (Table 3).

All genotypes identified in the studied *S. aureus* isolates were clustered into 7 *spa* complexes (Figure 1). The animal isolates were clustered into four complexes, namely, *spa*-CC034 (t011, t034, and t8588) belonging to ST-CC398, *spa*-CC1334 (t337, t1334, t1430, t8893, t12950, and t12952) within ST-CC9, *spa*-CC021 (t021, t318) within ST-CC30, and *spa*-CC499 (t084, t091, t4309, and t7568) within ST-CC7 and ST-CC15 (Figure 1). The *spa* genotypes of meat-derived *S. aureus* isolates were clustered into 7 complexes. Most isolates (73.4%) were grouped into *spa*-CC499 belonging to ST-CC15 and ST-CC7. Other numerous complexes included *spa*-CC273 (7.8%) and newly described *spa*-CC12954 (6.4%) both belonging to ST-CC1 (Figure 1, Table 3).

3.3. Characterization of MRSA Isolates. Thirty-six (17.8%) *S. aureus* isolates from pigs were classified as MRSA. All these isolates were *mecA*-positive and resistant to cefoxitin in the disc-diffusion method. None of them possessed *mecC* gene. All of the isolates were resistant to oxacillin with MIC ranged from 32 to 48 $\mu\text{g}/\text{mL}$. Additionally all of the MRSA isolates were resistant to penicillin and tetracycline and susceptible to gentamicin, erythromycin, ciprofloxacin, norfloxacin, and vancomycin. MRSA were isolated exclusively from pigs in slaughterhouse S1, originating from a single farm. All of them were classified to *spa* type t011 SCC*mecV*.

3.4. Characterization of BORSA Isolates. Twenty-eight (14%) *S. aureus* isolates from pigs and 21 (10%) from meat exhibited

borderline resistance to oxacillin. All of them were *mecA* and *mecC* negative, susceptible to cefoxitin and amoxicillin with clavulanic acid, and able to grow on ORSA plates containing minimum of 2 $\mu\text{g}/\text{mL}$ oxacillin. Seventeen (30%) and 3 (5%) of BORSA isolates grew on 3 and 4 $\mu\text{g}/\text{mL}$ oxacillin, respectively. MIC for oxacillin was <5 $\mu\text{g}/\text{mL}$ in isolates able to grow on 4 $\mu\text{g}/\text{mL}$ oxacillin. All BORSA isolates were positive in Cefinase test and harboured *blaZ* gene. *spa* t034 was found to dominate among BORSA from pigs (64%) and t091 among meat-derived BORSA (38%) (Table 4).

4. Discussion

Animal production models, that is, concentration of production in limited number of big holdings or in numerous small farms, are thought to influence the structure of *S. aureus* population [7]. Large European screening of pooled dust samples from pig breeding farms which focused on MRSA demonstrated considerable variation in terms of MRSA prevalence and their genotypes among EU countries [10]. Our previous research indicated low incidence of oxacillin-resistant *S. aureus* in food of animal origin in Poland [25]. This encouraged us to investigate the incidence and genotypes of *S. aureus* in pigs and pork meat in south-western Poland.

Our data indicate 19% mean incidence of *S. aureus* in pigs and 40% in pork meat. However, it should be stressed that depending on sampling session it varied from 0% to 48% in animals and from 0 to 44% in meat indicating significant variation in *S. aureus* occurrence among farms. As yet most research on incidence of *S. aureus* in pigs was focussed on MRSA [7]. Studies, like that by Vandendriessche et al. [26], carried out in Belgium, demonstrating MSSA occurrence (27%) and *spa* genotype structure among pigs are still rare.

TABLE 3: Clonal *spa* complexes of *S. aureus* isolates from pig nasal swabs and pork meat.

Cluster	<i>spa</i>	<i>spa</i> -CC	MLST-CC	n	Origin
1	t091	<i>spa</i> -CC 499	CC7*	108	Nasal swab (<i>n</i> = 17) and pork meat (<i>n</i> = 91)
1	t4309	<i>spa</i> -CC 499	CC15	32	Nasal swab (<i>n</i> = 1) and pork meat (<i>n</i> = 31)
1	t084	<i>spa</i> -CC 499	CC15	26	Nasal swab (<i>n</i> = 1) and pork meat (<i>n</i> = 25)
1	t499	<i>spa</i> -CC 499	CC15	12	Pork meat
1	t346	<i>spa</i> -CC 499	CC15	1	Pork meat
1	t289	<i>spa</i> -CC 499	CC7*	1	Pork meat
1	t774	<i>spa</i> -CC 499	CC15	1	Pork meat
1	t7568	<i>spa</i> -CC 499	CC15	1	Nasal swab
2	t337	<i>spa</i> -CC 1334	CC9	26	Nasal swab (<i>n</i> = 24) and pork meat (<i>n</i> = 2)
2	t1334	<i>spa</i> -CC 1334	CC9	7	Nasal swab
2	t1430	<i>spa</i> -CC 1334	CC9	4	Nasal swab
2	t12950	<i>spa</i> -CC 1334	CC9	2	Nasal swab
2	t8893	<i>spa</i> -CC 1334	CC9	1	Nasal swab
2	t12952	<i>spa</i> -CC 1334	CC9	1	Nasal swab
3	t13074	<i>spa</i> -CC 273	CC1	8	Pork meat
3	t1491	<i>spa</i> -CC 273	CC1	6	Pork meat
3	t273	<i>spa</i> -CC 273	CC1	3	Pork meat
4	t034	<i>spa</i> -CC 034	CC398	31	Nasal swab (<i>n</i> = 27) and pork meat (<i>n</i> = 4)
4	t011	<i>spa</i> -CC 034	CC398	37	Nasal swab
4	t8588	<i>spa</i> -CC 034	CC398	1	Nasal swab
5	t12954	<i>spa</i> -CC 12954	CC1*	12	Pork meat
5	t12955	<i>spa</i> -CC 12954	CC1*	1	Pork meat
5	t12953	<i>spa</i> -CC 12954	CC1*	1	Pork meat
6	t1333	<i>spa</i> -CC 021	CC30	1	Pork meat
6	t318	<i>spa</i> -CC 021	CC30	58	Nasal swab
6	t021	<i>spa</i> -CC 021	CC30	18	Nasal swab
7	t304	<i>spa</i> -CC 008	CC8	1	Pork meat
7	t1187	<i>spa</i> -CC 008	CC8	1	Pork meat
7	t008	<i>spa</i> -CC 008	CC8	1	Pork meat
Singleton	t3478		CC5	2	Pork meat
Singleton	t3380		CC1	2	Pork meat
Singleton	t3358		CC101*	2	Pork meat
Singleton	t015		CC45	1	Pork meat
Singleton	t078		CC25	1	Pork meat
Singleton	t127		CC1	1	Pork meat
Singleton	t156		CC12	1	Pork meat
Singleton	t267		CC97	1	Pork meat
Singleton	t9031		ST1027*	1	Pork meat
Singleton	t252		CC15	1	Nasal swab
Excluded	t118		ST2811*	1	Pork meat
Excluded	t519		CC7*	1	Pork meat
Excluded	t1939		CC398	1	Nasal swab
Excluded	t026		CC45	1	Nasal swab
Total <i>spa</i>	43	Total isolates	420		

MLST-CCs marked with asterisk (*) were determined in this study as described by Enright et al. (2000) [24]. For the remaining MLST-CCs, associations with the particular *spa* types were assessed through the Ridom SpaServer (<http://spa.ridom.de>) or from the relevant literature.

S. aureus genotypes from pigs studied here clustered into four clonal complexes, namely, CC30 (38% of all animal isolates), CC398 (32%), CC9 (19%), and CC7 (8%). High prevalence of MSSA from genetic lineages CC30 and CC398

has been already reported in pigs in Europe [26, 27]. Animal contamination with ST398 MRSA increased over last years. A number of evidences demonstrate the possibility of their transmission to humans [28]. Data from EFSA report indicate

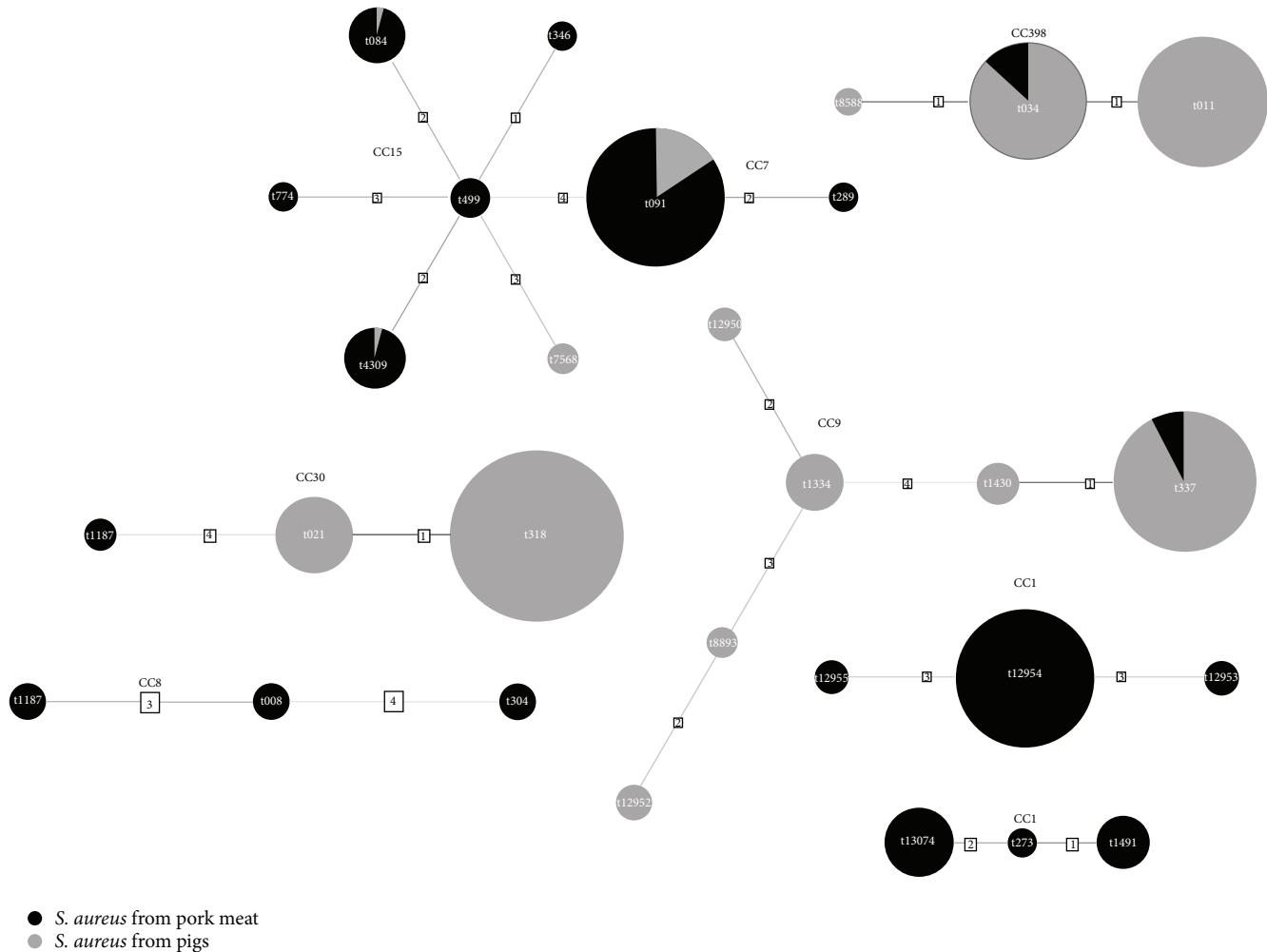
FIGURE 1: Cluster analysis of *spa* types of *S. aureus* isolates from pig nasal swabs and pork meat.

TABLE 4: Characteristics of BORSA isolates from pig nasal swabs and pork meat.

Oxacillin concentration permitting bacterial growth	<i>spa</i> type	Nasal swabs		<i>spa</i> type	Pork meat Number of isolates
		Number of isolates			
4 $\mu\text{g/mL}$	t034	1		t091	2
	t034	6		t091	6
	t011	1		t034	1
	t8588	1		t13074	1
	t337	1			
3 $\mu\text{g/mL}$	t034	11		t12954	4
	t318	1		t273	2
	t337	4		t1491	1
	t026	1		t034	1
	t1334	1		t127	1
2 $\mu\text{g/mL}$	t1187			t1187	1
	t13074			t13074	1
	t273				
	t1491				
			Total 28		Total 21

t011 MRSA as most frequent in Europe [10]. Similarly our MRSA isolates of *spa* type t011 constituted most numerous populations within CC398, but it should be emphasized that they were found at one sampling only. According to the only data on CC398 MRSA associated with pig environment in Poland their prevalence was not higher than 2% [10]. Here we demonstrate that although overall prevalence of genotype t011 MRSA in pigs was ca. 3%, their occurrence was likely restricted to single source. In turn, t034 MSSA was the most prevalent genotype (32%) belonging to CC398 in Denmark [27]. Our t034 MSSA isolates, although less numerous (12%), consistently occurred in a series of sessions, what may indicate wide dissemination of this genotype in Poland.

Transmission of CC398 from animals to food was not extensively investigated as yet. CC398 MRSA was already detected in milk and meat [28, 29]. According to some surveys pork meat contamination with CC398 strains was relatively frequent. Results of a Dutch survey report MRSA t011 isolates in 7% of pork meat [28]. Spanish report indicates 3% frequency of ST398 MRSA in raw pork [30]. We could not detect MRSA in pork meat; however our previous surveys on MRSA incidence in food of animal origin support very low frequency of these strains in Poland [25, 31]. Results presented here demonstrated, however, that MSSA and BORSA t034 isolates could be found in pork meat indicating potential of sporadic introduction of animal-associated genotypes into food chain.

Although the incidence of BORSA among human clinical isolates was reported to be about 5%, they have been implicated in community-acquired and hospital infections [32–34]. BORSA have already been detected in food primarily associated with ruminant milk [25, 35]. In turn, cows seem to be the only animal species in which incidence of BORSA was reported [36]. Genotypic structure of human BORSA population is largely unknown, and there are no data on animal BORSA genotypes. In the current study, 28 and 21 BORSA isolates from pigs and pork meat were identified, respectively. As much as 71% of animal BORSA isolates and 10% of meat isolates were assigned to CC398. These results illustrate the possibility of transmission of typical animal-associated, oxacillin-resistant *S. aureus* isolates to food. Remaining numerous BORSA genotypes belong to CC9 in animal isolates, as well as to CC1 and CC7 in meat isolates.

Another major *spa* cluster identified in this study within animal *S. aureus* isolates, including t1334, t337, t1430, t8893, t12950, and t2952, belongs to CC9. LA-MRSA and MSSA of CC9 are frequent in livestock in Asia [12, 37, 38]. Some reports confirm their low-rate occurrence in Europe [26, 27, 39]. As yet the only European animal *spa* types identified within ST9 include t1430, found in poultry in Netherlands [40], t337 isolated from pigs in Denmark and Belgium [26, 27], and t4794 MRSA from Italy [39]. In turn, *spa* types t337 and t899, representing CC9, were the main pig-derived MRSA in Thailand [38] and Hong Kong [12].

Incidence of t091 isolates, representing genotype belonging to ST7 (CC7), which consequently occurred in studied here pigs at several sampling sessions has not been already reported in this species, suggesting the possibility

of emergence of new genotype in livestock. t091 CA-MSSA together with t084 and t774 belonging to CC15, also found in studied animals, have been isolated only from human as yet [41] and recently found in broilers [26]. On the other side, isolates of CC7 and CC15 predominated in pork meat investigated here, accounting for 73% of total isolates derived from this product, suggesting additional most likely human source of meat contamination.

Isolates belonging to CC1 (almost 14%), including isolates assigned to new *spa*CC 12954 type, and CC8 (1.3%) were detected exclusively in meat. Although sporadic occurrence of these genotypes in livestock was already noted [10, 42, 43] isolation of these clones has been reported mainly from human [41, 44].

Only five genotypes, that is, t091, t034, t337, t084, and t4309, were isolated from both animals and pork meat. From these only t091 was identified with high frequency in both populations, while other genotypes consistently predominated in pigs (t034, t337) or meat (t084, t4309). Taking into account a significant diversity of bacterial genotypes revealed in consecutive samplings we cannot draw definite conclusions on the adaptation of studied genotypes to animal or food milieu. However, results of this survey generally demonstrating a weak genotypic relatedness of *S. aureus* from pigs and isolates from pork meat may indicate that certain genotypes can be introduced more readily than others into food chain. It seems that t091 genotype isolates can be better fitted to colonize both pigs and porcine food products. Although our data showed additional nonanimal source of meat contamination, as discussed above for CC1, CC7, CC8, and CC15 isolates, meat contamination may also be associated with *S. aureus* derived from niches other than pig nares, like skin or intestinal tract. Additional research should be conducted to shed light on structure of *S. aureus* carriage in animals.

5. Conclusions

Taking together, we first report on *spa* types and the oxacillin resistance of *S. aureus* isolates from pigs and pork meat in Poland. Besides *S. aureus* CC30, CC398, and CC9 already known to be widely distributed in pigs, genotype t091 belonging to CC7 was first reported to occur in this species. This is also the first report on the occurrence of CC398 BORSA isolates in pigs and MSSA and BORSA CC398 isolates in pork meat. CC7 isolates, including BORSA phenotype, together with isolates assigned to CC15 were shown to dominate in pork meat.

Conflict of Interests

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Research Article

An Activity of Thioacyl Derivatives of 4-Aminoquinolinium Salts towards Biofilm Producing and Planktonic Forms of Coagulase-Negative Staphylococci

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Microorganisms present in different environments have developed specific mechanisms of settling on various abiotic and biotic surfaces by forming a biofilm. It seems to be well justified to search for new compounds enabling biofilm reduction, which is highly resistant to antibiotics. This study was thus an initial assessment of the antibacterial activity of two new quinoline derivatives of a structure of 3-thioacyl 1-methyl 4-arylaminoquinolinium salts against coagulase-negative staphylococci (CoNS) isolated from a hospital environment, in a form of both biofilms and in planktonic form. Thirty-three stains of CoNS isolated from the hospital environment (air, surfaces) and seven reference strains from the ATCC collection were selected for the study. The mean MIC value for 1-methyl-3-benzoylthio-4-(4-chlorophenylamino)quinolinium chloride (4-chlorophenylamino derivative) was $42.60 \pm 19.91 \mu\text{g}/\text{mL}$, and in the case of strains subjected to 1-methyl-3-benzoylthio-4-(4-fluorophenylamino)quinolinium chloride (4-fluorophenylamino derivative) activity, the mean MIC value was $43.20 \pm 14.30 \mu\text{g}/\text{mL}$. The mean concentration of 4-chlorophenylamino derivative that inhibited biofilm formation was $86.18 \pm 30.64 \mu\text{g}/\text{mL}$. The mean concentration of 4-fluorophenylamino derivatives that inhibited biofilm formation was higher and amounted to $237.09 \pm 160.57 \mu\text{g}/\text{mL}$. Based on the results, both derivatives of the examined compounds exhibit high antimicrobial activity towards strains growing both in planktonic and biofilm form.

1. Introduction

Coagulase-negative staphylococci (CoNS) constitute a considerable part of the commensal flora of human skin and mucous membranes. These microorganisms are often isolated from nosocomial infections of the bloodstream, cardiovascular system, as well as infections of the eye, ear, nose, and throat [1]. Over the recent decades, the main representative of CoNS, *Staphylococcus epidermidis*, has become a significant opportunistic pathogen due to an increasing number of specialist medical procedures, including implanted objects.

Staphylococcus epidermidis is believed to be an important microorganism responsible for infections following surgical vascular grafts, infections after implantation of central venous catheters, heart valves, ventricular assist devices, coronary stents, neurological ventricular shunts, surgical wounds, arthroprostheses, or equipment used for fracture stabilization [2–6].

Microorganisms present in various environments have developed mechanisms of settling on various abiotic and biotic surfaces. The adhesive properties of bacteria may influence the degree of their invasiveness and the likelihood

of infection. Bacteria connecting to the surface form microcolonies anchored in the extracellular matrix [7]. Bacteria present in biofilms have developed a strategy of long-lasting microecosystem, representing a nonaggressive virulence. Therefore, biofilm-related infections may last for many months, extending even for the whole life; however, they rarely directly cause death [8].

A biofilm is a cyclically maturing, three-dimensional structure composed of about 85% of extracellular matrix and of about 15% of microorganism cell aggregates. The matrix is composed of polysaccharides, proteins, enzymes, DNA, bacterial glycolipids, water, and other environmental elements [9]. Because biofilm provides a reservoir for microbial cells, its dispersion enhances the risk of chronic and persistent infections. Likewise the matrix confers a protection against drugs and has environmental promoters that induce biofilm formation and contributes to drug resistance development [10, 11]. Biofilm structure promotes the antibiotic resistance through facilitated horizontal gene transfer due to the high microbial population density. Several mechanisms have been reported to contribute an increased antimicrobial resistance in biofilm structures [11, 12], including low diffusion, transmembrane passage of antibiotics across the polysaccharide matrix, physiological changes of bacteria due to slow growth rate and starvation responses (oxygen, nutrient deprivation, or environmental stress), phenotypic change of the cells forming the biofilm, the expression of efflux pumps that decrease intracellular antimicrobial concentration, and the emergence of persister cells which are multidrug-tolerant cells that have not acquired genetic resistance [13]. These factors contribute to biofilm cells being 1000-fold more resistant to antimicrobial agents than planktonic cells [11, 14, 15]. A well-known genetic element among the staphylococci with regard to biofilm formation is the *ica* operon, which encodes a polysaccharide intercellular adhesin (PIA), which is also often called poly-N-acetyl glucosamine (PNAG) according to its chemical structure [16–18]. The operon contains the *icaADBC* genes, in addition to the *icaR* gene, which exerts a regulatory function and is transcribed in the opposite direction. Earlier studies suggested a direct link between the presence of *ica* and biofilm formation. Once this operon is activated, four proteins are transcribed, *IcaA*, *IcaD*, *IcaB*, and *IcaC*, which are necessary for the biosynthesis of PIA [17, 19, 20]. *IcaA* encodes N-acetylglucosaminyltransferase, which synthesizes the PIA polymer. Sole expression of *icaA* induces only low enzymatic activity, but coexpression with *icaD* significantly increases the activity due to the phenotypic expression of the capsular polysaccharide. *IcaC* is responsible for formation of long chains, and *icaB* deacetylates the poly-N-acetylglucosamine molecule [17, 20]. Expression of locus *ica* genes is regulated by various environmental factors and regulatory proteins. PIA production and its deacetylation have been considered as key factors of virulence in *S. epidermidis* [20–22].

Mature biofilms have a specific three-dimensional structure, which is described as “towers” or “mushrooms” [23]. There are canals filled with liquid between the towers, and it is believed they play a significant function in nutrient supply to the cells in the deeper biofilm layers. Mechanisms

leading to canal formation and biofilm organization are not as well understood as those regulating intercellular adhesion. The results obtained from studies of *Pseudomonas aeruginosa* suggest a role in cell-cell signaling as part of a quorum-sensing system [24]. In staphylococci, expression of PIA exopolysaccharide in biofilms may to some degree contribute to biofilm formation. Various mechanisms of resistance to antibiotics are well known, such as antibiotic removal by antibiotic pumps, modification of their structure by enzymes or mutations of target sites [25, 26].

Various mechanisms of resistance of biofilm-forming strains to antibiotics are suggested. The first hypothesis is the slow or incomplete antibiotic penetration inside the biofilm structure. The second hypothesis conditions antibiotic activity on the chemical differentiation of the biofilm microenvironment. According to the third hypothesis, a subpopulation of highly protected microorganisms similar to spores is formed in biofilm structures. This hypothesis is confirmed in a study on newly formed biofilm structures, which are still too thin to present a mechanical barrier against antibiotic penetration [27, 28]. This hypothesis of spore formation by some cells living in a form of biofilm may be an explanation of lowered susceptibility of biofilm bacteria to various antibiotics, disinfection means, or a wide range of different chemical compounds [8, 25].

Modern chemotherapeutic compounds such as quinoline derivatives exhibit a wide antibacterial activity towards gram-positive bacteria such as *Staphylococcus aureus*, as well as towards gram-negative bacteria [29, 30] and also antifungal and antimalarial activity [29, 31]. Modification of the main structural fragment of a drug may lead to an improvement in its antimicrobial efficiency and strength as well as mode and direction of interaction.

The current treatment and control of biofilm is complicated, because antimicrobials have been developed against planktonically grown bacteria and microorganisms in metabolically active stage. Therefore, we have made an attempt to assess the activity of newly synthesized antibacterial agents 1-methyl-3-benzoylthio-4-(4-chlorophenylamino)quinolinium chloride (4-chlorophenylamino derivative) and 1-methyl-3-benzoylthio-4-(4-fluorophenylamino) quinolinium chloride (4-fluorophenylamino derivative) towards the strains of coagulase-negative staphylococci (CoNS), growing in a form of biofilm (BF) and in planktonic form (PF), isolated from a hospital environment. The investigated compounds were obtained as a result of an acylation of suitable derivatives of 1-methyl-4-aminoquinolinium-3-thiolates using benzoyl chloride [29, 30]. These compounds exhibit strong nucleophilic properties and easily undergo alkylation and acylation reactions on thiolate sulfur atom. The new compounds described, 4-chlorophenylamino derivative and 4-fluorophenylamino derivative, similar to 4-quinalones, are the quinoline derivatives; however their structure, compared to chinolones, is significantly different. 4-Aminoquinoline derivatives have been used as antimalarial drugs, while fluorine derivatives of 4-quinalones are an important class of antibiotics. The evaluated quinoline compounds contain the chlorine or fluoride in phenylamine substituent and not in quinoline ring as it is in case of quinolones. The examined

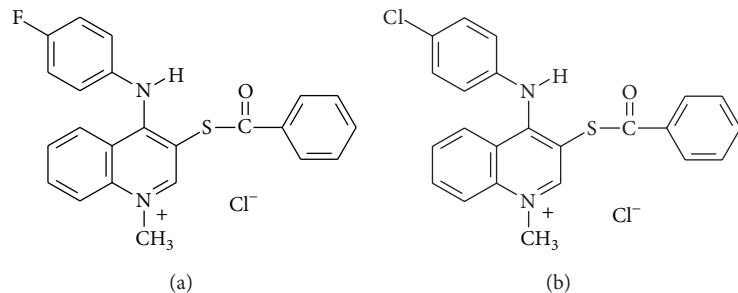


FIGURE 1: Structure of quinoline derivatives: (a) 1-methyl-3-benzoylthio-4-(4-fluorophenylamino) quinolinium chloride (4-fluorophenylamino derivative), (b) 1-methyl-3-benzoylthio-4-(4-chlorophenylamino)quinolinium chloride (4-chlorophenylamino derivative).

salts are the sulfate derivatives of 4-aminoquinoline, and considering their structure, they seem to be more similar to chloroquine (used for preventing malaria from *Plasmodium vivax*, *P. ovale*, and *P. malariae*) or ammonium salt.

Quinoline is a heterocyclic aromatic organic compound with the chemical formula C₉H₇N. This compound forms part of the structure of quinine, the malaria remedy found in cinchona bark and known since the time of the Incas. Quinolines and their derivatives occur in numerous natural products, many of which possess interesting physiological and biological properties. Design and preparation of potential quinoline-based antibacterial agents consist partially in a molecular hybridization approach that involves the coupling of two or more groups with relevant biological properties [32]. Figure 1 presents the structure of two examined compounds.

The search for new antibacterial compounds continues and other quinolines with similar structures may become available as efficient antibiotics against resistant microorganisms. The key reason of all these structural changes of quinoline derivatives was to improve physicochemical parameters of quinoline molecules, which may lead to an enhanced fit into the binding site. As the hospital strains become increasingly resistant to the standard antibiotics, there is an urgent need to understand the molecular mechanisms for new drug action and resistance so that novel antibacterial drugs can be designed. A number of modified quinolines compounds show some promise in this regard.

2. Material and Methods

2.1. Bacterial Strain. Thirty-three isolated of coagulase-negative staphylococci obtained from a hospital environment (surfaces, air) and eight reference strains from the ATCC collection (*S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 35984, *S. saprophyticus* ATCC 15305, *S. hominis* ATCC 27844, *S. haemolyticus* ATCC 29970, *S. capitis* subsp. *capitis* ATCC 35661, *S. warneri* ATCC 49454, and *S. lugdunensis* ATCC 49567) were selected for the study. The species of the strains were identified according to standard methodology with the use of biochemical APIStaph tests (bioMerieux, France) and stored for further analysis on TBE medium with the addition of 20% glycerol (Sigma) at -20°C reference strains from the ATCC collection were obtained from LCG Standards (Lomianki, Poland).

2.2. Investigation of Biofilm Production by the Congo Red Agar (CRA) Method. Phenotypic characterization of biofilm production was performed by culturing the CoNS isolates on CRA plates as described by Freeman et al. [33]. A special medium was prepared of brain-heart infusion broth (BHI-BTL Poland) supplemented with 5% sucrose and Congo red. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. According to the authors, biofilm producers form black, crusty colonies on CRA, whereas nonproducers form red colonies. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an intermediate result.

2.3. Biofilm Formation Studied by Microtiter Plate Assay (MP). We performed the microtiter plate assay described by Christensen et al. [34] with modifications. A suspension equivalent to McFarland 0.5 turbidity standard was prepared in Muller-Hinton broth (MHB-BTL, Poland) for each strain. The accuracy of bacterial counts in the suspension was confirmed by serial dilution in log steps. Aliquots of 100 μ L from each bacterial suspension were inoculated onto 96-well tissue microtiter plates. These were incubated at 37°C for 24 h in a normal atmosphere. The medium was then removed, and the wells were washed three times with phosphate-buffered saline (PBS, pH 7.2) to remove free-floating “planktonic” bacteria. An amount of 150 μ L of 0.1% crystal violet (Sigma, USA) was added to each well and left for 30 min at room temperature. The dye was removed, and this was followed by five washes with sterile deionized water. The preparations were destained with 200 μ L of 95% isopropanol in 1 M HCl for 5 min. Finally, 100 μ L of coloured isopropanol from each sample was transferred to another microtiter plate. The optical density (OD) of the suspension was measured at a wavelength of 490 nm with a Multitec SX microplate reader.

The ODs obtained were compared with those of the negative control (well without bacterial inoculum). We considered the $OD > 0.17$ to be positive. All the strains were tested four times, and the average value for each sample was calculated. The mean $A_{490} \pm SD$ values are presented.

The isolates were classified into two categories: nonadherent, optical density to or lower than 0.17 and adherent, optical density higher than 0.17. When the cut-off corresponded to nonadherent, the isolates were classified as negative and as positive when the cut-off corresponded to adherent.

2.4. Detection of *icaA* and *icaD* Genes Specific for Biofilm Production in *S. epidermidis* Strains. All isolates were stored at -80°C pending analysis and were subcultured on blood agar plates and checked for purity prior to DNA preparation. An Isolate Genomic DNA Mini Kit (BLIRT S.A., Poland) was used to isolate DNA from strains with the following modifications. Pure DNA was stored at -20°C .

PCR was used to detect the presence of *icaA* and *icaD*. The primer sequences and predicted product lengths for *icaA* and *icaD* were described by Ziebuhr et al. [35] and de Silva et al. [36]. The PCR reactions were performed in separate reactions using a 10x PCR RED master mix kit. The PCR cycling conditions used were 30 cycles of 30 s of denaturation at 95°C and 3 min of elongation at 72°C for all reactions, with an annealing for 1 min at 60°C (*icaA*) or 59°C (*icaD*). PCR was performed by using an MJ Mini Personal thermal cycler (BIORAD, Germany).

The PCR products were electrophoresed through 1.5% agarose gels and checked for size against molecular weight markers using 1Kb HypeLadderIV (BLIRT S.A., Poland).

2.5. Examination of Antistaphylococcal Activity of 1-Methyl-4-aminoquinolinium-3-thiolates. Determination of minimum inhibitory concentrations (MIC) of microorganisms was performed using the method of microdilutions on 96-well plates according to obligatory methodology [37].

The MIC value was determined by an incubation of the examined strains in 96-well plates for 20 hours at 37°C . Bacterial inocula were prepared in 0.9% sodium chloride from fresh cultures up to the moment of 0.5 McFarland's turbidity. Next, the inocula were diluted (1:100) in sterile Mueller-Hinton broth medium just before addition to the plates. The microorganisms were exposed to a serial dilution of 4-chlorophenylamino derivative and 4-fluorophenylamino derivative in the range from 1000 to $1\text{ }\mu\text{g/mL}$. In addition, the values of minimum and maximum MIC as well as MIC_{50} and MIC_{90} were determined. The examinations were conducted both for the strains forming biofilm and those not forming it.

2.6. Inhibition of Staphylococcal Biofilm Formation by 1-Methyl-4-aminoquinolinium-3-thiolates. The 1-methyl-4-aminoquinolinium-3-thiolates were tested for their potential to prevent biofilm formation of a biofilm producing *S. epidermidis* strain. The evaluation of minimum biofilm inhibiting concentrations (MBIC) was done colorimetrically from the metabolic activity of surviving CoNS strains cells treated with new quinoline compounds [38, 39]. The presence of viable cells in biofilm was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This "static," indirect viability assay measures enzymatic activity in actively respiring cells and is based on the formation of insoluble purple formazan due to the reduction of MTT by respiratory reductase of living staphylococcal cells. Therefore, it is a measure of cell viability and/or relative numbers of viable cells. The minimum biofilm inhibitory concentration (MBIC) is defined as the lowest concentration of an antimicrobial agent required to inhibit the formation of biofilms. Nonbiofilm-forming clinical isolate *S. epidermidis*

was used as negative control. The bioassay was performed in triplicate.

For assessing the biofilm growth inhibition effects of the 4-chlorophenylamino derivative and 4-fluorophenylamino derivative, they were added to the growth medium at the time of inoculation and the cells were allowed to form biofilm.

An aliquot of twofold serial dilutions (200 μL) was prepared in the 96-well microtiter plate containing tryptic soy broth (TSB, BTL, Łódź, Poland) supplemented with 2% D-glucose (TSBGlc), with final concentrations of 4-chlorophenylamino derivative ranging from 1000 $\mu\text{g/mL}$ to 1 $\mu\text{g/mL}$ for 4-fluorophenylamino derivative. Bacterial suspensions (100 μL ; 5×10^5 CFU/mL, final concentration) were then transferred into the plate. TSBGlc containing 0.2% DMSO was employed as a negative control. TSBGlc without the extract was used as the nontreated well and the medium with each concentration of the extracts was used as the blank control [40].

After incubation in 37°C for 24 hours the inhibitory effect was evaluated using MTT reduction assay. The supernatant was discarded and replaced with 200 μL of PBS supplemented with 50 μg MTT (Sigma-Aldrich, USA). The formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA), and the absorbance was determined at 570 nm (OD = 570 nm) with microplate reader (Thermo Electron Corp., Finland).

2.7. Statistical Analysis. All examinations of MIC and biofilms were performed four times, and the results were expressed as mean values \pm standard deviation (SD). Results from correlation assays were submitted to the *U* Mann-Whitney nonparametric comparing biofilm formation status and *ica* status, MP test, and CRA plate test. Results were considered significant when $P < 0.05$. For comparison of the MIC and MBIC results, the respective 50th percentile values (MIC_{50} and MBIC_{50}) and 90th percentile values (MIC_{90} and MBIC_{90}) were calculated, and the ranges were compared. Statistical analyses were performed using Statistica 10.0 PL (StatSoft).

3. Results

Analyses of the antimicrobial activity of 4-chlorophenylamino derivatives and 4-fluorophenylamino derivatives were conducted on 40 strains of coagulase-negative staphylococci, of which 33 strains were isolated from a hospital environment (air, surfaces) and seven reference strains were derived from the American Type Culture Collection. Among 33 of the identified CoNS environmental strains, 19 were identified as *S. epidermidis*, 5 strains as *S. haemolyticus*, 3 strains as *S. warneri*, 2 strains as *S. cohnii* subsp. *cohnii*, and 1 strain as *S. saprophyticus*, *S. kloosii*, *S. cohni* subsp. *urealyticum*, and as *S. capitis* subsp. *capitis*. Among the ATCC strains, the following were used in the study: *S. epidermidis* ATCC 12228, *S. saprophyticus* ATCC 15305, *S. hominis* subsp. *hominis* ATCC 27844, *S. haemolyticus* ATCC 29970, *S. capitis* subsp. *capitis* ATCC 35661, *S. warneri* ATCC 49454, and *S. lugdunensis* ATCC 49567.

By assessing the ability of biofilm formation using the CRA method, characteristic black crusty colonies were obtained only in the case of two (5%) strains, and intermediate colonies, grey ones in three (7.5%) cases. The examined reference strains did not exhibit an ability for biofilm formation using the CRA method (Table 1).

Among 40 strains of coagulase-negative staphylococci analyzed using the MP method, 11 (27.5%) strains were classified as forming a biofilm ($A_{490} > 0.17$). The other 22 examined strains and 7 reference strains did not form biofilms (Table 1). Among the 11 strains forming biofilms, 10 (90.9%) belonged to *S. epidermidis* species, while 1 strain (9.1%) was identified as *S. kloosii*.

After examining the presence of *icaA* and *icaD* genes in *S. epidermidis* isolated strains, the *icaAD* gene was noted in 11 strains (57.9%) (Table 1). Analyzing the results of an assessment of an ability of biofilm formation using CRA, MP methods, and determination of the presence of *icaA* and *icaD* genes, it may be concluded that 9 (90%) out of 10 *S. epidermidis* strains exhibiting phenotypical ability of biofilm formation using the MP method demonstrated the presence of at least one of these genes (*icaA*). In this one analyzed case, this may suggest the possibility of biofilm formation independent of the *icaADBC* operon. In one case, the presence of the *icaA* gene did not induce the phenotypical presence of biofilm in any of the methods used. Statistical analysis by the Kruskal-Wallis test showed a correlation among the phenotypical presence of biofilm and *ica* status ($P = 0.0017$).

Analyses of the MIC values for 40 strains of coagulase-negative staphylococci (Tables 2 and 3) showed a mean MIC value for 4-chlorophenylamino derivative of $42.60 \pm 19.91 \mu\text{g/mL}$, with a minimum value of $16 \mu\text{g/mL}$ and maximum of $64 \mu\text{g/mL}$. In the case of the strains subjected to 4-fluorophenylamino derivative, the mean MIC value was $43.20 \pm 14.30 \mu\text{g/mL}$, the minimum value was $16 \mu\text{g/mL}$, and the maximum was $64 \mu\text{g/mL}$. The values of MIC_{50} and MIC_{90} for all examined strains were $46 \mu\text{g/mL}$ and $64 \mu\text{g/mL}$, respectively, and were the same for 4-chlorophenylamino derivative and 4-fluorophenylamino derivatives.

Assessing the ability of biofilm inhibition by 4-chlorophenylamino derivative and 4-fluorophenylamino derivative, 11 strains exhibiting such ability phenotypically were selected for analysis using the MP method (Table 4). The analysis showed that biofilms grown in the presence of compounds had significantly lower activity as compared to an inhibitor control.

The mean concentration of 4-chlorophenylamino derivative inhibiting biofilm formation for the examined strains of coagulase-negative staphylococci was $86.18 \pm 30.64 \mu\text{g/mL}$, with a minimum of $40 \mu\text{g/mL}$ and maximum of $128 \mu\text{g/mL}$. In the case of 4-fluorophenylamino derivatives the mean concentration inhibiting biofilm formation was higher and amounted to $237.09 \pm 160.57 \mu\text{g/mL}$, while the minimum and maximum values were $56 \mu\text{g/mL}$ and $448 \mu\text{g/mL}$, respectively ($P < 0.001$). Values of MBIC_{50} and MBIC_{90} for 4-chlorophenylamino derivative were $96 \mu\text{g/mL}$ and $128 \mu\text{g/mL}$. In the case of 4-fluorophenylamino derivatives the MBIC_{50}

reached a value of $192 \mu\text{g/mL}$, and the MBIC_{90} value was $448 \mu\text{g/mL}$.

4. Discussion

The results of our study demonstrated the presence of the *icaA* and/or *icaD* genes and subsequent biofilm production in most *S. epidermidis* isolates (sensitivity 0.909; specificity 0.724). Taking into account the fact that biofilm structures are the main factor of virulence involved in infections with coagulase-negative staphylococci [1], it seems to be essential to determine their antibiotic activity considering infections caused by these microorganisms occur both in planktonic forms and those forming a biofilm. Scanning electron microscopy demonstrated that biofilm is visible as soon as after 24 hours of bacterial adhesion to the surface [41]. According to some observations, 99% of bacteria live in a form of bacterial biofilm, and only 1% in a free form not attached to the surface, for example, in body liquids [7].

Numerous studies were carried out with the attempt to compare the susceptibility of bacteria growing in a form of biofilm on selected antibiotics, as well as their equivalents in planktonic form; however the results were not validated and/or were unequivocal [42, 43]. Bacteria growing in a form of biofilm are from 100- up to 1000-fold more resistant than their planktonic forms [42–44].

An assessment of the bactericidal activity of six selected antibiotics on the planktonic and biofilm forms of three *S. epidermidis* strains and three *S. aureus* strains was conducted by Nishimura et al. [41]. In the case of clarithromycin, cefotaxime, erythromycin, and benzylpenicillin, the planktonic forms of the *S. epidermidis* strains demonstrated susceptibility to antibiotic concentrations below $0.5 \mu\text{g/mL}$, while in the case of vancomycin and cefmetazole this value was estimated at $1 \mu\text{g/mL}$. Conducting a similar analysis of biofilm-forming bacteria, Nishimura et al. demonstrated their susceptibility in concentrations not lower than $512 \mu\text{g/mL}$ to $1024 \mu\text{g/mL}$ [41].

The activity of various antibiotics including daptomycin, vancomycin, gentamicin, and rifampicin applied as a monotherapy and combined polytherapy against two clinical MRSA strains forming biofilm 1 was investigated by LaPlante and Woodmansee [45]. They noted that daptomycin and rifampicin had only slight differences in the ratio between MIC and MBEC. In the case of vancomycin, the MIC values for two examined strains forming biofilms were $2 \mu\text{g/mL}$ and $4 \mu\text{g/mL}$, respectively, while the MBEC values for the same strains were considerably higher and amounted to 128 mg/L and 64 mg/L , respectively. These strains were more susceptible to gentamicin with even higher differences between the MIC and MBEC values, and they were estimated as $0.5 \mu\text{g/mL}$ and 256 mg/L , respectively, for both strains.

Analysis of the susceptibility of eight *S. aureus* and CoNS strains forming biofilms with respect to azithromycin in a form of eye solution was conducted by Wu et al. [46]. The MIC values for eight examined strains ranged from $0.75 \mu\text{g/mL}$ to $>256 \mu\text{g/mL}$, and both strains were susceptible and resistant to azithromycin. Analyses of the susceptibility of biofilm forms of these strains to azithromycin in a form of eye

TABLE 1: Phenotypic and genotypic characterization data from 41 CoNS strains.

Strain number	Bacterial species	Biofilm status	MP test ^{*1} ($A_{490} \pm SD$)	CRA plate test ^{**2}	Presence of <i>icaA/icaD</i> gene ^{***2}
1	<i>S. saprophyticus</i>	Biofilm-negative	0.07 ± 0.01	r	ND
2	<i>S. kloosii</i>	Biofilm-positive	0.44 ± 0.08	b	ND
4	<i>S. cohnii</i> subsp. <i>urealyticum</i>	Biofilm-negative	0.11 ± 0.01	r	ND
5	<i>S. haemolyticus</i>	Biofilm-negative	0.08 ± 0.02	r	ND
14	<i>S. cohnii</i> subsp. <i>cohnii</i>	Biofilm-negative	0.10 ± 0.04	g	ND
24	<i>S. epidermidis</i>	Biofilm-positive	0.48 ± 0.06	r	+/-
25	<i>S. epidermidis</i>	Biofilm-positive	0.44 ± 0.10	r	+/-
26	<i>S. epidermidis</i>	Biofilm-positive	0.61 ± 0.03	r	+/+
27	<i>S. epidermidis</i>	Biofilm-negative	0.07 ± 0.02	r	-/+
28	<i>S. epidermidis</i>	Biofilm-negative	0.08 ± 0.01	r	-/-
32	<i>S. warneri</i>	Biofilm-negative	0.06 ± 0.01	r	ND
33	<i>S. epidermidis</i>	Biofilm-positive	0.19 ± 0.01	r	+/-
53	<i>S. epidermidis</i>	Biofilm-negative	0.13 ± 0.01	r	+/+
62	<i>S. cohnii</i> subsp. <i>cohnii</i>	Biofilm-negative	0.07 ± 0.02	g	ND
64	<i>S. epidermidis</i>	Biofilm-negative	0.08 ± 0.02	r	-/-
65	<i>S. epidermidis</i>	Biofilm-positive	0.27 ± 0.08	r	-/-
76	<i>S. epidermidis</i>	Biofilm-positive	0.47 ± 0.05	r	+/-
78	<i>S. epidermidis</i>	Biofilm-positive	0.53 ± 0.10	r	+/-
84	<i>S. epidermidis</i>	Biofilm-negative	0.09 ± 0.02	r	-/-
90	<i>S. epidermidis</i>	Biofilm-negative	0.07 ± 0.01	g	-/-
91	<i>S. epidermidis</i>	Biofilm-positive	0.45 ± 0.04	b	+/+
93	<i>S. haemolyticus</i>	Biofilm-negative	0.05 ± 0.00	r	ND
95	<i>S. epidermidis</i>	Biofilm-negative	0.10 ± 0.05	r	-/-
96	<i>S. haemolyticus</i>	Biofilm-negative	0.11 ± 0.02	r	ND
97	<i>S. epidermidis</i>	Biofilm-positive	0.46 ± 0.01	r	+/+
99	<i>S. epidermidis</i>	Biofilm-positive	0.55 ± 0.05	r	+/+
101	<i>S. haemolyticus</i>	Biofilm-negative	0.06 ± 0.01	r	ND
102	<i>S. epidermidis</i>	Biofilm-negative	0.06 ± 0.00	r	-/+
103	<i>S. capitis</i> subsp. <i>capitis</i>	Biofilm-negative	0.14 ± 0.03	r	ND
105	<i>S. haemolyticus</i>	Biofilm-negative	0.10 ± 0.03	r	ND
107	<i>S. epidermidis</i>	Biofilm-negative	0.07 ± 0.02	r	-/-
125	<i>S. warneri</i>	Biofilm-negative	0.08 ± 0.03	r	ND
139	<i>S. warneri</i>	Biofilm-negative	0.10 ± 0.04	r	ND
12228	<i>S. epidermidis</i> ATCC 12228	Biofilm-negative	0.11 ± 0.02	r	-/-
35984	<i>S. epidermidis</i> ATCC 35984	Biofilm-positive	0.56 ± 0.11	b	+/+
15305	<i>S. saprophyticus</i> ATCC 15305	Biofilm-negative	0.09 ± 0.03	r	ND
27844	<i>S. hominis</i> ATCC 27844	Biofilm-negative	0.06 ± 0.01	r	ND
29970	<i>S. haemolyticus</i> ATCC 29970	Biofilm-negative	0.08 ± 0.03	r	ND
35661	<i>S. capitis</i> subsp. <i>capitis</i> ATCC 35661	Biofilm-negative	0.08 ± 0.01	r	ND
49454	<i>S. warneri</i> ATCC 49454	Biofilm-negative	0.08 ± 0.02	r	ND
49576	<i>S. lugdunensis</i> ATCC 49576	Biofilm-negative	0.06 ± 0.01	r	ND

CRA: type of growth on Congo Red Agar medium, r: red colonies, b: black colonies, and g: grey colonies;

icaA/D: (+) presence of the gene, (-): absence of the gene, and ND: not detection.Correlation between biofilm status and ^{*1}MP test $r = P < 0.001$; ^{**2}CRA plate test $r = 0.13 (P = 0.785)$; ^{***2}*ica* status $r = 0.43 (P = 0.015)$. Results were considered significant when $P < 0.05$; ¹U Mann-Whitney nonparametric test; ²Rang Spearman test.

TABLE 2: Mean MIC values for CoNS strains subjected to an activity of 4-chlorophenylamino derivative and 4-fluorophenylamino derivative ($\mu\text{g/mL}$).

Bacterial species (number of strains)	4-Chlorophenylamino derivative MIC \pm SD	4-Fluorophenylamino derivative MIC \pm SD
<i>S. epidermidis</i> (19)	43 \pm 7.13	41 \pm 6.41
<i>S. haemolyticus</i> (5)	42 \pm 8.75	39 \pm 20.65
<i>S. warneri</i> (3)	31 \pm 8.83	43 \pm 0.00
<i>S. cohnii</i> subsp. <i>cohnii</i> (2)	28 \pm 4.62	36 \pm 13.90
<i>S. kloosii</i> (1)	48 \pm 18.48	16 \pm 0.00
<i>S. cohnii</i> subsp. <i>urealyticum</i> (1)	64 \pm 0.00	56 \pm 16.00
<i>S. saprophyticus</i> (1)	24 \pm 9.24	32 \pm 0.00
<i>S. capitis</i> subsp. <i>capitis</i> (1)	48 \pm 18.48	48 \pm 18.48
<i>S. epidermidis</i> ATCC 12228	28 \pm 8.00	64 \pm 0.00
<i>S. epidermidis</i> ATCC 35984	64 \pm 0.00	48 \pm 18.48
<i>S. saprophyticus</i> ATCC 15305	24 \pm 9.24	56 \pm 16.00
<i>S. hominis</i> ATCC 27844	64 \pm 0.00	48 \pm 18.48
<i>S. haemolyticus</i> ATCC 29970	64 \pm 0.00	56 \pm 16.00
<i>S. capitis</i> subsp. <i>capitis</i> ATCC 35661	48 \pm 18.48	48 \pm 18.48
<i>S. warneri</i> ATCC 49454	64 \pm 0.00	48 \pm 18.48
<i>S. lugdunensis</i> ATCC 49567	48 \pm 18.48	64 \pm 0.00

TABLE 3: Statistical analysis of obtained MIC values ($\mu\text{g/mL}$) 4-chlorophenylamino derivative and 4-fluorophenylamino derivative with respect to CoNS strains depending on growth form.

	Biofilm-negative strains MIC \pm SD ($\mu\text{g/mL}$) <i>n</i> = 29	Biofilm-positive strains MIC \pm SD ($\mu\text{g/mL}$) <i>n</i> = 12	<i>P</i>
4-Chlorophenylamino derivative	40.14 \pm 15.73	49.09 \pm 15.19	0.016
4-Fluorophenylamino derivative	45.52 \pm 13.79	37.09 \pm 14.43	0.018

Results were considered significant when $P < 0.05$; *U* Mann-Whitney nonparametric test.

TABLE 4: Values of minimum biofilm inhibitory concentration (MBIC) of the examined strains.

Bacterial species (number of strains)	4-Chlorophenylamino derivative MBIC \pm SD ($\mu\text{g/mL}$)	4-Fluorophenylamino derivative MBIC \pm SD ($\mu\text{g/mL}$)
<i>S. kloosii</i> (1)	44 \pm 24.00	56 \pm 16.00
<i>S. epidermidis</i> (9)	100 \pm 27.91	283 \pm 96.72
<i>S. epidermidis</i> ATCC 35984	128 \pm 0.00	384 \pm 147.80

solution (concentration of 0.25–1.0%) showed an inhibition of biofilm formation irrespective of the strains' susceptibility or resistance to azithromycin.

An analysis of various antibiotic (cefazolin, vancomycin, dicloxacillin, tetracycline, and rifampicin) activity mechanisms towards six strains of coagulase-negative staphylococci was conducted in turn by Cerca et al. [44]. When the target site of the antibiotic activity was the cell membrane, a reduction in biofilm formation was observed irrespective of the size of the antibiotic particle. Moreover, when the antibiotic target site was RNA or protein synthesis, a similar activity was noted for planktonic and biofilm forms. They concluded that phenotypical antibiotic resistance of bacterial

cells in a biofilm is predominantly related to the mechanism of antibiotic activity.

A significant variation in bactericidal activity between planktonic and biofilm forms of bacteria was also reported by El-Banna et al. [47]. Analyzing eight strains of staphylococci (three strains of *S. aureus* and five CoNS strains), they showed that, considering ciprofloxacin, the bactericidal value represented by the MIC was significantly different. For biofilm-forming strains, the MIC values were from 2- to 512-fold higher than those obtained for planktonic forms. These authors also obtained similar results by analyzing the activity of gentamicin towards the same bacterial strains. In the case of amoxicillin with clavulanic acid, the value of MBC for

biofilm forms was 4 to 64 times higher than the MBC results obtained for bacteria growing in planktonic form.

Comparing the minimum concentration inhibiting microorganism growth and the minimum concentration inhibiting biofilm formation for the examined CoNS strains, we demonstrated that in the case of 4-chlorophenylamino derivative the MIC values are only about 2-fold higher, and for 4-fluorophenylamino derivative they were approximately 6- to 10-fold higher ($P = 0.009$). Therefore, both derivatives of the examined compounds demonstrate high activity with respect to strains growing in a biofilm, and a clear predominance is demonstrated for 4-chlorophenylamino derivative.

The molecular basis of the action of these new compounds is not completely understood, but they are thought to interfere with bacterial DNA synthesis. Structure-activity relationship studies revealed that the antimicrobial activity in this heterocyclic class of quinoline molecules depends on the nature of the peripheral substituents and their spatial relationship within the quinoline skeleton. This type of quinolines may inhibit DNA synthesis by promoting cleavage of bacterial DNA in the DNA-enzyme complexes of type II topoisomerase DNA gyrase and the topoisomerase IV, resulting in rapid bacterial death [32].

The examined quinoline derivatives were able to inhibit biofilm formation, detach existing biofilms, and kill bacteria in biofilms of selected CoNS strains. Importantly, biofilms were similarly as sensitive as their planktonic counterparts, probably due to the dual activity of tested compounds on existing biofilms. MBIC values of thioacyl derivatives of 4aminoquinolinium salts against hospital isolates are being documented for the first time in this study which outlines a very sturdy basis for future investigations in pursuit to discover new anti-infectious agents. Further study is advised to elucidate the complex mode of action of newly synthesized compounds against biofilms of CoNS, particularly *S. epidermidis* and other clinically relevant microbes.

5. Conclusions

The administration of suboptimal chemotherapeutic concentrations, active only for planktonic forms, not the main infection factor, may be inefficient for pharmacological therapy. Therefore, it seems to be viable and purposeful to search for such antibacterial agents with similar activities towards planktonic and biofilm forms of bacteria. Considering the examined compounds, we conclude that only 4-chlorophenylamino derivative demonstrates such abilities. However, further analysis not only of antistaphylococcal activities and pharmacokinetics but also of drug interactions and chronic cumulative toxicities due to long-term administration is needed prior to clinical application of these drugs.

Conflict of Interests

The authors declare no potential conflict of interests. They confirm that all their affiliations with or financial involvements in or any financial conflict with the subject matter

discussed in the paper are completely disclosed (e.g., employment, consultancies, honoraria, expert testimony, grants, and royalties).

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Research Article

Discrepancies in Drug Susceptibility Test for Tuberculosis Patients Resulted from the Mixed Infection and the Testing System

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To find the potential reasons for the discrepancies in the drug susceptibility test (DST) of *M. tuberculosis* isolates, twenty paired isolates with disputed drug susceptibilities to isoniazid (INH) were selected according to the MGIT960 testing and Löwenstein-Jensen (L-J) proportion methods. Their MICs were confirmed again by broth microdilution method and by L-J proportion method. The spoligotyping results showed that, of all the 20 paired strains, 11 paired isolates belonged to the Beijing genotype and 6 paired isolates belonged to SIT1634, and that each of the remaining 3 paired isolates had two genotypes, namely, SIT1 and SIT1634. Those 3 paired isolates with different intrapair spoligotypes were further confirmed as mixed infection by the results that those three pairs of isolates with different 12 locus MIRU intrapair types and one pair carried different base pair at codon 315 (AGC versus AAC). Totally mutations in the *katG* gene were identified in 13 paired isolates. No mutations were found in the regulatory sequences and open reading frames (ORF) of the *inhA* and *ahpC* genes in any of the tested isolates. Those results showed that the different test systems and the mixed infection with particular genotypes of *M. tuberculosis* strains contributed to the drug susceptibility discrepancies.

1. Introduction

Performance of drug susceptibility testing (DST) to measure drug resistance is important not only before treatment, but also in the course of therapy to identify acquired resistance, especially in the areas with a high incidence of MDR-TB [1]. Conventional DST methods rely on egg-based (Löwenstein-Jensen; L-J) or agar-based (Middlebrook) media, but these are laborious and time-consuming procedures requiring 3 to 8 weeks to obtain results [2]. A number of new methods for DST, including the mycobacterial growth indicator tube (MGIT) [3], E test [4], and Alamar blue [5] methods, have been introduced over the last decade to detect mycobacteria rapidly and to improve their growth rates [6, 7].

The BACTEC MGIT960 method has been assessed in many countries and its degree of agreement with conventional DST methods in *M. tuberculosis* has been assessed [8–10]. Meta-analysis of published results revealed high accuracy and high predictive value associated with the use of BACTEC MGIT960 [11]. However, there are still discrepancies in the DST results obtained for different anti-TB drugs between BACTEC MGIT960 and other DST methods. The discrepancies in INH susceptibility between the MGIT960 and L-J proportion methods, for example, varied from 0% to 1% [9]; however, few investigations have been reported that addressed the possible mechanisms underlying the discrepancies between the MGIT960 system and L-J proportion methods.

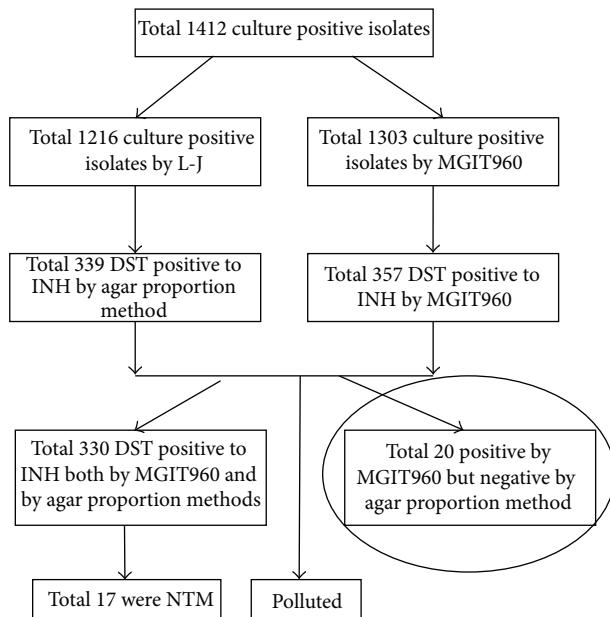


FIGURE 1: Strains selected in this experiment. A total of 1014 culture positive isolates were included in this study which were isolated in 2006. We focus on the INH as it is a very important antibiotic in curing tuberculosis. In this study of all the total 1412 culture positive isolates 1216 were positive on the L-J medium, of which 339 were resistant by L-J method to INH and 1303 isolates were positive by the MGIT960, of which 357 were resistant to INH by the MGIT960 system. Total 330 were DST positive to INH by both MGIT960 and agar proportion methods, of which 20 isolates with positive both by MGIT960 system but negative by agar proportion method were examined in this study.

Discrepancies can arise from many reasons, for example, different DST systems used, mixed infection with different *M. tuberculosis* strains, and last but not least, contamination. In this study, 20 paired isolates with disputed drug susceptibilities to INH were selected according to the MGIT960 testing and L-J proportion methods. The name of the “paired isolates” referred to the two isolates obtained separately from the cultures after the DST by MGIT960 and L-J proportion methods from the same sputum of the patient. The reasons for the DST discrepancies were analyzed by the spoligotyping and VNTR genotyping methods and drug resistance-related mutations tested in INH resistance-related genes.

2. Materials and Methods

2.1. Strains and Antibiotics. A total of 20 paired *M. tuberculosis* isolates with DST discrepancies were collected in Tianjin Haihe Hospital in the year of 2006 from total 1412 isolates (Figure 1). “paired isolates” were from the the culture of the MGIT960 and L-J proportion method, respectively, which was mentioned above. Meanwhile, 96 randomly selected isolates, whose MGIT960 and agar proportion DST results were in agreement, were also collected from the same hospital. The 20 paired *M. tuberculosis* isolates were determined to be sensitive to INH using the conventional L-J proportion method ($1\mu\text{g}/\text{mL}$) [12] but resistant to INH using the

BACTEC MGIT960 method ($0.1\mu\text{g}/\text{mL}$, Becton Dickinson Microbiology Systems, MD, USA) [9]. *M. tuberculosis* H37Rv (ATCC 27294) obtained from the Chinese National Reference Laboratory was used as a control.

2.2. Determination of the MIC of INH by Middlebrook 7H9 Broth Microdilution and L-J Agar Dilution. Resazurin was used as an indicator to test the MIC of INH in the Middlebrook 7H9 broth microdilution method [13]. Briefly, a $100\mu\text{L}$ volume of Middlebrook 7H9 broth containing 0.05% Tween 80 and 10% OADC (Sigma, USA) was dispensed into the wells of a 96-well cell culture plate (Corning Coast). INH concentrations, in Middlebrook 7H9 medium, were as follows: 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.6, and 1.8 mg/L . Recovered isolates were collected from L-J slants and homogenized. Turbidity was adjusted to the number 1 McFarland standard (approximately $1 \times 10^7\text{ CFU/mL}$) and the suspension is diluted 1:10 and $100\mu\text{L}$ of the dilution is added in each well that contains $100\mu\text{L}$ of the appropriate INH dilution. The final inoculum concentration was $5 \times 10^4\text{ CFU/mL}$. The plates were sealed and incubated at 37°C for one week. Twenty-five microliter of 0.02% resazurin (Sigma Chem. Co., USA) solution was then added to each well and the plates were incubated for an additional 2 days. A change in color from blue to pink indicated the growth of bacteria and the MIC was read as the minimum INH concentration that prevented the color change in the presence of resazurin.

Determination of the MIC of INH using the L-J proportion method followed the protocol of the Chinese Anti-Tuberculosis Association [12]. INH concentrations used in the L-J medium were 2.0, 1.8, 1.6, 1.2, 1.0, 0.8, 0.6, 0.4, and 0.2 mg/L . About 10^5 CFU were inoculated on the INH-containing medium slants and results were recorded after 5–6 weeks.

2.3. Genomic DNA Isolation, Polymerase Chain Reaction (PCR), and Sequence Analysis. Colonies were first removed from the recovering slants by scraping, resuspended in $500\mu\text{L}$ of TE (10 mM Tris, 1 mM EDTA (pH 8.0)), and killed by heating at 80°C for 30 min. The DNA extraction method, primers (from CyberSyn Co. Beijing, China), and PCR conditions were as described previously [14]. The primers were designed to amplify the *katG* gene, including the region around codon 315, the *inhA* regulatory region, the *inhA* ORF, and *oxyR-ahpC* regions (Table 1) [15, 16]. Both strands were sequenced for confirmation. Mutations were identified by BLAST comparisons with *M. tuberculosis* H37Rv as the reference (GenBank number NC_000962.3).

2.4. Molecular Typing by Spoligotyping and the 12-Locus MIRU Method. Spoligotyping was performed with a commercial kit (Isogen Bioscience BV, Maarssen, The Netherlands) according to the manufacturer’s instructions. Amplification of the direct variant regions for spoligotyping was performed essentially as described previously [17]. Interpretation of spoligotype patterns and assignment of octal codes were based on SITVIT2 database (Pasteur Institute of Guadeloupe, Parris, France), which is an updated version of the

TABLE 1: Primers used for PCR amplification in this study.

Gene	Forward primer, 5'-3'	Reverse primer, 5'-3'
<i>katG</i>	GCT GCT GTG GCC GGT CAA GA	CGT CCT TGG CGG TGT ATT GC
<i>inhA</i> reg	CCT CGC TGC CCA GAA AGG GA	ATC CCC CGG TTT CCT CCG GT
<i>inhA</i> ORF	GAA CTC GAC GTG CAA AAC	CAT CGA AGC ATA CGA ATA
<i>oxyR-ahpC</i>	CTG CGA CGG TGC TGG CACG	CAC GCT GCT GCG GGT GAT TGA T
MIRU and spoligotyping cluster for <i>M. tuberculosis</i> isolates		
Spoligotyping	GGT TTT GGG TCT GAC GAC	CCG AGA GGG GAC GGA AAC
MIRU02	TGG ACT TGC AGC AAT GGA CCA ACT	TAC TCG GAC GCC GGC TCA AAA T
MIRU04	GCG CGA GAG CCC GAA CTG C	GCG CAG CAG AAA CGT CAG C
MIRU10	GTT CTT GAC CAA CTG CAG TCG TCC	GCC ACC TTG GTG ATC AGC TAC CT
MIRU16	TCG GAG AGA TGC CCT TCG AGT TAG	CCC GTC GTG CAG CCC TGG TAC
MIRU20	TCG GAG AGA TGC CCT TCG AGT TAG	GGA GAC CGC GAC CAG GTA CTT GTA
MIRU23	CTG TCG ATG GCC GCA ACA AAA CG	AGC TCA ACG GGT TCG CCC TTT TGT C
MIRU24	CGA CCA AGA TGT GCA GGA ATA CAT	GGG CGA GTT GAG CTC ACA GAA
MIRU26	TAG GTC TAC CGT CGA AAT CTG TGA C	CAT AGG CGA CCA GGC GAA TAG
MIRU27	TCG AAA GCC TCT GCG TGC CAG TAA	GCG ATG TGA GCG TGC CAC TCA A
MIRU31	ACT GAT TGG CTT CAT ACG GCT TTA	GTG CCG ACC TGG TCT TGA T
MIRU39	CGC ATC GAC AAA CTG GAG CCA AAC	CGG AAA CGT CTA CGC CCC ACA CAT
MIRU40	GGG TTG CTG GAT GAC AAC GTG T	GGG TGA TCT CGG CGA AAT CAG ATA

previously released SpolDB4 database (<http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/tsSpoligo.jsp>), as previously described [18].

The numbers of tandem repeats (TRs) at each locus in the isolates were determined on the basis of the number of whole repeats in a PCR product of the size estimated from the gel [19]. Polymerase chain reaction assays for the 12 chosen loci were repeated and compared within and between gels to ensure consistent estimation of size and TR copy number [20].

3. Results

3.1. Genotyping Analysis. Genotyping analysis can determine not only whether an infection results from transmission of the given tuberculosis isolate, but also whether the infection involves more than one strain of *M. tuberculosis*. Results from our genotyping analysis showed that 10 paired isolates belong to the Spoligotype International Type SIT1 (Beijing genotype, 00000000003771) and 6 paired isolates belong to the Spoligotype International Type SIT1634 (MANU2, 77777777723771) (Table 2), a spoligotype that was not found in the 96 randomly selected clinical isolates (Table 3). Three paired isolates were mixtures of the SIT1 and SIT1634 spoligotypes, and one pair was a mixture of SIT1 and the SIT269 (Beijing genotype, 0000000000000771) spoligotypes. Compared with our set of 96 randomly selected isolates from Tianjin, only the Beijing and MANU genotypes were present and the percentage of the MANU genotype was extremely high (20 paired isolates: 15/40, 37.5%; 96 random clinical isolates: 3/96, 3.125%).

Results obtained by using the 12-locus MIRU method [19] showed that 20 pairs of isolates had 14 MIRU patterns. Both the spoligotyping and the MIRU patterns were different in

the isolates named as 6, 12, and 18 pairs, individually. The isolates named as 7 pairs had different spoligotypes, but the same MIRU type (Table 2).

3.2. MICs of the Tested Strains. To identify the differences between the liquid Middlebrook 7H9 and L-J proportion methods in DST, we tested the MICs of each of the 16 paired INH-resistant isolates and 4 pairs of isolates which consisted of different genotypes using both Middlebrook 7H9 broth microdilution and L-J proportion methods. The MICs of all the 24 tested isolates were determined to be greater than $0.1\ \mu\text{g/mL}$ (0.1 to $0.6\ \mu\text{g/mL}$) using the Middlebrook 7H9 broth microdilution method and greater than $0.3\ \mu\text{g/mL}$ (0.4 to $1.8\ \mu\text{g/mL}$) using the L-J proportion method (Table 4). The MICs of 5 pairs of the tested isolates using the L-J proportion method were higher than $1\ \mu\text{g/mL}$, the cutoff concentration for determining drug susceptibility in the L-J agar proportion method in this study (Table 4).

3.3. Sequence Analysis of the Putative INH-Target Genes. Mutations in the *katG* gene were identified in 13 paired isolates, of which each of 12 paired isolates carried the same mutations and one pair which showed a DST discrepancy by MGIT960 and L-J proportion methods carried different base pair at codon 315 (AGC versus AAC). The AGC315AAC mutation was found in 4 paired isolates, while 9 paired isolates carried the mutation AGC315ACC. The AGC315AAC and AGC 315ACC mutations were not associated with specificity to the Beijing or MANU2 genotypes among the tested isolates. Seven paired isolates did not contain mutations in the *katG* gene and no mutations were found in the regulatory sequences and open reading frames (ORF) of the *inhA* and *ahpC* genes in any of the tested isolates (Table 4).

TABLE 2: Genotypes of the 20 isolates with discrepancies in their INH DST as determined by the Middlebrook 7H9 broth microdilution and L-J agar dilution methods.

Pairs	Isolates	Spoligotyping pattern	MIRU pattern
1	2235	777777777723771	1241 2728 3422
	3010	777777777723771	1241 2728 3422
2	3195	000000000003771	1261 2718 3322
	2986	000000000003771	1261 2718 3322
3	3184	777777777723771	2261 2425 3322
	3255	777777777723771	2261 2425 3322
4	2577	000000000003771	1261 2718 3322
	549	000000000003771	1261 2718 3322
5	3478	000000000003771	1361 2618 3322
	3972	000000000003771	1361 2618 3322
6	322	777777777723771	1241 2728 3422
	501	000000000003771	1261 2718 3322
7	2671	000000000000771	1261 2719 3312
	1182	000000000003771	1261 2719 3312
8	2851	000000000003771	1241 2728 3422
	1563	000000000003771	1241 2728 3422
9	2566	777777777723771	1241 2728 3322
	497	777777777723771	1241 2728 3322
10	3079	777777777723771	1241 2728 3322
	2435	777777777723771	1241 2728 3322
11	3995	000000000003771	1261 2728 3322
	4835	000000000003771	1261 2728 3322
12	4394	000000000003771	1261 2718 3322
	4396	777777777723771	1241 2728 3422
13	4124	000000000003771	1361 2615 3322
	4198	000000000003771	1361 2615 3322
14	4192	000000000003771	2261 2615 3322
	4199	000000000003771	2261 2615 3322
15	4348	000000000003771	1261 2628 3321
	4355	000000000003771	1261 2628 3321
16	4482	777777777723771	1241 2618 3322
	1901	777777777723771	1241 2618 3322
17	4484	777777777723771	2261 2631 3321
	1914	777777777723771	2261 2631 3321
18	2098	777777777723771	2261 2631 3321
	2099	000000000003771	1241 2648 3322
19	2785	000000000003771	1241 2648 3422
	1554	000000000003771	1241 2648 3422
20	2789	000000000003771	1241 2648 3422
	1344	000000000003771	1241 2648 3422

Note: order of 12 MIRU loci is 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40.

4. Discussion

Different DST methods have been developed and are used in routine clinical practice such as the conventional L-J methods and the automated MB/BacT (Organon Teknika, Turnhout, Belgium), ESPII (Difco Laboratories, Detroit, Michigan), BACTEC 9000MB (Becton Dickenson Microbiology System,

TABLE 3: Spoligotyping patterns of the 96 randomly selected *M. tuberculosis* isolates.

Number of isolates	Shared types	Spoligotyping pattern
85	Beijing (SIT1)	000000000003771
2	Beijing-like (SIT269)	000000000000771
1	Beijing-like (SIT585)	000000000000031
2	T1 (SIT261)	737777773760771
1	T1 (SIT5)	000677777760771
1	T1 (SIT353)	777777774760771
1	MANU2 (SIT53)	777777777760771
1	Manu_ancestor (SIT523)	777777777777771
1	MANU2 (SIT1195)	777767477763771
1	U (SIT1200)	703777747777771

Sparks, MD), and BACTEC MGIT 960 (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) systems [5, 21–23]. The DST results would be influenced by many steps of the protocol, including the culture and the DST methods. In this study, we analyzed the discrepancy of the drug susceptibility test by the MGIT and L-J methods for the isolates collected from the culture by MGIT and L-J, respectively.

Except for the median time to report the DST results the *M. tuberculosis* complex culture positivity rates were also greatly different in MGIT and L-J [24], which indicated the possible culture preference to somewhat. And the detection time, accuracy, and performance capacity are also variable by different DST methods. Studies reported that the reasons for the different performance capacity among these methods mainly resulted from the different DST systems [23, 25]. The most obvious difference is the drug concentrations used for the DST. In MGIT system, the sensitive strains were susceptible to the INH less than the 0.1 $\mu\text{g}/\text{mL}$, while the concentration of the INH was 1 $\mu\text{g}/\text{mL}$ in L-J system in this study [12, 26, 27]. Of all the 20 paired cases 15 cases had MIC in borderlines between the MGIT and the DST methods, which was a usual reason for the discordant.

Many reports showed that there was a good concordance between DST on L-J and MGIT for INH in DST [25–27]. In this study, we still found that 20 paired isolates with the same genotypes individually showed the discrepancy in the drug susceptibilities to INH according to the MGIT960 testing and L-J proportion methods. Lawson et al. demonstrated that there was a substantial degree of agreement between the two methods, with similar INH and rifampicin DST patterns, but more frequent detection of streptomycin resistance and less frequent detection of ethambutol with L-J than MGIT-960. However, the differences were not statistically significant [25]. A multiple center evaluation showed that the discrepancies in INH susceptibility between the MGIT960 and L-J proportion methods varied from 0% to 1% [9].

Mixed infection with the different genotypes of *M. tuberculosis* in the same patient also affected the DST results even by the same testing systems [28, 29]. In this study heterogeneous genotypes were found in the isolates from each of the 4 patients. Three patients were infected by the different

TABLE 4: MIC of INH and the *katG*, *inhA*, and *oxyR-ahpC* mutations of the 20 pairs of *M. tuberculosis* isolates with DST discrepancies.

Pairs	Isolate*	7H9 Middlebrook ($\mu\text{g/mL}$)	L-J agar ($\mu\text{g/mL}$)	<i>katG315</i>	<i>inhA</i> reg	<i>inhA</i> ORF	<i>oxyR-ahpC</i>
1	2235	0.6	1	AAC	None	None	None
	3010	0.6	1	AAC	None	None	None
2	3195	0.1	1	AGC	None	None	None
	2986	0.1	1	AGC	None	None	None
3	3184	0.4	0.6	ACC	None	None	None
	3255	0.4	0.6	ACC	None	None	None
4	2577	0.2	0.4	AGC	None	None	None
	549	0.2	0.4	AGC	None	None	None
5	3478	0.6	1.2	ACC	None	None	None
	3972	0.6	1.2	ACC	None	None	None
6	322	0.4	1	ACC	None	None	None
	501	0.2	0.6	ACC	None	None	None
7	2671	0.6	1.2	AGC	None	None	None
	1182	0.4	1	AGC	None	None	None
8	2851	0.4	1	AAC	None	None	None
	1563	0.4	1	AAC	None	None	None
9	2566	0.4	1	ACC	None	None	None
	497	0.4	1	ACC	None	None	None
10	3079	0.6	1.4	AGC	None	None	None
	2435	0.6	1.4	AGC	None	None	None
11	3995	0.4	1	ACC	None	None	None
	4835	0.4	1	ACC	None	None	None
12	4394	0.4	0.8	ACC	None	None	None
	4396	0.4	0.8	ACC	None	None	None
13	4124	1	1.4	AAC	None	None	None
	4198	1	1.4	AAC	None	None	None
14	4192	0.4	1	ACC	None	None	None
	4199	0.4	1	ACC	None	None	None
15	4348	0.2	0.8	ACC	None	None	None
	4355	0.2	0.8	ACC	None	None	None
16	4482	0.4	1	AGC	None	None	None
	1901	0.4	1	AGC	None	None	None
17	4484	1	1.8	ACC	None	None	None
	1914	1	1.8	ACC	None	None	None
18	2098	0.4	1	AGC	None	None	None
	2099	0.4	1	AAC	None	None	None
19	2785	0.4	1	AAC	None	None	None
	1554	0.4	1	AAC	None	None	None
20	2789	0.2	0.6	AGC	None	None	None
	1344	0.2	0.6	AGC	None	None	None

Note: *katG315* is the predominant mutation. The wild type is AGC.

*16 isolates with consistent genotype in pair and 4 pairs of isolates (**bold**) with different genotypes in pair.

stains with Spoligotype International Type SIT1634 (Manu2) and Beijing genotypes and 1 patient was infected by the strains with two different Beijing genotypes. And also our test on the mutations of the putative INH-target genes, *katG*, *inhA*, and *ahpC* further confirmed one patient (number 18) with mixed infection by the heterogeneous genotypes (Table 4).

Some mycobacterial characteristics might be associated with particular genotypes. A well-known but controversial

example is that the Beijing family strains of *M. tuberculosis* are often associated with relapse [30], drug resistance [31], and an increased ability to cause disease, to be transmitted within certain geographic settings [32, 33]. The isolates with particular genotypes, such as Spoligotype International Type SIT1634 (Manu2) in this study, showed higher rate of resistance in MGIT960 system than in L-J system. In this study, we found that the percentage of “MANU” genotype

strains was markedly increased in paired isolates whose DST results showed discrepancies (37.5%) compared to the randomly selected clinical isolates (3.125%). An unusually high proportion of strains belonging to the “Manu” clade (27.15%) were also reported by Helal et al. [18]. Interestingly, Manu2 strains (SIT1634) have rarely been reported in Tianjin or even in China as a whole [34, 35] or in the SPOLDB4 database (excluding this study, $n = 3, 1$, from India and 2 from the USA).

In this study, all the 40 isolates were determined as resistant by MGIT and sensitive by L-J, of which twenty-seven isolates were found with mutations in *katG315* and 13 isolates were found with no mutations in *katG315* (Table 4). Those results of the mutations found in the INH-targeted genes supported that the DST result by the MGIT was more accurate than that by the L-J, and we also found that the MICs of some isolates by L-J agar method were very higher than those in the first execution in clinic, which indicated, to some extent, that the operation needs to be improved in proportion method on L-J agar.

5. Conclusion

Our study confirmed that the discrepancies of the DST in *M. tuberculosis* clinical isolates did exist for INH. One of the reasons for the discrepancy is the different test systems between the BACTEC MGIT960 system and the traditional L-J proportion method. Mixed infection by the strains with MANU2 and Beijing genotype patterns could also contribute to drug discrepancies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Zaoxian Mei and Zhaogang Sun contributed equally to this work.

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Research Article

Occurrence of Multidrug Resistant Extended Spectrum Beta-Lactamase-Producing Bacteria on Iceberg Lettuce Retailed for Human Consumption

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Antibiotic resistance in bacteria is a global problem exacerbated by the dissemination of resistant bacteria via uncooked food, such as green leafy vegetables. New strains of bacteria are emerging on a daily basis with novel expanded antibiotic resistance profiles. In this pilot study, we examined the occurrence of antibiotic resistant bacteria against five classes of antibiotics on iceberg lettuce retailed in local convenience stores in Rochester, Michigan. In this study, 138 morphologically distinct bacterial colonies from 9 iceberg lettuce samples were randomly picked and tested for antibiotic resistance. Among these isolates, the vast majority (86%) demonstrated resistance to cefotaxime, and among the resistant bacteria, the majority showed multiple drug resistance, particularly against cefotaxime, chloramphenicol, and tetracycline. Three bacterial isolates (2.17%) out of 138 were extended spectrum beta-lactamase (ESBL) producers. Two ESBL producers (T1 and T5) were identified as *Klebsiella pneumoniae*, an opportunistic pathogen with transferable sulfhydryl variable- (SHV-) and TEM-type ESBLs, respectively. The DNA sequence analysis of the *bla_{SHV}* detected in *K. pneumoniae* isolate T1 revealed 99% relatedness to *bla_{SHV}* genes found in clinical isolates. This implies that iceberg lettuce is a potential reservoir of newly emerging and evolving antibiotic resistant bacteria and its consumption poses serious threat to human health.

1. Introduction

Antibiotic use in the agriculture, aquaculture, and livestock industries has led to the emergence of antibiotic resistance genes (ARG) in the environment [1]. The use of antibiotics as growth promoters in the agriculture industry is particularly egregious; this use accounts for nearly half the 50 million pounds of antibiotics produced in the United States each year [2]. In addition, most antibiotics consumed by livestock or poultry are excreted as biologically active metabolites which can then select for and promote the growth of antibiotic resistant bacteria (ARB) [1]. Both antibiotics and ARB have been detected in animal waste, aquaculture, wastewater, river sediments, and farmland soil [3–6]. Low levels of antibiotics

have been detected in the leaves of growing plants cultivated using antibiotic contaminated soil, water, and sediments, further selecting and promoting the growth of ARB on green leafy vegetables [7]. The concurrence of the selection of ARB on green leafy vegetables and the increasing occurrence of foodborne pathogens on fresh produce is worrying.

There have been several recent outbreaks of foodborne illnesses associated with the consumption of fresh green leafy vegetables [8]. In 2011, 3842 human infections in Germany with enteropathogenic *E. coli* O104:H4 causing hemolytic uremic syndrome were associated with fenugreek seeds [9]. In the United States, spinach grown in Monterey County, California, infected with *E. coli* O157:H7 caused 15 deaths and over 100 hospitalizations [10]. Similarly, there was

an outbreak of Shiga-toxin-producing *E. coli* O157 on lettuce in Netherlands and Iceland in 2007, which resulted in at least 50 illnesses [11], and an outbreak of *Shigella sonnei* associated with iceberg lettuce in Europe in 1995 which resulted in over 100 confirmed cases of shigellosis [12]. Of particular interest to our study is the outbreak of *E. coli* O145 HUS associated with shredded romaine lettuce purchased in Michigan and Ohio in 2010 [13]. The increasing prevalence of foodborne pathogens on green leafy vegetables has been previously been attributed to the bacterial ability to be internalized from contaminated manure or water into the leafy plant tissue [14], specifically within the stomata [15]. These foodborne infections often cause gastrointestinal illnesses and severe cases are generally treated using beta-lactam antibiotics [16], particularly third and fourth generation cephalosporins. However, this treatment option is much less effective if the foodborne pathogens are ARB and extended spectrum beta-lactamase (ESBL) producers [17], as ESBL enzymes hydrolyze this newest generation of beta-lactam antibiotics.

In recent years, a growing number of studies have shown the emergence of bacterial strains resistant to beta-lactams and the main underlying mechanism is the production of beta-lactamase enzymes. The increasing prevalence of ESBL producers in particular is troubling, given their high correlation with multidrug resistance (defined as resistance to three or more classes of antibiotics) [18]. Additionally, the genes encoding beta-lactamases and other antibiotic resistance genes are often found on mobile genetic elements such as transposons and plasmids [19–21]. Thus, the ARB genes can be easily transferred from saprophytic bacteria to opportunistic pathogens. This transfer has already been demonstrated for ampicillin resistance encoded by ESBL genes in environmental bacteria found on lettuce [22] and spinach [23].

Since iceberg lettuce (IBL) is usually consumed rawly and has been identified as a source of foodborne pathogens by the World Health Organization, this study examined whether IBL leaves harbor ARB, specifically ESBL-positive bacteria. Bacteria found on green leafy vegetables are generally assumed to be harmless, but the expression of antibiotic resistance may suggest that the bacteria have other virulence properties [24]. Therefore, potential pathogens, like ESBL-producing *K. pneumoniae*, were further characterized for their ability to cause inflammation and cytotoxicity on human intestinal epithelial cell line CACO-2 [25]. Here we demonstrate that IBL retailed in Michigan carry ESBL-producing multidrug resistant bacteria with resistance genes on self-transmissible plasmids.

Some of these results were presented at the 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, California, Poster Session 088, Presentation C2-703.

2. Materials and Methods

2.1. Bacteriological Analysis of Iceberg Lettuce. In this pilot study, nine iceberg lettuce samples were purchased from local retail markets in Rochester, MI area. The lettuce samples were purchased from national distributors and all samples originated from the Salinas valley in California, a major

leafy vegetable-producing area in the United States [26]. The lettuce samples were stored at 4°C and processed for bacteriological analysis within 24 h of purchase. The samples were processed by first removing the outer leaves and then weighing 25 g of each sample and placing it in a sterile stomach bag with 100 mL of 0.1% peptone water. The stomach bag was sealed and kneaded in a stomacher at 150 rpm for 20 min. The resulting wash was then serially diluted 4 logs in 0.1% peptone water and 0.1 mL of log dilution was plated on tryptic soy agar (TSA, Becton Dickenson) and MacConkey (MAC, Becton Dickenson) plates with and without antibiotics [27]. The antibiotics used included CTX 64 μ g/mL, CIP 4 μ g/mL, GEN 16 μ g/mL, and TET 16 μ g/mL [28]. Antibiotic powders were purchased from Becton Dickenson. The quality of the antibiotic plates was confirmed using the quality control strains *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The number of CFU per gram of iceberg lettuce was determined for each IBL sample in order to determine the microbiological quality of the IBL samples. Single isolated bacterial colonies with distinct colony morphology and pigment production were randomly selected, picked, purified, and stored at -80°C for further analysis.

2.2. Biochemical Identification and Antibiotic Susceptibility Testing. The bacterial isolates from iceberg lettuce were identified using the BD Phoenix Automated Microbiology system [29] which showed correlation with manual identification methods [30]. Briefly, the Phoenix ID broth was inoculated with isolated colonies of bacteria and the bacterial suspension was adjusted to 0.5 optical density at 540 nm with Phoenix AST broth using Phoenix Auto Processor. The Phoenix NMIC/ID-124 combo panels were loaded onto the Phoenix 100 system and samples were processed according to the manufacturer's instructions. Each bacterial isolate was also tested against a panel of six antibiotics using Kirby-Bauer disk diffusion method following CLSI guidelines [28]. The antibiotic disks (Becton Dickenson) used were CTX 30 μ g, cefotaxime/clavulanic acid (CTX-CLA) 30/10 μ g, TET 30 μ g, chloramphenicol (CHL) 30 μ g, GEN 10 μ g, and CIP 5 μ g. Possible ESBL-positive bacteria were identified by their keyhole formation between the CTX and CTX-CA disks and confirmed using the CLSI phenotypic confirmatory test with ceftazidime (CAZ) 30 μ g, ceftazidime/clavulanic acid 30/10 μ g (CAZ-CLA), CTX, and CTX-CLA disks [31]. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively.

2.3. Genomic DNA Extraction and PCR Amplification. Total genomic DNA was extracted from each suspected ESBL-positive strain using a boiling method [32]. For the amplification of the gene sequences, each 50 μ L PCR reaction mixture contained 2 μ L of extracted DNA, 1 picomol of each primer, and 1X Taq-Pro Red Complete master mix (Denville Scientific, Metuchen, NJ) containing a final concentration of 1.5 mM MgCl₂. The primer sequences, annealing temperature, and expected size of the amplicon for ESBL gene sequences are summarized in Table 1. The PCR products were separated by electrophoresis on a 0.7% agarose-TAE gel containing

TABLE 1: Primer sequences used in PCR amplification of ESBL and 16S rRNA gene sequences.

Target gene	Primer sequences (5'-3')	T _m (°C)	Expected amplicon size (bp)	Reference
16S rRNA	F: AGAGTTGATCMTGGCTCAG R: AAGGAGGTGATCCAGCC	60	1400	[38]
bla _{SHV}	F: GGTTATGCGTTATTCGCC R: TTAGCGTTGCCAGTGCTC	60	867	[33]
bla _{TEM}	F: CCGTGTGCCCTTATTCC R: AGCACCATCTCAGCGA	56	800	[35]
bla _{CTX-M}	F: TTTGCGATGTGCAGTACCAAGTAA R: CTCCGCTGCCGGTTTATC	56	500	[35]
bla _{CTX-M-1}	F: AAAAATCACTGCGCCAGTTC R: AGCTTATTCCATGCCACGTT	56	415	[35]

ethidium bromide and photographed using UV light. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively, for SHV [33]. *K. pneumoniae* ATCC 700603 served as the positive control for the 16S rRNA amplification. PCR products that resulted in a band matching the expected size were purified using the QIAGEN PCR Products Purification kit following the manufacturer's instructions. The purified products were then cloned using the pGEM-T Easy Vector I system (Promega, Madison, WI) and sequenced in the ABI Prism 3730 DNA Analyzer (Applied Biosystems) at Wayne State University. The resulting sequences were aligned and compared to known beta-lactamase gene sequences in the BLAST databases at the NCBI website. A neighbor-joining phylogenetic tree was constructed in BioEdit using the bootstrap analysis run by the ClustalW Multiple Alignment tool.

2.4. Conjugal Transfer of Antibiotic Resistance to *E. coli*. Conjugal transfer experiments were done to determine whether beta-lactam resistance can be transferred from the donor environmental isolate to the sodium azide resistant recipient *E. coli* J53. Log cultures of donor and recipient were mixed in 1:1 ratio in 4 mL fresh TSB and the mixture was incubated at 25°C and 37°C for 24 h without shaking. The conjugal mix (0.1 mL) was spread on eosin methylene blue (EMB) agar containing 100 µg/mL sodium azide and 0.25 µg/mL ciprofloxacin. The transconjugants grown on selective media were purified and further tested for cotransference of antibiotic resistance and ESBL using the phenotypic disk diffusion tests recommended by CLSI [28].

2.5. Molecular Analysis, Isolation, and Hybridization Analysis of Plasmids. The plasmid DNA was extracted from *K. pneumoniae* ATCC 700603, *K. pneumoniae* T1, *K. pneumoniae* T5, *S. marcescens* M5, azide resistant *E. coli* J53, *E. coli* V517, and the transconjugants *E. coli* KCl, TC1, and TC5 using the QIAGEN Midiprep kit. The manufacturer's directions were followed for the purification of large, low copy number plasmids. The DNA was separated on a 1.0% agarose-TAE gel electrophoresed at 70 V for 1.5 h and then transferred to a positively charged nylon membrane (Roche) using the manufacturer's directions. Hybridization was carried out at 54°C using an 865 bp digoxigen-labeled bla_{SHV} probe created using

the PCR DIG-labeling kit (Roche) and the ESBL gene primers listed in Table 1 following the manufacturer's instructions.

2.6. Pulsed Field Gel Electrophoresis of Environmental and Clinical Strains of *K. pneumoniae*. Pulsed field gel electrophoresis was performed on T1 and T5 to determine the clonal relationship between these two environmental *K. pneumoniae* strains and clinical *K. pneumoniae* strains. The clinical strains used were isolated from a variety of sources in 2011, including urine, sputum, and wounds. The genomic DNA was prepared using standard procedures and then digested using XbaI (New England BioLabs) [36]. The resulting restriction patterns were interpreted using the criteria proposed by Tenover et al. [37] and our recent study [31].

2.7. Statistical Analysis. Student's *t*-tests were used to determine if there were any significant differences in the total bacterial counts and the ARB count for the iceberg lettuce samples and if there were significant differences in cytokine production between clinical and environmental strains. A one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to evaluate the differences in resistance patterns of the bacterial isolates. Statistical tests were performed using Minitab 16 and Microsoft Office Excel.

2.8. Nucleotide Sequence Accession Numbers. The nucleotide sequence of bla_{SHV} reported in this study has been deposited in GenBank under the accession number JX045654.

3. Results

3.1. Bacteriological Analysis of Iceberg Lettuce. In this preliminary study 9 IBL samples were purchased from the local Rochester retail market between November 2011 and February 2012. The total bacterial plate count was determined for each IBL sample and the average viable plate count was $2.74 \times 10^6 \pm 3.29 \times 10^6$ (mean \pm standard deviation, CFU/g). The average bacterial count for the total CTX-resistant community was $1.54 \times 10^5 \pm 2.79 \times 10^5$ (mean \pm standard deviation, CFU/g), while the average bacterial count for the total tetracycline-resistant community was $2.89 \times 10^2 \pm 5.75 \times 10^2$ (mean \pm standard deviation, CFU/g). No bacteria grew on

TABLE 2: Identification of 50 representative bacteria from the 138 bacteria selected from iceberg lettuce.

Genus	Number of isolates (%)	Species	Number of isolates (%)
<i>Pseudomonas</i>	36 (72%)	<i>fluorescens</i>	7 (14%)
		<i>oryzihabitans</i>	1 (2%)
		<i>putida</i>	5 (10%)
		Unidentified	23 (46%)
<i>Pantoea</i>	4 (8%)	<i>agglomerans</i>	4 (8%)
<i>Klebsiella</i>	2 (4%)	<i>pneumoniae</i>	2 (4%)
<i>Serratia</i>	2 (4%)	<i>marcescens</i>	2 (4%)
<i>Achromobacter</i>	2 (4%)	Unidentified	2 (4%)
<i>Pasteurella</i>	1 (2%)	<i>pneumotropica</i>	1 (2%)
<i>Suttonella</i>	1 (2%)	<i>indologenes</i>	1 (2%)
<i>Mannheimia</i>	1 (2%)	<i>haemolytica</i>	1 (2%)
<i>Cellulomonas</i>	1 (2%)	<i>turbata</i>	1 (2%)
Total	50 (100.00%)		50 (100.00%)

the plates containing high concentrations of GEN and CIP. All of the plates containing antibiotics had significantly lower plate counts (P value < 0.05) than those without antibiotics. Given that the microbiological safety standard for green leafy vegetables is 10^8 CFU/g, this suggests that all of the IBL samples were microbiologically safe for consumption. The predominant microbial community on iceberg lettuce was determined by cultivation of bacteria on TSA and MAC plates.

3.2. Biochemical Identification and Antibiotic Susceptibility Testing. The BD Phoenix Automated Microbiology system uses biochemical tests to identify bacteria and a total of 50 cultivable bacterial isolates were identified. The BD Phoenix identification system showed the presence of *Achromobacter* species, *K. pneumoniae*, *Mannheimia haemolytica*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas oryzihabitans*, and *S. marcescens*. *Pseudomonas* species was the most predominant bacteria found on IBL, as seen in Table 2. All nine IBL samples contained drug resistant bacterial isolates. Among the 138 bacterial isolates tested, 86% were resistant to CTX, 83% to CHL, 44% to TET, 5% to CIP, and 4% to GEN. In addition, the isolates showed different antibiotic resistant patterns. 6.5% were resistant to only one antibiotic; 40.6% were resistant to 2 antibiotics (CTX, CHL); 40.6% were resistant to 3 antibiotics (CTX, TET, and CHL); 0.71% was resistant to 4 antibiotics; and 0% was resistant to 5 antibiotics (Table 3). Three of the nine strains resistant to only one antibiotic were resistant to CTX. Only 3 (2.1%) were detected as ESBL producers. It is interesting to note that all 3 ESBL-positive strains were isolated from a single IBL sample. A one-way ANOVA on the resistance patterns of these isolates yielded highly significant variation among the iceberg lettuce samples (P value < 0.001). The Tukey test for the separation of means demonstrated that the *K. pneumoniae* isolates T1 and T5 had significantly higher antibiotic resistance than the other bacterial isolates. Both of these

isolates had the antibiotic resistance pattern CTX CHL GEN CIP, and the third ESBL-producing isolate, *S. marcescens*, demonstrated resistance against CTX and CIP.

3.3. Sequencing and Phylogenetic Analysis of ESBL Genes. The three ESBL-positive strains were designated T1, T5, and M5. T1 and T5 were both *K. pneumoniae* isolates which contained SHV-type and TEM-type ESBLs, respectively. M5 was a *S. marcescens* isolate that contained a CTX-M-1 beta-lactamase, as detected by PCR. The ESBL sequences amplified from these three isolates were compared against known sequences using the NCBI database BLASTn (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi#>). A unique 751 bp sequence from one *K. pneumoniae* (T1) strain was 99% identical with positions 44–799 of an 852 bp SHV-11 sequence from a clinical isolate of *K. pneumoniae* from Austria (GenBank sequence accession number JN676837) (Figure 1). This sequence is identical to the originally reported sequence except for a single nucleotide change (GAA→GGA at position 131) which encoded for a change in amino acid from glutamic acid to glycine at position 44 and a single nucleotide insertion (CGA→CCGA at position 792) which encoded for a change in 3 amino acids (S→E at position 265, M→Y at position 266, and A→G at position 267). Unlike the ESBL gene sequence from T1, the ESBL gene sequences from T5 and M5 were identical to previously characterized ESBLs. The phylogenetic analysis of SHV-11 amino acid sequences derived from ESBL genes present on pOU11 revealed the divergence from the existing sequences indicating an emergence of a variant of SHV gene family (Figure 2).

3.4. Conjugal Transfer of Antibiotic Resistance to *E. coli*. In order to determine the potential transfer of antibiotic resistance genes to other bacteria, conjugal transfer experiments were performed with *K. pneumoniae* T1 and T5 and *S. marcescens* M5 as the donors and sodium azide resistant *E. coli* J53 as the recipient at 25°C and 37°C (Figure 3). The reference strain *K. pneumoniae* ATCC 700603 was used as a positive control. A combination of antibiotic resistance traits was transferred from the donors T1 and T5 to the recipient *E. coli* (CTX CHL GEN CIP from T1 and CTX GEN CIP from T5) when transconjugants were selected on EMB plates containing ciprofloxacin. The frequency of conjugal transfer of pOU11 plasmid was 10^{-4} for both the *K. pneumoniae* donors into the recipient *E. coli* J53 Az^r. However, the transfer of antibiotic resistance from *S. marcescens* M5 to *E. coli* was not successful.

3.5. Southern Hybridization Analysis of Plasmids. The confirmation of the transfer of antibiotic resistance genes on a plasmid was done by plasmid analysis on a 1.0% agarose-TAE gel (Figure 4). Both the donors and the transconjugants carried plasmids of the same size. Similar results were seen for the other ESBL-containing *K. pneumoniae* isolate T5. The presence of a gene encoding an SHV-type ESBL was confirmed to be on a plasmid (pOU11) on the environmental isolate T1 with Southern blotting (Figure 4(b)) and comparison with the reference strain *K. pneumoniae* ATCC 700603.

TABLE 3: Multidrug resistance in bacterial isolates from iceberg lettuce.

Number of types antibiotic resistance	Number of isolates (%)	Antibiotic resistance patterns (number of isolates)
0	15 (10.9%)	
1	9 (6.5%)	CTX (3), CIP (2), CHL (2), GEN (2)
2	56 (40.6%)	CTX CHL (52), CTX TET (3), CTX CIP (1)
3	56 (40.6%)	CTX CHL TET (56)
4	2 (1.5%)	CTX CHL GEN CIP (2)
5	0 (0%)	

T1 SHV [JX045654]	LCIIS	LLATLPLAVH	ASPQPLEQIK	QSESQLSGRV	GMIEM	50
SHV-11 [AEZ49553]	LCIIS	LLATLPLAVH	ASPQPLEQIK	QSESQLSGRV	GMIEM	50
SHV-18 [AAF64386]	LCIIS	LLATLPLAVH	ASPQPLEQIK	ISESQLSGSV	GMIEM	50
TEM-1 [AAR25033]	VALIP	FIAAFCLPVF	AHPETLVVKV	DAEDQLGARV	GYIEL	50
CTX-M-1 [P28585]	TATVT	LLLGGSVPLYA	QTADVQQKLA	ELEHQSGGRV	GVAVD	50
T1 SHV [JX045654]	DLASG	RTLTAWRADE	RFPMMSTFKV	VLCGAVLARV	DAGDE	90
SHV-11 [AEZ49553]	DLASG	RTLTAWRADE	RFPMMSTFKV	VLCGAVLARV	DAGDE	90
SHV-18 [AAF64386]	DLASG	RTLTAWRADE	RFPMMSTFKV	VLCGAVLARV	DAGDE	90
TEM-1 [AAR25033]	DLNSG	KILEBSFRPEE	RFPMMSTFKV	DLCGAVLRSV	DAGDE	90
CTX-M-1 [P28585]	MAD-	NSQIQLWRADE	RFAMOSTSKV	MAVAAVLKKS	ESEPN	89
T1 SHV [JX045654]	QLERK	IHYRQQDLDV	YSPVSEKHLA	DGMTVGEELCA	AAITM	130
SHV-11 [AEZ49553]	QLERK	IHYRQQDLDV	YSPVSEKHLA	DGMTVGEELCA	AAITM	130
SHV-18 [AAF64386]	QLERK	IHYRQQDLDV	YSPVSEKHLA	DGMTVGEELCA	AAITM	130
TEM-1 [AAR25033]	QLCRR	IHYSQNDLVE	YSPVTEKHLT	DGMTVPELCS	AAITM	130
CTX-M-1 [P28585]	LNQR	VEIKKSLDLV	YNPIAEKHVD	GTMSLAElsa	AALQY	129
T1 SHV [JX045654]	SDNSA	ANLLLATVGG	PAGLTAFLRQ	IGDNVTRLDR	WETEL	170
SHV-11 [AEZ49553]	SDNSA	ANLLLATVGG	PAGLTAFLRQ	IGDNVTRLDR	WETEL	170
SHV-18 [AAF64386]	SDNSA	ANLLLATVGG	PAGLTAFLRQ	IGDNVTRLDR	WETEL	170
TEM-1 [AAR25033]	SDNTA	ANLLLTIGG	PKELTAFLHN	MGDHVTRLDR	WEPEL	170
CTX-M-1 [P28585]	SDNVA	MNLISHIVGG	PASVTAFAARQ	LGDETFRLDR	TEPTIL	169
T1 SHV [JX045654]	NEALP	GDARDTTTPA	SMAATLRKLL	TSQRSLARSQ	RQLLQ	210
SHV-11 [AEZ49553]	NEALP	GDARDTTTPA	SMAATLRKLL	TSQRSLARSQ	RQLLQ	210
SHV-18 [AAF64386]	NEALP	GDARDTTTPA	SMAATLRKLL	TSQRSLARSQ	RQLLQ	210
TEM-1 [AAR25033]	NEAIP	NDERDTTMPA	AMAATLRKLL	TCEULLLASR	QQLID	210
CTX-M-1 [P28585]	NAIIP	GDPRTTTSPPR	AMAATLRNLT	LKGALGDSQR	AQLVT	209
T1 SHV [JX045654]	WMVDD	RVAGPLIRSV	LPAGWFIADK	TGAGERGARG	IVALL	250
SHV-11 [AEZ49553]	WMVDD	RVAGPLIRSV	LPAGWFIADK	TGAGERGARG	IVALL	250
SHV-18 [AAF64386]	WMVDD	RVAGPLIRSV	LPAGWFIADK	TGAAKRGARG	IVALL	250
TEM-1 [AAR25033]	WMEAD	KVAGPLRSA	LPAGWFIADK	SGAGERGSRG	IIIAL	250
CTX-M-1 [P28585]	WMKGN	TTGAASIQAG	LPASWVVGDK	TGSGDYGTTN	DAVI	249
T1 SHV [JX045654]	GPNNK	AERIVVIYL	DTPAEYG			272
SHV-11 [AEZ49553]	GPNNK	AERIVVIYL	DTPASM			271
SHV-18 [AAF64386]	GPNNK	AERIVVIYL	DTPASMA			272
TEM-1 [AAR25033]	GPDGK	PSRIVVIYTT	GSOAPMD			272
CTX-M-1 [P28585]	WPKDR	APLILVFTYFT	QPQPKA			271

FIGURE 1: Amino acid sequence analysis of T1 SHV and other ESBLs. Amino acid substitutions are highlighted in red boxes. GenBank accession numbers are given in brackets and residue numbers are given in terms of the previously characterized SHV-11 from a clinical isolate in Austria (GenBank accession number AEZ49553).

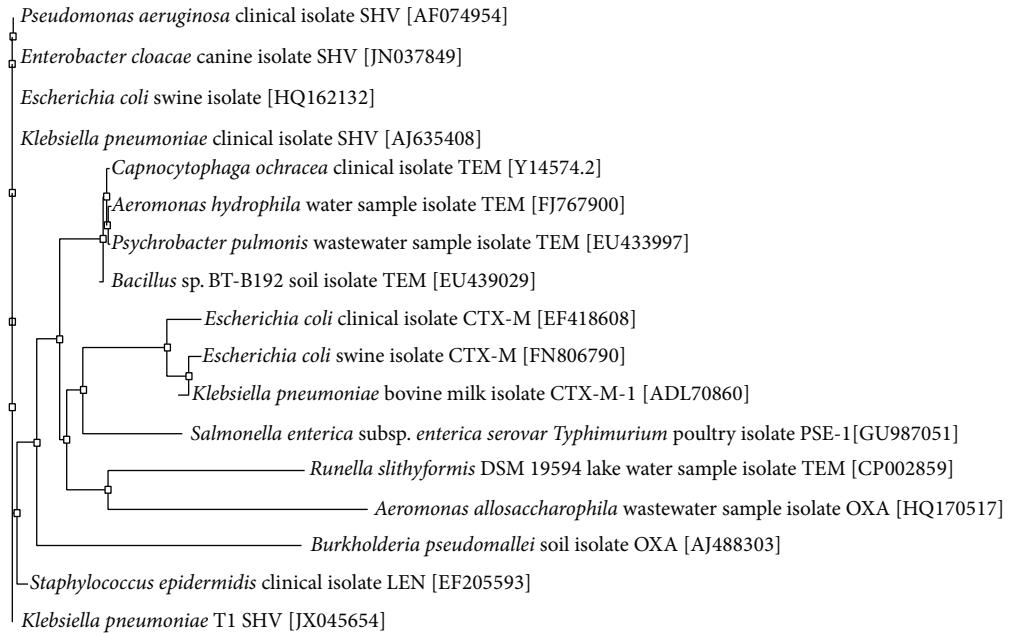


FIGURE 2: Phylogenetic tree of ESBL sequences. The phylogenetic tree represents a majority rule consensus tree based on protein similarity using neighbor joining. Bootstrap values (total 100) are calculated with neighbor joining and maximum likelihood methods. *Klebsiella pneumoniae* SHV is the outgroup.

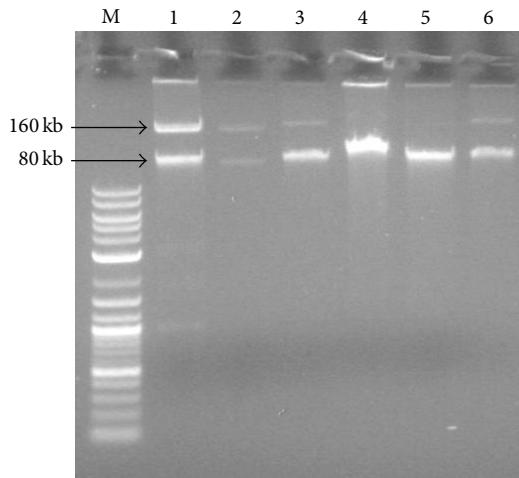


FIGURE 3: Plasmid profile of donors and transconjugants. Lane M: 1 kb linear DNA marker (New England BioLabs), Lane 1: *K. pneumoniae* ATCC 700603, Lane 2: *E. coli* transconjugant of *K. pneumoniae* ATCC 700603 mated at 37°C, Lane 3: *E. coli* transconjugant of *K. pneumoniae* ATCC 700603 mated at 25°C, Lane 4: *K. pneumoniae* T1, Lane 5: *E. coli* transconjugant of T1 mated at 37°C, and Lane 6: *E. coli* transconjugant of T1 mated at 25°C.

3.6. Pulsed Field Gel Electrophoresis of Environmental and Clinical Strains of *K. pneumoniae*. Pulsed field gel electrophoresis of the environmental *K. pneumoniae* strains was conducted to determine the clonal relationship between these strains and a variety of clinical strains. As shown in Figure 5,

genomic DNA of each isolate was spliced into 11 to 16 fragments and none of them showed similar PFGE pattern in the bands. Therefore, despite the relatedness of the ESBL gene sequences, the environmental strains of *K. pneumoniae* isolated in this study are of different clonal types compared to clinical strains.

4. Discussion

Despite the high percentage of ARB on IBL, we found a very low prevalence of ESBL producers which is consistent with previous studies on green leafy vegetables [23, 24, 38]. Similar to our findings that *K. pneumoniae* on IBL harbors ABR genes on self-transmissible plasmids, others have also shown the location of ABR genes on self-transmissible plasmids [23]. Interestingly, we found that *K. pneumoniae* isolate, T1, contained a unique ESBL gene sequence (SHV-type) that was for the first time detected in USA. The detection of this unique SHV-type gene sequence in this pilot study suggests that the IBL is a reservoir of emerging and novel ESBL genes. Moreover, the SHV gene sequence was 99% identical to a SHV-11 gene sequence from a clinical isolate of *K. pneumoniae* in Austria (GenBank accession AEZ49553). This gene sequence is also 99% similar to a gene sequence isolated from *Enterobacter cloacae* from a domestic dog, indicating that these sequences are widely disseminated (Figure 1). For example, Mesa and colleagues detected ESBL-producing bacteria belonging to family Enterobacteriaceae in human feces, wastewater, animal farms, and food [33]. They determined that the occurrence of ESBL-producing enterobacteria in food was relatively low, 0.4% compared to 2.1% in this study. However, it

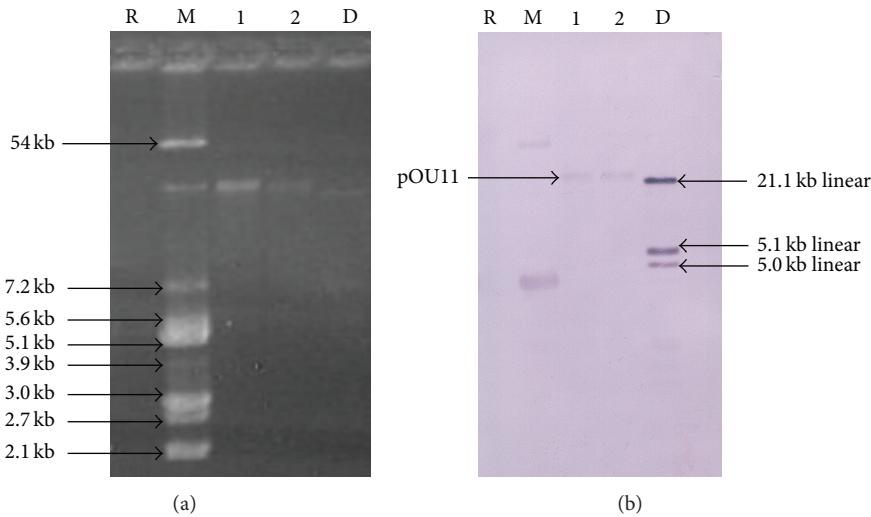


FIGURE 4: Gel electrophoresis and Southern hybridization of donors and transconjugants. (a) DNA isolated from an environmental strain of *K. pneumoniae* and reference strains of *E. coli* and *K. pneumoniae* and (b) Southern hybridization of an environmental strain of *K. pneumoniae* using the *bla_{SHV}* probe. Lane R: reagent control, Lane M: *E. coli* V517 plasmids, Lane 1: *K. pneumoniae* ATCC 700603 plasmids (used as a reference), Lane 2: *K. pneumoniae* T1 plasmids, and Lane D: DIG-labeled DNA ladder III (Roche), a linear DNA.

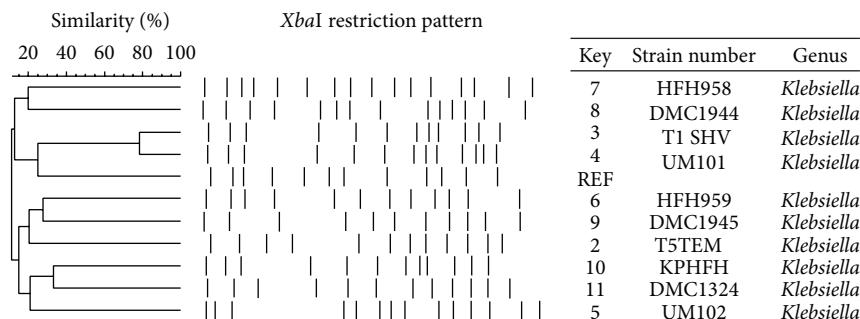


FIGURE 5: Pulsed field gel electrophoresis of clinical and environmental strains of *K. pneumoniae*. Strain HFH958 is an ESBL-positive clinical strain isolated from a wound; strain DMC1944 is an ESBL-positive clinical strain isolated from urine; strain T1 SHV is one of the ESBL-positive environmental strains discussed in this study; strain UM101 is an ESBL-negative clinical strain isolated from lungs; REF refers to the control strain *K. pneumoniae* ATCC 700603; strain HFH959 is an ESBL-positive clinical strain isolated from sputum; strain DMC1945 is an ESBL-positive clinical strain isolated from sputum; strain T5TEM is one of the ESBL-positive environmental strains discussed in this study; strain KPHFH is an ESBL-positive clinical strain; DMC1324 is an ESBL-negative clinical strain; UM102 is an ESBL-negative strain isolated from urine. All these *K. pneumoniae* strains were isolated in 2011.

must be noted that the Enterobacteriaceae studied by Mesa et al. were isolated primarily from cooked foods [33].

The first descriptions of ESBL genes involved point mutations of TEM- and SHV-type enzymes produced by clinical isolates, but the prevalence of CTX-M-type enzymes (which preferentially cleave cefotaxime over ceftazidime) has increased dramatically in the last two decades [17]. These three types of ESBL enzymes are considered the most prevalent and the most mutable, as there are now over 170 characterized SHV-type enzymes, 200 TEM-type enzymes, and 130 CTX-M-type enzymes. CTX-M-type enzymes are thought to have originated in an environmental strain of *Kluyvera* spp. and are frequently produced by environmental isolates. For instance, Raphael et al. found that gene sequences in Gram-negative saprophytes on spinach were 100% identical

to previously recognized CTX-M-type gene sequences from clinical isolates [38]. Our data also supports these findings, as we showed the presence of CTX-M-1-type beta-lactamase gene sequence in *Serratia marcescens*. Our phylogenetic analysis revealed that the IBL SHV-11 sequences did not match with known SHV genes (Figure 2).

To our knowledge, this is the first report of ESBL-producing enterobacteria on IBL retailed in Michigan markets in USA. Raphael et al. observed an ESBL incidence rate of 2.3% among bacterial isolates from spinach [38] and Bezanson et al. detected ESBL activity in 1.9% of the bacteria isolated from lettuce [22]. In contrast to previous studies on raw salad vegetables where low frequencies of multidrug resistant bacteria were observed (0–23%), we found that the vast majority of our isolates were resistant to multiple antibiotics

[22]. These studies excluded species with intrinsic resistance from antibiotic susceptibility tests, such as *Klebsiella* species from ampicillin susceptibility tests [34]. However, in other studies where isolates are not excluded on the basis of intrinsic resistance, high (95%) frequencies of multidrug resistant bacteria were seen on spinach [23]. Despite the high incidence of multidrug resistant bacteria on our IBL samples, we did not observe a high abundance of aminoglycoside or TET resistance in contrast to a recent study conducted in Costa Rica [39]. Rodriguez et al. observed proportional abundance rates of cultivable oxytetracycline-resistant and GEN-resistant isolates between 10% and 100%, but this abundance of antibiotic resistance can be explained by the regular application of GEN and oxytetracycline to the soil on these farms [39]. However, these antibiotics are not used regularly in the United States for the production of vegetables [6] and therefore we observed much lower occurrence of aminoglycoside and tetracycline resistance. Although these antibiotics are not used directly in agriculture, untreated manure or irrigation water containing these antibiotics has been shown to be a possible source for antibiotic resistance in bacteria on green leafy vegetables like iceberg lettuce [24]. In addition to high frequencies of ARB, iceberg lettuce has been implicated in multiple outbreaks of foodborne illnesses in Europe, particularly salmonellosis and shigellosis [12, 40]. Previous outbreaks have involved completely susceptible strains of foodborne pathogens [12]. However, further investigation revealed that several other fecal coliforms present on the source iceberg lettuce were ARB [12]. The presence of these bacteria and their antibiotic resistance determinants on IBL, which is commonly consumed rawly in hospital settings, is the cause for concern, since the ESBL-positive *K. pneumoniae* strains isolated in this study could act as nosocomial pathogens, particularly among immunocompromised patients [35]. The transferability of pOU11 encoding antibiotic resistance at 25°C and 37°C suggests a potential for the resistance to be transferred to other foodborne pathogens such as *Salmonella*, *Shigella*, *Aeromonas*, *Vibrio*, and *E. coli* O157:H7 in hospital and environment settings. Previous studies have shown the thermosensitivity and transferability of antibiotic resistant plasmids at wide range of temperatures are because of formation of temperature dependent synthesis of pili. Since fresh produce (including lettuce) is often served at room temperature (25 degrees Celsius), we studied whether this temperature allows for the transferability of DNA through mechanisms such as increased pili formation or type IV secretion systems [41]. At 37°C the virulence genes are derepressed [42] and our pOU11 plasmid effectively transfers at both temperatures (Figure 3). More research is needed to determine the exact mechanism of evolution and transfer of novel infectious genes expressing virulence properties and antibiotic resistance in ready-to-eat food.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Characterization of Multidrug Resistant Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* among Uropathogens of Pediatrics in North of Iran

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Escherichia coli remains as one of the most important bacteria causing infections in pediatrics and producing extended-spectrum beta-lactamases (ESBLs) making them resistant to beta-lactam antibiotics. In this study we aimed to genotype ESBL-producing *E. coli* isolates from pediatric patients for ESBL genes and determine their association with antimicrobial resistance. One hundred of the *E. coli* isolates were initially considered ESBL producing based on their MIC results. These isolates were then tested by polymerase chain reaction (PCR) for the presence or absence of *CTX*, *TEM*, *SHV*, *GES*, and *VEB* beta-lactamase genes. About 30.5% of isolated *E. coli* was ESBL-producing strain. The *TEM* gene was the most prevalent (49%) followed by *SHV* (44%), *CTX* (28%), *VEB* (8%), and *GES* (0%) genes. The ESBL-producing *E. coli* isolates were susceptible to carbapenems (66%) and amikacin (58%) and showed high resistance to cefixime (99%), colistin (82%), and ciprofloxacin (76%). In conclusion, carbapenems were the most effective antibiotics against ESBL-producing *E. coli* in urinary tract infection in North of Iran. The most prevalent gene is the *TEM*-type, but the other resistant genes and their antimicrobial resistance are on the rise.

1. Introduction

Multidrug resistant Gram-negative bacilli have been increasingly responsible for life-threatening infections all over the world [1, 2]. The extended-spectrum beta-lactamases (ESBLs) are class A plasmid mediated enzymes that hydrolyze oxyiminocephalosporin and monobactam antibiotics but are inhibited by clavulanic acid in vitro [3, 4]. Bacteria harboring ESBLs confer significant resistance to penicillin, narrow and extended-spectrum cephalosporin, and aztreonam

antibiotics. They also frequently show resistance to aminoglycosides, trimethoprim/sulfamethoxazole, and quinolones [5, 6]. Different types of ESBLs have been found in different countries. The *TEM* and *SHV* types were first reported from *Klebsiella pneumoniae* in Western Europe [7]. The *VEB* was first found in a single isolate of *E. coli* in Vietnam [5]. But recently, the *CTX* type (mainly cefotaximases) is being detected with increasing frequency, particularly in ESBL-producing *E. coli* [8]. The *GES-5* gene was first detected on plasmid in *Escherichia coli* from Greece in 2004 and later it

was isolated from *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa* [9]. The CTX-M enzymes are much more active against cefotaxime and ceftriaxone than against ceftazidime [3, 10, 11].

Genotypes of ESBLs producing isolates may be associated with the antibiotic resistance pattern, as it has been reported previously that the presence of CTX-M gene has been associated with the resistance to fluoroquinolones, aminoglycosides, and cotrimoxazole [11].

Extensive use of expanded-spectrum antibiotics is one of the most important factors associated with high resistance to antibiotics and high prevalence of ESBLs [12–14].

Since increasing the rate of multidrug resistant ESBL in North of Iran and antibiotics use varies in different regions and can cause variation in the prevalence of ESBL genotypes, we decided to determine the genotype of ESBL-producing *E. coli* in urinary tract infection regarding the CTX, TEM, SHV, GES, and VEB genes and their antimicrobial resistance in the North of Iran.

2. Material and Methods

2.1. Urine Collection and Bacterial Isolation. Urine samples were collected from pediatric patients during a 6-month period at Buali Sina Hospital (a tertiary referral pediatric hospital) in the North of Iran. Urine samples were collected by either midstream clean catch, suprapubic bladder aspiration (SPA), or transurethral bladder catheterization (TUBC) [15]. The samples were inoculated on 5% blood agar and MacConkey's agar and the *E. coli* isolates were identified by using standard methods.

2.2. Antimicrobial Susceptibility Testing. Antibiotic susceptibility was determined by the Kirby-Bauer disc diffusion test on Mueller Hinton agar plate and the results were evaluated according to the Clinical Laboratory Standards Institute (CLSI) guideline 2011 [16].

2.3. Detection of ESBL-Producing *E. coli* by MIC. The *E. coli* isolates were considered potential carrier of ESBL enzyme when they showed resistance to cefotaxime and ceftriaxone. The MIC (minimum inhibitory concentration) of fourteen antibiotics including ceftazidime, ceftriaxone, cefotaxime, ceftizoxime, cefepime, cefixime, gentamicin, amikacin, meropenem, imipenem, ciprofloxacin, cotrimoxazole, colistin, and piperacillin/tazobactam (Sigma Chemical Co., Germany) for resistant *E. coli* strains was determined by the agar dilution method [3]. The MIC was determined on Mueller Hinton agar with twofold dilutions of antibiotics concentration (from $0.5 \mu\text{g}/\text{mL}$ to $256 \mu\text{g}/\text{mL}$ and $10 \mu\text{L}$ of microbial suspension). Microbial growth was observed and documented after 24 hours of incubation at 35°C . The result was reported according to CLSI 2011 guidelines and divided into three categories: resistant, intermediate, and susceptible. The ESBL-producing *E. coli* isolates were considered resistant to both cefotaxime and ceftazidime if their MIC was $\geq 2 \mu\text{g}/\text{mL}$ in accordance with CLSI criteria [17].

2.4. DNA Isolation and Genotyping. A single colony from each ESBL-producing isolate was transferred into $100 \mu\text{L}$ of distilled water and the bacterial DNA was extracted by using a commercial DNA extraction kit (RTA, Ankara, Turkey). Bacterial genes associated with antimicrobial resistance phenotypes were detected by PCR amplification of target genes by using specific PCR primers (Table 1) in Eppendorf thermal cycler (Eppendorf, Germany). Table 1 shows primer sequences and specific thermal profile from TEM, SHV, CTX, VEB, and GES genes. PCR mixtures were prepared by using $5 \mu\text{L}$ template DNA, $12.5 \mu\text{L}$ PCR master mix; $1 \times$ PCR buffer [Tris-Cl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 1.5 mM MgCl_2] (pH 8.7), $200 \mu\text{M}$ dNTP, and $1 \mu\text{L}$ of each 10 pM primer and 0.5 U Taq DNA polymerase (Takara, Kyoto, Japan) in a final volume of $25 \mu\text{L}$. In all experiments, the following reference strains were used as positive controls: *K. pneumoniae* 7881 (CTX-M), *K. pneumoniae* 7881 strain (containing TEM and SHV), *P. aeruginosa* ATCC 27853 (VEB-1), and *K. pneumoniae* (GES) which was kindly provided by Professor P. Nordmann CHU Bicetre, France. A non-ESBL-producing strain (*E. coli* ATCC 25922) was used as a negative control.

3. Results

3.1. ESBL-Producing *E. coli*. Of 327 uropathogen *E. coli* isolates, one hundred (30.5%) were positive for extended-spectrum beta-lactamases. The ESBL isolates showed highest susceptibility to carbapenems (66%) and amikacin (58%) which is in accordance with 2011 CLSI criteria for MIC test (Table 2). The highest rate of resistance was observed for the following antibiotics: cefixime (99%), colistin (82%), and ciprofloxacin (76%).

3.2. Prevalence of ESBL Genes. The results of ESBL genotyping are shown in Figure 1. The TEM gene was the most prevalent (49%) followed by SHV (44%), CTX (28%), VEB (8%), and GES (0%) genes. None of our isolates carried the GES gene. About 12% of ESBL isolates were shown to have both TEM and CTX-M genes. Overall, 30% of isolates carried 2 resistant genes (Figure 2).

3.3. Association of Antimicrobial Resistance with Resistant Genes. Table 3 shows the presence or absence of resistant genes, susceptibility, and resistance to different antimicrobial agents. In most cases, there were not any significant differences regarding presence or absence of genes expression. Interestingly, higher resistance to cefotaxime, amikacin, and ceftriaxone was found in TEM negative group ($P = 0.04$, $P = 0.008$, and $P = 0.02$, resp.).

Resistance to cotrimoxazole, imipenem, amikacin, and third generation cephalosporins was observed more in CTX-M positive isolates than in CTX-M negative isolates. The presence of VEB gene was associated with higher resistance to carbapenems, gentamicin, and third generation cephalosporins. The presence of VEB gene was significantly associated with resistance to ceftazidime ($P = 0.05$). The presence of SHV gene was also associated with aminoglycosides and ciprofloxacin resistance. Our results also

TABLE 1: The sequences of primers and thermal condition used in PCR amplification.

Target genes	Primer used (5'-3')	Thermal cycling condition	PCR product size
TEM	TAATCAGTGAGGCACCTATCTC GAGTATTCAACATTCCGTGTC [18, 19]	94°C 3 min → 35 × [94°C 30 sec, 45°C 45 sec, 72°C 40 sec] → 72°C 7 min	800 bp
CTX	TTTGCATGTGCAGTACCAAGTAA CGATATCGTTGGTGGCATA [20]	94°C 5 min → 40 × [94°C 45 sec, 53.1°C 45 sec, 72°C 1 min] → 72°C 7 min	593 bp
VEB	CGACTTCCATTCCCCGATGC GGACTCTGCAACAAATACGC [9]	93°C 3 min → 40 × [93°C 1 min, 54.9°C 1 min, 72°C 1 min] → 72°C 7 min	585 bp
SHV	GGTTATGCGTTATTCGCC TTAGCGTTGCCAGTGCTC [21]	1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C, 1 min at 60°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	867 bp
GES	ATGCGTTCATTCACGCAC CTATTGTCGTGCTCAGG [22]	1 cycle of 5 min at 95°C; 30 cycles of 1 min at 95°C, 45 sec at 55°C, 1 min 30 sec at 72°C; 1 cycle of 8 min at 72°C	846 bp

TABLE 2: Percentage of antimicrobial susceptibility in ESBL-producing *E. coli* strains based on MIC results.

Antimicrobial agents	R (%)	I (%)	S (%)	CLSI breakpoints ($\mu\text{g/mL}$)	
				S	R
Cephalosporins					
Cefepime	67	13	20	≤ 8	≥ 32
Cefixime	99	0	1	≤ 0.25	≥ 1
Ceftriaxone	28	42	30	≤ 8	≥ 64
Ceftazidime	19	26	55	≤ 16	≥ 32
Ceftizoxime	46	27	27	≤ 8	≥ 64
Cefotaxime	13	40	47	≤ 8	≥ 64
Carbapenems					
Imipenem	23	11	66	≤ 4	≥ 16
Meropenem	18	15	67	≤ 4	≥ 16
Aminoglycosides					
Amikacin	34	8	58	≤ 16	≥ 64
Gentamicin	37	12	51	≤ 4	≥ 16
Others					
Ciprofloxacin	76	0	24	≤ 1	≥ 4
Colistin	82	0	18	≤ 2	≥ 4
Trimethoprim/sulfamethoxazole	65	7	28	$\leq 2/38$	$\geq 4/76$
Piperacillin/tazobactam	20	38	42	$\leq 16/4$	$\geq 128/4$

R: resistance, I: intermediate, S: sensitive, CLSI: Clinical Laboratory Standards Institute, and ESBL: extended-spectrum beta-lactamase.

showed a correlation between the presence of resistant genes and high rate of resistance to cefixime, colistin, and cefepime.

4. Discussion

In this study, attempt has been made to genotype the ESBL-producing *E. coli* isolates from pediatric patients for *CTX*, *TEM*, *SHV*, *GES*, and *VEB* genes and determine their association with antimicrobial resistance. The high prevalence of ESBL-producing *E. coli* (30.5%) and their high level of resistance to broad spectrum antimicrobial agents in ESBL-producing *E. coli* (e.g., 34% resistance to carbapenems) are

reported in this study. In addition, our study also highlights an association between the presence of *SHV* gene and resistance to aminoglycosides and fluoroquinolones antibiotics.

The prevalence of ESBL-producing *E. coli* isolates varies in different parts of the world and even among different hospitals within a country. The rate of prevalence in our center was about 30.5% which is close to the results reported by other studies in different regions of Iran [18, 23–25]. The rates of ESBL-producing *E. coli* were lower in other countries such as India (27%), Lebanon (13.3%), Korea (9.2%), and Turkey (17%) [26, 27].

In addition to beta-lactam antibiotics, ESBLs producing isolates are also resistant to other antimicrobial

TABLE 3: Association between gene expression and antimicrobial nonsusceptibility in ESBL-producing *E. coli*.

Antimicrobial agents	TEM		CTX		VEB		SHV	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Cephalosporins								
Cefepime	38 (77.6%)	42 (82.4%)	23 (82.1%)	57 (79.2%)	6 (75%)	74 (80.4%)	38 (86.4%)	42 (75%)
Cefixime	49 (100%)	50 (98%)	27 (96.4%)	72 (100%)	8 (100%)	91 (98.9%)	43 (97.7%)	56 (100%)
Ceftriaxone	29 (59.2%)	41 (80.4%)*	22 (78.6%)	48 (66.7%)	4 (50%)	66 (71.7%)	33 (75%)	37 (66.7%)
Ceftazidime	22 (44.9%)	23 (45.1%)	11 (39.3%)	34 (47.2%)	6 (75%)*	39 (42.4%)	17 (38.6%)	28 (50%)
Ceftizoxime	34 (69.4%)	39 (76.5%)	23 (82.1%)	50 (69.4%)	6 (75%)	67 (72.8%)	32 (12.7%)	41 (73.2%)
Cefotaxime	21 (42.9%)	32 (62.7%)*	18 (64.3%)	35 (48.6%)	5 (62.5%)	48 (52.2%)	21 (47.7%)	32 (57.1%)
Carbapenems								
Imipenem	14 (28.6%)	20 (39.2%)	11 (39.3%)	23 (31.9%)	3 (37.5%)	31 (33.7%)	15 (34.1%)	19 (34%)
Meropenem	12 (24.5%)	21 (42.2%)	9 (32.1%)	24 (33.3%)	3 (37.5%)	30 (32.6%)	13 (29.5%)	20 (35.7%)
Aminoglycosides								
Amikacin	14 (28.6%)	28 (54.9%)*	13 (46.4%)	29 (40.3%)	3 (37.5%)	39 (42.4%)	24 (54.5%)*	18 (32%)
Gentamicin	21 (42.9%)	28 (54.9%)	14 (50%)	35 (48.6%)	5 (62.5%)	44 (47.8%)	27 (61.4%)*	22 (39.3%)
Others								
Ciprofloxacin	34 (69.4%)	42 (82.4%)	21 (75%)	55 (76.4%)	5 (62.5%)	71 (77.2%)	39 (88.6%)*	37 (66%)
Colistin	39 (79.6)	43 (84.3%)	23 (82.1%)	59 (81.9%)	6 (75%)	76 (82.6%)	38 (86.4%)	44 (78.6%)
TMP/SXT	34 (69.4%)	38 (74.5%)	21 (75%)	51 (70.8%)	4 (50%)	68 (73.9%)	29 (65.9%)	43 (76.8%)
Pip/TBZ	29 (59.2%)	29 (59.2%)	15 (53.6%)	43 (59.7%)	4 (50%)	4 (50%)	25 (56.8%)	33 (59%)

R: resistance, I: intermediate, S: sensitive, and ESBL: extended-spectrum beta-lactamase.

*Significant differences ($P < 0.05$).

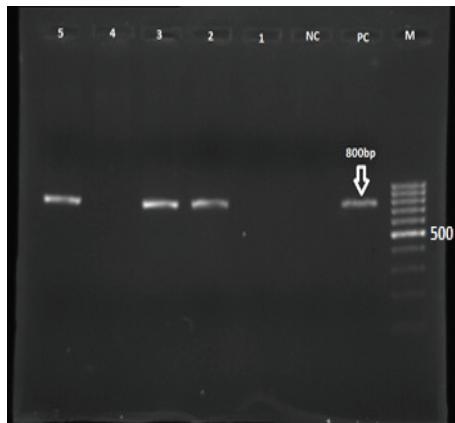


FIGURE 1: Agarose gel showing the 800 bp PCR fragments band for *TEM* gene from ESBL-producing *E. coli* isolates. Lanes: M: molecular weight marker (100 bp); PC: *K. pneumoniae* 7881 (positive control); NC: *E. coli* ATCC 25922 (negative control); 2, 3, and 5: *TEM* positive clinical samples; 1 and 4: *TEM* negative clinical samples.

agents, such as aminoglycosides, tetracycline, and trimethoprim/sulfamethoxazole [28]. The isolates showed high resistance to amikacin (34%), colistin (82%), and trimethoprim/sulfamethoxazole (65%) in our study. The study by Babypadmini and Appalaraju reported 74% resistance to trimethoprim/sulfamethoxazole and 91.6% resistance to fluoroquinolone in ESBL-producing *E. coli* pathogens by disk diffusion method [29], which is much higher than our results (65% resistance to trimethoprim/sulfamethoxazole and 76%

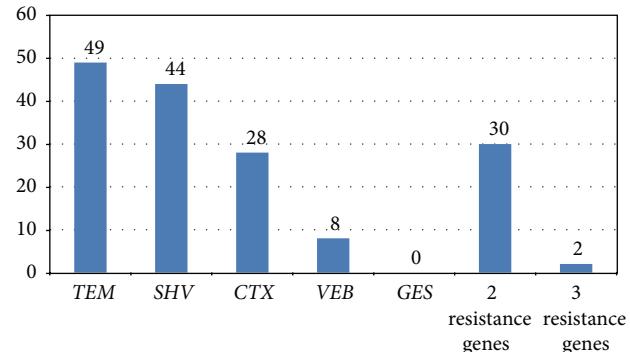


FIGURE 2: Distribution of *TEM*, *CTX*, *SHV*, *GES*, and *VEB* genes in ESBL-producing *E. coli* isolates.

resistance to fluoroquinolone). This difference may be due to use of different methods of evaluation for determining the susceptibility. We determined the antimicrobial resistance by the microdilution method which is more sensitive than disk diffusion method [22]. The results from other studies from Malaysia and Spain showed lower resistance to trimethoprim/sulfamethoxazole and ciprofloxacin in urine samples from adults than this study which may be due to different patient population (adults versus pediatrics). Totally, the increasing resistance of *E. coli* to trimethoprim makes this drug less effective as empiric treatment of UTI [30].

The ESBL-producing *E. coli* isolates in our study showed high resistance to colistin which is in accordance with the studies reported by Benenson et al. [31] and Ku et al.

[32]. Rapid increase in colistin resistance in *K. pneumoniae* strains was reported. Although some studies describe colistin activity against *Pseudomonas* and *Acinetobacter* isolates, the activity of colistin against ESBL-producing *E. coli* remains unclear [31, 33].

One of the most prominent and concerning findings in our study is the high resistance to broad spectrum antibiotics such as carbapenems which is in contrast with other studies that reported lower resistance (about 34%) to imipenem and meropenem in India [18], Malaysia [21], Columbia, Saudi Arabia [34], and Iran [24, 35, 36]. Although we found carbapenems as the most effective agent against the ESBL but the high rate of resistance, in comparison with other studies, is still very concerning. Recently, Alikhani et al. study in Iran showed 75% susceptibility among ESBL pathogens to carbapenems [20]. The main reason for large difference in the rate of resistance among different countries and different regions within the same country is due to the extensive use of broad spectrum antibiotics especially third generation cephalosporins and persistence of the resistant strains in health care facilities. Extensive usage of broad spectrum antibiotics specially third generation cephalosporins was reported by Salehfari et al. in our center. The rate of antibiotics consumption in our setting was significantly higher than other centers [37].

TEM was the most frequent resistant gene in ESBL-producing *E. coli* isolates in this study as it was also reported in several other studies [21, 24, 35]. *GES* resistant gene was not found in ESBL-producing *E. coli* isolates which is in line with the low frequency of this gene in ESBL-producing *E. coli* strains [36].

Analysis of the genotypes, antimicrobial resistance pattern, and MIC of different antimicrobial agents for ESBL-producing *E. coli* isolates showed a significantly higher resistance to ceftriaxone, cefotaxime, and amikacin in *TEM* genotype negative group. The study by French et al. showed that the *SHV* producing strains of *E. coli* were resistant to all aminoglycosides but sensitive to ciprofloxacin [38] but in this study the *SHV* genotype positive isolates were significantly resistant to both aminoglycosides and ciprofloxacin.

About 82% and 50% of *CTX-M* producing strains were resistant to quinolones and aminoglycosides, respectively, which was higher compared to Edelstein et al. study (21%) [39] but it was lower than the Mendonça et al. study (93%) [40]. The resistance to cefotaxime in *CTX-M* producers in our study was higher than those reported.

5. Conclusion

Although the *TEM* was found to be the most prevalent resistant gene, the prevalence of other resistant genes along with antimicrobial resistance is on the rise. Carbapenems were the most effective antibiotics against ESBL-producing *E. coli* in urinary tract infection in North of Iran. Considering the high prevalence of *SHV*, aminoglycosides and fluoroquinolones are not recommended for empiric therapy. The high rate of *SHV* and *VEB* transmission will result in

increasing the resistance to third generation cephalosporins, aminoglycosides, and fluoroquinolones.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Neem (*Azadirachta indica* A. Juss) Oil to Tackle Enteropathogenic *Escherichia coli*

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Neem (*Azadirachta indica* A. Juss) oil (NO) was assayed against forty-eight isolates of *Escherichia coli* by standardised disc diffusion test and microdilution test. By molecular biology characterization, fourteen isolates resulted in diarrheagenic *E. coli* with sixteen primer pairs that specifically amplify unique sequences of virulence genes and of 16S rRNA. The NO showed biological activity against all isolates. The bacterial growth inhibition zone by disc diffusion method (100 µL NO) ranged between 9.50 ± 0.70 and 30.00 ± 1.00 mm. The antibacterial activity was furthermore determined at lower NO concentrations (1:10–1:10,000). The percent of growth reduction ranged between 23.71 ± 1.00 and 99.70 ± 1.53 . The highest bacterial growth reduction was 1:10 NO concentration with 50 µL of bacterial suspension (ca. 1×10^6 CFU/mL). There is significant difference between the antibacterial activities against pathogenic and nonpathogenic *E. coli*, as well as NO and ciprofloxacin activities. Viable cells after the different NO concentration treatments were checked by molecular biology assay using PMA dye. On the basis of the obtained results, NO counteracts *E. coli* and also influences the virulence of *E. coli* viable cells after NO treatment. The NO metabolomic composition was obtained using fingerprint HPTLC.

1. Introduction

Zoonotic food- and waterborne pathogens began resistant to antibiotics. It is now evident that antimicrobial resistance is an environmental problem. Detectable antibiotic residues are present in waste water from water treatment plants [1], and antibiotic-resistant bacteria can be isolated from ground water and soil [2, 3]. The cause of contamination may be *inter alia* the consequence of farming practices. Use of antibiotics, as growth promoters or for prophylaxis in farm animals, selects resistant strains of enterobacteria in gastrointestinal tract. These resistant strains have been also isolated from food and consequently this represents the main way to spread in the human gastrointestinal tract [4, 5]. The increasing incidence of foodborne diseases, coupled with the resultant social and economic implications, causes a constant striving to produce safer feed and food, as to develop new natural antimicrobial agents [6–8].

Meat contamination by pathogen bacteria may have great health consequence and high impact on consumers. The most known cases are related to HUS, hemolytic uremic syndrome, that was first recognized in 1982 in USA and Canada, with outbreaks associated with fast food restaurants. People experienced gastroenteritis with bloody diarrhoea, caused by the lining of their microbiota. In 1993, a multistate outbreak generated international interest in this disease, popularized by the name “hamburger disease.” Hamburger disease is based on association with the consumption of ground beef patties containing a pathogen *Escherichia coli*. This should not be confused with the related benign *E. coli* that is in the gut of every mammal. Many strains of *E. coli* are part of the nonpathogenic facultative flora of intestinal tract of humans and other mammals. However, some of them induce diseases of the gastrointestinal and urinary tracts or may affect the central nervous system [9].

On the basis of their pathogenetic mechanism, diarrheagenic *E. coli* strains include ETEC (enterotoxigenic *E. coli*), EIEC (enteroinvasive *E. coli*), EHEC (enterohemorrhagic *E. coli*), EPEC (enteropathogenic *E. coli*), EAEC (enteroaggregative *E. coli*), and DAEC (diffusely adherent *E. coli*). All of them cause serious economic losses in farm animal herds and are widespread in newborns [10] in developed and developing countries. There is a wide range of transmission possibilities of these pathogens, including direct contact, food, drinks, environment, and others [11]. Epidemiology and clinical symptoms of the disease are similar in various animal species but the majority of strains are species-specific. They differ particularly in the type of the expressed surface “adherence” antigen (adhesin or pilus). These microorganisms produce two main types of virulence factors, that is, adhesins and enterotoxins.

In this work, a collection of *E. coli* isolates was considered. They were different in geographical origin and source of isolation and showed different pathogenetic characteristics.

Consumers look for meat products of upgraded sensory quality and increased functional and nutritional properties, as well as guaranteed safety but yet less processing, and fewer additives or “technological” interventions. Plant derived extracts, or phytocomplex, as effective antimicrobial agents, offer an alternative to synthetic food additives.

Neem (*Azadirachta indica* A. Juss) is considered one of the most promising trees of the 21st century, for its great potential in pest management, environment protection, and medicine [12]. Neem oil (NO) is the most important derived product with a great market worldwide. It contains about one hundred biologically active compounds. The most famous constituents are several nortriterpenes, named limonoids, that is, azadirachtin, nimbin, nimbidin, and nimbolide, besides the predominant oily constituents. NO is the most commercially relevant product obtained from the seeds. The neem cake is remaining after the extraction process.

In our previous studies, the antibacterial activity of NO against *E. coli* was investigated [13, 14]. The antibacterial activity resulted highest in comparison to the neem cake extract against meat spoilage microorganisms. The aim of the present work was to evaluate NO capability to cope with plastic genome of *E. coli*.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. Forty-eight strains of *E. coli* were considered. Among them, seventeen (FLC isolates) were from microorganism’s collection of the Fodder and Dairy Productions Research Centre of Lodi (CRA FLC) of CRA. They were isolated from milk and cheese. All strains were typed both phenotypically and genotypically. Phenotyping was made by the PhenePlate system for *E. coli* (PhP-EC, PhPPlate Microplate Techniques AB, Stockholm, Sweden) and genetic characterization by RAPD PCR technique [15].

Seventeen CVVI isolates were from microorganism’s collection of the Institute of Veterinary Research and Development of Central Vietnam, Vietnam. These microorganisms were isolated from faeces of calves affected by diarrhoea.

Ten NL isolates were from microorganism’s collection of the Department of Bacteriology of Wageningen UR Livestock Research, Wageningen University & Research Centre, Netherlands. They were isolated from faeces of piglets and calves. They are antigenically different and detectable using specific monoclonal antibodies towards different fimbria antigens by *in vitro* agglutination test [16]. Four reference strains were also considered (DSMZ and ATCC isolates). They were from international culture collections.

The cultivation/assay medium for *E. coli* was Minca + 1% Iso Vitalex Agar/Broth (Sifin, Berlin, Germany). Bacterial cultures for antibacterial testing were prepared by picking colony from 24-hour-old plates and suspending them in the broth medium (5 mL). Cultures were grown aerobically for 18 h at 37°C and 100 rpm. For antibacterial activity assay, 1 mL of each culture was diluted to 10⁵–10⁶ CFU/mL. The reference strains were grown on media and at the growth conditions as reported on products sheets.

2.2. Plant Extract. A commercial neem oil produced by Neem Italia (Manerba (BS), Italy) was used as test starting material (0.35% azadirachtin A). Total composition of the neem oil was checked by high performance thin layer chromatography [17].

Neem oil was diluted in Tween 80 (1:1 V/V; VWR, PBI International, MI, Italy) under agitation and sterilised by filtration through a 0.22 µm Millipore express filter (Millex-GP, Bedford, OH, USA) before use in the experiment.

2.3. HPTLC Assay

2.3.1. HPTLC System and Materials. The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of (i) Linomat 5 sample applicator using 100 µL syringes, connected to a nitrogen tank; (ii) ADC 2 chamber containing twin trough chamber 20 × 10 cm; (iii) immersion device III; (iv) TLC Plate Heater III; (v) TLC visualizer; (vi) TLC scanner 3 linked to winCATS software.

Solvents for extraction and HPLC grade solvents were purchased from Sigma-Aldrich and Carlo Erba (Milan, Italy). Glass plates 20 cm × 10 cm with glass-backed layers silica gel 60 (2 µm thickness) were from Merck (Darmstadt, Germany). Before use, plates were prewashed with methanol and dried for 3 min at 100°C. Standards used in the HPTLC analysis were isolated from neem cake (i.e., salannin, azadirachtin A, and unsaturated and saturated lipids) in previous research [18] and data concerning isolation and identification are not reported, but they are available per request. Limonoids standards concentration was 2 mM.

2.4. Sample Application. Filtered solutions were applied with nitrogen flow. Operating conditions were syringe delivery speed, 10 s µL⁻¹ (100 nL s⁻¹); injection volume, 2 µL; band width, 6 mm; distance from bottom, 15 mm.

2.5. Development. The HPTLC plates were developed in toluene : AcOEt 7 : 3 (v/v) as mobile phase (Figure 1), in the automatic and reproducibly developing chamber ADC 2,

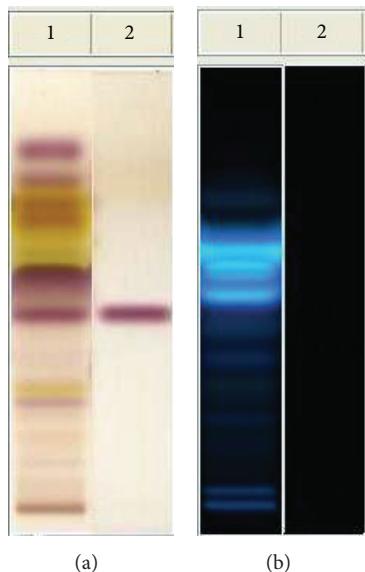


FIGURE 1: HPTLC analysis of neem oil EtOAc extract. Mobile phase: toluene : AcOEt 7 : 3 (v/v). Visualization: plate (a) (on the left) white light upper and lower; plate (b) (on the right) UV lamp at 366 nm. Derivatization: *p*-anisaldheyde. Track 1: neem oil; track 2: salannin.

saturated with the same mobile phase for 20 min at room temperature. The developing solvents (i.e., type of solvents and ratios) were carefully optimized before the analyses. The length of the chromatogram run was 80 mm from the point of application. The developed layers were allowed to dry in air for 5 min, derivatized with a selected solution, including *p*-anisaldheyde (1.5 mL *p*-anisaldheyde, 2.5 mL H₂SO₄, and 1 mL AcOH in 37 mL EtOH), dried in the open air, and then dipped into Macrogol reagent (1 g polyethylene glycol 400 in 20 mL of dichloromethane). Finally, the plates were warmed for 5 min at 120°C before inspection. All treated plates were inspected by a CAMAG TLC visualizer under a UV light at 254 or 366 nm or under reflectance and transmission white light (WRT), respectively, before and after derivatization.

2.6. Molecular Biology Characterization of the *E. coli* Isolates. Two primer pairs that amplify specific *E. coli* 16S rRNA sequences and fourteen primer pairs that specifically amplify target gene coding for virulence factors (adhesins and toxins) were employed to characterize the *E. coli* isolates considered in this study (Table 1). The PCR reaction mixtures and conditions are those as reported in the literature (Table 1).

The amplification products' sizes, coordinates, and accession numbers of each primer pair are shown in Table 2. Amplified products (7 μL) were analyzed by electrophoresis in 2% or 3% agarose gels buffered in 0.5x TBE (TBE buffer: 90 mM tris(hydroxymethyl)aminomethane, 90 mM boric acid, and 3 mM ethylenediaminetetraacetate Na salt, pH 8.3, Sigma-Aldrich, Milano, Italy) against a 50 bp, 100 bp, and 1 Kb ladder used as size marker (Invitrogen, Milano, Italia) and visualized by UV light at 260 nm (Fotodine 3-3102 Celbio,

Milano, Italy) after staining with ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, EtBr, Sigma-Aldrich, Milano, Italy).

2.7. Assessment of Antibacterial Activity. The antibacterial activity of the NO was assayed using standardized disc diffusion agar and microdilution methods. Disc diffusion method was carried out according to the standard method by Bauer et al. [19]. Bacteria cultures adjusted to 0.5 McFarland standard were used to lawn Muller Hinton agar plates evenly using a sterile swab. The agar plates were dried for 15 minutes. The discs impregnated with NO (100 μL) were placed on the agar surface. Each test plate comprises three discs. The discs were placed equidistant to each other. Muller Hinton agar plates were set also up with positive control, which is the antibiotic ciprofloxacin (CFX) (100 μL wt/v) (hydrochloride monohydrate 1 mg/mL, Bayer, Milano, Italy) and Tween 80 (TWN) (VWR International PBI Srl, Milano, Italy, 1 mg/mL) as negative control. The plates were then incubated at 37°C for 18 h. After the incubation, the plates and those considered as controls were examined for inhibition zone. The inhibition zones were then measured using calipers and were recorded. The plates were done in triplicate for each bacterial isolate and the experiment was performed twice. The results were recorded as mean ± S.D. of the duplicate experiment. Differences between means of data were compared by LSD calculated using the SAS.

The antibacterial activity of NO was also evaluated using microdilution method in conventional sterile polystyrene microplates (Corning, Euroclone SpA, Milan, Italy). Each well of the microplate was filled with 100 μL of sterile suitable liquid media for each bacterial isolate considered, 50 μL of inoculum and amounts of extract at lower concentrations (1:10–1:10,000) were added. Control treatment without NO was used in the experiment. The microplates were incubated at 37°C for 24 h. Bacterial growth was determined by OD reading at 630 nm/10 mm pathlength with an ELISA microplate reader (Dynatech ML-3000, Pina de Ebro, Spain). Bacterial cell concentration was transformed to cells/mL using the reference curve equation.

The reference curve was constructed by diluting at 1:100 each bacterial isolate. Counting the number of bacterial cells of an aliquot of this dilution was done using a Neubauer chamber (Celeromics, Vedano al Lambro, MI, Italy). Finally, cell concentrations were transformed to a percentage of bacterial inhibition. The percentage of bacterial growth reduction (GR%) was estimated using as reference the control treatment (*T* = without extract) as

$$\text{GR\%} = \frac{C - T}{C} \times 100. \quad (1)$$

Three replicates were considered. The results were recorded as mean ± S.D. of the duplicate experiment. Differences between means of data were compared by least significant difference (LSD) calculated using the SAS.

TABLE 1: Primer pairs used to specifically amplify target gene coding for virulence factors (1–9 = toxins; 9–15 fimbriae) of *E. coli* and 16S rRNA (16–17).

Target gene coding for virulence factors	Oligonucleotide sequences of primers	Reference
(1) LT	F 5'-ATT TAC GGC GTT ACT ATC CTC-3' R 5'-TTT TGG TCT CGG TCA GAT ATG-3'	[25]
(2) Sta	F 5'-TCC GTG AAA CAA CAT GAC GG-3' R 5'-ATA ACA TCC AGC ACA GGC AG-3'	[26]
(3) STb	F 5'-GCC TAT GCA TCT ACA CAA TC-3' R 5'-TGA GAA ATG GAC AAT GTC CG-3'	[26]
(4) Stx1all	F 5'-CGC TGA ATG TCA TTC GCT CTG C-3' R 5'-CGT GGT ATA GCT ACT GTC ACC-3'	[27]
(5) Stx2all	F 5'-CTT CGG TAT CCT ATT CCC GG-3' R 5'-CTG CTG TGA CAG TGA CAA AAC GC-3'	[27]
(6) Stx2e	F 5'-ATG AAG AAG ATG TTT ATA GCG-3' R 5'-TCA GTT AAA CTT CAC CTG GGC-3'	[25]
(7) EAST1	F 5'-CCA TCA ACA CAG TAT ATC CGA-3' R 5'-GGT CGC GAG TGA CGG CTT TGT-3'	[28]
(8) eae	F 5'-GGA ACG GCA GAG GTT AAT CTGCAG-3' R 5'-GGC GCT CAT CAT AGT CTTTC-3'	[27]
(9) hlyA	F 5'-AGCTGCAAGTGCAGGGTCTG-3' R 5'-TACGGGTTATGCCTGCAAGTTCAC-3'	[29]
(10) F4 (K88)	F 5'-GCT GCA TCT GCT GCA TCT GGTATG G-3' R 5'-CCA CTG AGT GCT GGTAGT TAC AGC C-3'	[30]
(11) F5 (K99)	F 5'-TGC GAC TAC CAA TGC TTC TG-3' R 5'-TAT CCA CCA TTA GAC GGA GC-3'	[26]
(12) F6 (P987)	F 5'-TCT GCT CTT AAA GCT ACT GG-3' R 5'-AAC TCC ACC GTT TGT ATC AG-3'	[25]
(13) F17	F 5'-GGG CTG ACA GAG GAG GTG GGGC-3' R 5'-CCC GGC GAC AAC TTC ATCACC GG-3'	[30]
(14) F18	F 5'-GTG AAA AGA CTA GTG TTT ATT TC-3' R 5'-CTT GTA AGT AAC CGC GTA AGC-3'	[31]
(15) F41	F 5'-GAG GGA CTT TCA TCT TTT AG-3' R 5'-AGT CCA TTC CAT TTA TAG GC-3'	[26]
(16) E16SI	F 5'-CCCCCTGGACGAAGACTCAC-3' R 5'-ACCGCTGGCAACAAAGGATA -3'	[29]
(17) E16SII	F 5'-AGAGTTTGATGGCTCAG-3' R 5'-GGACTACCAGGGTATCTAAT-3'	[31]

3. Results and Discussion

3.1. Molecular Biology Characterization of the *E. coli* Isolates. The molecular biology characterization of the forty-eight *E. coli* isolates showed that fourteen isolates were diarrheagenic *E. coli*. They were ten *E. coli* isolated from feces of calves and piglets and four from calves collected, respectively, in Netherlands and Central Vietnam. Their virulence characteristics are reported in Table 3.

3.2. HPTLC Assay. The NO metabolomic fingerprint shows characteristic sequence of metabolites according to the polarity of constituents. The identification of the raw material was assured by the presence of salannin ($R_f = 0.42$), which is a typical marker of neem. In comparison with the spot of azadirachtin ($R_f = 0.23$), salannin appears as the main limonoid spot. Spots concerning lipids are present at R_f

values at ca. 0.80, due to unsaturated fatty acids and fatty alcohols, and at R_f ca. 0.50, due to saturated and unsaturated triglycerides. The most interesting feature of the plate concerns the presence of compounds with high fluorescent reaction at between R_f 0.55 and 0.66, which are perfectly visible at 366 nm after derivatization with *p*-anisaldehyde. These spots can be attributed to compounds with high conjugated unsaturation in polycyclic aromatic structures, very different from those of the nortriterpenes limonoids, so far considered responsible for the activity. Therefore, more studies are necessary to decide about the importance of antibacterial activity of these substances in the phytocomplex.

3.3. NO Antibacterial Activity. The results obtained show that NO has a broad spectrum of antibacterial activities against the tested *E. coli* isolates. As shown in Table 4, the antibacterial activity was evaluated based on the diameters

TABLE 2: List of primer pairs' amplification products, coordinates, and accession numbers.

Target gene coding for virulence factors	Amplicon (bp)	Primer coordinates	Accession number
(1) LT	281	27–47, 287–307	S60731
(2) STa	244	267–286, 492–510	M58746
(3) STb	279	515–534, 773–793	AY028790
(4) Stx1all	302	113–134, 394–414	M17358
(5) Stx2all	516	50–69, 543–565	M59432
(6) Stx2e	264	1176–1196, 1419–1439	M36727
(7) EAST1	111	2–24, 94–114	S81691
(8) eae	775	1441–1460, 2193–2215	AF022236
(9) hylA	569	867–885, 1435–1412	X79839
(10) F4 (K88)	792	31–54, 798–822	M29374
(11) F5 (K99)	450	45–64, 475–494	M35282
(12) F6 (P987)	333	193–212, 506–525	M35257
(13) F17	411	289–310, 677–699	AF055313
(14) F18	510	1–23, 490–510	M61713
(15) F41	431	154–173, 565–584	X14354
(16) E16SI	401	1628–170, 2063–2082	AB035924
(17) E16SII	798	8–27, 798–805	J01859

TABLE 3: Molecular characterisation of enteropathogenic *E. coli* and reference strains considered in this study.

<i>E. coli</i> isolate collection's designation	Surface antigen	Toxins	Fimbriae
(1) CVVI K10B	nd	STb, LT, EAST1	F4
(2) CVVI KH10	nd	STa, STb	F18
(3) NLK99	O8K85K99	nr1	F5
(4) NLP987	O64 : K; 9877	STa+	F6
(5) CVVI E12b	nd	STa	F5, F41
(6) CVVI E10	nd	STa	F5, F41
(7) NLK99-1	O8 : K25 : K99	nr	F5
(8) NLK99-3	O101 : K28 : K99	nr	F5
(9) NLK99-5	O9 : K30 : K99	nr	F5
(10) NLK99-7	O101 : K32 : K99	nr	F5
(11) NLK99-9	O9 : K35 : K99	nr	F5
(12) NLK99-11	O9 : K37 : K99	nr	F5
(13) NLK99-15	O20 : K? : K99	nr	F5
(14) NLK99-19	O101 : K? : K99	nr	F5
(15) DSMZ8696	O55 : H6	nr	Nr
(16) DSMZ9025	—	—	—
(17) DSMZ10973	O6	nr	nr
(18) ATCC3559	—	—	—

CVVI: Central Vietnam Veterinary Institute; NL: Department of Bacteriology and Animal Science, University of Wageningen, Netherlands; DSMZ: Leibniz-Institut DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; ATCC: American Type Culture Collection.

of clear inhibition zone surrounding the paper discs soaked with 100 μ L of neem oil. The NO average GIZ mm range from 9.50 ± 0.70 to 30.00 ± 1.00 . The NO GIZ varies between enteropathogenic and nonenteropathogenic *E. coli* being, respectively, 24.33 ± 0.58 – 30.00 ± 1.00 and 9.50 ± 0.70 – 21.53 ± 1.53 . It is significantly ($P < 0.05$) different with respect to the antibiotic activity. However, the *E. coli* isolate FLC1167 (from milk) resulted to be less susceptible and the *E. coli* isolate

NLP097/F5 (from piglet feces) the most susceptible to NO treatment (100 μ L) among all tested bacteria using the disc diffusion method.

The CFX GIZ range is 0.00 ± 0.00 – 32.65 ± 75 . The enteropathogenic *E. coli* resulted to be resistant or less susceptible to CFX than the nonenteropathogenic *E. coli*, showing a GIZ range of, respectively, 0.00 ± 0.00 – 18.24 ± 1.68 mm and 21.64 ± 0.94 – 32.65 ± 75 mm. The isolates

TABLE 4: Antibacterial activity of neem oil (NO) against forty-eight *Escherichia coli* isolates revealed as growth inhibition zone (mm).

<i>E. coli</i> isolates	Growth inhibition zone (mm)*			
	NO (100 μ L)	TWN (100 μ L)	WTR (100 μ L)	CFX (100 μ L)
(1) FLC 1056	11.33 \pm 0.58 b	—	—	30.41 \pm 0.20 a
(2) FLC 1247	16.13 \pm 1.15 b	—	—	30.52 \pm 1.07 a
(3) FLC 1059	15.83 \pm 1.13 b	—	—	29.62 \pm 1.00 a
(4) FLC 1243	19.00 \pm 1.00 b	—	—	31.53 \pm 0.67 a
(5) FLC 1048	12.33 \pm 0.58 b	—	—	29.42 \pm 0.58 a
(6) FLC 1167	9.50 \pm 0.70 b	—	—	30.61 \pm 1.21 a
(7) FLC 1249	13.33 \pm 0.58 b	—	—	29.61 \pm 1.11 a
(8) FLC 1055	14.53 \pm 1.25 b	—	—	31.75 \pm 0.82 a
(9) FLC 1054	16.23 \pm 1.18 b	—	—	31.41 \pm 0.76 a
(10) FLC 1085	18.00 \pm 1.00 b	—	—	30.53 \pm 1.17 a
(11) FLC 1244	15.33 \pm 0.48 b	—	—	28.86 \pm 1.00 a
(12) FLC 1165	19.50 \pm 0.70 b	—	—	31.33 \pm 0.67 a
(13) FLC 1086	11.33 \pm 0.58 b	—	—	29.82 \pm 0.48 a
(14) FLC 1053	14.53 \pm 1.15 b	—	—	32.65 \pm 1.39 a
(15) FLC 1095	16.83 \pm 1.18 b	—	—	29.05 \pm 1.22 a
(16) FLC 1219	10.70 \pm 1.00 b	—	—	32.75 \pm 0.55 a
(17) FLC 1235	13.23 \pm 0.88 b	—	—	30.15 \pm 0.55 a
(18) DSM8696	13.50 \pm 0.50 b	—	—	26.21 \pm 1.00 a
(19) DSM9025	13.33 \pm 0.58 b	—	—	21.64 \pm 0.94 a
(20) DSM10973	13.53 \pm 1.25 b	—	—	29.14 \pm 1.75 a
(21) ATCC33559	13.83 \pm 1.18 b	—	—	32.12 \pm 1.09 a
(22) CVVI E210	13.00 \pm 1.00 a	—	—	25.83 \pm 1.65 a
(23) CVVI E173	12.23 \pm 0.58 b	—	—	32.35 \pm 1.49 a
(24) CVVI E12b	27.50 \pm 0.50 b	—	—	11.25 \pm 0.68 a
(25) CVVI E16	14.33 \pm 0.88 b	—	—	27.54 \pm 1.45 a
(26) CVVI E320	21.53 \pm 1.35 b	—	—	28.75 \pm 1.86 a
(27) CVVI E130	11.83 \pm 1.78 b	—	—	29.64 \pm 0.87 a
(28) CVVI E48	10.00 \pm 1.40 a	—	—	29.31 \pm 0.27 a
(29) CVVI KH10	26.33 \pm 0.53 b	—	—	15.34 \pm 0.66 a
(30) CVVI K10B	27.50 \pm 0.56 b	—	—	0
(31) CVVI E298	11.33 \pm 0.48 b	—	—	23.90 \pm 1.69 a
(32) CVVI E273	13.53 \pm 1.75 b	—	—	29.59 \pm 1.77 b
(33) CVVI K436	13.14 \pm 1.68 b	—	—	30.21 \pm 1.38 a
(34) CVVI E98	11.00 \pm 1.00 a	—	—	26.91 \pm 1.56 a
(35) CVVI E77	14.33 \pm 0.58 b	—	—	23.93 \pm 0.59 a
(36) CVVI E148	13.50 \pm 0.50 b	—	—	28.71 \pm 0.87 a
(37) CVVI E10	24.33 \pm 0.58 b	—	—	10.35 \pm 1.11 a
(38) CVVI E215	16.53 \pm 1.15 b	—	—	26.41 \pm 1.40 a
(39) NLK99/F5	29.83 \pm 1.18 b	—	—	16, 21 \pm 0.89 a
(40) NLP987/F5	30.00 \pm 1.00 a	—	—	13.21 \pm 1.15 a
(41) NLK99-1*	21.73 \pm 1.35 b	—	—	15.34 \pm 1.37 a
(42) NLK99-3*	28.33 \pm 1.50 b	—	—	0
(43) NLK99-5*	25.00 \pm 1.10 a	—	—	9.54 \pm 1.11 a
(44) NLK99-7*	26.33 \pm 0.58 b	—	—	14.25 \pm 1.11 a
(45) NLK99-9*	27.50 \pm 0.50 b	—	—	11.26 \pm 1.78 a
(46) NLK99-11*	29.53 \pm 1.25 b	—	—	16.24 \pm 1.68 a
(47) NLK99-15*	28.83 \pm 1.38 b	—	—	16.35 \pm 1.11 a
(48) NLK99-19*	25.00 \pm 1.70 a	—	—	15.53 \pm 0.84 a

Three paper discs per plate and three plates for each bacterium were considered. The experiment was repeated twice. Values are given as mean \pm S.D. Values in a row followed by different lowercased letters are significantly different at $P \leq 0.05$.

CVVIK10B (from calf feces) and NLK99-3* (from calf feces) both revealed resistance to CFX. No GIZ was detected in plates treated with negative controls (TWN and WTR).

As shown in Table 5, the percent bacterial GR revealed at 100 μ L, 10 μ L, 1 μ L, and 0.1 μ L NO concentrations was in the range 23.71 ± 1.00 – 99.70 ± 1.53 ; 21.61 ± 0.56 – 91.63 ± 0.08 ; 17.58 ± 1.33 – 69.57 ± 0.00 ; and 11.18 ± 0.89 – 67.58 ± 0.89 .

There is a significant difference of antibacterial activity among the isolates and the NO concentrations tested (Table 5). The highest percent bacterial GRs were detected at 100 μ L NO and they concerned mainly enteropathogenic *E. coli* isolates. Amplicons of the expected sizes from virulence genes of enteropathogenic *E. coli* isolates were not detected when bacterial viable cells were checked in samples treated with 100 μ L and 10 μ L NO concentrations. On the contrary, amplicons of the expected size were revealed in the same samples using primer pair numbers 16 and 17, as reported in Table 1, that specifically amplify unique sequences of *E. coli* 16S rRNA.

The antibiotic activity of ciprofloxacin is to bind and inhibit bacterial topoisomerase types II and IV, thus being able to interfere with the bacterial processes of replication, transcription, and DNA repair.

An increasing ciprofloxacin resistance of *E. coli* isolates was reported [20] according several epidemiological studies. *E. coli*, the most commonly isolated bacterium in clinical samples from patients affected by different severity of diarrheal symptoms, shows high antibiotic resistance [21, 22]. Diarrheagenic *E. coli*, considered in the experiment, showed a resistance or less susceptibility to ciprofloxacin, in comparison with the other nonenteropathogenic isolates tested.

The viable cells of the fourteen diarrheagenic *E. coli* were checked after NO treatment with primer pairs listed in Table 1 and PMA dye. The dye propidium monoazide (PMA Biotium Inc., Hayward, CA, USA) is a photoreactive dye with high affinity for DNA. The dye intercalates into DNA and forms a covalent linkage upon exposure to intense visible light. It is cell membrane impermeable. When a sample comprising both live and dead bacteria is treated with PMA, only dead cells are susceptible to DNA modification due to their compromised cell membranes [23, 24]. Therefore, selective detection of the sole live cells is achieved.

The fourteen ciprofloxacin resistant/less susceptible diarrheagenic *E. coli* seem to lose their virulence after NO treatment, because amplicons were obtained only with the primer pairs numbers 16 and 17 (Table 1). This let us suppose that antibacterial activity acts on adhesion factor and membrane and its permeability with possible loss of extrachromosomal DNA.

4. Conclusions and Future Implications

Studies of new antimicrobials from plant derived extracts and agroindustrial byproducts as antimicrobials and preservatives are important issues in applied microbiology and biotechnology, for both implementing and improving effective alternative technologies to tackle antimicrobial

resistance. The potential use of plant natural antimicrobials would require amendments of several different legal texts involving areas such as food additives, food packaging, and hygiene. Anyway, the applications could concern either the natural preservation in the food industries or an accessible and safe alternative to synthetic antimicrobial drugs.

Abbreviations

ATCC:	American Type Culture Collection
CFX:	Antibiotic: hydrochloride monohydrate
CRA:	Agricultural Research Council, Italy
CVVI:	Central Vietnam Veterinary Institute
DAEC:	Diffusely adherent <i>E. coli</i>
DSMZ:	Leibniz-Institut DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EAEC:	Enteroaggregative <i>E. coli</i>
EHEC:	Enterohemorrhagic <i>E. coli</i>
EIEC:	Enteroinvasive <i>E. coli</i>
ELISA:	Enzyme-linked immunosorbent assay
EPEC:	Enteropathogenic <i>E. coli</i>
ETEC:	Enterotoxigenic <i>E. coli</i>
GIZ:	Growth inhibition zone
HPTLC:	High performance thin layer chromatography
HUS:	Hemolytic uremic syndrome
LSD:	Least significant difference
NCE:	Neem cake extract
NL:	Department of Bacteriology and Animal Science, University of Wageningen, Netherlands
NO:	Neem oil
OD:	Optical density
PMA:	Propidium monoazide
RAPD PCR:	Random amplified polymorphic DNA polymerase chain reaction
Rf:	Ratio between the migration distance of substance and the migration distance of solvent front
SAS:	Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA)
SD:	Standard deviation
TWN:	Tween 80
WTR:	Sterile distilled water.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Paola Del Serrone carried out research concept and design, collection and/or assembly of biological assay data, collection and/or assembly of molecular biology assay data, data analysis and interpretation, statistical analysis, writing the paper, critical revision of the paper, and the final approval of the

TABLE 5: Bacterial growth reduction (%) at 24 h in liquid medium with different concentrations of NO, using as reference the control treatment (without NO).

<i>E. coli</i> isolates	Percent growth reduction zone (%)			
	NO (100 µL)	NO (10 µL)	NO (1 µL)	NO (0.1 µL)
(1) FLC 1056	39.25 ± 1.43 c	35.61 ± 1.00 b	21.67 ± 1.33 a	11.31 ± 2.08 a
(2) FLC 1247	25.51 ± 1.15 c	24.70 ± 1.00 b	21.88 ± 1.33 a	11.86 ± 1.00 a
(3) FLC 1059	31.51 ± 1.15 c	25.70 ± 1.00 b	24.58 ± 1.33 a	14.86 ± 1.00 a
(4) FLC 1243	38.90 ± 1.00 d	28.79 ± 1.00 c	29.40 ± 0.00 b	16.68 ± 1.20 a
(5) FLC 1048	25.60 ± 1.53 d	23.73 ± 2.08 b	23.69 ± 2.00 b	12.83 ± 1.73 a
(6) FLC 1167	23.71 ± 1.00 b	21.61 ± 0.58 a	21.27 ± 0.00 a	11.18 ± 0.89 a
(7) FLC 1249	29.65 ± 1.53 b	28.61 ± 1.00 a	28.77 ± 1.33 a	18.51 ± 2.08 a
(8) FLC 1055	34.51 ± 1.15 c	29.70 ± 1.00 c	24.38 ± 1.33 a	14.86 ± 1.00 a
(9) FLC 1054	31.51 ± 1.15 d	29.70 ± 1.00 c	25.78 ± 1.33 a	14.86 ± 1.00 a
(10) FLC 1085	39.90 ± 1.00 c	28.79 ± 1.00 b	28.10 ± 0.00 b	15.58 ± 1.20 a
(11) FLC 1244	35.70 ± 1.53 d	28.73 ± 2.08 b	28.59 ± 2.00 b	12.63 ± 1.73 a
(12) FLC 1165	39.71 ± 1.00 c	29.61 ± 0.58 c	29.17 ± 0.00 b	16.48 ± 0.89 a
(13) FLC 1086	39.55 ± 1.53 c	28.61 ± 1.00 c	27.87 ± 1.33 b	12.21 ± 2.08 a
(14) FLC 1053	34.51 ± 1.15 c	89.70 ± 1.00 c	27.58 ± 1.33 b	14.86 ± 1.00 a
(15) FLC 1095	31.51 ± 1.15 c	29.70 ± 1.00 b	25.18 ± 1.33 a	14.86 ± 1.00 a
(16) FLC 1219	38.95 ± 1.00 b	28.79 ± 1.00 b	28.10 ± 0.00 b	16.68 ± 1.20 a
(17) FLC 1235	36.70 ± 1.53 d	27.73 ± 2.08 c	27.79 ± 2.00 b	12.83 ± 1.73 a
(18) DSM8696	39.71 ± 1.00 c	29.61 ± 0.58 c	29.67 ± 0.00 b	17.58 ± 0.89 a
(19) DSM9025	39.65 ± 1.53 c	28.61 ± 1.00 c	26.17 ± 1.33 b	18.10 ± 2.08 a
(20) DSM10973	34.51 ± 1.15 c	29.70 ± 1.00 c	23.28 ± 1.33 b	14.86 ± 1.00 a
(21) ATCC33559	31.51 ± 1.15 d	29.70 ± 1.00 c	21.58 ± 1.33 b	14.86 ± 1.00 a
(22) CVVI E210	88.90 ± 1.00 c	88.79 ± 1.00 c	69.20 ± 0.00 b	14.78 ± 1.20 a
(23) CVVI E173	36.50 ± 1.53 d	27.73 ± 2.08 c	27.99 ± 2.00 b	22.83 ± 1.73 a
(24) CVVI E126	89.81 ± 1.00 c	89.61 ± 0.58 c	69.37 ± 0.00 b	44.58 ± 0.89 a
(25) CVVI E16	38.55 ± 1.53 c	28.61 ± 1.00 c	27.57 ± 1.33 b	11.51 ± 2.08 a
(26) CVVI E320	38.51 ± 1.15 c	29.70 ± 1.00 c	27.18 ± 1.33 b	14.86 ± 1.00 a
(27) CVVI E130	30.71 ± 1.15 c	29.70 ± 1.00 c	27.68 ± 1.33 a	14.46 ± 1.00 a
(28) CVVI E48	38.60 ± 1.00 c	28.79 ± 1.00 c	29.20 ± 0.00 a	16.68 ± 1.20 a
(29) CVVI KH10	99.60 ± 1.53 d	81.73 ± 2.08 c	68.39 ± 2.00 b	62.33 ± 1.73 a
(30) CVVI K10B	89.81 ± 1.00 c	89.61 ± 0.58 c	69.37 ± 0.00 b	60.58 ± 0.89 a
(31) CVVI E298	39.75 ± 1.53 c	28.61 ± 1.00 c	27.57 ± 1.33 b	20.11 ± 2.08 a
(32) CVVI E273	34.51 ± 1.15 c	29.70 ± 1.00 c	26.58 ± 1.33 b	22.86 ± 1.00 a
(33) CVVI K436	31.85 ± 1.15 d	29.70 ± 1.00 c	27.18 ± 1.33 b	15.86 ± 1.00 a
(34) CVVI E98	37.93 ± 1.00 c	28.79 ± 1.00 c	29.50 ± 0.00 b	16.68 ± 1.20 a
(35) CVVI E77	33.69 ± 1.53 d	21.73 ± 2.08 c	27.29 ± 2.00 b	19.83 ± 1.73 a
(36) CVVI E148	39.71 ± 1.00 c	29.61 ± 0.58 c	29.57 ± 0.00 b	22.58 ± 0.89 a
(37) CVVI E10	89.65 ± 1.53 c	88.61 ± 1.00 c	61.67 ± 1.33 b	50.79 ± 2.08 a
(38) CVVI E215	34.51 ± 1.15 c	29.70 ± 1.00 c	17.58 ± 1.33 b	21.86 ± 1.00 a
(39) NLK99/F5	91.51 ± 1.15 d	89.70 ± 1.00 c	67.68 ± 1.33 b	61.86 ± 1.00 a
(40) NL12B/F5	88.90 ± 1.00 c	88.79 ± 1.00 c	69.60 ± 0.00 b	63.68 ± 1.20 a
(41) NLK99-1*	97.70 ± 1.53 d	81.73 ± 2.08 c	68.69 ± 2.00 b	62.83 ± 1.73 a
(42) NLK99-3*	89.71 ± 1.00 c	79.61 ± 0.58 c	69.57 ± 0.00 b	67.58 ± 0.89 a
(43) NLK99-5*	89.65 ± 1.53 c	78.61 ± 1.00 c	67.67 ± 1.33 b	50.81 ± 2.08 a
(44) NLK99-7*	84.51 ± 1.15 c	79.70 ± 1.00 c	67.58 ± 1.33 b	44.86 ± 1.00 a
(45) NLK99-9*	91.51 ± 1.15 c	79.70 ± 1.00 c	67.28 ± 1.13 b	64.86 ± 1.00 a
(46) NLK99-11*	88.90 ± 1.00 c	78.79 ± 1.00 c	69.60 ± 0.00 b	66.68 ± 1.20 a
(47) NLK99-15*	99.70 ± 1.53 d	91.63 ± 0.28 c	68.69 ± 2.00 b	62.83 ± 1.73 a
(48) NLK99-19*	89.71 ± 1.00 c	79.61 ± 0.58 c	69.57 ± 0.00 b	67.58 ± 0.89 a

Three plates for each bacterium were considered. The experiment was repeated twice. Values are given as mean ± S.D. Values in a row followed by different lowercased letters are significantly different at $P \leq 0.05$.

paper. Chiara Toniolo was responsible for collection and/or assembly of chemical data, data analysis and interpretation, critical revision of the paper, and final approval of the paper. Marcello Nicoletti carried out collection and/or assembly of chemical data, data analysis and interpretation, writing the paper, critical revision of the paper, and final approval of the paper.

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Research Article

Diffusion and Persistence of Multidrug Resistant *Salmonella* Typhimurium Strains Phage Type DT120 in Southern Italy

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Sixty-two multidrug resistant *Salmonella enterica* serovar Typhimurium strains isolated from 255 clinical strains collected in Southern Italy in 2006–2008 were characterised for antimicrobial resistance genes, pulsotype, and phage type. Most strains (83.9%) were resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (ACSSuT) encoded in 88.5% by the *Salmonella* genomic island (SGII) and in 11.5% by the InH-like integron (*bla*_{OXA-30}-*aadA1*) and *catA1*, *sull*, and *tet(B)* genes. STYMXB.0061 (75%) and DT120 (84.6%) were the prevalent pulsotype and phage type identified in these strains, respectively. Five other resistance patterns were found either in single or in a low number of isolates. The pandemic clone DT104 (ACSSuT encoded by SGII) has been identified in Italy since 1992, while strains DT120 (ACSSuT encoded by SGII) have never been previously reported in Italy. In Europe, clinical strains DT120 have been reported from sporadic outbreaks linked to the consumption of pork products. However, none of these strains were STYMXB.0061 and SGII positive. The prevalent identification and persistence of DT120 isolates would suggest, in Southern Italy, a phage type shifting of the pandemic DT104 clone pulsotype STYMXB.0061. Additionally, these findings raise epidemiological concern about the potential diffusion of these emerging multidrug resistant (SGI linked) DT120 strains.

1. Introduction

In recent times, diseases caused by foodborne pathogens have been the object of an increasing number of studies [1]. This is owing to the rates of morbidity and mortality that can be achieved by such diseases and the impact that they have in both public health and social economic costs. Among the different bacteria responsible for foodborne diseases, *Salmonella enterica* represents one of the leading causes of such infections with *S. Enteritidis* and *S. Typhimurium* being the most common serovars isolated from humans in Europe and the USA [2]. However, while in Europe *S. Enteritidis* still remains the predominant serovar from clinical cases, in Italy it is the serovar *S. Typhimurium* which is prevalent [3].

In the past decades, the emergence and spread of *Salmonella* multidrug resistant (MDR) strains have also contributed to increasing the impact that these pathogens have had in public health. The insurgence of MDR has generally been linked to acquisition of resistance genes located on plasmids, transposons, genomic islands, and integrons. For example, the multidrug resistance exhibited by the pandemic *S. Typhimurium* definitive phage type 104, (DT104), is due to resistance genes located within a chromosomal genomic island termed SGII composed of a 43 kbp region in which lies a 13 kbp gene cluster of *aadA2*, *floR*, *tet(G)*, *bla*_{PSE-1}, and *sull* encoding resistance for streptomycin, chloramphenicol, tetracycline, ampicillin, and sulfamethoxazole, respectively [4].

The constant increase of antimicrobial resistance is a global scale problem and it raises concern on the choice of antimicrobials available for the treatment of bacterial infections, particularly for those pathogens of major social relevance. It is thus necessary to implement measures that may contribute to better assessing the extent of resistance-gene diffusion among these pathogens. Both monitoring and characterisation of the antimicrobial susceptibility are undoubtedly valuable investigation systems that fulfill the purpose of such measures. Antimicrobial resistance is usually acquired by point mutations in the bacterial genome or by horizontal transfer of genetic elements harbouring resistance genes. Additionally, gene capture and expression elements called integrons have recently been shown to play an important role in acquisition and dissemination of resistance genes. Among the different classes of integrons, class 1 is the most detected and found strongly associated with capture of antimicrobial resistance genes. Class 1 integrons are then recognised as major contributors to the problem of multidrug-resistant pathogens [5]. In *S. enterica*, class 1 integrons are common and greatly contribute to the extent of antimicrobial resistance in this species [6].

If genetic characterization of antimicrobial resistances was combined with molecular strain typing, it would greatly benefit knowledge on the spread of resistance genes among the clonal groups of *Salmonella* strains principally identified in the clinical field. Phage typing and pulsed-field gel electrophoresis (PFGE) have become universally recognized as the reference methods for *Salmonella* strain subtyping.

In Italy, until 2006, the most common phage types identified among MDR clinical *S. Typhimurium* strains were DT104, U302, and DT7 [3]. The first was strongly associated with isolates pulsotype STYMXB.0061 resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline, while strains U302 and DT7 were pulsotype STYMXB.0079 and resistant to ampicillin, streptomycin, sulfamethoxazole, and tetracycline. However, in Southern Italy (where *Salmonella* infections are endemic), data on resistance genes and clonal relationship among the MDR strains isolated from clinical cases is poor [7]. In this study, MDR *S. Typhimurium* strains isolated from several hospitals, from 2006 to 2008, were phage typed and characterised by pulsed field gel electrophoresis (PFGE). The genetic basis of the antimicrobial resistance was also established.

2. Materials and Methods

2.1. Bacterial Isolates and Antimicrobial Susceptibility Testing. From January 2006 to December 2008, four hundred and seventy-seven *Salmonella* strains were isolated from clinical cases which occurred in Southern Italy. Two hundred and fifty-five were *S. enterica* serovar Typhimurium. Strains were isolated from six different hospitals scattered among the Italian neighbouring regions of Apulia and Basilicata. Biochemical identification of *Salmonella* was confirmed by an automated system (Microscan, dade Behring, Milan, Italy) and serotyping was performed by agglutination tests

with specific antisera O and H antigens (Bio-Rad Laboratories, Italy). Classification was performed according to the Kauffmann-White scheme [8].

All isolates were included in the database of the Enter-net Surveillance Network at the Institute of Hygiene of the University of Bari (Italy) coordinated by the Istituto Superiore di Sanità, Rome (Italy). There were sixty-two bacteria that were resistant to at least one agent in three or more of the antimicrobial categories tested (namely, aminoglycosides, folate pathway inhibitors, penicillins, phenicols, and tetracyclines) and so defined as MDR [11].

Antimicrobial susceptibility testing was carried out by the agar disk diffusion test on Mueller-Hinton agar (Oxoid, Milan, Italy), following the Clinical and Laboratory Standards Institute (CLSI) guidelines [12]. The antimicrobial disks were ampicillin (A; 10 µg), chloramphenicol (C; 30 µg), kanamycin (K; 30 µg), streptomycin (S; 10 µg), sulfamethoxazole (Su; 25 µg), tetracycline (T; 30 µg), and trimethoprim (Tp 5 µg). *E. coli* ATCC 25922 was used as a quality control strain.

2.2. PCR Detection of SGI, Class 1 Integrons, and Antimicrobial Resistance Genes. Primers used to detect SGII, class 1 integrons, and the antimicrobial resistance genes *aadA1*, *aadA2*, and *strAB* (encoding resistance for streptomycin; category aminoglycosides), *bla_{PSE-1}* and *bla_{TEM}* (encoding resistance for ampicillin; category penicillins), *floR* (encoding resistance for chloramphenicol; category phenicols), *sul1* and *sul2* (encoding resistance for sulfamethoxazole; category folate pathway inhibitors), and *tet(B)*, *tet(C)*, and *tet(G)* (encoding resistance for tetracycline; category tetracyclines) were as reported previously [9, 10]. Primers to detect the antimicrobial resistance genes *bla_{OXA-30}* (encoding resistance for ampicillin), *catA1* (encoding resistance for chloramphenicol), *dfrA1* and *dfrA12* (encoding resistance for trimethoprim; category folate pathway inhibitors), and *tet(A)* and *tet(D)* (encoding resistance for tetracycline) were designated in this study. All primers are listed in Table 1. The primers employed in the current study were based on sequences available in GenBank and designed by using the Primer3 (version 4.0.0) software (<http://primer3.wi.mit.edu>). Genomic DNA was extracted as previously described [13]. PCRs were performed in a total volume of 25 µL containing 50 to 100 ng of total DNA, 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂; pH8.3), 200 µM of each deoxynucleoside triphosphate (dNTP), 20 µM of each primer, and 1U *Taq* polymerase (Takara Bio Inc., Otsu, Shiga, Japan).

Antimicrobial gene cassettes integrated into class 1 integrons were amplified with primers 5CS-F and 3CS-R and cloned into pGEM-T Easy vector (Promega, Milan, Italy) in accordance with the manufacturer's instructions. *E. coli* JM109 was used as a recipient strain. The cloned products were purified using the Pure Yield Plasmid Miniprep System (Promega) and sequenced by the Big Dye Terminator method (BMR Genomics, Padova, Italy). The resulting DNA sequences were analysed for similarity by using the BLAST program available on the NCBI BLAST homepage (<http://www.ncbi.nlm.nih.gov/BLAST>).

TABLE I: Primers used for PCR amplification of resistance genes.

Primer	5'-3' sequence	Gene target	Amplicon size (bp)	TA	Reference
aadA1-F	TTTGATCAACGACCTTTGGAAAC				
aadA-R	GGACAACGTAAGCACTACATTG	<i>aadA1</i> and <i>aadA2</i>	294	58°C	[9]
blaoxa30-F	ATTATCTACAGCAGGCCAGTGCATC				
blaoxa30-R	TTCGACCCCAAGTTCTGTAAAGTC	<i>bla</i> _{OXA-30}	716	63°C	This study
blaPSE-1-F	GGATTACAATGGCAATCAGCGCTTCC				
blaPSE-1-R	AATCGCATCATTGCGCTCTGCCATTG	<i>bla</i> _{PSE-1}	658	65°C	[10]
blaTEM1-F	TGAAGATCAGTTGGGTGCACGAGTGG				
blaTEM1-R	AGTTGCCTGACTCCCCGTCGTAGA	<i>bla</i> _{TEM-1}	700	63°C	[9]
catA1-F	CACCGTTGATATATCCAATGGCATCGT				
catA1-R	CTGCCGACATGGAAGCCATCACAAAC	<i>catA1</i>	582	62°C	This study
dfrA1-F	TATTCCATGGAGTGCCAAAGGTGAAC				
dfrA1-R	GAGGCGAAGTCTTGGGAAAACTGG	<i>dfrA1</i>	375	63°C	This study
dfrA12-F	CAGTACGCATTTATCTCGTTGCTGCG				
dfrA12-R	CAAGCTCGAATTCTGTTTCGTTGAGC	<i>dfrA12</i>	417	63°C	This study
floR-F	CTCCTTCGACATCCTCGCTTCAGTG				
floR-R	AGAACAGCGAAGAAGGTGCCATACCG	<i>floR</i>	636	64°C	[10]
sull-F	TCGGCATTCTGAATCTCACCGAGGAC				
sull-R	AAATTTCGCGAGGGTTCCGAGAAGG	<i>sul1</i>	786	64°C	[10]
sul2-F	GACAGTTCTCGATGGAGGCCGTA				
sul2-R	GTGTGCGGATGAAGTCAGCTCCACCT	<i>sul2</i>	700	64°C	[10]
strA-F	TGGTTGCCTGTCAGAGGCCGAGAAC				
strB-R	ATCGACGTCCAGCGCACGAGAGAAC	<i>strAB</i>	1466	63°C	[9]
tetA-F	CCTGATCGTAATTCTGAGCACTGTCG				
tetA-R	CACCCGTTCCACGTTTATAGAAC	<i>tet(A)</i>	1089	63°C	This study
tetB-F	CAGGTTATCTTGCTCCTGGCTTGG				
tetB-R	TTGAGGGGTTAACATGAAGGTATCG	<i>tet(B)</i>	1014	63°C	[9]
tetC-F	GGATATCGTCATTCCGACAGCATCG				
tetC-R	GATAATGGCCTGCTCTCGCCGAAAC	<i>tet(C)</i>	745	63°C	[9]
tetD-F	ACTGTCCAATGTGCTGTGGATTTGT				
tetD-R	CTGACCAGCACACCCCTGTAGTTTCC	<i>tet(D)</i>	747	63°C	This study
tetG-F	GAGCCGCAGTCGATTACACGATTATG				
tetG-R	CAACAGAACGGGAAACACCATCCATC	<i>tet(G)</i>	680	64°C	[10]
intI1-F	CGAACCGAACAGGGCTTATGTCCACTG				
intI1-R	CATCGTCGTAGAGACGTCGGAATGG	<i>intII</i>	838	63°C	[10]
5CS-F	GCCTCGGGCATCCAAGCAGCAAGC				
3CS-R	CTTGACCTGATAGTTGGCTGTGAGCAA	5'CS <i>attI1</i> end 3'CS	variable	65°C	This study
U7-L12	ACACCTTGAGCAGGGCAAAG	<i>tdhF</i>			
LJ-R1	AGTTCTAAAGGTTCGTAGTCG	<i>int</i>	500	60°C	[4]
104-RJ	TGACGAGCTGAAGCGAATTG	S044			
C9-L	AGCAAGTGTGCGTAATTG	<i>int2</i>	515	60°C	[4]

2.3. PFGE and Phage Typing. Genomic restriction was performed according to the standardized PulseNet *Salmonella* protocol [14]. Agarose-embedded DNA was digested with 40 U of *Xba*I for 3 h at 37°C. The restriction fragments were separated by electrophoresis in Tris-borate-EDTA (44.5 mM Tris-borate, 1 mM EDTA; pH 8.0) at 14°C for 20 h using a CHEF-DR III (Bio-Rad, Milan, Italy). Electrophoresis conditions were as follows: 6 V/cm, angle of 120°C, for 20 h with pulse times of 2.2 to 63.8 s. The *Salmonella* Braenderup H9812 strain was used as a molecular standard. The PFGE agarose

gels were stained with ethidium bromide (40 µg/mL) and the DNA band images were acquired by the Gel Doc-It photo documentation system (Gel Doc-It photo documentation system, UVP, Upland, CA, USA).

PFGE profiles in digital tagged image file format (TIFF files) were analyzed with the BioNumerics software package v.6.6 (Applied Maths, Sint-Martens-Latem, Belgium) [15]. DNA profiles differing in one or more DNA fragments were considered as distinct patterns. Strains with a coefficient of similarity ≥90% were classified as genetically closely related.

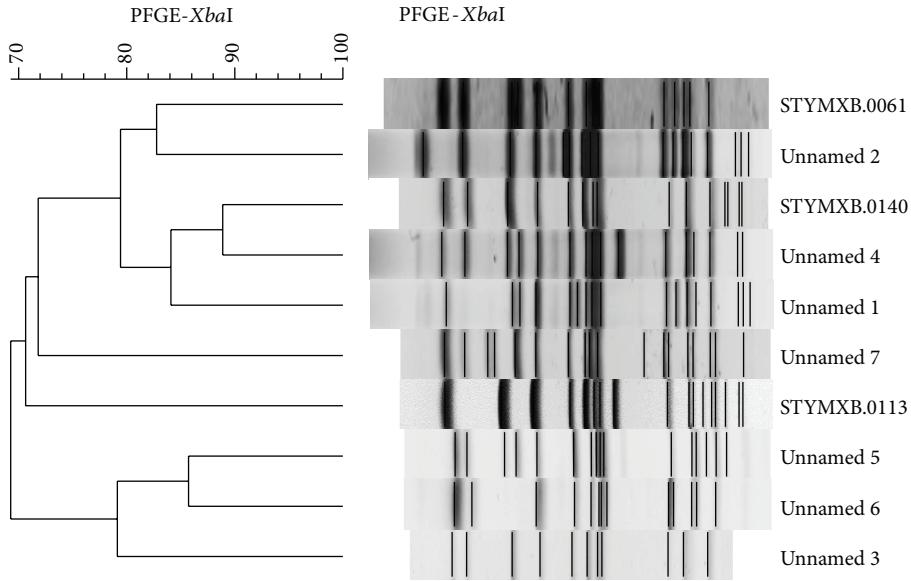


FIGURE 1: PFGE profiles and cluster analysis of *Salmonella enterica* serotype typhimurium strains analysed in this study. All representative variants of single PFGE patterns are included.

All PFGE profiles were compared with those included in the PulseNet-Europe international database and named with a six letter code followed by a four digit numerical identifier, for example, STYMXB.0006. Dendrogram and cluster analysis were performed using algorithms available within the BioNumerics software package v.6.6 (Figure 1). Percent similarity between different chromosomal fingerprints was scored by the Dice coefficient. The unweighted pair group method with arithmetic means (UPGMA), with a 1.00% tolerance limit and 1.00% optimisation, was used to obtain the dendrogram. DNA profiles differing by one or more DNA fragments were considered as distinct patterns. Phage typing was performed according to the standard procedure [16].

3. Results

3.1. Antimicrobial Susceptibility, Pulsotype, and Phage Typing. Six distinct resistance patterns were identified within the 62 MDR *S. typhimurium* strains analysed in this study (Table 2). The resistance pattern to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim (termed resistance pattern ACSSuT) was that prevalent (83.9%), being identified in 52 strains. All others resistance patterns were identified either in single (ASSuT, ASSuTp, and AKSSuT) or in a low number of isolates (ASuTTp and ACSSuTTp).

Strains were characterised by genomic *Xba*I restriction patterns and resolved through a pulsed field gel electrophoresis and phage typing. PFGE profiles were compared with those included in the PulseNet-Europe international database and specific pulsotypes were assigned when the coefficient of similarity had a value $\geq 90\%$. Among the 52 strains with resistance pattern ACSSuT, 39 were pulsotype STYMXB.0061 with phage types distributed as follows: DT120 (84.6%), U302 (13.5%), and RDNC (1.9%). The remaining 13 strains with

resistance pattern ACSSuT were pulsotypes STYMXB.0140 (4) or unnamed (9), and all of them were classified as phage type DT120. Five strains resistance pattern ASuTTp were pulsotype STYMXB.0113: two strains were classified DT35 and three DT193. Two isolates exhibiting the resistance pattern ACSSuTTp and the single isolates with resistance patterns ASSuT, ASSuTp, and AKSSuT were pulsotype unnamed and phage types DT120, DT7var, DT1, and U302, respectively.

3.2. Resistance Genes Associated with SGII. Identification of SGII and associated resistance genes was performed by PCR. Among the 52 strains with resistance pattern ACSSuT, 46 were positive for the presence of SGII. Resistance genes (*aadA2*, *floR*, *tet(G)*, *bla_{PSE-1}*, and *sull*) usually harboured by SGII were identified and their genetic organization proved indistinguishable from that reported for SGII [4]. SGII and associated resistance genes were also identified in strains with resistance pattern ACSSuTTp and found identical to that identified in strains with resistance pattern ACSSuT. Strains with resistance patterns ASuTTp, AKSSuT, ASSuT, and ASSuTp were all negative for the presence of SGII.

3.3. Resistance Genes and Class 1 Integrons. Class 1 integrons were identified in all strains with resistance patterns ACSSuT and ACSSuTTp. Two amplicons of 1,009 bp and 1,197 bp were obtained among the 46 strains with resistance pattern ACSSuT and SGII positive. The nucleotide sequence of the gene cassettes was determined and two open reading frames (ORFs) of 852 and 987 bp were detected and found homologous to *aadA2* and *bla_{PSE-1}*, respectively. PCR analysis was further extended for detection of *sull* and its linkage to class 1 integron. The *sull* gene was found associated only with the class 1 integron harbouring the *bla_{PSE-1}* gene cassette. These data further confirmed the presence of SGII. An amplicon of 2,013 bp was obtained from the remaining 6 strains with

TABLE 2: Antimicrobial susceptibility, phage types, pulsotypes, PCR detection of SGII, class 1 integrons, and resistance genes in *S. Typhimurium* strains isolated in Southern Italy in 2006–2008.

PulseNet Europe nomenclature	Phage type (no. of strains)	Year (no. of strains)	Resistance pattern ^a	Class 1 integrons			SGII	
				<i>intI1</i>	Gene cassette(s)	Resistance gene(s)	Left junction	Right junction
STYMXB.0061	DT120 (31)	2006 (9); 2007 (13); 2008 (9)	ACSSuT	+	<i>aadA2; bla_{PSE-1}</i>	<i>floR; sulI; tet(G)</i>	+	+
		2006 (1); 2008 (4)		+	"	"	+	+
		2008 (2)		+	"	"	+	-
	RDNC (1)	2006 (1)	"	+	"	"	+	+
STYMXB.0113	ST193 (3)	2006	ASuTTp	-	-	<i>bla_{TEM}; sul2; tet(A); dfrA12</i>	-	-
	DT35 (2) ^b	"	"	-	-	<i>bla_{TEM}; sul2; tet(A)</i>	-	-
STYMXB.0140	DT120 (4)	2007 (4)	ACSSuT	+	<i>bla_{OXA-30}-aadA1</i>	<i>catA1; sulI; tet(B)</i>	-	-
Unnamed 1	DT120 (9)	2006 (2); 2007 (3)	ACSSuT	+	<i>aadA2; bla_{PSE-1}</i>	<i>floR; sulI; tet(G)</i>	+	+
Unnamed 2	"	2008 (2)	"	+	"	"	+	+
Unnamed 3	"	2008 (2)	"	+	<i>bla_{OXA-30}-aadA1</i>	<i>catA1; sulI; tet(B)</i>	-	-
Unnamed 4	"	2007 (2)	ACSSuTTp	+	<i>aadA2; bla_{PSE-1}</i> <i>dfrA1-aadA1</i>	<i>floR; sulI; tet(G)</i>	+	+
Unnamed 5	DT7var ^c	2007 (1)	AKSSuT	-	-	<i>bla_{TEM}; sul2; strAB; tet(B)</i>	-	-
Unnamed 6	DT1	2008 (1)	ASSuT	-	-	<i>bla_{TEM}; sul2; strAB; tet(A)</i>	-	-
Unnamed 7	U302 ^d	2008 (1)	ASSuTp	-	-	<i>bla_{TEM}; sul2; dfrA12</i>	-	-

^aA, ampicillin; C, chloramphenicol; K, kanamycin; S streptomycin; Su, sulfamethoxazole; T, tetracycline; and Tp, trimethoprim.

^bThe resistance to Tp was not identified.

^cThe resistance to K was not identified.

^dThe resistance to S and Tp was not identified.

RDNC: the reaction pattern is not conforming to a recognised phage type.

Key: +, positive; -, negative.

resistance pattern ACSSuT (SGII negative). Two ORFs of 813 and 987 bp, organised as a gene cassette array, were identified and found homologous to *bla_{OXA-30}* and *aadA1*. The presence of both *bla_{OXA-30}* and *aadA1*, in these 6 isolates, was also confirmed by PCR and *sulI* was found associated with the gene cassette array. Detection of *catA1* and *tet(B)* completed the analysis of the resistance genes in these 6 isolates.

The strains with resistance pattern ACSSuTTp additional to SGII, indistinguishable from that identified in strains with resistance pattern ACSSuT, harboured another class I integron element. The gene cassette amplicon (1,534 bp) was sequenced and two ORFs of 474 and 789 bp were identified. The ORFs were found homologous to *dfrA1* and *aadA1*, respectively.

In strains with resistance pattern ASuTTp, the identified resistance genes were *bla_{TEM}*, *sul2*, *tet(B)*, and *dfrA12*. In the single strains with resistance patterns AKSSuT, ASSuT, and ASSuTp, the resistance genes were *bla_{TEM}*, *sul2*, *strAB*, *tet(B)*; *bla_{TEM}*, *sul2*, *strAB*, *tet(A)*; and *bla_{TEM}*, *sul2*, *dfrA12*,

respectively. The genetic basis for resistances to kanamycin (resistance pattern AKSSuT) and streptomycin (resistance pattern ASSuTp) was not identified.

4. Discussion

Salmonella infections still remain a major public health concern in many countries with an estimated incidence of 15.1 cases per 100,000 persons in the United States and 42.2 cases per 100,000 persons in the EU [17]. In Italy, the average annual incidence is 14 cases per 100,000 persons [18]. However, since *Salmonella* infections are generally based on the identification of *Salmonella* strains from stool culture, data officially reported are likely to represent only a fraction of infections which really occur. The difference between reported cases and those probably occurring has recently been investigated by a study that assessed the seroincidence of *Salmonella* infections in eight European countries, Italy included [18]. The study demonstrated the misleading data

on the incidence of *Salmonella* infections based only on numbers of stool culture-confirmed cases. Hence, the need to integrate epidemiological data with those gained by novel methods of investigation such as the assessment of cases by seroincidence.

Salmonella is also the leading cause of hospitalization from foodborne pathogens and the increase of antimicrobial resistance often represents a problem for the choice of a proper antimicrobial therapy (if required). A clear example is provided by the insurgence in the mid-1980s (in the United Kingdom) of the MDR *S. Typhimurium* phage type 104, resistance pattern ACSSuT encoded by SGII, and its subsequent diffusion. Shortly after its emergence, detection of the MDR *S. Typhimurium* DT104 constantly increased among 29 countries which took part in a survey conducted from 1992 to 2001 [19]. In the following years, this clone became virtually pandemic.

In Italy, a survey conducted from 1992 to 2000 highlighted the presence of this pandemic clone from 1992 [20]. Detection of the MDR *S. Typhimurium* DT104, resistance pattern ACSSuT, remained constant from 2002 to 2004 accounting for 20% of the *S. Typhimurium* strains of clinical origin [21].

In this study, we report the characterisation of 62 MDR *S. Typhimurium* strains isolated from clinical cases which occurred in Southern Italy from 2004 to 2006. The incidence of *S. Typhimurium* was of 53.5% (255 strains out of the 477), data that confirmed the prevalent detection of this serovar in Italy. Multidrug resistance was detected in 24.3% of the *S. Typhimurium* strains. STYMXB.0061 was the prevalent (62.9%) identified pulsotype. This datum is in agreement with that reported by other studies STYMXB.0061 being one of the most common profiles identified in *S. Typhimurium* [22] and in Italy reported as the second PFGE profile detected in human isolates from 2003 to 2006 [23]. STYMXB.0061 is strongly associated with the phage type 104 and this relationship is documented by a number of studies on *S. Typhimurium* strains isolated from both human and animal sources [10, 22, 23]. SGII, first detected in *S. Typhimurium* strains phage type 104, has also been identified in strains with phage types related to DT104 such as DT120, U302, or DT12 [24]. Strains DT120 or DT12 with resistance pattern ACSSuT exhibited PFGE profiles identical to those shown by isolates DT104 with resistance pattern ACSSuT and distinct from profiles exhibited by strains of the same phage types [25]. Such a feature has been supposed due to a change in phage susceptibility in a small proportion of *S. Typhimurium* strains phage type 104 with resistance pattern ACSSuT rather than to horizontal gene transfer of resistance determinants. Additionally, phage typing has widely been used for strain characterisation, and in surveillance and outbreaks investigation [26]. Interestingly, in our survey, none of the isolates STYMXB.0061 with resistance pattern ACSSuT was DT104. Thirty-one (79.5%) were phage type DT120 and seven (15.2%) phage type U302. The prevalent identification of phage type DT120 would suggest, at least in Southern Italy, a phage type shifting of the *S. Typhimurium* strain DT104 (resistance pattern ACSSuT, pulsotype STYMXB.0061) of clinical origin. This possibility is also supported by the virtual absence of phage type DT120 detection in *S. Typhimurium* strains of

human and non-human sources isolated in Italy at least until 2000 [27, 28]. In a subsequent study performed in Italy (2002–2004) on *S. Typhimurium* strains isolated from human and animal sources, DT120 was rarely detected among the 367 identified MDR strains (resistance pattern ACSSuT). Only 7 isolates were DT120 while 196 (72.3%) were DT104. Additionally, for all of these 7 strains, neither source of isolation nor antimicrobial resistance genes were specified [3].

MDR *S. Typhimurium* DT120 strains (e.g. resistant to ASSuT, encoded by *bla*_{TEM}, *strAB*, *sul2* and *tet(B)*) have recently emerged in European countries, with pigs being the likely reservoir of infection [29]. Indeed, in a survey on the prevalence of *Salmonella* in slaughtered pigs, conducted in the EU from 2006 to 2007, the serovars Typhimurium and Derby were those most frequently identified [30].

The potential impact of *S. Typhimurium* DT120 in public health has recently been highlighted by clinical outbreaks which occurred in Denmark in 2008 and the UK in 2011. Infections linked to the consumption of pork or pork products [31–34]. Moreover, in Greece, DT120 is associated with the most frequently occurring clones in human and pigs (2006–2011) [33]. However, none of these DT120 isolates was STYMXB.0061 (STYMXB.0083 was the pulsotype detected from the outbreak in UK, while STYMXB.0010 was that identified in Denmark and Greece) and, above all, the antimicrobial resistance patterns (mainly ASSuT and ACSSuTTp) were not linked to the presence of SGII.

In Italy, the phage type DT120 has commonly been detected from finishing pigs in slaughterhouses [35]. However, these data come from slaughterhouses located in Northern Italy while data from Southern Italy, to the best of our knowledge, are still unavailable. Additionally, none of the *S. Typhimurium* DT120 strains isolated from slaughterhouses in Northern Italy was resistance pattern ACSSuT and no data was available on their pulsotype. It would be interesting to investigate the molecular and phenotypic features of *S. Typhimurium* strains isolated from swine samples in Southern Italy. This might help determine possible epidemiological routes that have contributed to the spread of *S. Typhimurium* strains phage type DT120 that we detected from the clinical isolates.

The integrons *dfrA1-aadA1* and *bla*_{OXA-30}-*aadA1* have been identified in *S. Typhimurium* strains isolated from different geographic areas [6]. The identification of distinct groups of integrons together with phage typing and molecular strain characterisation represents valuable markers to assess possible clonal expansion. Interestingly, the above types of integrons were all identified in MDR strains DT120 with different pulsotype (STYMXB.0113 or Unnamed) highlighting the presence of distinct DT120 clones among clinical cases in Southern Italy.

5. Conclusions

In this study, we first described the extent of the antimicrobial resistance genes and associated genetic elements among clinical MDR *S. Typhimurium* strains isolated from an Italian area where *Salmonella* infections are endemic. Data

presented suggest, in this endemic area, a phage type shifting of the pandemic clone DT104 (resistance pattern ACSSuT) to DT120. Such a shifting, to the best of our knowledge, has occurred recently since no clinical *S. Typhimurium* strains DT120 (resistance pattern ACSSuT encoded by SGI encoded) was not reported until 2004. The extent and persistence of these findings raise concerns in public health about the possible diffusion of these emerging DT120 clones.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Emerging Rapid Resistance Testing Methods for Clinical Microbiology Laboratories and Their Potential Impact on Patient Management

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Atypical and multidrug resistance, especially ESBL and carbapenemase expressing Enterobacteriaceae, is globally spreading. Therefore, it becomes increasingly difficult to achieve therapeutic success by calculated antibiotic therapy. Consequently, rapid antibiotic resistance testing is essential. Various molecular and mass spectrometry-based approaches have been introduced in diagnostic microbiology to speed up the providing of reliable resistance data. PCR- and sequencing-based approaches are the most expensive but the most frequently applied modes of testing, suitable for the detection of resistance genes even from primary material. Next generation sequencing, based either on assessment of allelic single nucleotide polymorphisms or on the detection of nonubiquitous resistance mechanisms might allow for sequence-based bacterial resistance testing comparable to viral resistance testing on the long term. Fluorescence *in situ* hybridization (FISH), based on specific binding of fluorescence-labeled oligonucleotide probes, provides a less expensive molecular bridging technique. It is particularly useful for detection of resistance mechanisms based on mutations in ribosomal RNA. Approaches based on MALDI-TOF-MS, alone or in combination with molecular techniques, like PCR/electrospray ionization MS or minisequencing provide the fastest resistance results from pure colonies or even primary samples with a growing number of protocols. This review details the various approaches of rapid resistance testing, their pros and cons, and their potential use for the diagnostic laboratory.

1. Introduction

Generation of antimicrobial susceptibility patterns remains one of the most important tasks of clinical microbiology laboratories. The effective calculated antimicrobial therapy of infectious disease patients is consistently challenged by the rapidly rising prevalence of resistant and multidrug- or even pandrug-resistant pathogens worldwide. In recent years, this trend was accompanied by a shift from Gram-positive to Gram-negative bacteria like multidrug-resistant Enterobacteriaceae strains (MRE; resistant to three or more classes of antibiotics) as well as multidrug-resistant

nonfermenters (*Pseudomonas aeruginosa* and *Acinetobacter baumannii*) [1–4]. In particular, carbapenemase expressing Enterobacteriaceae coresistant to non-beta-lactam antibiotics like quinolones, aminoglycosides, colistin, and fosfomycin are a recent major public health concern [5–8]. Colonization by MRE is highly region and patient group specific. For example, in the French capital Paris, a tenfold increase in the intestinal colonization rate of healthy individuals with extended-spectrum beta-lactamase- (ESBL-) producing bacteria was observed during the last half decade [9]. ESBL colonization was with 4.6%, particularly in French children aged 6–24 months, significantly above average [10]. Long

lasting persistence of MRE, as demonstrated by a Swedish and a French study, contributes to the increase in the MRE prevalence, sometimes even years after infection [11, 12]. A median MRE-colonization period of 12.5 months could be detected in a cohort of newborn children in Norway [13]. An English study was able to verify persistence of resistance genes even in the absence of antibiotic pressure [14]. Furthermore, the colonization rate also differs between healthy subjects and patients at risk. It could be demonstrated in a Korean endemic area that 20.3% of healthy individuals were colonized with ESBL producers, while high-risk patients were colonized in 42.5% of cases [15]. However, the risk of faecal colonization depends mainly on the local prevalence. For example, an ESBL prevalence of 65.7% has been demonstrated in healthy adults in Thailand [16], while another study showed an ESBL prevalence of 11.3% in outpatients in England [17]. Farm animals are another reservoir for multidrug-resistant bacteria. A survey in the Netherlands demonstrated that chickens are colonized with ESBL-producing Enterobacteriaceae to more than 70%, while swine and cattle are known reservoirs for livestock-associated methicillin resistant *Staphylococcus aureus* (laMRSA) [18].

The multiresistances of the Gram-negative bacteria represent a major challenge for the traditional culture-based microbiology. Furthermore, the limited treatment options for a calculated therapy and therewith the risk of an inappropriate therapy are an intensifying factor of this problem [19]. As a consequence morbidity and mortality of outpatient and nosocomial-acquired infections with multidrug-resistant Gram-negative bacteria are significantly increased. Similarly, *Mycobacterium tuberculosis* has posed a serious health threat as a result of multidrug resistance. In its 2013 global report on tuberculosis, WHO estimates that 3.6% (95% confidence interval: 2.1–5.2%) of new cases and 20.2% (95% confidence interval: 13.3–27.2%) of previously treated cases had multidrug-resistant (MDR) tuberculosis (defined as tuberculosis caused by *M. tuberculosis* isolates that are resistant to rifampicin and isoniazid) and 1.3 million TB deaths [20]. On the other hand, CMV resistance has been reported to be on the rise in transplant recipients [21, 22].

Information on antimicrobial susceptibility aids a clinician in prescribing an appropriate antimicrobial drug for a particular infection. Due to the rapid rise in antimicrobial resistance worldwide [1], it is becoming increasingly important for a clinician to rapidly receive information on the antimicrobial susceptibility profile of the isolated pathogen for appropriate treatment to be initiated. Traditionally, clinical microbiology laboratories have relied on phenotypic methods to determine the antibiotic susceptibility profiles of pathogens [23]. These methods remain useful and have advantages such as low costs as well as being easy to perform and having established interpretation criteria. But they lack the ability to generate timely susceptibility results, hence delaying initiation of treatment [24]. Furthermore, currently there is a need to establish adequate and standardized screening and isolation procedures for carbapenemase-producing bacteria especially in risk patients as well as in patients

in which MRE colonization/infection has been previously shown. These limitations have been found to have consequences in patient management; for example, delay in the initiation of antibacterial treatment has led to increases in mortality [25] as well as in hospitalization time [26] and make it challenging to implement the back-end approach of the antimicrobial stewardship program, which has shown rewarding results in patient management and the fight against antimicrobial resistance [27].

In response to the limitations of phenotypic methods and the desires to improve patient management and curb the spread of antimicrobial resistance, rapid antimicrobial susceptibility testing methods are continuously developed. These methods have been found to identify a pathogen and its antimicrobial susceptibility profile within a short period of time. There are basically five different ways to accelerate susceptibility testing in clinical diagnostics: (I) bypassing conventional culture by direct detection of the pathogen or resistance mechanism in the primary sample; (II) bypassing plate or broth culture dependent susceptibility testing (secondary culture); (III) avoiding time consuming work steps/methods; (IV) increasing the sensitivity to the detection of the infectious agent; that means detecting the infectious agent in earlier disease stages at lower viral or microbial loads; and (V) earlier detection of an evolving drug resistance during treatment in spreading less susceptible quasispecies.

For example, real-time quantitative PCR (qPCR) has made it possible to detect multidrug-resistant tuberculosis (MDR TB) in a sample within an hour, hence immediately initiating appropriate treatment and control measures [28]. Also, MALDI-TOF mass spectrometry (MS) has made it possible to detect the most pathogens in a sample within minutes with high sensitivity and specificity [29]. In addition, these methods have made it possible to control the spread of resistant strains, reduce the length of patient stay in hospitals, and enhance the implementation of antimicrobial stewardship programs.

In this review, we detail the rapid antimicrobial susceptibility testing methods that have been developed recently. They include classical agglutination assays; molecular testing methods, for example, qPCR, DNA microarrays, Luminex xMAP assays, and next generation sequencing; fluorescence *in situ* hybridization (FISH); and mass spectrometry-based methods, for example, phyloproteomics, assays using stable isotope labeling of amino acids, mass spectrometric beta-lactamase assays, PCR/electrospray ionization-mass spectrometry (PCR/ESI MS), minisequencing, and mass spectrometry-based comparative sequence analysis (MSCSA). In addition, we discuss the impact that these techniques are likely to bring for the patient management and the reduction of antimicrobial resistance.

2. Agglutination Assays as Rapid Culture-Associated Options

Agglutination assays are based on a suspension of microparticles coated with specific antibodies, leading to agglutination

in contact with their specific antigens. Such procedures are useful for a preliminary resistance screening from pure bacterial colonies if the resistance mechanism of interest is associated with a single antigen only, which is expressed on the surface of the pathogen. Accordingly, agglutination assays are unfeasible for the screening for complex resistance patterns, which may be associated with multiple structurally different families of enzymes as in the case of extended-spectrum beta-lactamases (ESBL) or carbapenemases in Gram-negative rod-shaped bacteria.

Agglutination assays for the rapid identification of bacterial resistance patterns are widely restricted to the identification of the penicillin binding protein 2a (PBP-2a), the major resistance determinant of Methicillin resistant *Staphylococcus aureus* (MRSA). Different agglutination kits show specificities of 91.3% to 100% if applied to MRSA colony material [30–32]. The sensitivity is even more restricted, ranging between 82.7% and 94.1% [30–32]. If sufficient quantities of colony material are used, agglutination testing allows for the identification of small-colony variant MRSA strains as well [33].

The lack of sensitivity seems to be associated with certain staphylococcal cassette chromosome (SCC-mecA) types with type IV scoring particularly poor [31]. Furthermore, agglutination kits are only positive if methicillin resistance is due to the *mecA* gene. If *mecC*, a divergent *mecA* homologue, is the cause of the resistance, agglutination usually fails as observed for 10 out of 10 *mecC*-positive, live-stock associated MRSA strains [34].

Of note, agglutination based PBP-2a testing is possible from liquid sample materials as well. However, the sensitivity is poor. From blood culture pellets, PBP-2a agglutination showed sensitivity of only 18% in a recent study. In contrast, specificity was excellent with 100% [35].

3. Genotypic Antimicrobial Resistant Detection Methods

The usage of genotypic methods in the rapid detection of antimicrobial resistance genes is gradually shifting from academic research laboratories to diagnostic laboratories and point-of-care testing. The attractiveness of these methods in determination of antimicrobial resistance has been attributed to two factors: firstly, their capability to generate results within a short time as compared to phenotypic methods; secondly, their capability to detect antimicrobial genes directly from the patient sample without necessarily waiting for culture results [36]. These two attributes aid clinicians in prescribing appropriate treatment to patients at the opportune time, hence making a positive contribution to antimicrobial stewardship programs [27]. However, genotypic tools for the detection of antimicrobial resistance may generate false negative results due to (i) their inability to detect new resistance mechanisms or (ii) false-positive results, because they may detect inactive or incomplete resistance genes in a specimen, which have not inferred resistance to the antimicrobial drug under test [37].

Current genotypic methods that are used for the rapid detection of antimicrobial resistance genes include (i) nucleic acid amplification methods, particularly real-time quantitative PCR (qPCR); (ii) DNA hybridization based methods, particularly DNA microarrays; (iii) Luminex xMAP technology; and (iv) next generation sequencing methods. Below is a brief description on the application of each of these molecular methods for the rapid detection of antimicrobial resistance.

3.1. Nucleic Acid Amplification Methods. Recently, one of the PCR techniques that has received a wide application in clinical microbiology is the quantitative real-time PCR (qPCR) technique [38]. This has been attributed to its flexibility and capability to rapidly and simultaneously identify multiple pathogens in a clinical specimen and the presence of antimicrobial resistance genes in the identified pathogens [39]. As a result, numerous qPCR assays for rapid identification of pathogens in clinical specimens have been developed but most of the available qPCR assays for detection of microbial resistance genes are limited to the detection of antibiotic resistance. In short, most of the available commercial qPCR assays detect the presence of *mecA* and *mecC*, which confer methicillin resistance in *S. aureus*; the *vanA* and *vanB* genes, which confer glycopeptide resistance; and genes that encode extended-spectrum β-lactamases (for detailed review on each assay see Maurin, 2012 [39]). One outstanding feature of all these qPCR assays is their capability to simultaneously and accurately detect resistance genes within a remarkably shorter time period of 4–6 hours. Similarly, qPCR assays for rapid detection of resistance against rifampin (RIF) and isoniazid (INH) have been introduced. Ramirez and coworkers have recently combined qPCR and high-resolution melt (HRM) technology to develop an assay, which rapidly and simultaneously identifies multidrug-resistant *M. tuberculosis*, mutations in the *rpoB* gene conferring resistance to RIF, and mutations in the *katG* and *inhA* genes conferring resistance to INH [28]. This assay produces results within 6 hours as compared to GenoType MTBDRplus assay (Hain Lifescience GmbH, Germany) and culture susceptibility testing, which take 8 hours and 56 days to generate results. In the recent time, several in-house qPCR assays for rapid and simultaneous detection of genes encoding *Klebsiella pneumoniae* carbapenemase (*bla_{KPC}*) and New Delhi metallo-β-lactamase (*bla_{NDM}*) in Gram-negative rod-shaped bacteria [40–43] have been introduced. Similarly, several in-house qPCR assays for rapid and simultaneous detection of *bla_{OXA-48}*, *bla_{VIM}*, and *bla_{IMP}* carbapenemase genes in Enterobacteriaceae have been established [44–46].

PCR-based MRSA testing has found wide applications in microbiological routine laboratories. Next to in-house assays, commercially available molecular MRSA testing platforms comprise, for example, BD GeneOhm MRSA (Becton Dickinson, Heidelberg, Germany), GT MRSA Direct/GQ MRSA (Hain Lifescience, Nehren, Germany), Hyplex StaphyloResist (Amplex, Gießen, Germany), LightCycler (Roche Diagnostics Ltd., Rotkreuz, Switzerland) kits like LC MRSA Advanced, Cepheid Xpert/Gene Expert (Cepheid, Sunnyvale, CA, USA), and TIB Molbiol LightMix MRSA (TIB Molbiol,

Berlin, Germany). All test systems showed reliable results in a recent external laboratory control evaluation in Germany [47]. Similarly, commercial PCR assays for the detection of ESBL-associated $\text{bla}_{\text{CTX-M}}$ beta-lactamases and only partially ESBL-associated bla_{TEM} and bla_{SHV} as well as OXA1-type carbapenemases (the latter combined in a consensus run) were introduced (Amplex, Gießen, Germany) [48]. Similar multiplex PCR systems are available for the most frequently detected carbapenemases, which are particularly useful for the follow-up during hospital outbreak events (Amplex, Gießen, Germany), even from primary sample materials [49, 50]. The switch of molecular carbanemase detection to robust loop-mediated isothermal amplification (LAMP) [46] allows for commercial point-of-care testing (POCT) compatible test solutions for bedside testing, for example, the eazyplex Super-BugCRE system (Amplex, Gießen, Germany) which provides results within 10 minutes. However, the great number of different possible cephalosporin and carbapenem resistance mechanisms finally exceeds any multiplexing capacity if completeness is aspired.

Nevertheless, in addition to rapid and simultaneous providing of reliable results, qPCR has been found to be affordable, sensitive, specific, user friendly, not space demanding, and deliverable [37–39, 51]. Due to these attributes, qPCR has found various applications in point-of-care testing (POCT). For example, the Xpert MTB/RIF test (Cepheid, Sunnyvale, CA, USA) is a qPCR-based assay that has been developed to rapidly and simultaneously detect *M. tuberculosis* and rifampicin (USAN: rifampin) resistance. To evaluate its usefulness in POCT, a large multicentre study involving 6069 cases from six unrelated sites was performed. In this study, Xpert MTB/RIF detected rifampicin resistance cases in 1 hour as compared to line-probe assay and phenotypic drug susceptibility testing that detected the same cases in 20 days and 106 days, respectively [52]. As mentioned above, a similar GenXpert-based POCT test for MRSA screening from clinical sample materials is available as well.

Multiplex PCR assays have also been developed to rapidly and simultaneously identify multiple pathogens in clinical specimens as well as the presence of antimicrobial resistance genes in the identified pathogens. Strommenger and coworkers developed a multiplex PCR, which simultaneously detects 9 resistance genes in *S. aureus* directly from clinical specimen within 6 hours [53]. These 9 resistance genes include *mecA* (methicillin resistance), *aacA-aphD* (aminoglycoside resistance), *tetK*, *tetM* (tetracycline resistance), *ermA*, *ermC* (macrolide-lincosamide-streptogramin B resistance), *vataA*, *vatB*, and *vatC* (streptogramin A resistance) [53].

Like qPCR, multiplex PCR assays were used as POC tests to facilitate patient management. One example is the multiplex PCR-based Unyvero Pneumonia Application (UPA) assay (Curetis AG, Holzgerlingen, Germany) that has been developed to rapidly and simultaneously detect 18 bacterial species, *Pneumocystis jirovecii* and 22 resistance markers from respiratory specimens (<http://www.curetis.com/>). In one of the studies showing its suitability for POC testing, the UPA assay detected multiple antibiotic resistances within 1 hour (as compared to phenotypic methods that took 96 hours) in a group of 56 hospitalized patients with respiratory tract

infections who were under treatment. This finding influenced the modification of treatment in fifteen patients with severe pneumonia leading to their recovery [54]. The UPA assay is, of course, not able to replace conventional testing due to its design because it is not able to detect further microbial species and resistance mechanisms besides the implemented ones.

The continuous development of PCR-based assays with the capability to rapidly and simultaneously detect pathogens and presence of resistance genes in specimens coupled with their application in POCT may further improve the management of patients as long as appropriate quality control is ensured.

3.2. DNA Microarray Technology. The biggest challenge associated with the unprecedented rise of antimicrobial drug resistance worldwide is the scarce availability of assays that are able to rapidly and simultaneously identify a causative pathogen and generate its antimicrobial resistance profile. Recent oligonucleotide-based DNA microarrays match this challenge. In a recent study, Zhang and coworkers described that CapitalBio DNA microarray (CapitalBio Corp.) could in a mean time of 5.8 hours simultaneously identify *Mycobacterium* species and detect mutations that confer isoniazid (INH) and rifampicin (RMP) resistance in specimens collected from spinal tuberculosis patients as compared to conventional culture and drug susceptibility testing which took a mean time of 56.8 days [55]. Briefly, oligonucleotide probes, which had been designed to identify *Mycobacterium* species based on 16S rRNA sequences and mutations of *rpoB*, *inhA*, and *katG* that confer INH and RMP resistance, were covalently linked to the surface of aldehyde-activated slides. DNA was extracted from specimens. PCR was used to amplify the resistance genes and amplicons hybridized on the slides. The emitted fluorescent signals were analyzed. Guo and coworkers evaluated the ability of a biochip, which is based on the same principle to rapidly and simultaneously identify multidrug-resistant *M. tuberculosis* (MRTB) and mutations of *rpoB*, *inhA*, and *katG* that confer INH and RMP resistance in clinical sputum specimens [56]. This group found that the biochip could in a mean time of 6 hours simultaneously identify *M. tuberculosis* and detect mutations that confer INH and RMP resistance.

Recent reports have also reported the availability of Check-Point's ESBL/KPC DNA microarray for the identification and detection of extended-spectrum β -lactamases (ESBLs) and *Klebsiella pneumoniae* carbapenemases (KPC carbapenemases) [57, 58]. This array uses a methodology known as multiplex ligation detection to identify ESBL-associated or at least partially ESBL-associated genes (bla_{TEM} , bla_{SHV} , and $\text{bla}_{\text{CTX-M}}$) and bla_{KPC} genes (for details see [57]). In a study to evaluate the rapidness at which this array could identify and detect these genes, Naas and coworkers found that Check-Point's ESBL/KPC DNA microarray could identify them in 7–8 hours as compared to conventional susceptibility testing that took a mean time of 54 hours. Similar results were observed by Willemsen and coworkers in a study that was aimed at evaluating the rapidness at

which this array could identify and detect these ESBL/KPC genes in hospitals in the Netherlands [58]. In addition to detecting and identifying ESBL/KPC resistance in gastrointestinal tract infections caused by Enterobacteriaceae, Check-Point's ESBL/KPC DNA microarray has also been used to detect and identify KPC resistance in hospital-acquired pneumonia caused by *Klebsiella pneumoniae* [59]. Based on these experiences, the Check-MDR CT 102 DNA microarray for the detection of the most prevalent carbapenemase genes (bla_{NDM} , bla_{VIM} , bla_{KPC} , $\text{bla}_{\text{OXA-48}}$, and bla_{IMP}) and extended-spectrum β -lactamase- (ESBL-) related gene families (bla_{SHV} , bla_{TEM} , and $\text{bla}_{\text{CTX-M}}$) has been developed. The evaluation of the rapidness of the Check-MDR CT 102 DNA microarray to detect these genes has shown that it yields results 5 hours faster than Check-Point's ESBL/KPC DNA microarray [60].

At present, the DNA microarray technology is mostly used in the routine detection of antimicrobial resistance of TB and HIV [61–66]. The routine use of systems such as MVplex (Genaco Biomedical Products, Huntsville, USA) and StaphPlex systems (Genaco Biomedical Products, Huntsville, USA), which combine both qPCR and DNA microarray technology, suggest that independent DNA microarray technology might find further applications in the routine clinical microbiology [67, 68]. The MVplex system detects the *nuc*, *mecA*, (SCCmec)-*orfX*, *vanA*, *vanB*, *ddl*, and *tuf* genes to screen for MRSA in nasal swabs [69], and the related StaphPlex system performs simultaneous species-level identification (*nuc* versus *tuf*) and detection of *mecA*, *aacA*, *ermA*, *ermC*, *tetM*, and *tetK* as well as Panton-Valentine leukocidin (PVL) for the rapid detection and characterization of staphylococci directly from positive blood culture bottles [70].

3.3. Luminex xMAP Technology. The description of cooccurring single nucleotide polymorphism (SNP) mutations in antimicrobial resistance associated genes allows for targeted resistance testing. For example, unequivocally genetic studies have proven that there are 5 different mutations in quinolone resistance-determining region (QRDR) of *gyrA*, *gyrB*, and *parE* within *Salmonella typhi* [71]. Similar studies have also shown distinct mutations in the quinolone resistance-determining region (QRDR) of *gyrA* within *Campylobacter jejuni* and *Campylobacter coli* [72].

Rapid simultaneous detection of cooccurring single nucleotide polymorphism (SNP) mutations in antimicrobial resistance associated genes remains, however, challenging. Most molecular assays such as qPCR and pyrosequencing lack the capability to simultaneously detect cooccurring single nucleotide polymorphism (SNP) mutations in different genes in a given specimen [73]. However, this challenge has been overcome by Luminex xMAP Technology, a multiplexing technology, which allows for simultaneous detection of multiple nucleic acid sequences in a single reaction [74]. During operation, microtiter plates are loaded with microspheres, that is, coated and color-coded beads. The microspheres are mixed with purified nucleic acids of the test organism and allowed to hybridize, emitting monochromatic light, which the Luminex analyzer reads and interprets. At

present, this technology has been used to simultaneously detect 11 mutations in *gyrA*, *gyrB*, and *parE* of *Salmonella Typhi* and *Salmonella Paratyphi A* [75]. Further, it has been used to simultaneously detect mutations in *gyrA* of *C. jejuni* and *C. coli* [76]. In comparison to sequencing and microarray technology, Luminex xMAP Technology has been found to be flexible, rapid, and cost effective [74–76].

3.4. Next Generation Sequencing (NGS). Near whole genome sequencing (WGS) or next generation sequencing (NGS) allows for the assessment of bacterial genomes within several hours. A variety of different technological solutions have been introduced, including laser printer sized benchtop devices like 454 GS Junior (Roche, Basel, Switzerland), MiSeq (Illumina, San Diego, CA, USA), and Ion Torrent PGM (Life Technologies, Grand Island, NY, USA). In a previous analysis, the MiSeq (Illumina) system scored best regarding both throughput per run and error rates, while both the 454 GS Junior (Roche) and the Ion Torrent PGM (Life Technologies) systems were prone to homopolymer-associated indel errors [77].

Result interpretation of whole bacterial genomes is based on either allelic comparisons [78] or single nucleotide polymorphism (SNP) analysis [79]. Data assessment and interpretation can be facilitated by commercial software packages like SeqSphere+ (Ridom BIOINFORMATICS Ltd., Münster, Germany) or BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium).

NGS allows for resistance identification by the presence of the underlying mechanism rather than just in pharmacodynamic terms [80], so it may revolutionize microbial resistance testing on the long term. This comprises the identification and characterization of resistance genes encoding for extended-spectrum β -lactamases (e.g., $\text{bla}_{\text{CTX-M}}$, bla_{TEM} , and bla_{SHV}), plasmid-mediated AmpCs (e.g., bla_{CMY}), quinolone resistance (e.g., mutations in *gyrA*, *parC*, or *qnr* elements), aminoglycoside resistance (e.g., aminoglycosides modifying enzymes, 16S rRNA methylases), or carbapenemases (e.g., bla_{KPC} , bla_{NDM}) [81].

NGS-based resistance testing is of particular interest for slowly growing infectious agents with atypical resistance patterns like multidrug-resistant (MDR) or extensive-drug resistance (XDR) *M. tuberculosis*, for which rapid identification or exclusion of resistance determinants is of high relevance for the therapeutic approach. Ion Torrent full-gene sequencing with consecutive complete genetic analysis within 5 days (Table 5) allowed for reliable resistance detection in *M. tuberculosis* isolates of Burmese, Hmong, and Indian immigrants in the USA [82]. Similar WGS data were described for drug-resistant strains from Russia, harbouring almost all known drug-resistance associated mutations [83]. In a direct comparison of Ion Torrent sequencing with phenotypic Bectec MGIT 960 (Becton Dickinson, Franklin Lakes, NJ, USA) analysis and genotypic Hain line-probe assay (LPA) (Hain Lifescience Ltd., Nehren, Germany), there was complete concordance of NGS to phenotypic resistance and genotypic *rpoB* and *katG* results for the analyzed *M. tuberculosis* isolates. Even more, Ion Torrent sequencing detected

uncommon substitutions and previously uncharacterized resistance mutations in *rpoB*, *rrs*, and *pncA* [84]. Further, NGS is able to discriminate mixed mycobacterial genotypes in patient isolates based on single nucleotide variations (SNVs) [85]. So it might be suitable to identify resistance mutations in genotypes that occur in minor proportions only.

However, NGS-based resistance testing is not restricted to mycobacteria. Recently, NGS was used to identify transmissible plasmids in multidrug-resistant *E. coli* isolates expressing an ESBL phenotype and transferring their cefotaxime resistance marker at high frequency in laboratory conjugation experiments [86]. High-throughput sequencing successfully proved to be a valuable tool for tracing resistance plasmids in the course of outbreaks as well [87]. However, a commercial NGS assay (Hospital Acquired Infection BioDetection System, Pathogenica, Boston, MA, USA) for investigations of outbreaks with ESBL-positive Enterobacteriaceae showed good sensitivity (98%) but failed to discriminate between ESBL and non-ESBL TEM and SHV beta-lactamases or to specify CTX-M genes by group [88].

Current obstacles to a routine use of NGS technologies in diagnostic microbiology and resistance testing comprise costs and scarcely available user-friendly bioinformatics platforms [89]. Nevertheless, NGS technologies provide high-resolution genotyping in a short time frame of only two to five days [89]. Therefore, NGS/WGS in the microbiological laboratory will be the logical next step for the routine diagnosis of infection and the prediction of antimicrobial susceptibility [90], potentially replacing traditional cultural approaches on the intermediate or long term.

4. Fluorescence *In Situ* Hybridization (FISH) for the Detection of Bacterial Resistance

FISH (fluorescence *in situ* hybridization) is a cheap and convenient option for the identification and resistance testing of bacterial pathogens. Traditional FISH is based on specific hybridization of short, usually 18–25 bases long, fluorescent-labelled, single-stranded oligonucleotide probes to ribosomal RNA (rRNA) of the target organism with subsequent analysis under the fluorescence microscope, usually allowing for the identification of microbes at genus or species level. In principle, each kind of intracellular RNA can be hybridized with FISH probes. However, rRNA is particularly well suited as a FISH target, because ribosomes are numerous in a protein-synthesizing cell, thus allowing for a boosting of fluorescence intensity [91].

This traditional FISH method is both rapid and easy to standardize, so it can be applied for molecular rapid testing. Small modifications of the procedure comprise the use of patent-protected, commercial peptide nucleic acid (PNA) probes or probes containing locked nucleic acids (LNA) instead of simple single-stranded DNA probes. PNA-FISH technology reduces nonspecific probe attachment due to the electrically neutral backbone of the oligonucleotides and is recommendable for routine diagnostics due to a higher degree of standardization. However, patent-protected PNA

probes are expensive, although they are well suited for the diagnostic routine setting [92].

FISH is particularly suitable for the detection of resistance determinants if two prerequisites are guaranteed. Ribosomally mediated resistance, for example, affecting antibiotic drugs like macrolide or linezolid, is well suited, because ribosomal RNA copies are numerous in living cells, allowing for bright fluorescence signals. Further, FISH can be successfully applied if only one or few variable bases provide resistance, so there is no need for a large number of probes in the probe panel.

These prerequisites are fulfilled in case of clarithromycin resistance testing in *Helicobacter pylori*. Therefore, FISH-based resistance testing was early evaluated for this indication [93]. Clarithromycin in *H. pylori* is basically mediated by three point mutations in the ribosomal 23S rRNA [94] which can be addressed by three described FISH probes: ClaR1, ClaR2, and ClaR3 [93] (Table 1). While ClaR1 is associated with a minimum inhibitory concentration (MIC) of >64 mg/L, ClaR2 and ClaR3 are associated with varying MICs between 8 mg/L and 64 mg/L [94].

The FISH probes for clarithromycin resistance testing in *H. pylori* were successfully applied to bacteria both from culture and in biotic material and extensively assessed in various studies [93, 95–97]. Reliable test results can even be achieved in formalin-fixed, paraffin-embedded tissue after adequate deparaffination [98]. The combined use of probes labelled with different fluorescence molecules allows for the identification of coinfections with clarithromycin-sensitive and -resistant *H. pylori* strains by FISH [99].

Commercial test providers distributed the robust and easy-to-apply procedure. In one study with such a commercial test kit [100], a sensitivity of 90% and a specificity of 100% were achieved for the detection of clarithromycin-resistant *H. pylori* within biotic material. In another study, occasional false-positive *H. pylori* detections were generated [101], although the results of FISH-based resistance testing of correctly identified *H. pylori* proved to be reliable. Recently, a PNA probe-based approach for clarithromycin resistance testing in *H. pylori* showed perfect matching with PCR/sequencing in a retrospective study with formalin-fixed, paraffin-embedded tissues (Table 2) [102].

Similar to *H. pylori*, FISH-based clarithromycin resistance testing could be successfully demonstrated for thermotolerant *Campylobacter* spp. with a wild-type probe and a clarithromycin resistance probe targeting the A2059G mutation in the 23S rRNA gene (Table 3). The observed sensitivity and specificity with culture material were 100% [103].

Comparable to clarithromycin resistance, linezolid resistance is ribosomally mediated. In enterococci, it is typically caused by a 2567G>T base substitution in the 23S rRNA (Table 4). In a collection of 106 enterococcal isolates, a corresponding linezolid resistance FISH assay succeeded in predicting phenotypic resistance in 100% of cases [104]. Even a single mutated allele was associated with strong fluorescence signals.

First successful attempts of FISH-based resistance testing were described for non-rRNA-based resistance mechanisms

TABLE 1: DNA-FISH-probes detecting clarithromycin resistance in *H. pylori*, Rüssmann et al., 2001a [93].

Target	Probe	Probe sequence
Wild type	ClaWT	5'-CGG-GGT-CTT-TCC-GTC-TT-3'
Clarithromycin resistance mutation 1 (A2143G)	ClaR1	5'-CGG-GGT-CTT-CCC-GTC-TT-3'
Clarithromycin resistance mutation 2 (A2144G)	ClaR2	5'-CGG-GGT-CTC-TCC-GTC-TT-3'
Clarithromycin resistance mutation 3 (A2143C)	ClaR3	5'-CGG-GGT-CTT-GCC-GTC-TT-3'

TABLE 2: PNA-FISH-probes detecting clarithromycin resistance in *H. pylori*, Cerqueira et al., 2013 [102], shortened versions of the DNA-FISH-probes from Table 1.

Target	Probe	Probe sequence
Wild type	HpWT	5'-GGT-CTT-TCC-GTC-T-3'
Clarithromycin resistance mutation 1 (A2143G)	Hp2	5'-GTC-TTC-CCG-TCT-T-3'
Clarithromycin resistance mutation 2 (A2144G)	Hp1	5'-GTC-TCT-CCG-TCT-T-3'
Clarithromycin resistance mutation 3 (A2143C)	Hp3	5'-GTC-TTG-CCG-TCT-T-3'

TABLE 3: DNA-FISH-probes detecting clarithromycin resistance in thermotolerant *Campylobacter* spp., Haas et al., 2008 [103]. Of note, probe C wt 23S is identical with probe ClaWT, probe C res 23S 2059A>G with probe ClaR2 (Table 1).

Target	Probe	Probe sequence
Wild type	C wt 23S	5'-CGG-GGT-CTT-TCC-GTC-TT-3'
Clarithromycin resistance mutation (A2059G)	C res 23S 2059A>G	5'-CGG-GGT-CTC-TCC-GTC-TT-3'

TABLE 4: DNA-FISH-probes detecting linezolid resistance in enterococci. Locked nucleic acids (LNA) were used at the mismatch position (**bold**, underlined print) within in probes.

Target	Probe	Probe sequence
Wild type	LZD-WT	5'-CCC-AGC- <u>T</u> CG-CGT-GC-3'
Linezolid resistance mutation (G2567T)	LZD-res	5'-CCC-AGC- <u>T</u> AG-CGT-GC-3'

TABLE 5: Approximate turn-around-time, investment costs, reagent costs, and necessity of skilled personnel of different rapid diagnostic test procedures.

Rapid diagnostic procedure	Turn-around-time	Investment costs	Reagents costs (per sample)	Necessity of skilled personnel
Agglutination assays	<5 minutes	—	<1.00€	Low
Fluorescence <i>in situ</i> hybridization	1-2 hours	<15,000.00€	1.00–8.00€	Intermediate
Real-time PCR (including DNA preparation)	4–6 hours	35,000.00–60,000.00€	15.00–25.00€	Strongly depending on the test system
Loop-mediated isothermal amplification (LAMP) assays	<1 hour	2,000.00–4,000.00€	15.00–25.00€	Intermediate
Next generation sequencing (NGS)	2–5 days	350,000.00–750,000.00€	75.00–800.00€	Very high
Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)	<5 minutes	75,000.00–300,000.00€	<1.00€	High

as well. FISH-based detection of *bla*_{SHV-238/240}, one of the genes coding for extended-spectrum β-lactamases (ESBL), is an example of a non-rRNA-based FISH protocol for detecting a particular resistance determinant using the probe 5'-GAC-CGG-AGC-TAG-CAA-GCG-3' [105]. However, the ESBL phenotype can be associated with a variety of different alleles, so this particular probe will be of use only in case of a specific suspicion, for example, during an outbreak. Accordingly, such

a procedure will be reserved for very few if any indications in the diagnostic routine.

Further progression of FISH technology comprises signal-amplified, catalyzed reported deposition (CARD) FISH; doubly labeled oligonucleotide probe- (DOPE-) based FISH; combinatorial labelling and spectral imaging (CLASI) FISH; and the combination of FISH with other diagnostic approaches as well as FISH procedures for gene identification,

requiring *in situ* amplification of the respective gene as in case of the rolling circle amplification (RCA) FISH [106]. RCA-FISH was successfully applied for the identification of the *mecA* gene in Methicillin resistant *Staphylococcus aureus* (MRSA) based on the *mecA*-probes MR-1 5'-AAG-GAG-GAT-ATT-GAT-GAA-AAA-GA-3' and MR-2 5'-GGA-AGA-AAA-ATA-TTA-TTT-CCA-AAG-AAA-A-3' [107].

FISH-based detection of resistance determinants is a promising diagnostic approach due to its rapidity, convenience, and cost effectiveness. The associated rapid detection of antimicrobial resistance may lead to early resistance-adapted optimization of antimicrobial therapy with associated benefits for the patient's health. The main advantage of FISH is its potential use for resistance testing directly from primary material including tissue with low effort. So FISH can also be applied in resource-limited settings where expensive technologies are not available (Figure 1). In contrast to PCR, FISH can also attribute a particular resistance mechanism to a microscopically observed bacterium.

However, so far, FISH is restricted to very few indications for which protocols have been described. As a further drawback, standardization of FISH-based resistance testing is widely missing. If applied from primary sample materials like tissue, tissue autofluorescence has to be considered, requiring considerable experience to interpret such diagnostic results. To reduce potential interpretation errors, FISH from tissue further requires counterstaining with a pan-eubacterial FISH probe and nonspecific DNA staining, for example, with DAPI (4',6-diamidino-2-phenylindole), to confirm the presence of nucleic acids of the detected pathogens as recently demanded [108].

Given all these limitations, FISH for resistance testing will presumably stay a bridging technology until amplification-based technologies will be available as easy-to-apply and cost-efficient benchtop systems on the market.

5. Direct Fluorescent Imaging of Resistance Determinants by Fluorescence Resonance Energy Transfer (FRET)

Nonnucleotide probes labelled with reporter and quencher molecules, allowing for fluorescence energy transfer (FRET), can be used to detect enzymatic resistance mechanisms as described for β -lactamases [109]. After enzymatic hydrolyzation of probes to separate the quencher from the reporter, the hydrolyzed probes attach the resistance enzymes as reactive electrophiles. However, this mechanism has so far been only described for β -lactamases in a proof-of-principle analysis [109] and broad evaluation studies are missing. Its practical relevance for the microbiological routine diagnostics will require further evaluation.

6. Mass Spectrometric Approaches

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry- (MALDI-TOF MS-) based intact cell mass spectrometry (ICMS) has recently advanced to the standard method for species identification for cultured bacteria

and fungi [24, 110–114]. Promising approaches have been made using ICMS spectra for subspecies identification [115]. This technique bears a high potential for the fast identification of susceptibility associated biomarker ions that is lately only marginally realized in clinical routine diagnostics. Thus, phyloproteomic approaches help to identify indirectly mostly chromosomal encoded resistance genes by identifying phylogenetic relatedness [116–121]. MS can be used to detect changes in the bacterial or fungal proteome induced by exposition to antimicrobials [24, 122–124]. Whole proteome changes in consequence of exposition to antimicrobials can be also detected using stable isotope labeled amino acids (SILAC) [125, 126]. One very promising approach is the so-called mass spectrometric beta-lactamase (MSBL) assay [127–131], which is based on the mass spectrometric detection of hydrolyzed beta-lactams. Finally there is the combination of genotypic and mass spectrometric methods: PCR amplicons can be characterized by PCR/electrospray ionization-mass spectrometry (PCR/ESI MS) [132], and minisequencing [133, 134] and mass spectrometry-based comparative sequence analysis [135, 136] can be used to detect susceptibility changes associated with point mutations.

6.1. Prediction of Broad Spectrum Resistant Clonal Groups by Phyloproteomics. MALDI-TOF MS-based intact cell mass spectrometry (ICMS) is potentially able to characterize strains at the subspecies level and could act as useful tool for taxonomy and epidemiology [137, 138]. For the discrimination of representative strains particular biomarker ions that were completely present or absent as well as shifts in biomarker masses in a particular subset of strains were considered. Using different mathematical algorithms, it was, for example, feasible to discriminate *Salmonella enterica* ssp. *enterica* serovar Typhi from other less virulent *Salmonella enterica* ssp. *enterica* serotypes [139], to distinguish *Campylobacter jejuni* MLST-ST22 and ST45 from other MLST sequence types [140] or to perform phyloproteomic analysis of *Rhodococcus erythropolis* [141], *Pseudomonas putida* [142], or *Neisseria meningitidis* [143].

The first approaches to associate MS fingerprints with susceptibility patterns were designed to differentiate methicillin susceptible *Staphylococcus aureus* (MSSA) from methicillin resistant *Staphylococcus aureus* (MRSA) [144–148]. These were mostly not standardized and hardly reproducible. But relatively good reproducibility was demonstrated for the discrimination of the five major MRSA clonal complexes CC5, CC8, CC22, CC30, and CC45 corresponding to the five major PFGE MRSA types regardless of their methicillin sensitivity [149, 150]. A study by Lu and coworkers identified a set of biomarkers that were able to distinguish between methicillin resistant and vancomycin-intermediate *S. aureus* (VISA) strains and vancomycin-susceptible *S. aureus* strains, as well as between SCCmec types IV and V isolates and SCCmec types I–III isolates [151]. Further studies demonstrated that isogenic *S. aureus* lacking or artificially harboring SCCmec could not be distinguished in a mass range from 2000 to 15000 m/z [152], whereas isogenic MRSA, which spontaneously reverted to MSSA, could be discriminated by MALDI-TOF MS [153].

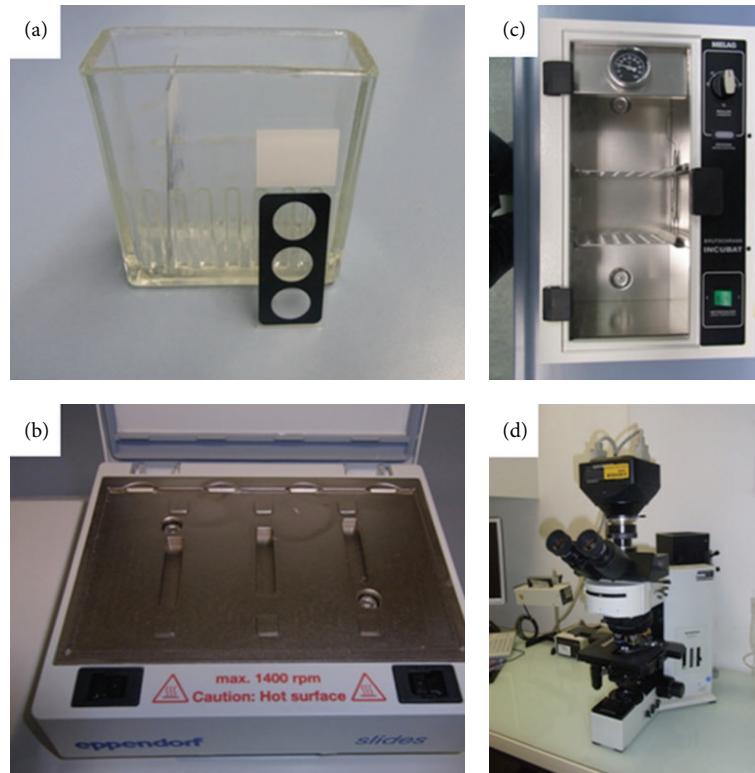


FIGURE 1: Little equipment—as here exemplified by material from the Institute for Microbiology, Virology and Hygiene, University Medical Center Rostock—is required for performing FISH analyses. (a) Glass apparatus for fixing and washing of slides. (b) Slide chamber, allowing for a rapid and steady heat transmission. (c) Incubator for the washing step. (d) Multichannel fluorescence microscope.

One study from New Zealand showed that the discrimination of *vanB* positive vancomycin-resistant *Enterococcus faecium* (VRE) and vancomycin-susceptible *E. faecium* using ICMS fingerprinting is feasible [121], but these findings were not reproducible in other areas. Thus it was speculated that this was just reflecting the specific epidemiological situation in New Zealand [125].

Other studies on *Clostridium difficile* demonstrated a sufficient discriminatory power of MALDI-TOF MS spectra analysis to recognize the PCR ribotypes 001, 027, and 126/078 [116]. Phyloproteomic analysis is a sufficient tool to identify high-virulent or multidrug-resistant strains of particular bacterial species if their virulence or their resistance is associated with phylogenetic and therewith phyloproteomic relatedness. Thus it is an up-and-coming technique not only for epidemiological surveys but also for individual patient management.

Compared to Gram-positive bacteria, Gram-negative bacteria are particularly problematic because their resistance genes are often encoded on plasmids, which can be easily exchanged with other Gram-negative bacteria even across species boundaries [154]. But some of the extended beta-lactamase genes (ESBL) and carbapenemases are associated with particular bacterial clonal complexes. *Klebsiella pneumoniae* ST258 (expressing KPC carbapenemase) and *E. coli* ST131, ST69, ST405, and ST393 (expressing ESBL) [155] belong to these clonal complexes.

Similar phyloproteomic analysis has been successfully demonstrated to discriminate between different subsets of *E. coli* strains [156]. Coupling MALDI-TOF MS with multivariate data analysis allows for discriminating ESBL-expressing *E. coli* B2 ST131 and D (ST69, ST393, and ST405) from other *E. coli* strains [117, 118].

One likely problem in the calculated treatment of *Bacteroides fragilis* infections is the possibility that some strains express a high-potential metallo-β-lactamase encoded by the gene *cfa* [157]. The microbial species *B. fragilis* is subdivided into two divisions (I and II) and usually only isolates of division II harbor *cfa*. Recently, two independent studies identified a set of biomarkers or precisely shifts in biomarker masses that help to distinguish both divisions using MALDI-TOF MS coupled with a cluster algorithm [119, 120].

6.2. Detection of Whole Proteome Changes Induced by Echinocandins. Echinocandins, namely, anidulafungin, caspofungin, and micafungin, are the treatment of choice for invasive and systemic infections with *Candida* and *Aspergillus* species. They also comprise important reserve antimicrobial agents especially in the case of infections with azole-resistant strains, for example, *Aspergillus* species. Due to the increasing use of echinocandins in the treatment of fungal infections, the prevalence of echinocandin-resistant isolates caused by mutations in the *fks1-3* (hypersensitive for the immunosuppressant FK560) genes increases [158]. Thus,

rapid identification of azole and echinocandin susceptibility are needful for a successful therapy of systemic mycoses.

In a pioneer study, the feasibility of MALDI-TOF MS-based testing to estimate fluconazole susceptibility of *Candida albicans* was shown by Marinach and coworkers [122]. During the test procedure, *Candida* cells were incubated for 24 hours in liquid medium containing different concentrations of fluconazole. After harvesting and acid extraction of the *Candida* cell pellets, the supernatants were spotted on a MALDI-TOF target plate and mass spectra were recorded. Comparable to the estimation of minimal inhibitory concentrations (MIC), the so-called minimal profile changing concentration (MPCC), the lowest concentration of fluconazole at which changes in the mass spectrum were recordable, was estimated by comparing the mass spectra of the particular suspensions of the fluconazole dilution series. Remarkably, MPCC differed only in one dilution step from the MIC and therewith it is a comparably sufficient parameter reflecting antimicrobial susceptibility [122].

de Carolis and coworkers adapted this procedure to test *C. albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, *Aspergillus fumigatus*, and *Aspergillus flavus* for echinocandin MICs that are due to mutations in *fks1* and, in the case of *C. glabrata*, also in *fks2* [123]. Additionally, they accelerated the data analysis by applying composite correlation index (CCI) analysis. The CCI value was calculated in comparison to reference spectra of the two extreme concentrations [123].

This procedure was further optimized by Vella and coworkers [124]. They reduced the incubation period down to 3 hours by incubating the yeast cell suspension without as well as with two different echinocandin concentrations corresponding to intermediate and complete resistance [124].

6.3. Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC). The successful application of mass spectrometry (MS) in the detection of antimicrobial resistance has also opened a door for the entry of another quantitative proteomics approach known as SILAC into the era of rapid detection of antibiotic resistance. This approach is based on the principle that proteins are made up of amino acids. Hence, cells grown in media supplemented with amino acids incorporate these amino acids into their cellular proteome [125]. In addition, protein profiles of a metabolically active cell reveal its metabolic activities at a specific time. Already established SILAC antimicrobial detection protocols to detect antibiotic resistance involve the growth of three cultures of the test strain. The first culture is grown in medium with normal (light) essential amino acids, the second culture is grown in media supplemented with labeled (heavy) essential amino acids, and the third culture is grown in media supplemented with both labeled (heavy) essential amino acids and the analyzed antimicrobial drug. These three cultures are mixed, their proteomes are extracted and measured by MS, and the peaks are compared. The test strain is classified as susceptible if its protein peak profile is similar to that of the first culture. On the other hand, it is classified as resistant if its protein peak profile is similar to the second culture [159]. This approach has been successfully used to differentiate

methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) [160]. Also, it has been successfully used to test the susceptibility of *P. aeruginosa* to three antibiotics of different classes with different modes of action: meropenem (β -lactam antibiotic), tobramycin (aminoglycoside), and ciprofloxacin (fluoroquinolone) [126]. In both cases, the results were assessed after 2 to 4 hours and the results were comparable to those obtained from minimum inhibitory concentration (MIC) testing. In addition to these advantages, SILAC is easy and straightforward to perform. For this reason, very soon it may be used to detect antimicrobial resistance in antiviral, antifungal, and antiparasitic drugs.

6.4. Mass Spectrometric β -Lactamase Assay. In contrast to the aforementioned mass spectrometric assays, the mass spectrometric β -lactamase assay (MSBL) is not based on the analysis of the bacterial proteome. The MSBL is based on the direct mass spectrometric detection of β -lactamase metabolites [127–131]. The procedure is as follows. First bacteria are suspended in a buffered solution with and for reference without a β -lactam antibiotic. This suspension is incubated for 1 to 3 hours. After centrifugation, the supernatants are analyzed by MALDI-TOF MS. Specific peaks (mass shifts) for intact and hydrolyzed β -lactams indicate functional presence of β -lactamases. It was demonstrated that the MSBL delivers results within 2.5 hours for bacteria inactivating ampicillin, piperacillin, cefotaxime, ceftazidime, ertapenem, imipenem, and meropenem [131]. Thus, particularly NDM-1, VIM-1/2, KPC-1-3, OXA-48, OXA-162, and IMP carbapenemase expression by Enterobacteriaceae, *Acinetobacter baumannii*, and *Pseudomonas* spp. was detectable [128, 130].

With a total turn-around-time after positive primary bacterial culture of circa 4 hours, this method is significantly faster than culture-based susceptibility testing [127–131].

6.5. Mass Spectrometric Analysis of PCR Products: PCR/ESI MS. PCR/electrospray ionization-mass spectrometry (PCR/ESI MS) combines, nucleic acid amplification with mass spectrometric analysis of the amplicons, which are brought into a gas phase using electrospray ionization. The major advantage of this technique is its high multiplexing capacity that enables the parallel detection of a wide panel of resistance genes. It was demonstrated that PCR/ESI MS is able to accurately detect nine different KPC carbapenemases ($\text{bla}_{\text{KPC-2-10}}$) [132] as well as the *gyrA* and *parC* point mutations, which are associated with quinolone resistance in *A. baumannii* [161].

Also because of its high multiplexing capacity, PCR/ESI MS is a suitable tool for simultaneous (sub)species identification and resistance gene detection, which is of particular importance for the treatment of mycobacterial infections. On the one hand, it is necessary to distinguish nontuberculosis mycobacteria (NTM) from *M. tuberculosis*; on the other hand, multidrug-resistant tuberculosis (MDR-TB) strains must be detected. PCR/ESI MS-based assays have been developed to facilitate NTM species identification and parallel detection of resistance genes associated with rifampicin,

isoniazid, ethambutol, and fluoroquinolone resistance in TB and NTM [162]. Moreover, there are enormous time savings compared to traditional mycobacterial culture and resistance testing via the agar proportion method [162–164].

The high sensitivity of PCR/ESI MS in the detection of hard-to-culture or even nonculturable bacteria makes it a reliable method for the direct detection of pathogens in hardly acquirable samples like heart valves [165] as well as for surveillance studies [166, 167].

6.6. Minisequencing-Primer Extension Followed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Analysis (PEX/MALDI-TOF). Another method that was also adapted for the rapid detection of ganciclovir resistance in HCMV (human cytomegalovirus) by Zürcher and coworkers is single nucleotide primer extension (also known as minisequencing or PinPoint assay) followed by matrix-assisted laser desorption/ionization time-of-flight analysis (PEX/MALDI-TOF) [134]. In general, the combination of PEX and MALDI-TOF MS is a cost-efficient high-throughput method for the detection of single nucleotide polymorphisms (SNPs) [133]. The PEX/MALDI-TOF workflow using patient plasma is as follows [134].

For the primer extension reaction, the reverse PEX primer (5'-CTT-GCC-GTT-CTC-CAA-C-3') was added in high concentration. The 3'-end of the primer is located directly at the site of mutation (A594V; GCG/wild type → GTG/mutant) to be detected. The extension reaction catalyzed by a DNA polymerase is terminated in the case of a wild-type allele just after one nucleotide complementary to the mutated nucleotide and in the case of a mutant after two nucleotides by a dideoxynucleotide (ddNTP). Because of the molecular weight difference in consequence of the varying mass increase of the PEX primer, mutant and wild type can be discriminated using MALDI-TOF MS [133].

According to current standards, HCMV resistance testing is performed using Sanger sequencing [168]. By monitoring a patient cohort of five individuals using Sanger sequencing and PEX/MALDI-TOF, Zürcher et al. could demonstrate that the PEX/MALDI-TOF method is much more sensitive than the Sanger method. PEX/MALDI-TOF requires the presence of only 20%–30% of the ganciclovir unsusceptible HCMV quasispecies to reliably detect the resistance mutation [134]. In consequence, this method was able to detect the appearance of the UL97 resistance mutation already ten days after the “last wild-type only constitution,” whereas Sanger sequencing detected the appearance of the resistant subpopulation at day 20 [134]. Consequently, a ganciclovir therapy can be monitored by PEX/MALDI-TOF more contemporary. A necessary change in therapy may be done earlier, and critical time for the preservation of the graft and the patient can be saved.

A comparable test setup was designed to detect TEM-type ESBL in Enterobacteriaceae [169]. Conversion of TEM penicillinases to TEM-type ESBL is mostly due to amino acid substitutions at Ambler's positions: Glu104, Arg164, and Gly238 [170]. To detect these SNPs in the *bla_{TEM}* genes, a set of seven internal primers have been designed to bind near

the three codons of Ambler's positions in such a way that the masses of all possible reactions products are maximally distant from each other and are easy to distinguish in the mass spectrum. All primers are used in one multiplex reaction. Thus it is feasible to detect different types of TEM-type ESBL in one reaction [169].

Other minisequencing protocols have been established to detect fluoroquinolone resistance related SNPs in *N. gonorrhoeae* [171], clarithromycin resistance in *Helicobacter pylori* [172], and rifampin and isoniazid-resistance in *M. tuberculosis* [173].

6.7. MSCSA-Mass Spectrometry-Based Comparative Sequence Analysis to Detect Ganciclovir Resistance. Mass spectrometry-based comparative sequence analysis (MSCSA) was initially established by Honisch and coworkers (SEQUENOM, San Diego, USA) for the genotyping of bacteria using mass spectrometric fingerprinting of the standard multilocus sequence typing (MLST) loci [135].

The MSCSA principle was adapted to facilitate the detection of mutations in the UL97 gene to detect ganciclovir resistance of human cytomegalovirus (HCMV) [136].

HCMV reactivation occurs frequently in consequence of immune suppression especially after stem cell and solid organ transplantation [174]. Thus, HCMV infection may lead to graft dysfunction or even rejection. To counteract this, antiviral treatment with the analogue of 2'-deoxy-guanosine ganciclovir is indicated [175]. Under therapy, which may span several months, it is necessary to monitor the emergence of resistance and possibly switch to other drugs such as the more toxic foscarnet [176]. Ganciclovir resistance is typically a consequence of single nucleotide polymorphisms in the 3'-region of the UL97 kinase gene encoding a viral kinase, which activates ganciclovir by phosphorylation [177].

These UL97 single nucleotide polymorphisms are detected by MSCSA as follows: after DNA isolation from EDTA-plasma samples, the 3'-region of the UL97 is amplified in two amplicons using T7-promotor-tagged forward primers and SP6-tagged reverse primers. Both amplicons are *in vitro* transcribed in two separate reactions using T7 and SP6 RNA polymerase followed by cytosine or uracil specific RNaseA cleavage of plus and minus strand RNA transcripts. After this, all four obtained RNaseA cleavage products are transferred to a SpectroCHIP array (SEQUENOM, San Diego, USA). MALDI-TOF mass spectra are recorded and *in silico* compared to calculated MS spectra of reference sequences. Based on the obtained data, the UL97 sequence can be assembled and thereby the presence of a ganciclovir resistance associated single nucleotide polymorphism can be detected [136]. Due to the automation of post-PCR processing and analysis as well as reduced hands-on time, acceleration of the detection process of ganciclovir resistance can be achieved.

7. Conclusions and Outlook

To solve the increasing problem of a worldwide rising prevalence of infections due to multidrug- or even pan-drug-resistant bacteria, medical microbiology has to establish a

new generation of rapid resistance testing assays. The key features of these new assays should be significant reduction of turn-around-time (Table 5) and a high multiplexing capacity, because of the already mentioned shift from Gram-positive to Gram-negative multidrug-resistant bacteria in recent years with various resistance mechanisms [1–4]. So, MRSA detection simply means detection of the penicillin binding protein 2A (PBP2A), the SCCmec genetic element, respectively [178]. Detection of vancomycin-resistant *S. aureus* (VRSA) as well as vancomycin-resistant enterococci (VRE) means the detection of Van-A, Van-B, and rarely Van-C [179].

In contrast to this situation in Gram-positive bacteria, multidrug resistance in Gram-negative bacteria is due to the expression of extended-spectrum β -lactamases (ESBLs), carbapenemases, aminoglycoside-blocking 16S rRNA methylases, and many other mechanisms associated with several hundreds of gene variants/mutations [4–8]. The more these resistance genes can be detected in parallel, the higher the probability of an exact determination of a particular susceptibility pattern is.

But rapid resistance testing is only one key to the solution of this problem, especially because the multiplexing capacities of the individual assays are limited and the costs are too high. Thus, resistance surveillance programs are and have been established at different levels: hospital-wide, regional, and international. For example, some hospitals introduced a general ESBL screening in analogy to the MRSA screening in high-risk groups. In recent years, various studies were carried out to identify the ESBL-transmission rate in maximum care hospitals and in households with ESBL-colonized individuals. The studies showed that the ESBL-transmission rate of 1.5% to 4.5% is relatively low if compliance with standard hygiene measures is guaranteed [180, 181]. In contrast, the ESBL-transmission rate in households with common food preparation was 25% and therewith comparable high as the MRSA-transmission rate [181, 182]. A prospective study demonstrated a relatively high prevalence of 15% for ESBL-producing Enterobacteriaceae on admission, but these strains were involved in only 10% of the infections at admission time [183]. Such regional surveillance studies form the basis for national and international surveillance statistics such as those published by the European Antimicrobial Resistance Surveillance Network (EARS-Net). Such surveillance studies on the prevalence of certain ESBL and carbapenemase subtypes can contribute to the identification of resistance mechanisms of the quantitatively biggest importance, which should be included in Gram-negative test panels. Thus, appropriate surveillance studies contribute to the solution of the problem of limited multiplexing capacity at least partially.

As recently predicted, next generation sequencing (NGS) with its high multiplexing capacity will soon be part of routine diagnostics, more and more replacing cultural approaches as an accurate and cheap procedure in routine clinical microbiology practice. This will include sequence-based resistance testing and additional detection of particular virulence factors, making culture unnecessary on the intermediate or long term [184]. The generation of microbial sequence data for

“short term” patient management will revolutionize infectiology and diagnostic microbiology, allowing for deeper and more rapid insights into the patients’ infectious pathologies [90]. As a high-resolution tool, high-throughput sequencing has the potential to optimize both diagnostics and patient care [185]. NGS will affect antibiotic stewardship [80] by defining resistance by the presence of a mechanism rather than just in pharmacodynamic terms as it is performed right now. Present obstacles include the imperfect correlation of genotype and phenotype; further, technical challenges have to be overcome [80]. However, as NGS becomes increasingly cost effective and convenient, it bears the potential to replace the so far multiple and complex procedures in a microbiological routine laboratory by just a single, straightforward, and most efficient workflow [184].

Besides NGS, mass spectrometry will be the second key technique in rapid medical microbiology. The integration of subtype specific mass spectra databases in MS associated software packages will enable the identification of high-virulent strains within very short time periods. The mass spectrometric β -lactamase assay (MSBL) as well as adaptations to other anti-microbiota classes will expectantly advance to helpful tools of the diagnostic microbiologist. Finally, the combination of both nucleic acid amplification and mass spectrometric analysis, for example, in PCR/ESI MS assays with its high multiplexing capacity, has the potential to enter routine diagnostic in the coming years.

Nevertheless, these highly sophisticated and expensive diagnostic solutions will hardly be available in resource-limited countries, for example, in the sub-Saharan tropics, where multidrug resistance is nevertheless on the rise [186]. Cheap and easy-to-perform rapid molecular techniques like fluorescence *in situ* hybridization (FISH) might be an option for such settings [187] until MALDI-TOF MS or sequence-based approaches become more affordable and easy to apply. The rapid and correct choice of adequate antibiotic therapy will decide on the survival of critically ill patients with infectious diseases, for example, sepsis patients [188, 189]. In times of decreasing susceptibility to antimicrobial drugs, this choice gets increasingly complicated. So the words of the ancient German infectious disease specialist Robert Koch become more and more true: “If a doctor walks behind his/her patient’s coffin, sometime cause follows consequence.” (Original German text of the witticism: “Wenn ein Arzt hinter dem Sarg seines Patienten geht, so folgt manchmal die Ursache der Wirkung.”) Reliable information on the resistance patterns of etiologically relevant pathogens has to be rapidly available to avoid this final consequence as frequently as possible.

Conflict of Interests

The authors declare that there is no conflict of interests according to the guidelines of the International Committee of Medical Journal Editors.

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Research Article

Molecular Identification and Quantification of Tetracycline and Erythromycin Resistance Genes in Spanish and Italian Retail Cheeses

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Large antibiotic resistance gene pools in the microbiota of foods may ultimately pose a risk for human health. This study reports the identification and quantification of tetracycline- and erythromycin-resistant populations, resistance genes, and gene diversity in traditional Spanish and Italian cheeses, via culturing, conventional PCR, real-time quantitative PCR (qPCR), and denaturing gradient gel electrophoresis (DGGE). The numbers of resistant bacteria varied widely among the antibiotics and the different cheese varieties; in some cheeses, all the bacterial populations seemed to be resistant. Up to eight antibiotic resistance genes were sought by gene-specific PCR, six with respect to tetracycline, that is, *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)*, *tet(S)*, and *tet(W)*, and two with respect to erythromycin, that is, *erm(B)* and *erm(F)*. The most common resistance genes in the analysed cheeses were *tet(S)*, *tet(W)*, *tet(M)*, and *erm(B)*. The copy numbers of these genes, as quantified by qPCR, ranged widely between cheeses (from 4.94 to $10.18 \log_{10}/g$). DGGE analysis revealed distinct banding profiles and two polymorphic nucleotide positions for *tet(W)*-carrying cheeses, though the similarity of the sequences suggests this *tet(W)* to have a monophyletic origin. Traditional cheeses would therefore appear to act as reservoirs for large numbers of many types of antibiotic resistance determinants.

1. Introduction

Antibiotic resistance (AR) is a natural phenomenon, the appearance of which predates the clinical use of antibiotics [1, 2]. Unfortunately, the widespread use and misuse of antibiotics in clinical and nonclinical environments for more than seven decades have provided optimal conditions for the appearance, mobilization, and concentration of highly efficient resistance systems in bacteria [3]. The transfer of AR genes into human and animal pathogens could ultimately lead to a failure of antibiotic therapy [4]. Mobilization among bacterial species is facilitated by AR genes being commonly located on mobile genetic elements such as transposons and plasmids, which have high horizontal transfer capacity [5]. The presence of antibiotics in the environment not

only provides a positive selection for resistant pathogens, but also exerts an evolutive pressure on components of the commensal microbiota [6]. Under these conditions, the commensal bacteria in food could become a reservoir for AR determinants that could then further be disseminated via the food chain [7–9].

Determining the prevalence of AR genes in a given environment, and their characterization, requires the isolation and identification of the resistant bacteria, followed by a molecular analysis of their AR determinants [10–12]. The identification and quantification of AR genes directly in environmental samples, that is, without culturing biases, would be useful [13, 14]. Culture-independent analysis is also faster and more accurate than culture-based methods. Indeed, several AR identification and AR gene quantification techniques

that require no culturing have recently been developed, such as denaturing gradient gel electrophoresis (DGGE) [15, 16], real-time quantitative PCR (qPCR) [13, 14, 17], construction and functional analysis of gene libraries [18–20], AR gene microarrays [21, 22], and analysis of metagenomic sequences [23, 24]. Knowledge of the types and loads of AR genes in foods could ultimately be of help to estimate the risk of their transmission during cheese manufacturing and after consumption. To our knowledge, few attempts have been made to directly analyze AR gene numbers and diversity in food samples.

The aims of the present work were to identify the microbial populations resistant to tetracycline and erythromycin in commercial Spanish and Italian cheeses and to quantify their antibiotic resistance genes, using culturing and molecular techniques. These antibiotics were selected as a model due to the fact that resistance against tetracycline and erythromycin has been extensively documented among food-borne bacteria, including strains of lactic acid bacteria species.

2. Material and Methods

2.1. Cheese Sampling. Twenty commercial cheeses—10 Spanish (Cabrales, Zamorano, Majorero, Mahón, Torta del Casar, Manchego, Ibores, Garrotxa, De La Mesta, and Ibérico) and 10 Italian (Gorgonzola “dolce,” Gorgonzola “picante,” Caprino, Quartirolo Lombardo, Pecorino Sardo, Grana Padano, Montasio, Monte Veronese, Asiago, and Taleggio)—were bought at retail stores. These cheese types vary in terms of the technology used to manufacture them (artisanal (without starters), industrial (using commercial starters), the type of milk used (cow, goat, ewe, or mixtures), the treatment to which that milk is subject (raw, pasteurised), and ripening time (from one to 24 months)).

2.2. Plate Counts of Cultivable Antibiotic-Resistant Bacteria. Cubes of ten grams of cheese from the centre were homogenised with 90 mL of a 2% (w/v) sterilised sodium citrate solution prewarmed at 45°C for 1 min in a Colworth Stomacher 400 (Seward Ltd., London, UK). Cheese homogenates were tenfold diluted in Ringer’s solution (Merck, Darmstadt, Germany) and the dilutions were plated in duplicate on selective and nonselective agarified media. Total aerobic mesophilic bacteria (including aerotolerant and facultative anaerobes) were enumerated on Plate Count Milk Agar (PCMA; Merck), Gram-positive bacteria such as lactic acid bacteria (LAB) were enumerated using de Man Rogosa and Sharpe agar (MRS; Merck), enterococci on Slanetz and Bartley agar (SB; Merck), and staphylococci on Baird-Parker agar (BP; Merck). Tetracycline and erythromycin resistant populations were counted using the same nonselective selective and media containing tetracycline ($15 \mu\text{g mL}^{-1}$) and erythromycin ($8 \mu\text{g mL}^{-1}$) (Sigma-Aldrich, St. Louis, MO, USA). Antibiotic concentrations were considered eight- to tenfolds higher than the usual resistance levels of susceptible populations [29]. This concentration, which is much lower than that provided by dedicated resistance mechanisms [5, 29], was considered to avoid unspecific growth of susceptible bacteria. Plates were then incubated at 30°C (PCMA and

MRS media) and 45°C (SB medium) for 72 h and at 37°C (BP medium) for 48 h.

2.3. Total DNA Extraction and Purification. Cheese samples (5 grams) were homogenized with 45 mL of a 2% sterile sodium citrate solution and incubated at 37°C for 3 h in the presence of 1 mg mL^{-1} pronase (Sigma-Aldrich) and 100 μL β -mercaptoethanol (Merck). Total microbial cells were harvested by centrifugation at 5000 $\times g$ for 20 min and disrupted using 0.1 mm glass beads (Sigma-Aldrich) as reported elsewhere [30]. Genomic DNA was purified by phenol and phenol/chloroform extractions and precipitated with 2-propanol (all chemicals from Sigma-Aldrich). Finally, it was suspended in sterile water containing 5–15 mg mL^{-1} of RNase (Sigma-Aldrich).

Total DNA was also isolated from pure cultures of control strains carrying known AR genes: *Staphylococcus epidermidis* SE36 [*tet(K)*], *Enterococcus faecium* ET51 [*tet(L)*], *Lactococcus lactis* IPLA 31008 [*tet(M)*], *Enterococcus faecalis* Jtet [*tet(O)*], *Enterococcus* spp. ET15 [*tet(S)*], *Bifidobacterium longum* B93 [*tet(W)*], *Lactobacillus johnsonii* G41 [*erm(B)*], and *Bacteroides fragilis* 79a [*erm(F)*]. Genomic microbial DNA from these strains was purified from 1 mL of an overnight culture in brain heart infusion broth (BHI; Merck) using the Kit GenElute bacterial genomic DNA (Sigma-Aldrich). The recovered DNA was then stored at –20°C until analysis.

2.4. PCR Detection of Tetracycline and Erythromycin Resistance Genes. The presence of tetracycline and erythromycin resistance genes was examined in DNA from the cheese samples and from control strains by standard PCR using both universal primers for genes encoding tetracycline resistance through ribosomal protection proteins (RPPs), such as *tet(M)*, *tet(O)*, *tet(S)*, and *tet(W)*, and gene-specific primer pairs for genes encoding tetracycline resistance [*tet(K)* and *tet(L)*] and erythromycin resistance [*erm(B)* and *erm(F)*] (Table 1). The PCR conditions used for amplification were those reported in Table 1. Positive (DNA from appropriate control strains) and negative (no template DNA) controls were subjected to amplification under the same conditions.

2.5. Real-Time Quantitative PCR

2.5.1. Design of Primers. Updated tetracycline and erythromycin resistance genes in databases were compiled for designing of new primers for the qPCR analysis. Nucleotide sequences encoding tetracycline resistance in the form of RPPs and efflux pumps, and erythromycin resistance, were downloaded from the GenBank database (see Table 1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2014/746859>) and aligned with one another using Mega 5 software. Conserved regions at appropriate distances were used for designing the primers employed in qPCR reactions (Table 1). This was achieved using Primer Express 3.0 software (Applied Biosystems, Carlsbad, CA, USA).

2.5.2. qPCR Conditions. qPCR analyses were performed to quantify tetracycline (*tet*) and erythromycin (*erm*) resistance

TABLE 1: Sequence and properties of the PCR primers used in this work.

Application/primer	Target gene	Sequence (5'-3')	Annealing T ^a (°C)	Amplicon size (bp)	Reference
Conventional PCR					
Tet-F	<i>tet</i> ^a	GCTCA(T/C)GTTGA(T/C)GCAGGAA	50	1292	[25]
Tet-R		AGGATTGGCGG(C/G)ACTTC(G/T)A			
TetK-F	<i>tet</i> (K)	TTATGGTGGTTGTAGCTAGAAA	55	348	[15]
TetK-R		AAAGGGTTAGAAACTCTTGAAA			
TetL-F	<i>tet</i> (L)	GTMGTTGCGCGCTATATTCC	55	696	[15]
TetL-R		GTGAAMGRWAGCCCACCTAA			
DI-F	<i>tet</i> (M)	GAYACICCIIGGICAYRTIGAYTT	55	1513	[26]
TetM-R		CACCGAGCAGGGATTTCTCCAC			
TetO-F	<i>tet</i> (O)	AATGAAGATTCCGACAATT	55	781	[26]
TetO-R		CTCATGCGTTGTAGTATTCCA			
TetS-F	<i>tet</i> (S)	ATCAAGATATTAAGGAC	55	573	[26]
TetS-R		TTCTCTATGTGGTAATC			
TetW-F	<i>tet</i> (W)	AAGCGGCAGTCACTCCCTTCC	50	1150	[11]
Tet-R		AGGATTGGCGG(C/G)ACTTC(G/T)A			
ErmB-F	<i>erm</i> (B)	GAAAAGGTACTCAACCAAATA	50	639	[27]
ErmB-R		AGTAACGGTACTTAAATTGTTAC			
ErmF-F	<i>erm</i> (F)	CGGGTCAGCACTTTACTATTG	50	466	[27]
ErmF-R		GGACCTACCTCATAGACAAG			
Real-time qPCR					
TBA-F	16S rDNA	CGGCAACGAGCGCAACCC	60	130	[28]
TBA-R		CCATTGTAGCACGTGTAGCC			
TetK-qPCR-F	<i>tet</i> (K)	TGCTGCATTCCCTTCACTGA	60	69	This study
TetK-qPCR-R		GCTTGCCTTGTTTTTCTTGTAA			
TetL-qPCR-F	<i>tet</i> (L)	GGGTAAAGCATTTGGCTTATTGG	60	63	This study
tetL-qPCR-R		ATCGCTGGACCGACTCCTT			
TetM-qPCR-F	<i>tet</i> (M)	CAGAATTAGGAAGCGTGACAA	60	67	This study
TetM-qPCR-R		CCTCTCTGACGTTCTAAAGCGTAT			
TetO-qPCR-F	<i>tet</i> (O)	AATGTCAGAACGGAACAGGAAGAA	60	59	This study
TetO-qPCR-R		CGTGATAAACGGGAAATAACGTT			
TetS-qPCR-F	<i>tet</i> (S)	CGAGGTCATTCTCATTGGTGAA	60	84	This study
TetS-qPCR-R		CAGACACTGCGTCCATTGTAAA			
TetW-qPCR-F	<i>tet</i> (W)	ACGGCAGCGCAAAGAGAA	60	60	This study
TetW-qPCR-R		CGGGTCAGTATCCGCAAGTT			
ErmB-qPCR-F	<i>erm</i> (B)	GGATTCTACAAGCGTACCTTGGA	60	69	This study
ErmB-qPCR-R		AATCGAGACTTGAGTGTGCAAGAG			
ErmF-qPCR-F	<i>erm</i> (F)	TGATGCCGAAATGTTCAAGT	60	63	This study
ErmF-qPCR-R		AAAGGAAATTCGGAACGTCAA			
DGGE assays					
TetM-F	<i>tet</i> (M)	ACAGAAAGCTTATTATATAAC	55	171	[15]
GC ^b -TetM-R		TGGCGTGTCTATGATGTTCAC			
TetW-F	<i>tet</i> (W)	GAGAGCCTGCTATATGCCAGC	64	168	[15]
GC-TetW-R		GGCGTATCCACAATGTTAAC			

^a*tet*, genes encoding ribosome protection proteins.^bSequence of the GC clamp: CGCCCGGGGCGGCCCGGGGGGGGGGGCACGGGGGG.

genes in the total microbial DNA from the cheese samples. Amplifications were performed in an ABI Prism Fast 7500 sequence detection system (Applied Biosystems). Each 20 μL qPCR reaction included 4 μL of extracted DNA (approximately 200 ng), 10 μL of SYBR Green PCR Master Mix

(containing ROX as a passive reference), and 900 nM of each primer. The following cycling conditions were used: 2 min at 50°C, an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 60 s at 60°C. Baseline and threshold calculations were performed using ABI Prism

Fast 7500 software. Melting temperature analyses and size estimations of the PCR products were performed on agarose gels to check for nonspecific amplification.

2.5.3. Construction of qPCR Standards. Conventional PCR reactions, as described above, were used to generate gene-specific amplicons used as template DNA standards for qPCR. Amplicons were purified using a gel/PCR extraction kit (ATP Biotech, Banciao City, Taiwan) and quantified fluorometrically using a Gen5 Microplate dsDNA Quantitation Kit (Biotek Instruments, Winooski, VT, USA). The number of DNA molecules was calculated based on the size and mass of the amplicons. Tenfold serial dilutions of the amplicons were prepared and used following the procedure by Yu et al. [17]. To ensure accuracy in copy number quantification, a complementary control standard curve was obtained using DNA from known numbers of *B. longum* B93 cells (enumerated in MRS agar plates containing 0.25% cysteine following incubation in anaerobiosis at 37°C for 72 h); this strain carries a single copy of *tet(W)* [31]. Dilutions of total DNA from the cells were subjected to qPCR analysis as above.

2.5.4. qPCR Expression Data. Absolute gene copy number was expressed as the number of copies of resistant genes per g⁻¹, whereas relative copy number was expressed as the number of resistant genes per million copies of total bacteria ribosomal genes, as determined with the universal prokaryotic primers TBA-F and TBA-R (Table 1). Absolute abundance was calculated based on the results obtained for the corresponding standard of each resistant gene. Relative abundance was calculated using $E^{\Delta Ct}$, where E is the efficiency of the primer according to the slope of standard curve ($E = 10^{-1/\text{slope}}$) and ΔCt is the Ct value of the gene target (tetracycline and erythromycin resistance genes) normalized against the Ct value of the total bacterial numbers in the samples. For the relative quantification, the copy number of the 16S rRNA genes per cell was averaged to five. The efficiency E of each pair of primers was as follows: 1.94 for *tet(K)*, 1.97 for *tet(L)*, 1.97 for *tet(M)*, 2.03 for *tet(O)*, 2.02 for *tet(S)* and 2.03 for *tet(W)*, and 1.94 for *erm(B)* and 1.99 for *erm(F)*.

2.6. Denaturing Gradient Gel Electrophoresis Analysis. DNA from cheeses and strains harbouring known *tet* genes (*Lactococcus lactis* IPLA 31008 [*tet(M)*], *Enterococcus faecalis* Jtet [*tet(O)*]), *Enterococcus* spp. ET15 [*tet(S)*], and *Bifidobacterium longum* B93 [*tet(W)*]) was amplified by PCR using DGGE primers (Table 1), employing the PCR conditions described elsewhere [15]. DGGE analysis of the amplified *tet* genes was performed as previously reported [16] with slight modifications. Briefly, DGGE was performed in a DCode apparatus (Bio-Rad, Richmond, CA, USA) at 60°C on 8% polyacrylamide gels with a formamide-urea denaturing gradient of 15–50%. Electrophoresis was conducted at 150 V for 2 h and 200 V for 1 h. After electrophoresis, gels were stained in an ethidium bromide solution (0.5 µg mL⁻¹), and the DNA bands were visualized and captured using a Gbox system and GeneSys software (Syngene, Cambridge, UK). After isolation and reamplification with the same primers without the GC clamp and identical PCR conditions, bands

from the acrylamide gels were identified by sequencing and sequence comparison. Online similarity searches were performed by using the BLAST tool in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>).

3. Results

3.1. Prevalence of AR Populations in Spanish and Italian Cheeses. The Spanish and Italian cheese samples were subjected to conventional microbiological analysis on selective and differential media supplemented or not with tetracycline and erythromycin. Populations of tetracycline-resistant (*Tet^r*) and/or erythromycin-resistant bacteria (*Erm^r*) were detected on most counting media for both Spanish and Italian cheeses made from raw or pasteurised milk (Tables 2 and 3, resp.). The AR bacterial counts varied widely depending on the microbial group and type of cheese. In general, Italian cheeses (Table 3) returned lower *Tet^r* and *Erm^r* counts than Spanish cheeses (Table 2). Table 2 shows the total number of mesophilic, aerobic, *Tet^r*, and *Erm^r* bacteria (enumerated in PCMA) to reach a maximum of 6.79 log₁₀ cfu g⁻¹ and 7.15 log₁₀ cfu g⁻¹, respectively, in samples of Cabrales. Microbial counts in this cheese type showed the antibiotic-resistant populations to generally be two to three log₁₀ units lower than in antibiotic-free media; such results were only returned by this cheese.

3.2. Prevalence of AR Genes in Spanish and Italian Cheeses. Table 4 shows the resistance genes identified by PCR in total DNA from the different cheeses using specific primers for six tetracycline and two erythromycin resistance genes. Two or more AR genes were detected in all cheeses with the exception of Mahón and Gorgonzola “dolce” cheeses, both of which returned a single AR gene (*tet(W)* and *tet(K)*, resp.). Indeed, the Spanish De La Mesta cheese harboured all eight AR genes examined in this work, while the Italian Asiago cheese harboured seven (all but *tet(L)*). The most common gene encoding tetracycline resistance was *tet(S)*, which was identified in nine Spanish and six Italian cheeses, followed by *tet(W)* in seven Spanish and four Italian cheeses. In contrast, *tet(L)* and *tet(O)* were the least widespread of the tetracycline resistance determinants; these were detected in three Spanish cheeses and in two Spanish and two Italian cheeses, respectively. The *erm(B)* gene was detected in all but three cheese types, while the *erm(F)* gene was identified in seven of the 10 Italian cheeses, but only in one Spanish cheese (De La Mesta).

3.3. Quantification of Tetracycline and Erythromycin Resistance Genes by qPCR. Samples positive for AR genes in gene-specific PCR were subjected to qPCR to quantify those identified. A standard curve encompassing 10³ through 10¹⁰ gene copies per reaction was produced using total DNA from *B. longum* B93 as a control ($r^2 \geq 0.996$). The qPCR detection limit determined from both the control DNA of *B. longum* and the serial dilutions of gene-specific amplicons was estimated to be about 10⁴ copies per gram of cheese. Independent reactions were performed in triplicate; high

TABLE 2: Microbial counts (\log_{10} cfu g $^{-1}$) of the total and antibiotic resistant bacterial populations found in samples of ten Spanish cheeses.

Culture medium ^a	Cheese									
	Cabrales*	Zamorano*	Majorero*	Mahón	Torta del Casar*	Manchego	Ibores*	Garrotxa	De La Mesta*	Ibérico
PCMA	7.74	6.64	9.23	6.49	8.18	8.21	8.26	7.58	6.01	8.62
PCMA + Tc	6.76	2.74	4.26	<2.0 ^b	5.98	3.21	5.76	3.68	5.46	4.70
PCMA + Erm	7.15	3.92	4.36	3.84	5.82	3.16	5.65	4.72	3.91	3.62
MRS	7.20	6.53	4.53	6.36	7.93	6.21	8.12	7.40	5.08	7.92
MRS + Tc	6.79	3.64	4.11	<2.0	4.96	<2.0	4.77	3.12	<2.0	<2.0
MRS + Erm	6.90	4.18	4.03	<2.0	4.89	<2.0	5.67	3.22	<2.0	<2.0
SB	5.83	4.07	2.95	<2.0	5.67	<2.0	5.85	3.13	4.82	5.04
SB + Tc	5.08	2.30	<2.0	<2.0	4.82	<2.0	3.20	<2.0	2.81	4.24
SB + Erm	5.18	3.04	<2.0	<2.0	4.95	<2.0	<2.0	<2.0	3.61	<2.0
BP	5.34	3.53	3.91	3.74	5.30	<2.0	4.27	4.26	4.18	4.72
BP + Tc	4.71	2.78	<2.0	<2.0	4.84	<2.0	2.98	3.20	<2.0	<2.0
BP + Erm	5.00	2.98	3.56	<2.0	4.95	<2.0	<2.0	4.47	<2.0	<2.0

^aCulture media used for counting of different bacteria groups: PCMA for total mesophilic bacteria, MRS for lactic acid bacteria, SB for enterococci, and BP for staphylococci and micrococci. Antibiotics utilized to supplement the culture media: Tc, tetracycline and Erm, erythromycin.

^bCounts below the detection limit ($2.0 \log_{10}$ cfu g $^{-1}$).

*Cheeses made from raw milk.

TABLE 3: Microbial counts (\log_{10} cfu g $^{-1}$) of the total and antibiotic resistant bacterial populations found in samples of ten Italian cheeses.

Culture medium ^a	Cheese									
	Gorgonzola "dolce"	Gorgonzola "piccante"	Caprino*	Quartiolo Lombardo	Pecorino Sardo*	Grana Padano*	Montasio*	Monte Veronese*	Asiago*	Taleggio*
PCMA	7.78	8.70	8.23	6.00	6.00	8.04	7.38	8.63	7.36	8.34
PCMA + Tc	5.04	4.48	3.00	2.05	<2.0 ^b	3.60	<2.0	3.00	4.60	<2.0
PCMA + Erm	6.00	<2.0	<2.0	2.85	5.30	<2.0	<2.0	5.45	2.48	2.70
MRS	6.40	7.60	8.30	6.38	5.30	7.00	7.63	8.15	5.00	6.70
MRS + Tc	4.08	<2.0	<2.0	<2.0	<2.0	3.60	2.00	<2.0	3.67	<2.0
MRS + Erm	3.08	3.01	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	3.73	<2.0
SB	5.46	5.21	<2.0	6.00	4.88	3.60	4.41	3.20	5.30	2.00
SB + Tc	<2.0	<2.0	<2.0	2.30	<2.0	3.78	2.00	2.78	2.00	<2.0
SB + Erm	2.48	<2.0	<2.0	<2.0	2.00	<2.0	2.00	<2.0	<2.0	<2.0
BPA	5.95	5.44	<2.0	3.90	<2.0	3.97	2.60	3.78	<2.0	2.85
BP + Tc	3.78	2.48	<2.0	3.23	<2.0	3.60	2.00	<2.0	<2.0	<2.0
BP + Erm	3.00	<2.0	<2.0	2.48	<2.0	<2.0	<2.0	3.00	<2.0	<2.0

^aCulture media used for counting of different bacteria groups: PCMA for total mesophilic bacteria, MRS for lactic acid bacteria, SB for enterococci, and BP for staphylococci and micrococci. Antibiotics utilized to supplement the culture media: Tc, tetracycline and Erm, erythromycin.

^bCounts below the detection limit ($2.0 \log_{10}$ cfu g $^{-1}$).

*Cheeses made from raw milk.

reproducibility was always obtained (average standard deviation ≤ 0.3). The amplification of AR genes with conventional gene-specific PCR and qPCR primers returned similar results for the Spanish cheeses (Table 4; Figures 1 and 2). Indeed, only two samples showed qPCR amplification failures for genes previously detected by gene-specific PCR among the Spanish cheeses (one *tet(K)* and one *erm(B)* gene). In contrast, among the Italian cheeses, eight samples showing AR genes in gene-specific PCR analysis (three for *erm(F)*, two for each *erm(B)* and *tet(W)*, plus one for *tet(S)*) failed to show the same with qPCR-specific primers.

The copy number of the different tetracycline (Figure 1) and erythromycin (Figure 2) resistance genes varied widely among the cheese types. Absolute copy numbers of AR genes (\log_{10}/g of cheese) ranged from 4.94 (*erm(F)* in Asiago cheese) to 10.18 (*tet(S)* in Torta del Casar cheese). On average, the copy number of the different AR genes, quantified by qPCR analysis, was 6.6 ± 1.2 copies per gram of cheese. Figures 1 and 2 show the number of each resistance gene as a percentage (%) of the total bacterial loads in the cheeses as determined by 16S rDNA analysis. In this way, intersample variations in gene copy number between cheeses are clearly

TABLE 4: Tetracycline and erythromycin resistance genes detected by conventional PCR in Spanish and Italian cheeses.

Cheese	Gene							
	<i>tet(K)</i>	<i>tet(L)</i>	<i>tet(M)</i>	<i>tet(O)</i>	<i>tet(S)</i>	<i>tet(W)</i>	Erythromycin resistance gene	
							<i>erm(B)</i>	
Spanish cheeses								
Cabrales*	+	+	+	-	+	+	+	-
Zamorano*	+	+	+	-	+	+	+	-
Majorero*	-	-	-	-	+	-	+	-
Mahón	-	-	-	-	-	+	-	-
Torta del Casar*	-	-	+	+	+	+	+	-
Manchego	-	-	-	-	+	-	+	-
Ibores*	-	-	+	-	+	+	+	-
Garrotxa	-	-	-	-	+	+	+	-
De La Mesta*	+	+	+	+	+	+	+	+
Ibérico	-	-	+	-	+	-	+	-
Italian cheeses								
Gorgonzola "dolce"	-	-	-	-	-	-	+	-
Gorgonzola "piccante"	+	-	-	-	-	-	+	+
Caprino*	-	-	-	+	+	+	+	+
Quartiolo Lombardo	-	-	-	-	+	+	-	-
Pecorino Sardo*	+	-	+	-	+	+	-	-
Grana Padano*	-	-	-	-	-	-	+	+
Montasio*	-	-	-	-	-	-	+	+
Monte Veronese*	-	-	-	-	+	-	+	+
Asiago*	+	-	+	+	+	+	+	+
Taleggio*	-	-	-	-	+	-	+	+

*Cheeses made from raw milk.

depicted. In general agreement with the microbial counting results, fewer AR gene copies were found in the Italian than in the Spanish cheeses.

3.4. Denaturing Gradient Gel Electrophoresis (DGGE) Analysis. DGGE analyses were undertaken to assess the intragenic sequence variability of two of the most prevalent AR genes, *tet(W)* and *tet(M)*. Besides differences in gene copy number, sequence variability can be a cause of amplification failure in gene-specific PCR and qPCR. Figure 3 shows the DGGE profiles obtained for *tet(W)* using total DNA from Spanish cheese samples as a template. The DGGE primers returned amplifications in six of the seven cheese samples deemed positive by gene-specific PCR (all but Mahón cheese). A prominent band was present in all samples analysed, the mobility of which was comparable to that produced when using as a template DNA from the *B. longum* control strain carrying a chromosomal *tet(W)* gene. Ten other bands of minor intensity were also observed across the cheeses (up to six bands in one sample; Figure 3, line 1), of which eight could be reamplified and sequenced. Sequence comparison showed these bands to be of identical nucleotide sequence to *tet(W)*, except at two polymorphic positions. These polymorphic bands migrated differently on the gel. However,

surprisingly, some bands from different positions showed identical sequences.

DGGE analysis was also performed with specific primers for *tet(M)* using DNA from cheeses in which this gene had been identified. In this case, no differences were seen among the DGGE profiles. All the profiles obtained were composed of one intense and two weak bands, but all these bands were also produced when DNA from a control strain carrying a single-copy *tet(M)* gene was used as a template (data not shown).

4. Discussion

It is acknowledged that the misuse and overuse of antibiotics in agriculture, aquaculture, animal husbandry, and the clinic have caused an increase in AR bacteria, the consequence of the selection pressure exerted in these environments [3, 32]. AR in dairy products has traditionally been examined by conventional methods of microbial analysis. Indeed, in the present work, the tetracycline and erythromycin resistant populations of 20 Spanish and Italian traditional cheeses were first identified by culturing. High numbers of *Tet^r* and *Erm^r* bacteria were found in most samples, suggesting cheese to be an important vehicle of AR genes within the food

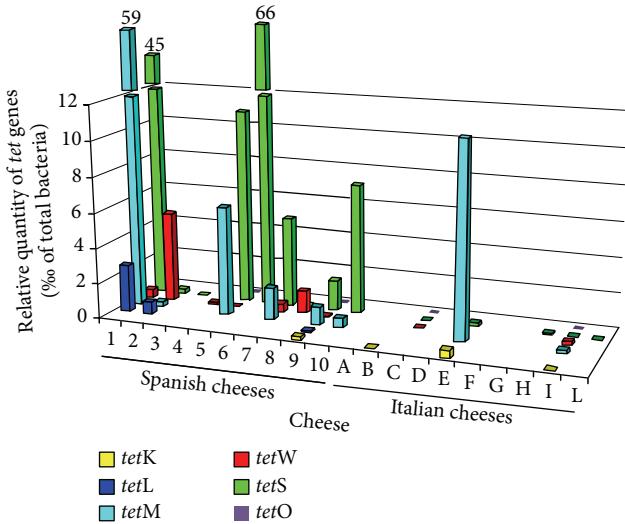


FIGURE 1: Bars diagram depicting the percentage copy number (%) of different tetracycline resistance genes quantified by qPCR as a function of the total bacteria in Spanish (1 to 10) and Italian (A to L) cheeses. The order of both Spanish and Italian cheese types is the same as in the tables.

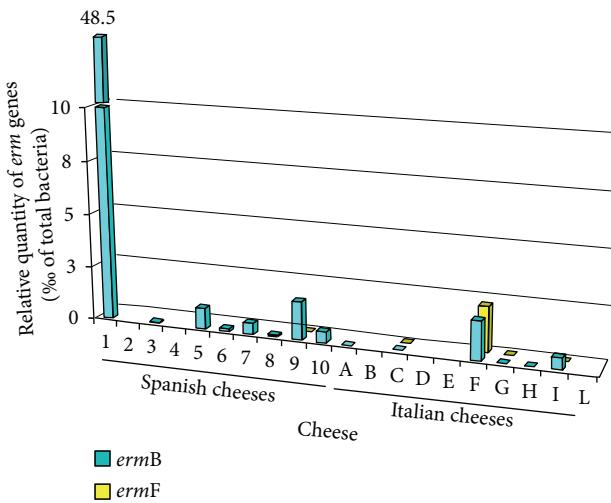


FIGURE 2: Bars diagram depicting the percentage copy number (%) of *erm*(B) and *erm*(F) erythromycin resistance genes quantified by qPCR as a function of the total bacteria in Spanish (1 to 10) and Italian (A to L) cheeses. The order of both Spanish and Italian cheese types is the same as in the tables.

chain. Similar ratios between counts of AR and antibiotic susceptible bacteria have previously been reported in other cheese types, such as Mozzarella [33] and Cheddar [8].

No significant differences were seen in AR counts between cheeses made from raw or pasteurised milk or between cheeses made from milk of different species. These results agree with previous reports that indicate reductions in the presence of AR bacteria only in sterile foods [4]. Pasteurisation may reduce the numbers and types of environmental bacteria, but traditional cheesemaking is open to contamination, in which organisms from many sources can

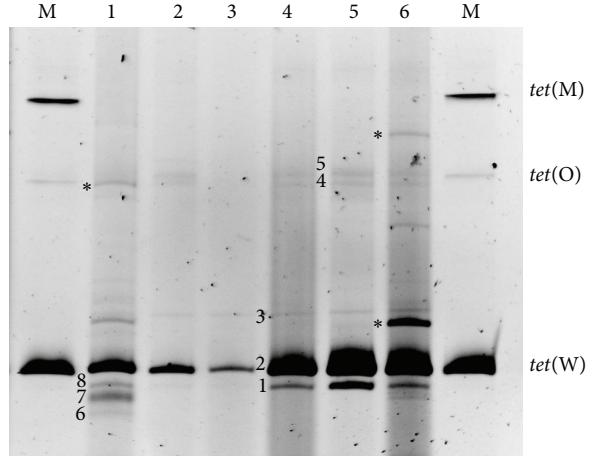


FIGURE 3: PCR-DGGE analysis of total DNA from Spanish cheeses using specific DGGE-primer for the tetracycline resistance *tet*(W) gene. Lanes from 1 to 6, Cabrales, Zamorano, Torta del Casar, Ibores, Garrotxa, and De La Mesta. Lanes M, DGGE markers consisting of amplicons obtained with specific DGGE primers for *tet*(M), *tet*(O), and *tet*(W), and using as template total DNA from bacterial strains harboring the respective tetracycline resistance gene. The asterisks denote bands that did no reamplification and, consequently, could not be identified.

gain access to and develop in the cheese. The Italian cheeses contained smaller AR populations than the Spanish ones. The curd-cooking step in some Italian cheeses may reduce the number of nonstarter microorganisms (e.g., enterococci and staphylococci), which have been shown to contain a plethora of resistance types [34–36]. Further, AR counts seemed to be inversely correlated with ripening time (data not shown), which is usually longer for Italian cheeses.

The presence of a wide range of tetracycline and erythromycin resistance genes in microbial populations from different environments has been reported elsewhere [13–15, 37, 38]. In the present study, the most abundant *tet* and *erm* genes were *tet*(S), *tet*(W), *tet*(M), and *erm*(B). In addition, *erm*(F) was widespread among the Italian cheeses.

The detection of *tet* and *erm* resistance genes by qPCR has frequently been used to monitor AR gene loads in different environments [13, 39–41]. However, it has been little used with dairy products [14, 37]. In the present work, great variation was seen between the cheeses in terms of the total abundance of *Tet*^r and *Erm*^r genes. Absolute copy numbers higher than 5 log₁₀ units have previously been reported for different *tet* genes [37]. In the present work, gene copy numbers were much higher than the corresponding AR bacterial counts in antibiotic-containing media. Similar results have recently been reported for *tet*(A) and *tet*(B) in hake using qPCR and Taqman probes [42]. This might be a reflection of the actual copy number of the genes in AR bacteria (e.g., they may be present in high copy number plasmids) or due to the fact that DNA-based techniques do not distinguish between live, viable-but-noncultivable, and dead bacteria [13, 14, 37, 42]. Despite this, qPCR results showed a similar trend to plate counting. Lower gene copy numbers and AR bacterial counts were always recorded for the Italian cheeses. Intragenic

variability, amplification yields by different primer pairs, and/or the high qPCR detection limit (of 10^4 copies per gram of cheese) might explain the discrepancies between the results obtained by this technique and those by gene-specific PCR.

As mentioned earlier, in addition to its use for tracking bacterial populations, the DGGE technique has already been used to analyse the polymorphism of tetracycline resistance genes in several environments [15, 16]. However, to our knowledge, this is the first attempt to use this molecular tool to estimate the diversity of *tet* genes in dairy products. A canonical *tet(W)* and *tet(M)* sequence accounted for the majority of genes in all cheeses. Nevertheless, single-nucleotide differences at two positions in the analysed segment of *tet(W)* were noted. Whether polymorphic sequences are carried by different bacterial species remains unknown. Nevertheless, the low sequence divergence suggests that *tet* resistance genes in cheese have a monophyletic origin and are spread among the majority bacterial populations through horizontal transfer. This has already been reported for *tet* genes in bacterial populations from other environments [43, 44].

5. Conclusions

The Spanish and Italian cheeses analyzed in this work showed wide variation in their AR bacterial populations, AR gene diversity, and AR gene loads for resistance to tetracycline and erythromycin. The results of this research are of basic and applied interest. Methods and techniques can be extended to study resistance against other groups of antibiotics of currently higher clinical significance, such as β -lactams and aminoglycosides. On average, more than three different AR genes were detected in every cheese type. Indeed, all eight *erm* and *tet* resistance genes searched for were identified in one Spanish cheese (De La Mesta) and seven in one Italian cheese (Asiago). The diversity of genes and their large copy numbers can be considered as a biological hazard with a likely, yet undefined, risk of horizontal transfer. To fully assess this risk would require the genetic characteristics of the bacteria carrying these AR genes and the location of these genes in the genome (chromosome, plasmid, transposon, integrons, etc.) to be known. As some other foods of animal origin, cheeses might be key players on the spread of AR genes via the food chain. This is certainly a biological hazard, which anticipates a transfer to susceptible bacteria during cheese manufacture or after consumption. Therefore, improvements in hygiene in animal husbandry, milk production, and cheese manufacturing practices may contribute to preventing the spread of these (and maybe others) AR determinants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Differences in Extended-Spectrum Beta-Lactamase Producing *Escherichia coli* Virulence Factor Genes in the Baltic Sea Region

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The aim of this study was to compare the prevalence of different virulence factor (VF) genes in extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* strains isolated from the Baltic Sea region. A total of 432 strains of phenotypically ESBL positive *E. coli* were collected from 20 institutions located in Estonia, Latvia, Lithuania, and the region of St. Petersburg in Russia from January to May 2012 and analyzed for phylogenetic group and prevalence of 23 VF genes. The strains were collected from clinical material (urine, blood, wound, and respiratory tract). Bacterial isolates were compared according to phylogenetic group, clinical material, and geographical origin. Most of the VF genes were concentrated within phylogenetic group B2 and/or D. When comparing strains isolated from different countries, it was found that strains originating from Estonia and Latvia belonged mainly to group B2 and strains from Lithuania and Russia mainly to groups B2 and D. The P-fimbrial adhesin gene *papEF* was more prevalent in Russian strains, colicin gene *cvaC* in Lithuanian strains, and capsular gene *kpsMTII* in Latvian strains; serum resistant gene *traT* was less prevalent in Estonian strains. The regional differences of VF genes remained statistically significant after taking into account the phylogenetic distribution in the countries.

1. Introduction

Escherichia coli strains, which are important to humans, can be classified into 3 groups: commensal strains, intestinal pathogenic (enteric or diarrheagenic) strains, and extraintestinal pathogenic *E. coli* (ExPEC) strains [1]. ExPEC strains can cause infections in almost every organ or anatomical site, typically urinary tract infections, neonatal meningitis, intra-abdominal infections, pneumonia, soft-tissue infections, and bacteremia [1, 2].

Pathogenic *E. coli* clones have acquired specific virulence factors (VF), which confer an increased ability to adapt to

new niches and allow them to cause a broad spectrum of diseases [3]. ExPEC isolates have functionally similar VF profiles and clonal background, and they are distinct from commensal and intestinal pathogenic *E. coli* strains [4]. VF-s of ExPEC include different adhesins, toxins, capsules, siderophores, invasins, and antibiotic resistance. These VF-s contribute to colonization and invasion into host tissues, avoidance to immune responses, and antimicrobial drugs and acquiring nutrients from the host [5, 6]. The management of infections caused by *E. coli* is complicated due to the increasing resistance to antibiotics. Extended-spectrum β -lactamase (ESBL) production is a common mechanism of resistance to

third-generation cephalosporins in *E. coli*, associated with the frequent use of β -lactam antibiotics in treatment of serious *E. coli* infections [5].

E. coli strains can be divided into four main phylogenetic groups: A, B1, B2, and D [7]. The strains causing extraintestinal infections belonging preferentially to group B2 and to a lesser extent to group D. Commensal strains largely belong to groups A and B1 [8, 9]. However, the distribution of phylogenetic groups may vary in different geographic regions. It depends on the climatic zone and environmental factors [10].

The aim of this study was to compare the phylogenetic distribution and prevalence of different VF-s in extended-spectrum β -lactamase producing extraintestinal *E. coli* strains isolated from the Baltic Sea region.

Some information presented in this paper was previously demonstrated at 23rd ECCMID held on 27–30, April, in Berlin, Germany [11].

2. Materials and Methods

2.1. Strains. A total of 423 strains of phenotypically ESBL positive *Escherichia coli* were investigated. All consecutive ESBL positive strains were collected from patients from 20 institutions located in Estonia ($n = 5$), Latvia ($n = 4$), Lithuania ($n = 3$), and the region of St. Petersburg in Russia (further referred to as Russian strains) ($n = 8$) from January to May 2012. The strains were isolated from different clinical materials (Table 1), identified as *E. coli* by Matrix Assisted Laser Desorption/Ionization Time of Flight instrument (MALDI-TOF; Bruker Daltonik GmbH, Germany) and ESBL production was confirmed by ROSCO ESBL kit (Rosco Diagnostics A/S, Denmark) [12].

2.2. Phylogenetic Analysis. All bacterial strains were assigned to one of the four main *E. coli* phylogenetic groups (A, B1, B2, and D) according to PCR-based method published by Clermont et al. [7].

2.3. Virulence Genotyping. All bacterial isolates were screened for 23 VF genes coding for adhesins (*papAH*, *papC*, *papEF*, *papGI*, *papGII*, *papGIII*, *fimH*, *sfa/focDE*, *focG*, *nfaE*, and *bmaE*), toxins (*hlyA*, *cvaC*, and *cdtB*), capsule synthesis (*kpsMTII*, *kpsMTIII*, *kpsMT KI*, and *rfc*), siderophore systems (*fyuA* and *iutA*), invasin (*ibeA*), pathogenicity island (PAI) marker of highly virulent uropathogenic *E. coli* strain CFT073, which is used as a marker for uropathogenic PAIs [13], and serum resistance (*traT*).

In order to determine 23 VF genes, four previously described multiplex-PCR primer sets were used: (i) PAI, *papAH*, *fimH*, *kpsMTIII*, *papEF*, and *ibeA*; (ii) *fyuA*, *bmaE*, *sfa/focDE*, *iutA*, *papGIII*, and *kpsMT KI*; (iii) *hlyA*, *rfc*, *nfaE*, *papGI*, *kpsMTII*, and *papC*; (iv) *cvaC*, *cdtB*, *focG*, *traT*, and *papGII* [14].

Total bacterial DNA was purified using PureLink Pro96 Genomic DNA Kit (Invitrogen, USA). Amplification was done in 25 μ L mixtures containing 2 μ L (200 ng) of template DNA, 1x HotStart PCR buffer (Thermo Scientific, USA),

0.2 μ M of 4 dNTPs, 0.6 μ M of each primer, 2.5 mM of MgCl₂, and 1U HotStart DNA polymerase (Thermo Scientific, USA).

PCR conditions were as follows: 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C for primer set (i) and 30 s at 63°C for primer sets (ii)–(iv), 1.30 min at 72°C, and finally 15 min incubation at 72°C. The PCR products were analyzed by electrophoresis (150 V, 400 mA for 1.5 h) in 2% agarose gel prepared in 1xTris-acetate-EDTA (TAE) buffer, stained with ethidium bromide (0.5 μ g/mL).

For each of the detected genes, one PCR product was sequenced and compared with *E. coli* DNA sequences on NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/>) in order to control whether primers bind on correct region in bacterial DNA. Controlled DNA samples were further used as positive controls.

2.4. Statistical Analysis. Fisher's exact test was used to compare the prevalence of the 23 individual VF genes between strains isolated from different countries, materials, and strains belonging to different phylogenetic groups. Comparisons of VF scores were assessed using Mann-Whitney U test. VF score was calculated as sum of virulence genes detected, adjusted for multiple detection of the *pap*, *sfa*, *foc*, and *kpsMTII* operon (*papAH*, *papEF*, *papC*, *kpsMT KI*, and *focG* were not taken into account). In order to assess the impact of phylogenetic group and country of origin to the VF score while controlling for the possible contribution from the other variable in the model, the mutually adjusted odds ratios with 95% confidence intervals were calculated using fractional logit models [15]. The models were fitted for (1) all VF genes (adjusted for multiple detection of the *pap*, *sfa*, *foc*, and *kpsMTII* operons), (2) adhesin genes (adjusted for multiple detection of *pap*, *sfa*, and *foc* operons), (3) capsule synthesis genes (adjusted for multiple detection of *kpsMTII* operon), (4) toxin genes, and (5) genes coding for siderophores. The group least likely to carry VF gene was chosen as reference group. The criterion for significance was taken as $P < 0.05$.

3. Results

3.1. VF Genes. All of the 423 extraintestinal ESBL-producing *E. coli* isolates contained at least one of the VF genes studied. In total, 26 (6%) strains contained 1–3 VF genes, 208 (49%) strains 4–6 VF genes, 164 (39%) strains 7–9 VF genes, and 24 (6%) strains 10–13 VF genes. No strain contained more than 13 out of 23 studied VF genes.

The prevalence of VF genes ranged from 0% (*bmaE* and *papGI*) to 94% (*fimH*) (Table 2).

3.2. Comparison of VF Genes in Strains according to Phylogenetic Group. Among the 423 *E. coli* isolates, 26 (6%) strains belonged to phylogenetic group A, 17 (4%) strains to group B1, 300 (71%) strains to group B2, and 80 (19%) strains to group D.

The different phylogenetic groups exhibited disparate VF scores (mean \pm standard deviation): group A 3.9 ± 1.6 , group B 4.8 ± 1.5 , group B2 6.0 ± 1.3 , and group D 5.3 ± 1.3 . Strains

TABLE 1: Number of ESBL producing *E. coli* strains isolated from different countries and materials.

Origin of strains (<i>n</i>)	Estonia (<i>n</i> = 149)	Latvia (<i>n</i> = 112)	Lithuania (<i>n</i> = 35)	Russia (<i>n</i> = 127)
Urine (<i>n</i> = 266)	110 (74%)	52 (46%)	19 (54%)	85 (67%)
Blood (<i>n</i> = 27)	7 (5%)	8 (7%)	7 (20%)	5 (4%)
Wound (<i>n</i> = 92)	23 (15%)	37 (33%)	7 (20%)	25 (20%)
Respiratory tract (<i>n</i> = 38)	9 (6%)	15 (13%)	2 (6%)	12 (9%)

TABLE 2: The prevalence of 23 VF genes among 423 extraintestinal ESBL producing *E. coli* strains, isolated from the Baltic Sea region.

VF	Gene	Prevalence (% of total)
Adhesins	<i>bmaE</i>	0 (0)
	<i>fimH</i>	399 (94)
	<i>focG</i>	5 (1)
	<i>nfaE</i>	2 (0.5)
	<i>papAH</i>	65 (15)
	<i>papC</i>	101 (24)
	<i>papEF</i>	67 (16)
	<i>papGI</i>	0 (0)
	<i>papGII</i>	71 (17)
Toxins	<i>papGIII</i>	8 (2)
	<i>sfa/focDE</i>	45 (11)
Toxins	<i>hlyA</i>	68 (16)
	<i>cvaC</i>	49 (12)
	<i>cdtB</i>	2 (0.5)
Capsule	<i>kpsMTIII</i>	251 (59)
	<i>kpsMT K1</i>	22 (5)
	<i>rfc</i>	5 (1)
	<i>kpsMTII</i>	204 (48)
Siderophores	<i>fyuA</i>	352 (83)
	<i>iutA</i>	341 (81)
Invasin	<i>ibeA</i>	37 (9)
Pathogenicity island	<i>PAI</i>	215 (51)
Serum resistance	<i>traT</i>	364 (86)

belonging to group B2 were found to carry significantly more VF genes than strains belonging to A, B1, and D ($P < 0.001$; $P < 0.001$; $P < 0.001$). Strains belonging to group A were found to carry significantly less ($P < 0.001$) VF genes than strains belonging to group D.

Most of the genes were found to be more prevalent in groups B2 and/or D (Table 3). In group B2, capsular gene *kpsMTII*, siderophore gene *fyuA* and pathogenicity island marker *PAI* were more prevalent and at the same time toxin gene *cvaC* was less prevalent as compared to the other three groups. P-fimbrial adhesin gene *papEF* was more prevalent in group D when compared to the other groups.

3.3. Comparison of VF Genes in Strains according to Clinical Material. When comparing *E. coli* strains according to

clinical material, no statistical differences in phylogenetic distribution were found. There were differences in prevalence of 3 VF genes: P-fimbrial adhesin gene *papGII* was found more frequently in strains isolated from respiratory tract than in strains from urine (34.2% versus 14.3%; $P = 0.004$), and capsular gene *kpsMTII* was also found more frequently in strains isolated from respiratory tract than in strains from blood (60.5% versus 29.6%; $P = 0.02$). In strains isolated from wound, siderophore gene *iutA* was found more frequently than in strains isolated from urine and blood (90.2% versus 77.8% and 70.4%; $P = 0.01$ and $P = 0.02$).

3.4. Comparison of VF Genes in Strains according to Geographical Origin. The ESBL-producing *E. coli* strains in Estonia and Latvia belonged mostly to phylogroup B2 and in Lithuania and Russia to groups B2 and D (Table 4).

There were differences in prevalence of 15 VF genes. Compared to the other 3 countries, P-fimbrial adhesin gene *papEF* was more prevalent in Russian strains, toxin gene *cvcC* was more prevalent in Lithuanian strains, capsular gene *kpsMTII* was more prevalent in Latvian strains, and serum resistance gene *traT* was less prevalent in Estonian strains (Table 5).

3.5. What Affects the Existence of VF Genes: Phylogenetic Group or Origin of Strains? In fractional logit models, some regional differences remained statistically significant after taking into account the phylogenetic distribution in the countries (Table 6). The odds ratio (OR) of all appointed VF genes was higher in strains isolated from Latvia (OR 1.1, 95% CI: 1.0–1.2) and the OR of adhesin genes was higher in strains isolated from Russia (OR 1.2, 95% CI: 1.0–1.3) compared to strains isolated from Estonia. The capsule synthesis genes were least represented in Russian strains; strains isolated from Latvia (OR 1.8, 95% CI: 1.4–2.3) and Lithuania (OR 1.4, 95% CI 1.0–1.9) were carrying significantly more capsule synthesis genes compared to Russian strains. Siderophores were more than twice as likely to be represented in Russian (OR 2.7, 95% CI 1.4–5.4) than in Lithuanian strains.

The VF scores were also associated with the phylogenetic group after adjusting for country of origin. Strains belonging to the phylogenetic group A were least likely to carry VF genes (Table 6). The OR for all VF genes and adhesin genes was significantly higher for strains belonging to phylogenetic groups B1 (OR 1.3, 95% CI 1.0–1.7 and OR 1.4, 95% CI 1.0–1.9), B2 (OR 1.8, 95% CI 1.5–2.2 and OR 1.8, 95% CI 1.3–2.3), and D (OR 1.5, 95% CI 1.2–1.9 and OR 1.6, 95% CI 1.2–2.1). In phylogenetic group B2, the OR of capsule synthesis genes

TABLE 3: VF-s exhibiting significant prevalence differences according to phylogenetic distribution among 423 extraintestinal ESBL producing *E. coli* strains, isolated from Baltic Sea region.

VF	Gene	Number of isolates (% of total)				<i>P</i>
		A ^a (n = 26)	B1 ^b (n = 17)	B2 ^c (n = 300)	D ^d (n = 80)	
Adhesins	<i>papAH</i>	3 (12)	0 (0)	44 (15)	19 (24)	0.02 ^{bd}
	<i>papC</i>	4 (15)	0 (0)	73 (24)	24 (30)	<0.02 ^{bc,bd}
	<i>papEF</i>	1 (4)	0 (0)	44 (15)	22 (27)	<0.02 ^{ad,bd,cd}
	<i>papGII</i>	1 (4)	1 (6)	50 (17)	19 (24)	0.02 ^{ad}
Toxins	<i>fimH</i>	19 (73)	16 (94)	288 (96)	76 (95)	<0.001 ^{ac,ad}
	<i>hlyA</i>	0 (0)	0 (0)	53 (18)	15 (19)	<0.02 ^{ac,ad}
	<i>cvaC</i>	7 (27)	5 (29)	23 (8)	14 (17)	0.005 ^{ac} ; <0.02 ^{bc,cd}
Capsule	<i>kpsMTII</i>	2 (8)	3 (18)	174 (58)	25 (31)	<0.001 ^{ac,bc,cd} ; 0.02 ^{ad}
Siderophore	<i>fyuA</i>	15 (58)	11 (65)	263 (88)	63 (79)	<0.001 ^{ac} ; <0.05 ^{ad,bc,cd}
	<i>iutA</i>	17 (65)	13 (76)	242 (81)	69 (86)	0.04 ^{ad}
Pathogenicity island	<i>PAI</i>	3 (11)	5 (29)	186 (62)	21 (26)	<0.001 ^{ac,cd} 0.01 ^{bc}
Serum resistance	<i>traT</i>	18 (69)	14 (82)	262 (87)	70 (87)	<0.04 ^{ac,ad}

^{a,b,c,d}Indicate strains isolated from different phylogenetic groups.

TABLE 4: Phylogenetic distribution of 423 extraintestinal ESBL producing *E. coli* strains isolated from different countries.

Phylogenetic group	Number of isolates (% of total)				<i>P</i>
	Estonia ^a (n = 149)	Latvia ^b (n = 112)	Lithuania ^c (n = 35)	Russia ^d (n = 127)	
A	11 (7)	3 (3)	3 (9)	9 (7)	—
B1	3 (2)	2 (2)	1 (3)	11 (9)	<0.02 ^{ad,bd}
B2	121 (81)	100 (89)	22 (63)	57 (45)	0.02 ^{ac} ; <0.001 ^{ad,bc,bd}
D	14 (9)	7 (6)	9 (26)	50 (39)	0.02 ^{ac} ; <0.003 ^{ad,bc,bd}

^{a,b,c,d}Indicate strains isolated from different countries.

(OR 1.7; 95% CI: 1.2–2.5) and siderophores (OR 3.5; 95% CI: 1.7–7.1) was higher than in group A. In phylogenetic groups B2 and D, siderophore genes were twice as likely (OR 3.5, 95% CI 1.7–7.1 and OR 2.4, 95% CI 1.1–5.3) as in groups B1 or A.

4. Discussion

In this study, we characterized the collection of 423 extraintestinal phenotypically ESBL positive *E. coli* strains with respect to phylogenetic groups and 23 VF genes. To our knowledge, the present study is the first to assess the phylogenetic distribution and prevalence of VF genes within the *E. coli* strains isolated from the Baltic Sea region.

ESBL positive *E. coli* strains belonged mostly to phylogenetic groups B2 and D, which contained more VF genes compared to groups A and B1. *E. coli* strains isolated from Estonia and Latvia belonged mostly to phylogenetic group B2 and strains isolated from Lithuania and Russia mainly to

groups B2 and D. Phylogenetic group and country of origin were associated with prevalence of VF genes, whereas clinical materials from which the strains were isolated were not.

Most of the studied ESBL positive *E. coli* strains belonged to phylogenetic group B2 (71%) and group D was the second most common phylogenetic group (19%). Our results support some previous results obtained with ESBL positive *E. coli* strains isolated from different clinical material (blood, urine, wound, and sputum) [16, 17] but do not match the observations made by Branger et al. and Rodrigues-Baño et al., where group B2 was represented, respectively, only in 36.4% and in 15.4% of the ESBL-producing strains isolated from different clinical materials and strains causing bloodstream infections [18, 19]. They explain the scarce prevalence of group B2 in their studies with higher antibiotic resistance of strains belonging to other phylogroups than B2. Also, the occurrence of resistance encoding integrons has been found more frequent in *E. coli* strains of phylogenetic group B2 compared to non-B2 strains [20]. Strains belonging to

TABLE 5: VF-s exhibiting significant prevalence differences according to geographical origin among 423 ESBL producing *E. coli* strains.

VF	Gene	Number of isolates (% of total)				<i>P</i>
		Estonia ^a (n = 149)	Latvia ^b (n = 112)	Lithuania ^c (n = 35)	Russia ^d (n = 127)	
Adhesins	<i>papAH</i>	14 (9)	8 (7)	5 (14)	38 (30)	$\leq 0.001^{ad,bd}$
	<i>papEF</i>	13 (9)	10 (9)	3 (9)	41 (32)	$\leq 0.001^{ad,cd};$ 0.005^{bd}
	<i>papC</i>	20 (13)	29 (26)	6 (17)	46 (36)	$\leq 0.05^{ab,cd};$ 0.001^{ad}
	<i>papGII</i>	3 (2)	24 (21)	4 (11)	40 (31)	$\leq 0.02^{ac,cd};$ $\leq 0.001^{ab,ad}$
	<i>sfa/focDE</i>	28 (19)	9 (8)	5 (14)	3 (2)	$\leq 0.02^{ab,cd};$ 0.001^{ad}
Toxins	<i>focG</i>	2 (1)	1 (1)	2 (6)	0 (0)	0.05 ^{cd}
	<i>hlyA</i>	24 (16)	12 (11)	4 (11)	28 (22)	0.02 ^{bd}
	<i>cvaC</i>	18 (12)	13 (12)	10 (29)	8 (6)	$\leq 0.03^{ac,bc};$ $\leq 0.001^{cd}$
Capsule	<i>kpsMTII</i>	78 (52)	77 (69)	9 (26)	40 (31)	$\leq 0.008^{ab,ac,ad};$ $\leq 0.001^{bc,bd}$
	<i>kpsMTIII</i>	81 (54)	80 (71)	27 (77)	63 (50)	0.01 ^{ac} ; $\leq 0.007^{ab,bd,cd}$
Siderophores	<i>rfc</i>	0 (0)	0 (0)	1 (3)	4 (3)	0.04 ^{ad}
	<i>fyuA</i>	125 (84)	90 (80)	25 (71)	112 (88)	0.03 ^{cd}
	<i>iutA</i>	106 (71)	101 (90)	28 (74)	108 (85)	0.02 ^{bc} ; $\leq 0.001^{ab,ad}$
Invasin	<i>ibeA</i>	27 (18)	3 (3)	4 (11)	3 (2)	0.04 ^{cd} ; $\leq 0.001^{ab,ad}$
Serum resistance	<i>traT</i>	110 (74)	110 (98)	33 (94)	111 (87)	$\leq 0.007^{ac,ad,bd}$ 0.001 ^{ab}

^{a,b,c,d}Indicate strains isolated from different countries.

TABLE 6: Estimated odds ratios (OR) and 95% confidence intervals (CI) for carrying of all VF genes and genes belonging to specific VF groups.

VF genes	OR (95% CI)							
	Countries				Phylogenetic groups			
	Estonia	Latvia	Lithuania	Russia	A	B1	B2	D
All VF genes	1	1.1 (1.0–1.2)*	1.1 (0.9–1.2)	1.1 (0.9–1.2)	1	1.3 (1.0–1.7)*	1.8 (1.5–2.2)*	1.5 (1.2–1.9)*
Adhesins	1	1.0 (0.9–1.2)	1.1 (0.9–1.4)	1.2 (1.0–1.3)*	1	1.4 (1.0–1.9)*	1.8 (1.3–2.3)*	1.6 (1.2–2.1)*
Capsule synthesis genes	1.3 (0.9–1.7)	1.8 (1.4–2.3)*	1.4 (1.0–1.9)*	1	1	1.2 (0.6–2.2)	1.7 (1.2–2.5)*	1.4 (0.9–2.1)
Toxins	1.3 (0.8–2.2)	1	1.8 (0.9–3.6)	1.1 (0.6–2.0)	1.0 (0.5–2.1)	1.2 (0.5–2.8)	1	1.5 (0.9–2.3)
Siderophores	1.2 (0.6–2.2)	1.8 (0.9–3.7)	1	2.7 (1.4–5.4)*	1	1.2 (0.5–3.0)	3.5 (1.7–7.1)*	2.4 (1.1–5.3)*

* Statistically significant OR.

phylogroups A, B1, and D express significantly less VF genes and invade more commonly compromised hosts; hence, less VF-s would be required to cause infections in such patients. Antibiotic resistance gives such strains an advantage to cause infections; previous antibiotic treatment was common, which would have been selected for ESBL *E. coli* [18, 19].

The distribution of VF genes in different phylogenetic groups was not even. As reported previously, most of

the genes, in which case differences in distribution were observed, were more prevalent in phylogenetic groups B2 and/or D [19, 21–23]. *E. coli* strains belonging to group B2 showed the highest virulence score, which is concordant with previous studies [19, 21, 23]. In group B2, siderophore gene *fyuA*, capsular gene *kpsMTII*, and pathogenicity island marker *PAI* were more prevalent and colicin gene *cvaC* was less prevalent than in other phylogenetic groups. Carattoli

et al. found pathogenicity island in all ESBL positive *E. coli* strains, whereas the great majority of the strains belonged to phylogenetic group B2 [24]. In strains belonging to phylogenetic group D, P-fimbrial adhesin gene *papEF* was more prevalent than in strains from other groups.

We found the virulence gene profile to be significantly associated with the geographical origin of the strain. There were differences in prevalence of 15 out of 23 VF studied genes among *E. coli* strains isolated from different countries. An explanation for this may be because of the different phylogenetic distribution of *E. coli* strains originating from different countries in the Baltic Sea region; as in the current study and previous studies by other authors, the distribution of VF genes has been found to differ between phylogroups [16, 17, 19]. *E. coli* strains originating from Estonia and Latvia showed a similar distribution to phylogenetic groups: more strains belonged to group B2 and less to groups B1 and D in comparison with Lithuanian and Russian strains. Strains isolated from Lithuania and Russia belonged to groups D and B2.

Some differences in prevalence of VF genes seemed to be explainable by differences among phylogenetic distribution: P-fimbrial adhesin (*pap*) genes occurred more frequently in Russian strains. The difference in prevalence of *pap*-genes could be explained by differences in the phylogenetic distribution of strains, because half of the Russian *E. coli* strains belonged to group D and we found that *pap*-genes are associated with group D. However, after adjusting for phylogenetic distribution, we found that Russian strains showed higher statistical probability of containing adhesin genes, which indicates that there could be also other explanations for higher prevalence of *pap*-genes in Russian strains. The same could be said about capsular gene *kpsMTII*, which was found to be associated with group B2 and occurred more frequently in Latvian strains, where prevalence of group B2 was the highest. But again we found that the odds ratio of capsular genes in Latvian strains was the highest, so higher prevalence of *kpsMTII* might not be explainable by differences in phylogenetic distribution. Although comparing prevalence of siderophore genes *fyuA* and *iutA* did not show higher proportion of these genes in Russian strains, we found that when differences in phylogenetic distribution were taken into account, siderophore genes were more than twice as likely to be represented in Russian strains.

To our knowledge, there are only a few studies that compare the prevalence of virulence genes in ExPEC strains isolated from different countries [25, 26]. Grude et al. compared *E. coli* strains that were isolated from patients with bacteriuria from Russia and Norway. They found differences in phylogenetic distribution and virulence gene profile: Russian isolates belonged mainly to phylogenetic group A, while phylogroups B2 and D were predominant among the Norwegian isolates. Norwegian isolates also had a significantly higher number of virulence genes compared to isolates from Russia [25]. Differences observed could be due to geographic and climatic factors, as it has been found that they play an important role in structuring *E. coli* population worldwide, including also commensal populations [10, 27]. As the geographic region observed in the current study is small (Baltic countries and

region of St. Petersburg in Russia) and countries involved have a similar climate, then differences found are probably caused by other factors.

Our study revealed no significant relatedness between infection site and phylogenetic distribution or virulence gene profile of studied ESBL producing *E. coli* strains. This indicates the importance of host factors in the process of infection development. We only found significant differences in prevalence of 3 VF genes (*papGII*, *kpsMTII*, and *iutA*) and no significant differences in phylogenetic distribution. Lee et al. had similar results: they found that distribution of phylogenetic groups was similar between isolates from different clinical materials (blood and urine), but in contrast to the current study, they observed differences in prevalence of other genes (*fyuA*, *traT*, and *PAI*), which were more prevalent in strains isolated from blood than in strains from urine [23]. There is not enough data about VF genes of ExPEC strains isolated from other infections than urinary tract infections, bacteremia, and neonatal meningitis, but some authors have described that strains which colonize respiratory tract and other body locations could be similar to strains isolated from blood and urine. This could explain the scarceness of differences among strains isolated from different clinical materials [28, 29].

In conclusion, our study indicates that the prevalence of particular virulence factors in extraintestinal extended-spectrum beta-lactamase producing *E. coli* strains is associated with phylogenetic group and geographical origin of strains rather than infection site. The regional differences of VF genes in ESBL positive *E. coli* strains remained statistically significant after taking into account the phylogenetic distribution in the countries.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Implementation of a Computerized Decision Support System to Improve the Appropriateness of Antibiotic Therapy Using Local Microbiologic Data

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A prospective quasi-experimental study was undertaken in 218 patients with suspicion of nosocomial infection hospitalized in a polyvalent ICU where a new electronic device (GERB) has been designed for antibiotic prescriptions. Two GERB-based applications were developed to provide local resistance maps (LRMs) and preliminary microbiological reports with therapeutic recommendation (PMRTRs). Both applications used the data in the Laboratory Information System of the Microbiology Department to report on the optimal empiric therapeutic option, based on the most likely susceptibility profile of the microorganisms potentially responsible for infection in patients and taking into account the local epidemiology of the hospital department/unit. LRMs were used for antibiotic prescription in 20.2% of the patients and PMRTRs in 78.2%, and active antibiotics against the finally identified bacteria were prescribed in 80.0% of the former group and 82.4% of the latter. When neither LRMs nor PMRTRs were considered for empiric treatment prescription, only around 40% of the antibiotics prescribed were active. Hence, the percentage appropriateness of the empiric antibiotic treatments was significantly higher when LRM or PMRTR guidelines were followed rather than other criteria. LRMs and PMRTRs applications are dynamic, highly accessible, and readily interpreted instruments that contribute to the appropriateness of empiric antibiotic treatments.

1. Introduction

Microorganism resistance to antibiotics changes over time and varies according to geographic area, hospital, or even hospital department [1, 2]. Various studies on severe infections in hospitalized patients, especially those in intensive care units (ICUs), have associated an inappropriate initial antibiotic treatment with increases in bacterial resistance, morbidity-mortality, hospital stay, and hospital costs [3–12]. Selection of the appropriate empiric antibiotic treatment requires knowledge of changes in the etiology of infectious processes and

in antibiotic resistance patterns in each hospital area [13, 14].

The selection of empiric antibiotic therapy is generally based on updated clinical practice guidelines or therapeutic recommendations developed by expert groups from scientific societies [15–17], which must be adapted to the epidemiologic characteristics of each country or healthcare area [13, 18]. Once microbiological results are confirmed and the satisfactory clinical progression of patients is observed, it is recommended to deescalate antibiotic therapy when possible in accordance with the antibiograms of the identified bacteria [3, 6, 10, 11, 15, 19].

Hospital antibiograms are commonly used to monitor local trends in antimicrobial resistance and to prepare antibiotic policies for guiding targeted empiric therapy [20]. Thus, besides the identification and study of microorganism susceptibility, the microbiology laboratory has a role in periodically updating results for designing empiric antibiotic treatment guidelines adapted to the local microbial epidemiology [21, 22]. These guidelines should be based on the best available clinical evidence and on the resistance profiles in each healthcare setting [6, 18]. They need to be constantly updated, taking account of the clinical usefulness of treatments, the ease of their management, and consensus agreements among professionals [21]. Guidelines are considered to be more useful when defined and implemented by a multidisciplinary team and adequately disseminated and promoted, followed by evaluation of their acceptance and implementation [23, 24]. Ideally, these guidelines should be developed for each hospital department, and there is a particular need to avoid needless antibiotic administration for a suspected nosocomial infection in the ICU [25].

The objectives of our study were to design, develop, and implement a new computer application based on the local epidemiologic analysis of bacterial susceptibility to antibiotics and to assess the usefulness to physicians of the information that it offers for selecting the most appropriate antibiotic treatment in ICU patients with suspicion of nosocomial infection.

2. Patients and Methods

This study was conducted over a three-year period in a third-level 821-bed hospital, *Complejo Hospitalario Torrecárdenas* (CHT), serving 350,000 inhabitants and eight primary care districts in the province of Almería (southeast Spain).

2.1. Study Design. A prospective, quasi-experimental study was conducted in three stages between January 2008 and December 2010. During the first six months, a new computer-assisted program was developed for antibiotic selection. Between July and October 2008, four information sessions and two round table discussions were conducted with the specialized physicians and nursing staff of the ICU and Departments of Microbiology, Preventive Medicine, and Pharmacy of the hospital to promote the new program and train participating physicians in its application. Finally, between October 2008 and December 2010, the new system was implemented for antibiotic prescription in the ICU, the patients in the study were followed up, and the results were analyzed.

2.2. A Computer-Assisted Program for Antibiotic Selection. We developed an application, based on Microsoft.NET Framework with Visual C# and SQL, with Open DataBase Connectivity (ODBC) to the Laboratory Information System (LIS) of the Hospital Microbiology Department in order to provide real-time analysis and updating of data obtained from microbiological studies. The application, designated *Guía Electrónica de Resistencias Bacterianas* (GERB), was

installed in a central server. The database is automatically updated and allows consultation of all antibiograms recorded in the LIS, extracting data according to different selection criteria (e.g., date or date interval, patients, samples, diagnoses, microorganisms isolated, antibiotics tested, and hospital departments), and creating graphics to facilitate interpretation and visualization on the computer screens in the network.

Two GERB-based computer applications were developed: local resistance maps (LRMs) and preliminary microbiological reports with therapeutic recommendation (PMRTRs). Both guidelines consider the epidemiology, the evidence-based best treatment options for the most prevalent bacterial pathogens, and the local-specific antibacterial pathogen susceptibility.

2.2.1. Local Resistance Maps (LRMs). These maps graphically depict the accumulated susceptibility data in the LIS for all bacteria identified in samples from ICU patients during the previous year and from the patients who have undergone antibiograms. The GERB system is used to create three types of LRM for the empiric treatment of *lower respiratory tract infections*, based on antibiograms for bacteria isolated from samples from the lower respiratory tract; *urinary tract infections*, based on antibiograms for bacteria isolated from urine samples; and *bacteremias*, based on antibiograms of bacteria isolated from blood cultures. The antibiotics included in the LRMs were selected by consensus among the ICU physicians; their inclusion required a minimum of 30 *in vitro* assays. The percentage of bacteria susceptible to each antibiotic was depicted by using a color code: green: susceptible, yellow: intermediately susceptible, and red: resistant.

Physicians had ready access to the maps *via* touch screens located in the ICUs that were connected to the hospital intranet and automatically updated every 24 h, incorporating any new records entered into the GERB system.

2.2.2. Preliminary Microbiological Reports with Therapeutic Recommendation (PMRTRs). A PMRTR was issued when the microbiology laboratory reported a sample to be positive according to the culture results but before a definitive identification and antibiogram. The requesting ICU physician was informed about the microorganism genus or isolated microorganism or, when not available, the result of Gram staining and was given therapeutic recommendations. These named the antibiotics with highest activity against the microorganisms presumably involved and against the specific infectious disease of the patient, according to the information in the GERB, and they included the most favorable pharmacokinetics and pharmacodynamics properties according to the infection focus. This signed report was sent directly to a dedicated remote printer in the ICU.

2.3. Patients. Inclusion criteria for the patients in the polyvalent 24-bed ICU were as follows: (i) *suspicion of nosocomial infection*, defined as developing ≥ 48 hrs after ICU admission, in the lower respiratory tract, urinary tract, or blood (bacteremia), based on clinical symptoms and results

of laboratory tests or radiologic exam. The focus was microbiologically defined as the lower respiratory tract or urinary tracts when the corresponding microbiological cultures were positive, regardless of the presence of bloodstream infection; bacteremia was defined by the isolation of one or more high-grade pathogens in a blood culture specimen or the identification of a common skin contaminant or skin flora in at least two separate blood culture specimens from different sites in the same patient; and (ii) *susceptibility to antibiotic treatment*. Exclusion criteria were presence of signs of infection or being in incubation period at admission, referral from another hospital department or health center, and age under 14 yrs.

The Acute Physiology and Chronic Health Evaluation (APACHE) II score [26] was determined for each patient, considering the worst reading in the first 24 h of ICU stay, in order to evaluate the severity of illness and calculate the predicted mortality rate.

2.4. Antibiotic Selection Criteria. Antibiotic prescription was structured in three levels, with the aim of treating patients in the shortest time possible with the most appropriate antibiotic according to the infection focus and clinical situation. The first level was the implementation of an empiric antibiotic treatment in patients with clinical suspicion of infection. For this purpose, ICU physicians had access via the touch screen in the unit to the LRM, which depicted the percentage activity of different antibiotics against the microorganisms usually detected in each infectious process, allowing them the possibility of prescribing the treatment in accordance with these data (guidelines). In the second action level, after the putative isolation or identification of one or more microorganisms in a sample from the patient, the physician received a PMRTR prepared by the microbiology specialists (see above). Finally, after identification of the bacteria, the microbiology laboratory issued a definitive report with the corresponding antibiogram.

The study was conducted under the following conditions: (i) the information provided by these GERB applications was not binding in any case. Physicians were not obliged to use the LRM and/or PMRTR guidelines and could base their selection of antibiotic therapy on exclusively clinical criteria (in accordance with the guidelines of the Hospital Infections Committee); (ii) the selection of antibiotic treatment was always adapted to the clinical situation of patients and took account of any therapeutic limitations, including allergies, drug interactions, and toxicity, considering renal and hepatic function, administration routes, dose, dose intervals, and so forth; (iii) in the LRM and/or PMRTR guidelines, an antibiotic was recommended when active against $\geq 75\%$ of all microorganisms isolated in the same infection focus during the previous 12 months; (iv) broad-spectrum antibiotics were used for severe infections (especially in low respiratory tract infections and bacteremias); (v) after receipt of the PMRTR, the physician was able to modify or maintain the initial empiric treatment; and (vi) after receipt of the definitive microbiological report, with bacterial identification and corresponding antibiogram, deescalation was conducted when indicated, selecting the most appropriate antibiotic(s)

according to clinical, microbiological, and pharmacological criteria. No study was made of the reasons for the therapeutic decisions taken by the physicians in this study.

Antibiotic treatment was considered appropriate when at least one of the prescribed antibiotics was active *in vitro* against the isolated microorganism(s) and the drug regimen was in accordance with current medical standards. The appropriateness of the therapeutic option and/or antibiotic prescription was assessed by comparing each of the antibiotics recommended and/or prescribed in a patient with the definitive antibiogram of the microorganism(s) finally identified as the causal agent, when available. An antibiotic with synergic activity, for example, aminoglycosides, was not considered appropriate when it was the only antibiotic active against the isolate *in vitro*.

2.5. Data Collection. Data were gathered in all studied patients on admission date, sex, age, main diagnosis, personal history of interest (allergies, other diseases, previous medication, etc.), chronic organ failure (hepatic, renal, pulmonary, cardiovascular, and immunosuppression, as defined by APACHE II), clinical progress during hospital stay using a semiquantitative scale [27], analytical results (full blood count, biochemistry, cultures, etc.), and daily body temperature. Other variables recorded were the empiric antibiotic treatment selected (indicating whether LRM guidelines were followed or not), any treatment change (indicating whether PMRTR recommendation was followed), the dose, dosing frequency, administration route, possible toxicity, total hospital stay, and date of discharge or death. Patients were followed up until their death or ICU discharge.

2.6. Statistical Analysis. SPSS 17.0 for Windows was used for the data analyses. Pearson's chi-square test (with continuity correction when required) was used to compare the appropriateness of prescribed antibiotic treatments according to the application of clinical criteria, LRM guidelines, or PMRTR recommendations and to compare patient mortality rates in each of these situations and when no empiric treatment was administered. Fisher's exact test in a 2×2 tables was used when the sample size was too small and conditions for Pearson's chi-square test application were not met. The Student's *t*-test was employed to compare the mean days of ICU stay as a function of the criteria used for empiric antibiotic treatment prescription (clinical, LRM, or PMRTR) and the receipt or not empiric treatment. The Mann-Whitney *U* test was used when the distribution of a variable was nonnormal according to the results of a previously applied Shapiro-Wilk test. $P < 0.05$ was considered significant in all tests.

3. Results

Between October 2008 and December 2010, 218 patients in the ICU of our hospital met the study eligibility criteria, 139 males (63.8%) and 79 females (36.2%). The mean APACHE II score of the study cohort was 16.9 ± 7.5 (range, 2–40).

TABLE 1: Distribution by sample type of the 262 microorganisms isolated in the 137 patients.

Microorganism	Respiratory samples	Urine	Blood cultures	Total
<i>Acinetobacter baumannii</i>	2	1	3	6
<i>Bacteroides fragilis</i>			3	3
<i>Candida albicans</i>	4	5		9
<i>Candida parapsilosis</i>	4	1	1	2
<i>Candida tropicalis</i>			2	2
<i>Citrobacter koseri</i>	5		1	6
<i>Enterobacter aerogenes</i>	4		1	5
<i>Enterobacter cloacae</i>	10			10
<i>Enterobacter sakazakii</i>	1			1
<i>Enterococcus faecalis</i>	2		7	9
<i>Enterococcus faecium</i>	1		1	2
<i>Escherichia coli</i>	22	7	2	31
<i>Haemophilus influenza</i>	8			8
<i>Klebsiella oxytoca</i>	4			4
<i>Klebsiella pneumoniae</i>	18	3		21
<i>Morganella morganii</i>	1			1
<i>Proteus mirabilis</i>	7	3	1	11
<i>Pseudomonas aeruginosa</i>	29	5	5	39
<i>Pseudomonas stutzeri</i>	1			1
<i>Serratia liquefaciens</i>		1		1
<i>Serratia marcescens</i>	7		2	9
<i>Serratia plymuthica</i>	1			1
<i>Staphylococcus aureus</i>	32		6	38
<i>Staphylococcus epidermidis</i>			10	10
<i>Staphylococcus hominis</i>			4	4
<i>Stenotrophomonas maltophilia</i>	12			12
<i>Streptococcus grupo viridans</i>			2	2
<i>Streptococcus pneumoniae</i>	14			14

Microbiological documentation of infection was obtained in 137 patients (62.8%) (Table 1), with the identification of 262 different microorganisms from 185 respiratory samples, 26 urine samples, and 51 blood cultures (without considering duplicates in the same sample type). Gram-negative bacteria (63.7%) were the most frequent, followed by Gram-positive bacteria (30.2%), fungi (5.0%), and anaerobes (1.1%). A single microorganism was isolated in 68 (49.6%) of the patients (43 microorganisms in respiratory samples, 2 in urine samples, and 23 in blood cultures), while multiple microorganisms were isolated from the same or different samples in the remaining 69 patients. Microorganisms were isolated from respiratory samples alone in 74 patients, from blood cultures alone in 24 patients, and from urine samples alone in 2 patients; in the remaining 37 patients, microorganisms were isolated from two or more samples from different infection foci. No microorganisms were isolated in culture in 81 (37.2%) of the patients, whose clinical suspicion of infection was not microbiologically confirmed.

3.1. Assessment of Appropriateness of Antibiotic Prescriptions that Follow LRM Guidelines. Empiric antibiotic treatment was implemented for suspicion of nosocomial infection in 173 of the 218 study patients (79.4%), but LRM guidelines were only followed in 44 of these (25.4%) (Table 2). When clinical criteria alone were adopted, the most frequently prescribed antibiotics were amoxicillin-clavulanic acid, vancomycin, levofloxacin, carbapenems (meropenem or imipenem), and ceftriaxone. When LRM guidelines were followed, the most frequently prescribed antibiotics were carbapenems, vancomycin, piperacillin-tazobactam, amikacin, and linezolid.

After sample culture, microorganisms were isolated in 77 of the 129 patients (59.7%) prescribed according to clinical criteria and in 15 (34.1%) of the 44 patients prescribed in accordance with LRM guidelines.

The empiric treatment was a single antibiotic in 76 (43.9%) of the 173 patients (amoxicillin-clavulanic acid in 43 [56.6%] of cases); two antibiotics in 47 (27.2%) of the 173 patients, three in 41 (23.7%), and four in 9 (5.2%). Monotherapy was prescribed in 51.8% of patients treated according to clinical criteria (amoxicillin-clavulanic acid in 64.2% of cases) versus 20.5% of those treated according to LRM. The appropriateness of the empiric antibiotic treatments was evaluated by analyzing the antibiotics prescribed in the 92 patients for whom an antibiogram of the isolated microorganism was available. In the 77 of these patients treated according to clinical criteria, 36.4% of the antibiotics prescribed to this group proved to be active against the isolated bacteria, in comparison to 80.0% of the 15 patients treated according to LRM guidelines. Hence, the percentage appropriateness of the empiric antibiotic treatment was significantly higher ($P = 0.005$) when LRM guidelines were followed.

3.2. Assessment of Appropriateness of PMRTR Recommendations. The microbiology laboratory issued 139 PMRTRs for 96 (44.0%) of the 218 patients in the study, with a total of 362 recommendations for antibiotic therapy (Table 2). When Gram-negative bacilli were isolated in culture, the most frequently recommended antibiotics were imipenem, amikacin, and piperacillin-tazobactam; when Gram-positive cocci in clusters were isolated, they were linezolid and vancomycin,

TABLE 2: Distribution of the antibiotics administered to the 173 patients receiving empiric treatment of the antibiotics recommended by PMRTR (362 recommendations) and of the antibiotics administered to the 87 patients whose treatment was modified after PMRTR emission.

Antibiotic	Patients who received empirical treatment (173 patients)		Antibiotic recommended by PMRTR (362 recommendations)			Treatment modified after PMRTR emission (87 patients)		
	Empirical treatment according to clinical criteria (129 patients)	Empirical treatment according to LRM (44 patients)	When Gram-negative bacillus was isolated	When Gram-positive cocci in clusters were isolated	When Gram-positive cocci in chains were isolated	Treatment modified according to clinical criteria but not PMRTR (19 patients)	Treatment modified according to PMRTR (68 patients)	
Amoxicillin-clavulanic acid	52 (22.3%)	1 (1.0%)	2 (0.8%)	—	—	3 (7.9%)	1 (0.8%)	
Piperacillin-tazobactam	6 (2.6%)	15 (15.6%)	61 (23.6%)	—	—	—	17 (12.9%)	
Cefazolin	7 (3.0%)	2 (2.1%)	—	—	—	—	—	
Ceftriaxone	24 (10.4%)	2 (2.1%)	2 (0.8%)	—	—	6 (15.8%)	3 (2.3%)	
Cefotaxime	8 (3.4%)	1 (1.0%)	13 (5.0%)	—	7 (15.6%)	1 (2.6%)	7 (5.3%)	
Ceftazidime	5 (2.1%)	1 (1.0%)	5 (1.9%)	—	—	2 (5.3%)	3 (2.3%)	
Cefepime	1 (0.4%)	1 (1.0%)	13 (5.0%)	—	—	—	2 (1.5%)	
Imipenem	10 (4.3%)	7 (7.3%)	68 (26.4%)	—	8 (17.8%)	2 (5.3%)	14 (10.6%)	
Meropenem	15 (6.4%)	12 (12.6%)	7 (2.7%)	—	—	3 (7.9%)	16 (12.1%)	
Levofloxacin	25 (10.7%)	5 (5.2%)	20 (7.8%)	—	10 (22.2%)	2 (5.3%)	7 (5.3%)	
Ciprofloxacin	3 (1.3%)	—	1 (0.4%)	—	—	—	—	
Amikacin	14 (6.0%)	15 (15.6%)	61 (23.6%)	—	—	3 (7.9%)	26 (19.6%)	
Tobramycin	12 (5.2%)	5 (5.2%)	2 (0.8%)	—	—	6 (15.8%)	3 (2.3%)	
Gentamicin	2 (0.9%)	—	—	—	—	2 (5.3%)	—	
Vancomycin	28 (12.0%)	18 (18.8%)	—	27 (45.8%)	10 (22.2%)	23 (17.4%)	4 (10.4%)	
Linezolid	10 (4.3%)	6 (6.3%)	—	28 (47.5%)	2 (4.4%)	2 (5.3%)	10 (7.6%)	
Other	11 (4.7%)	5 (5.2%)	3 (1.2%)	4 (6.7%)	8 (17.8%)	1 (2.6%)	—	
Total	233 (100%)	96 (100%)	258 (100%)	59 (100%)	45 (100%)	38 (100%)	132 (100%)	

LRM: local resistance map; PMRTR: preliminary microbiological reports with therapeutic recommendation.

and when Gram-positive cocci in chains were isolated, they were vancomycin and levofloxacin. The appropriateness of PMRTR therapeutic recommendations was evaluated by comparing each of the 362 recommended antibiotics with the definitive antibiogram of the microorganism(s) eventually identified in each patient, which showed that 90.3% of recommended antibiotics were active against the identified bacterium/bacteria.

3.3. Assessment of Appropriateness of Antibiotic Prescription after Receipt of PMRTR Recommendations. Antibiotic treatment prescription recommendations were followed in 68 (70.8%) of the 96 patients for whom a PMRTR was issued, leading to the modification of initial empiric treatment in 36 patients (52.9%), its maintenance in 4 patients (5.9%), or the commencement of treatment in 28 previously untreated patients (41.2%). Clinical criteria rather than the received PMRTR were followed in 19 patients (19.8%). In the remaining 9 patients (9.4%), PMRTRs were issued after ICU discharge or death.

Table 2 shows that when the criteria were exclusively clinical, the most frequently prescribed antibiotics were ceftriaxone, tobramycin, carbapenems, and vancomycin, in this order. However, when PMRTR recommendations were followed, the most frequent were carbapenems, amikacin, vancomycin, piperacillin-tazobactam, and linezolid. Combined therapy with two antibiotics was predominant both in the prescriptions following clinical criteria (mainly ceftriaxone plus tobramycin) and in those following PMRTR (mainly carbapenem plus amikacin).

The appropriateness of antibiotic prescriptions after PMRTR receipt was assessed by comparing the antibiotic prescribed to each patient with the definitive antibiogram of the microorganism(s) finally identified in each sample. According to the definitive antibiogram, 42.1% of the antibiotics prescribed following clinical criteria were active against the isolated bacteria, whereas 82.4% of those prescribed in accordance with PMRTR guidelines were active. The percentage appropriateness of antibiotic treatment prescription was therefore significantly higher ($P = 0.001$) when PMRTR was followed.

3.4. Global Assessment of GERB Use

3.4.1. GERB Use and Level of Associated Appropriateness. As noted above, LRM were followed for the prescription of empiric antibiotic therapy in 44 (20.2%) of the 218 patients in the study and were not followed in 174 (79.8%) patients. Active antibiotics against the isolated bacteria were prescribed in 80.0% of the former group but in only 36.4% of the latter. Out of the 87 patients with available PMRTR, the recommendations were followed in 68 (78.2%) but not in 21 (21.8%) patients. Active antibiotics were prescribed against isolated bacteria in 82.4% of the former cases but in only 42.1% of the latter.

LRM and PMRTR were both followed in only 8 (9.2%) of the 87 patients for whom they were both available (LRM

for empiric treatment prescription, then PMRTR for modification of the initial treatment). Solely clinical criteria were adopted in 32 (36.8%) of the patients, while either LRM or PMRTR were followed in the remaining 47 (54.0%).

3.4.2. Mortality and Hospital Stay. The influence of GERB (LRM and PMRTR) was evaluated on the two main clinical variables: mortality and days of ICU stay. This analysis only included the 137 patients with diagnostic certainty of infection, that is, when a clinically significant microorganism was isolated.

The mean ICU stay of patients who received empiric treatment following LRM guidelines was 13.8 days, with a mortality rate of 20.0%; their mean ICU admission APACHE II score was 17.7. The mean ICU stay of patients who received empiric treatment following clinical criteria was 19.5 days, with a mortality rate of 27.3%; their mean ICU admission APACHE II score was 17.6. There were no significant differences between patients receiving empiric treatment according to LRM guidelines or clinical criteria in mortality ($P = 0.751$) or days of stay ($P = 0.156$), even when nonsurvivors were included ($P = 0.519$).

The mean ICU stay of patients treated according to PMRTR recommendations was 19.7 days, with a mortality of 29.4%; their mean ICU admission APACHE II score was 18.0. The mean ICU stay of patients treated according to clinical criteria was 20.1, with a mortality of 36.8%; their mean ICU admission APACHE II score was 19.0. There were no significant differences between patients treated according to PMRTR recommendations or clinical criteria in mortality ($P = 0.735$) or days of stay ($P = 0.943$), even when nonsurvivors were included ($P = 0.219$).

4. Discussion

Physicians should prescribe an appropriate empiric antibiotic treatment in patients with clinical suspicion of infection. The criteria adopted are usually based on their own experience or on guidelines that are often developed in another setting, even in another country. Hence, therapeutic treatment is frequently not adapted to the microbial epidemiology of the specific healthcare area, which may favor therapeutic failure. Numerous publications by scientific societies and healthcare institutions have emphasized the need to consider local epidemiology in the development of therapeutic guidelines for the prescription of empiric antibiotics [15–17, 21, 22].

Computerized decision support systems (CDSS) are clinical consultation systems that assist physicians in diagnostic and therapeutic decision making by analyzing patient and population data. They have proven effective to improve medical care, reduce prescription errors, and enhance compliance with recommendations [28, 29]. These programs do not replace clinical judgment but rather increase the information available for physicians to be able to make correct decisions [22]. A systematic review associated successful CDSS implementation with the integration of the system in the clinical process and with the availability of recommendations at the time and place of decision making [30].

Evans et al. [28] evaluated the effects of a computerized anti-infective-management program for real-time patient-specific recommendations on the type of antimicrobial, dose, administration route, and treatment duration, finding it to be useful in surgical prophylaxis and in targeted and empiric treatments; use of this program also significantly reduced the number of days that patients received antimicrobial treatment in the ICU. Thursky et al. [31] associated the utilization of a real-time microbiology browser and CDSS for antibiotic prescription with a reduction in total antibiotic prescriptions, especially in the most widely prescribed broad-spectrum antibiotics.

In the present study, GERB-derived LRM s permitted the rates of bacterial resistance to antibiotics to be monitored, based on the information in the LIS of the microbiology laboratory. In general, this application permits (i) structuring of epidemiologic data by hospital area and by infectious disease, (ii) daily and automatic updating with new laboratory results, (iii) presentation of the information in a web environment, and (iv) presentation in readily interpreted graphics of data on bacterial resistance to the antibiotics habitually used in the treatment of a given infectious disease. After positive cultures are obtained, PMRTRs provide a preliminary report on the putative identification of isolated microorganism(s), issuing therapeutic recommendations based on their most likely susceptibility profile according to the local epidemiology of the hospital unit and the specific infectious disease in question.

According to the present results, the utilization of LRM s and PMRTRs contributed to the adaptation of antibiotic treatments, favoring the administration of the most active antibiotics in clinical situation. The lower percentage appropriateness of empiric antibiotic treatments that followed clinical criteria was related to the prescription of monotherapy antibiotics, especially amoxicillin-clavulanic acid, or to the use of combinations of narrow-spectrum antibiotics with high microorganism resistance rates. The considerable increase in percentage appropriateness with treatments following LRM and/or PMRTR guidelines was associated with the prescription of antibiotics with very low resistance rates.

A major challenge in evaluating expert systems that support therapeutic decision making concerns the adherence of physicians to their use, which was relatively low in the present study, especially in relation to LRM guidelines. Physicians may be reluctant to abandon their own criteria or well-established antimicrobial therapy guidelines with recognized prestige, especially in the prescription of empiric treatments [32]. The much higher adherence to PMRTR may be attributable to its provision of an explicit recommendation in a printed report with the signature of a microbiology specialist. Adherence to the GERB applications was stronger when the clinical situation of the patient was more severe, finding a mean APACHE II score of 21 in the eight patients for whom LRM and PMRTR were followed, or when the recommendation was to continue with the same antibiotic therapy.

The results of this study did not support the hypothesis that application of these GERB applications would significantly reduce the mortality rate and length of ICU stay.

Previous studies also found no significant reduction in mortality after the development and implementation of local treatment protocols, although these were associated with an improvement in empiric therapy adaptation and a reduction in the antibiotic treatment duration [13, 25].

In common with other investigations of measures designed to improve antibiotic use, it was not possible to conduct a randomized controlled trial, and the design of our prospective study was therefore quasi-experimental. Patients were not managed with a specific protocol, and it was therefore not possible to control for all relevant clinical variables. We cannot rule out the influence of unmeasured variables and we did not evaluate the response to antibiotic therapy according to predefined clinical variables. A further limitation was the difficulty in assessing the clinical impact of the GERB applications, because no microorganism was isolated in a large percentage (37.2%) of patients; therefore, although there was suspicion of infection, there was no microbiological confirmation. It is likely that a large number of the empiric treatments, following either clinical criteria or LRM, were not for a true bacterial infection, although the early onset of antibiotic treatment may possibly have avoided growth of the microorganism in culture. The selection of one antibiotic or another would not have determined the final outcome in the first situation but may have done so in the second. In fact, it is possible that the lower number of patients in which a given microorganism was isolated when empiric treatment was based on LRM guidelines (34.1%) is related to the high appropriateness rates for antibiotic prescriptions in line with these guidelines. Finally, our assessment of the appropriateness of antibiotic treatments did not consider the isolation of other microorganisms against which these treatments are not active. This is the case of fungi, such as *Candida* spp., which only represented 5% of the microorganisms identified.

In conclusion, these new GERB applications offer dynamic, highly accessible, and easily interpreted instruments to assist physicians in the selection of antibiotic treatment. Their implementation increases the percentage of patients administered with an appropriate initial empiric therapy. It would be of interest to perform a similar study in different hospital departments over the same time period in order to examine variations among them.

Conflict of Interests

Roche Diagnostics, S.L. acquired the rights for the commercial use of the *Guía Electrónica de Resistencias Bacterianas* (GERB) by a license agreement with the Servicio Andaluz de Salud, University of Granada, and University of Almeria.

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Research Article

Antibacterial Activity of *Pseudonocardia* sp. JB05, a Rare Salty Soil Actinomycete against *Staphylococcus aureus*

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Staphylococcus aureus is a Gram-positive bacterium that causes many harmful and life-threatening diseases. Some strains of this bacterium are resistant to available antibiotics. This study was designed to evaluate the ability of indigenous actinomycetes to produce antibacterial compounds against *S. aureus* and characterize the structure of the resultant antibacterial compounds. Therefore, a slightly modified agar well diffusion method was used to determine the antibacterial activity of actinomycete isolates against the test microorganisms. The bacterial extracts with antibacterial activity were fractionated by silica gel and G-25 sephadex column chromatography. Also, the active fractions were analyzed by thin layer chromatography. Finally, the partial structure of the resultant antibacterial compound was characterized by Fourier transform infrared spectroscopy. One of the isolates, which had a broad spectrum and high antibacterial activity, was designated as *Pseudonocardia* sp. JB05, based on the results of biochemical and 16S rDNA gene sequence analysis. Minimum inhibitory concentration for this bacterium was 40 AU mL⁻¹ against *S. aureus*. The antibacterial activity of this bacterium was stable after autoclaving, 10% SDS, boiling, and proteinase K. Thin layer chromatography, using anthrone reagent, showed the presence of carbohydrates in the purified antibacterial compound. Finally, FT-IR spectrum of the active compound illustrated hydroxyl groups, hydrocarbon skeleton, and double bond of polygenic compounds in its structure. To the best of our knowledge, this is the first report describing the efficient antibacterial activity by a local strain of *Pseudonocardia*. The results presented in this work, although at the initial stage in bioactive product characterization, will possibly contribute toward the *Pseudonocardia* scale-up for the production and identification of the antibacterial compounds.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a facultative anaerobic, Gram-positive coccus. This bacterium is the most common cause of a wide variety of illnesses, such as impetigo, pimples, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, as well as life-threatening diseases, such as pneumonia, osteomyelitis, meningitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. It can affect almost any parts of the body, including skin, connective tissue, respiratory, bone, joint, and endovascular regions. One estimate indicated that nearly 500,000 patients

are admitted to American hospitals annually due to staphylococcal infection [1]. Penicillin is a common medication used to treat an *S. aureus* infection. However, the problem of penicillin resistance is extremely pronounced in most countries, where first-line therapy is most commonly a penicillinase-resistant β -lactam antibiotic such as oxacillin or flucloxacillin [2]. Soil bacteria belonging to actinomycetes group are one of the best sources of bioactive natural compounds used as antibiotics, pesticides, pharmaceuticals, herbicides, antiparasitics, and enzymes [3, 4]. About 13,000 biologically active secondary metabolites have been discovered from actinomycetes, and from which 70% have already been

purified [5]. Within actinomycetes, *Streptomyces* is the most investigated genus because of either commercial interests or dominance of the genus on dilution plates and its facility of isolation [6].

It has been found that among actinomycetes the frequencies of isolation for *Streptomyces*, *Actinoplanes*, *Actinomadura*, *Microbispora*, *Micromonospora*, *Nocardia*, *Pseudonocardia*, *Streptosporangium*, *Thermoactinomyce*, and *Thermomonospora* were 95.3, 0.2, 0.1, 0.18, 1.4, 1.98, 0.06, 0.10, and 0.14%, respectively.

Non-Streptomyces actinomycetes (NSA) are a group of actinomycetes that were reviewed by El-Tarably and Sivasithamparam [6]. H. A. Lechevalier and M. P. Lechevalier (1967) isolated 5000 actinomycetes from the soil and found that the genera of NSA, which were evaluated as rare, *Thermomonospora*, *Actinoplanes*, *Microbispora*, *Thermoactinomyces*, *Streptosporangium*, *Micropolyspora*, *Pseudonocardia*, and *Microellobosporia*, form less than 0.2% of the total isolates [7].

The family of Pseudonocardiaceae contains seven genera (*Actinopolyspora*, *Amycolata*, *Amycolatopsis*, *Kibdelosporangium*, *Pseudonocardia*, *Saccharomonospora*, and *Saccharopolyspora*) [8], several of them with industrial interest in bioconversion processes as antibiotic producers, including erythromycin, vancomycin, and rifamycin [9]. Li et al. (2011) reported the production of three new diazaanthraquinone derivatives by the strain SCSIO 01299, a marine actinomycete member of the genus *Pseudonocardia*, isolated from deep-sea sediment of the South China Sea. They found that some of these compounds exhibited potent cytotoxic activities against three tumor cell lines (SF-268, MCF-7, and NCI-H460) with IC₅₀ values between 0.01 and 0.21 μM and also showed antibacterial activities against *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Bacillus thuringiensis* SCSIO BT01, with minimum inhibitory concentration (MIC) values of 1–4 μg mL⁻¹ [10].

As it was referred to earlier, actinomycetes produce many useful antibiotics against a large number of pathogenic bacteria. In the last decade, the screening for new secondary metabolites with antibacterial activity has also focused on minor groups of actinomycetes, including species that are difficult to isolate and culture, and those that grow under extreme conditions (i.e., alkaline and acidic conditions) [11]. Therefore, this study was designed to evaluate the ability of indigenous actinomycete strain(s) to produce antibacterial compound(s) against *S. aureus* in order to identify potent antibacterial compound(s).

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. For the isolation of actinomycetes, twenty-three soil samples were collected from different saline and alkaline affected soils located in the vicinity of Hoze-Soltan, Qom, Iran.

Four different media, Water Yeast Extract Agar (WYE): 0.25 g of yeast extract, 0.5 g of K₂HPO₄, and 18 g of agar per liter of tap water, Starch Casein Agar (SCA): Soluble starch, 10.0 g; K₂HPO₄, 2.0 g; KNO₃, 2.0 g; NaCl, 2.0 g; Casein, 0.3 g;

MgSO₄·7H₂O, 0.05 g; CaCO₃, 0.02 g; FeSO₄·7H₂O, 0.01 g, and agar 15.0 g, pH 8, Czapek Dox Agar (Sucrose, 30.0 g; NaNO₃, 3.0 g; K₂HPO₄, 1.0 g; KCl, 0.5 g; MgSO₄·7H₂O, 0.5 g; FeSO₄·7H₂O, 0.01 g; and agar 15.0 g; pH 7), and ISP₂ (Malt extract, 10.0 g; Yeast extract, 4.0 g; Glucose, 4.0 g, and agar 20.0 g; pH 7) were used for identification, isolation, and preservation of actinomycetes. All chemical reagents and solvents were provided from Merck, Germany.

To provide antibacterial compounds secreted out into the culture medium, actinomycete isolates were cultured into ISP₂ broth medium and incubated on a rotary shaker at 160 rpm for 14 days at 30°C.

Test bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, *B. pustulii*, *Escherichia coli*, *Klebsiella* sp., *Pseudomonas aeruginosa*, *Xanthomonas* sp., and *Acetinobacter* sp., provided from the Persian Type Culture Collection of the Iranian Research Organization for Science and Technology (IROST) (<http://portal.irost.org/persian/PTCC/>), were grown overnight in Luria-Bertani (LB) medium at 30°C.

The preliminary identification of isolates were carried out based on the result of biochemical conventional tests analysis [12]. Morphology and Gram stain were also determined by the use of a light microscope (1,000x) (Zeiss, Argentina S.A.).

2.2. Antibacterial Activity Assay. A slightly modified agar well diffusion method [13] was adopted to determine the antibacterial activity of twenty-six actinomycete isolates against the test microorganisms with three replicates. Briefly, eight millimeter wells were made in Muller Hinton agar plates and inoculated with the test microorganisms. The same concentration of the diluted extracts of each actinomycete isolates then was poured into the wells. After 15-, 24- and 48-hour incubation at 28°C, the antibacterial activity of isolates was evaluated by measuring the diameter of the clear inhibition zones around the wells. The minimum inhibitory concentrations (MICs) were determined using the microplate dilution method.

The protein concentration of cell lysates was determined by the Bradford's method [14].

2.3. Preparation of DNA and Amplification of 16S rDNA Gene. DNA was prepared using a DNA extraction kit (QIAamp DNA Mini Kit, USA) and 16S rRNA encoding gene, amplified using primers S-C-Act-235-S-20 (5'-CGCGGCCTATCAGCTTGTG-3') and S-C-Act-878-A-19 (5'-CCGTACTCCCCAGGGGG-3') [15]. PCR reactions were carried out in 25 μL reaction mixtures containing PCR buffer 10X (2.5 μL), 10 mM dNTPs (0.5 μL), 1.5 mM MgCl₂ (0.75 μL), 2 μM of each primer, 1 unit of Taq DNA polymerase, and 50 ng of genomic DNA. All the PCR reagents were purchased from Fermentas (St. Leon Rot, Lithuania). PCR was conducted in a DNA Thermal Cycler (Techne Flexigen, Minneapolis, MN, USA) under the following conditions: 3 min initial denaturation at 94°C, 30 cycles of 40 s denaturation at 94°C, primer annealing at 58°C for 40 s, and 45 s of elongation at 72°C, followed by a final extension at 72°C for 10 min. PCR products were loaded on 0.8% agarose gel to ensure that the size range of amplified

16S rDNA fragments were between 500 and 625 bp. 16S rDNA genes of the antibacterial-producing bacteria were sequenced and the nucleotide sequences were deposited in GenBank database. The 16S rDNA sequences were used to search the GenBank database using a nucleotide blast algorithm (<http://blast.ncbi.nlm.nih.gov/>) to display the closest matches to the 16S rDNA sequences for known species. Sequences were aligned with illustrative actinomycete 16S rDNA sequences and a phylogenetic tree was constructed by the neighbor-joining method, using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 [16, 17]. One thousand (1,000) bootstrap replications were used to evaluate the branched supporting values.

2.4. Purification and Partial Characterization of Antibacterial Compounds. The following procedure was used to extract and isolate antibacterial compound(s) from *Pseudonocardia* sp. JB05. Briefly, 50 mL of supernatant of bacterial culture was mixed with the same volume of absolute ethanol (Merck, Germany) and centrifuged at 2500 g to remove any bacterial cells. The cell free supernatant was concentrated (20X) using a vacuum centrifugation (Speed Vac AES 1010, Savant). The concentrated supernatant was dissolved with 1 mL of distilled water (dH₂O), gently vortexed, and extracted sequentially with dH₂O, n-hexane and/or ethyl acetate [18].

The antibacterial activity of the extracts was checked using an agar-well diffusion method against *S. aureus* as above mentioned [13].

The extracts with antibacterial activity were fractionated by silica gel column chromatography (610 × 16 mm) in dH₂O (0.5 mL min⁻¹). The active fractions were pooled and loaded onto G25 column chromatography and analyzed by thin layer chromatography (TLC) on silica gel plates (SiO₂, Merck) with acetonitrile (ACN): methanol (MeOH): dH₂O (6:1:3, v/v/v) [18]. The anthrone reagent (0.1 g anthrone, 50 mL pure H₂SO₄, and 5 mL dH₂O) was sprayed onto the silica plate to detect the soluble sugars. Anthrone is a tricyclic aromatic ketone that is used for the colorimetric determination of carbohydrates [19].

The partial structure of the resultant antibacterial compounds was then characterized by Fourier transform infrared spectroscopy (FTIR). The spectra were also scanned in the range of 400 to 4000 cm⁻¹ and plotted as intensity versus wavelength [20, 21].

2.5. Stability of Antimicrobial Compounds. The stability of antibacterial compounds was tested by treating them with proteinase K, 10% sodium dodecyl sulfate (SDS), and boiling. An experiment based on a completely randomized factorial design with three replicates was performed and the effect of two factors A: four cell free supernatants (A₁: number 012-1, A₂: number 012-2, A₃: number 010-31, and A₄: number 025-26) and B: destructive agents (B₁: proteinase K, B₂: 10% SDS and B₃: boiling) were investigated. The bacterial effective extracts were exposed to destructive agents for enough time and their antibacterial activities were then examined against *S. aureus* as described earlier; all data were analyzed by JMP7

software (SAS Institute Inc). Mean comparison was carried out by Tukey's LSD method.

3. Results and Discussion

3.1. Selection and Identification of Actinomycetes. Sporulation of actinomycetes makes them easy to identify on agar plates. From among four media (WYE, SCA, CzapekDox Agar, and ISP₂), WYE was the best medium for the isolation of actinomycetes. In general, this medium is poor in organic carbon, which effectively controlled fungal and eubacterial growth and thus helping in the isolation of the slower growing actinomycetes [22]. Since WYE and YCED media were especially effective for the isolation of actinomycetes, they were used predominantly [23]. In this research, sixty actinomycetes were isolated from different parts of salty and alkaline soil samples collected from Hoze-soltan in Qom, Iran. The isolates were identified based on the result of morphological and biochemical conventional tests analysis. Accordingly, the isolate number 010-31 was designated as *Pseudonocardia* sp. JB05 (Table 1).

3.2. Antibacterial Assay. Antibacterial activity assay was performed using well diffusion method. Among the sixty isolated actinomycetes, only four strains (number 012-1, number 012-2, number 010-31, and number 025-26) showed antibacterial activity against at least one test microorganisms (Figure 1(a)). Strain number 010-31 had the highest inhibitory effect on the growth of all tested pathogenic bacteria, especially on *S. aureus* (Table 1). pH and temperature ranges for the growth of isolate number 010-31 were 6–10 and 27–32°C, respectively, with an optimal growth on pH 8 and 30°C, respectively. It was also capable to grow in WYE medium containing 5% w/v NaCl.

3.3. Molecular Analysis and Phylogenetic Studies. Actinomycetes specific primers were used to amplify 16S rDNA gene (500–625 bp). These primers that were earlier used by Stach [15], these specific primers, can easily identify actinomycetes from other bacteria (Figure 1(b)). The 16S rDNA sequences of the four antibacterial-producing isolates (number 012-1, number 012-2, number 010-31, and number 025-26) were blasted using megablast tool of GenBank (<http://www.ncbi.nlm.nih.gov/>) and were deposited in the NCBI database as *Streptomyces* sp. JB07 (HQ896734), *Pseudonocardia* sp. JB06 (HQ896733), *Pseudonocardia* sp. JB05 (HQ896675), and *Pseudonocardia* sp. JB02 (HQ398191). Analysis of phylogenetic tree by using 16S rDNA sequences had led to the formation of three main clades, including *Streptomyces*, *Pseudonocardia*, and *Nocardiopsis* (Figure 2). These isolated bacteria belong to the two main families of actinomycetes; Streptomycetaceae, with a frequency of 95.3%, and *Pseudonocardiaceae*, with a frequency of 0.06% [24].

3.4. Purification and Partial Characterization of Antibacterial Compound. Antimicrobial purification was performed for *Pseudonocardia* sp. JB05 extract because of its highest

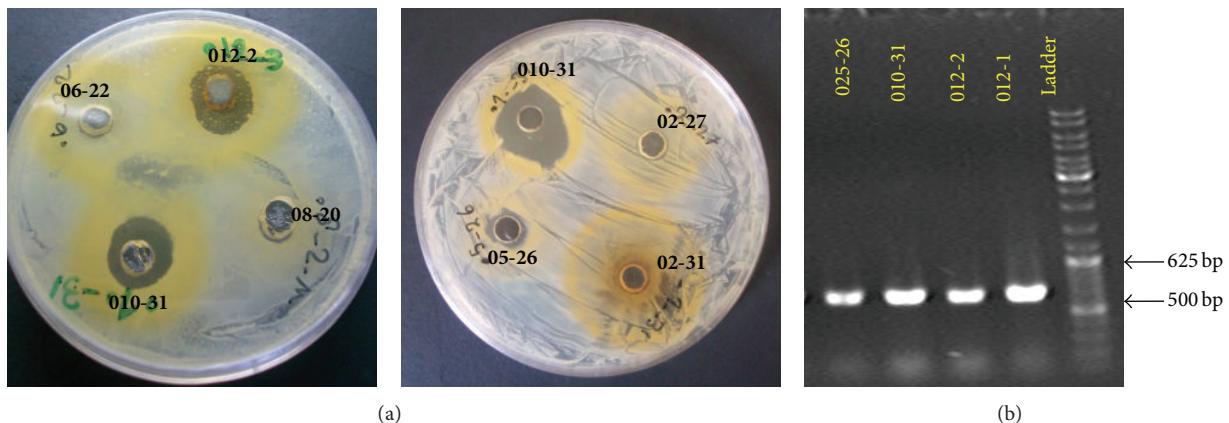


FIGURE 1: Screening of actinomycete isolates based on (a) their antibacterial activity against *S. aureus* and (b) their 16S rDNA gene fragments. From right to left: gel electrophoresis of 1 kb DNA ladder and the PCR products of 16S rDNA gene fragments. Numbers shown above each lane or on each plate are the numbers of the actinomycete isolates.

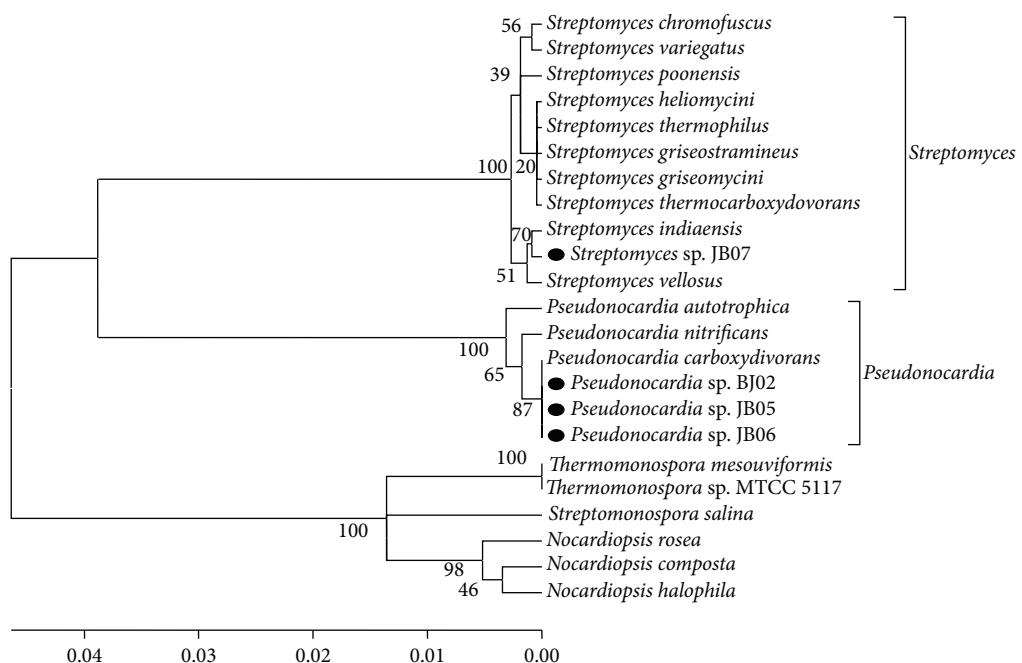


FIGURE 2: Phylogenetic tree of the 16S rDNA nucleotide sequences. Numbers above branches represent bootstrap values (1000 replicates) using neighbor joining.

inhibition zone against *S. aureus*. The antibacterial supernatant was vacuum-evaporated to dryness and then extracted with dH₂O and/or organic solvents (n-hexane and/or ethyl acetate). Our results showed that the dH₂O extract was the most active extract against *S. aureus*, compared to those extracted using organic solvents. As an appropriate polar solvent, dH₂O was used to extract the compound before being compared with the one from the organic solvent [18]. The dH₂O extract was fractionated according to the following procedure: a total of 15 fractions were separated through a silica gel column (Figure 3(a)). When evaluated by an inhibition test, fractions numbers 3, 4, 5, and 6 indicated a clear inhibition zone (Figure 3(b)). They were

pooled and loaded on G25 column chromatography and, after fractionation, fraction number 5 showed the largest clear zone against *S. aureus*.

Thin layer chromatography analysis revealed a blue-green band by using the anthrone reagent with R_f value of 0.8 which indicates the presence of carbohydrates in the purified antibacterial compound.

Finally, an IR spectrum was obtained on a Bruker tensor 27 Fourier transform infrared spectroscopy (FT-IR) instrument. Accordingly, the FT-IR spectrum of dH₂O extracts of *Pseudonocardia* sp. JB05 exhibited absorption around 3410 cm⁻¹, which indicates hydroxyl groups, while the absorption at 2800–2915 cm⁻¹ and at 1600 cm⁻¹ indicates

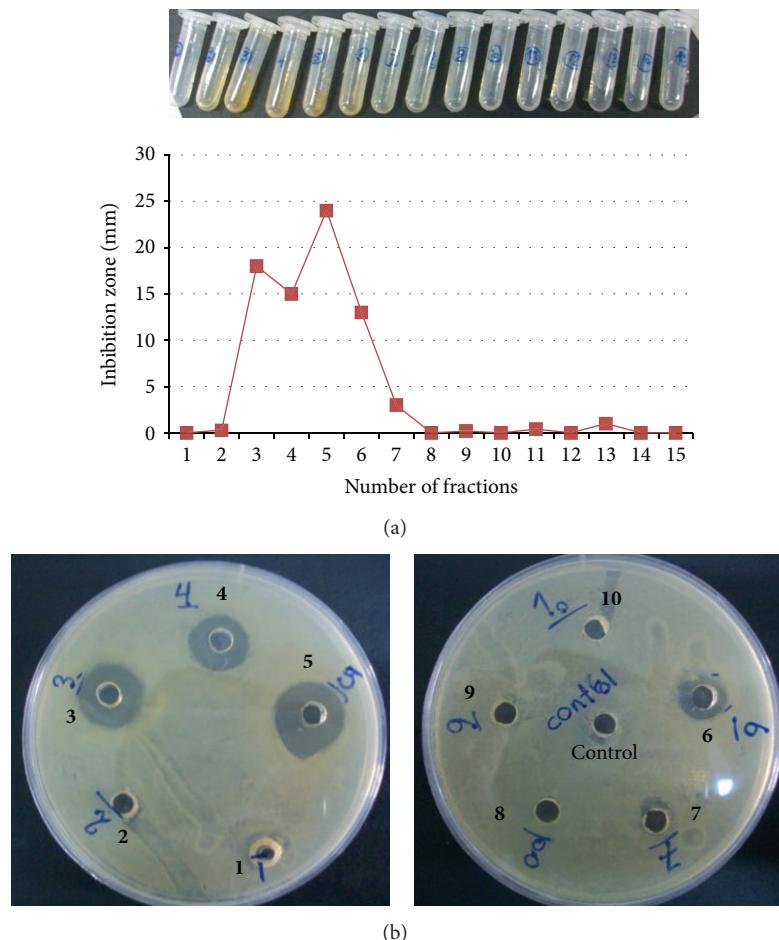


FIGURE 3: (a) Antibacterial activity of fractions numbers 1–15 of *Pseudonocardia* sp. JB05 extract. (b) Inhibition clear halo zone of *S. aureus* around the antibacterial fractions numbers 3, 4, 5, and 6 on agar plates.

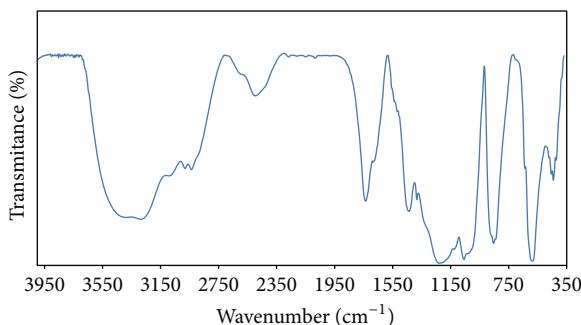


FIGURE 4: FT-IR analysis of the antibacterial compound isolated from *Pseudonocardia* sp. JB05.

the presence of hydrocarbon skeleton and a double bond of polygenic compounds, respectively (Figure 4). Almost similar trend was observed in the FT-IR spectrum of ethyl acetate extract of *Streptomyces albidoflavus* PU23 [21]. The spectrum exhibited absorption bands at 3296 and 1031.8 cm^{-1} , which is indicator of hydroxyl groups, while absorption at 1639 cm^{-1} indicates the presence of double bonds.

3.5. Protein Assay. Bradford analysis [14] using a standard curve showed that the amount of protein in the antibacterial active fractions was $0.0011 \mu\text{g}/\mu\text{L}$.

3.6. Stability of Antibacterial Compound. The effects of proteinase K, surfactant (10% SDS), and boiling on the antibacterial activity of four antibacterial extracts against *S. aureus* were examined. The results showed that none of these above mentioned conditions had any significant effect on the antibacterial activity of the extract of bacterium number A₃ (*Pseudonocardia* sp. JB05) (Table 1) (Figure 5). Also, autoclaving had no effect on antibacterial activity of this bacterium (Figure 5). MIC of *Pseudonocardia* sp. JB05 antibacterial compound was found to be 40 AU mL^{-1} against *S. aureus*.

4. Conclusion

S. aureus causes minor diseases, including life-threatening diseases. Some species of this bacterium are resistant to available antibiotics, such as beta-lactam antibiotics. Therefore, a new antibacterial compound is necessary to control

TABLE 1: Morphological and biochemical characteristics and antibacterial activity of *Pseudonocardia* sp. JB05.

Properties	<i>Pseudonocardia</i> sp. JB05
Morphological characteristics	
Sporophor morphology	Straight
Color of aerial mycelium	Brown
Color of substrate mycelium	Reddish brown
Spore mass	Brown
Biochemical characteristics	
Gram staining	+
H ₂ S production	-
Nitrate reduction	-
Urease	+
Catalase	++
Chitinase	-
Starch hydrolysis	+
Melanin production	-
Antibacterial effect on indicator strains	
<i>Staphylococcus aureus</i>	++
<i>Bacillus subtilis</i>	±
<i>Pseudomonas aeruginosa</i>	-
<i>Klebsiella</i> sp.	-
<i>Xanthomonas citri</i>	-
<i>Acinetobacter</i> sp.	-
<i>Escherichia coli</i>	-
<i>Bacillus pusteurii</i>	+

++: high activity; +: positive effect; -: negative effect.

++ = >20 mm, + = 10–20 mm, ± = 1–10 mm, - = 0 mm, and N = non determined.

the activity of this pathogenic bacterium. The present study was undertaken to evaluate the beneficial antibacterial effect of *Pseudonocardia* sp. JB05 on some pathogenic bacteria, especially *S. aureus*.

Different indigenous bacterial strains were isolated from alkaline soils of Hoz-Soltan, Qom, Iran, and compared for their ability to produce antibacterial compounds. Our results indicate that the strain *Pseudonocardia* sp. JB05 is the most effective candidate because of the antibacterial activity presented. The determination of JB05 antibacterial compounds indicated a minimum inhibitory concentration (MIC) of 40 AU mL⁻¹ against *Staphylococcus aureus*.

The results also demonstrated that antibacterial activity by *Pseudonocardia* sp. JB05 was not influenced by the presence of surfactant (10% SDS), proteinase K, and boiling.

Thin layer chromatography, using anthrone reagent, showed the presence of carbohydrates in the purified antibacterial compound. Also, FT-IR spectrum of the active compound illustrated hydroxyl groups, hydrocarbon skeleton, and double bond of polygenic compounds in its structure. To the best of our knowledge, this is the first report describing the efficient antibacterial activity by a local strain of *Pseudonocardia*. The results presented in this work, although at the initial stage in bioactive product characterization,

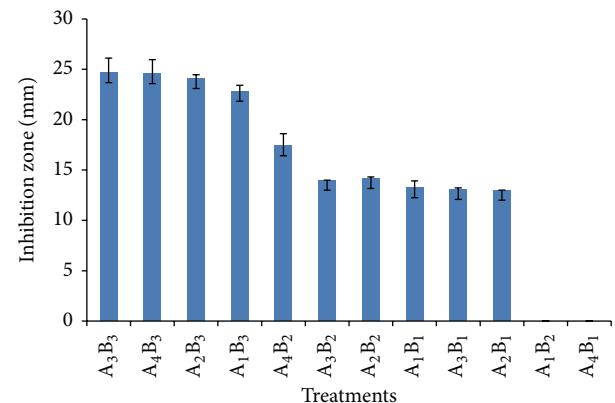


FIGURE 5: Effect of proteinase K, surfactant (10% SDS), and boiling on the antibacterial activity of four antibacterial compounds against *S. aureus*. A₁: *Streptomyces* sp. JB07 (No. 012-1), A₂: *Pseudonocardia* sp. JB06 (No. 012-2), A₃: *Pseudonocardia* sp. JB05 (No. 010-31), A₄: *Pseudonocardia* sp. JB02 (No. 025-26) and B₁: proteinase K, B₂: boiling, B₃: 10% SDS.

will possibly contribute toward the *Pseudonocardia* scale-up for the production and identification of the antibacterial compounds.

Conflict of Interests

There is no potential conflict of interests for each author, concerning the submitted paper.

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Research Article

***Helicobacter pylori* in Vegetables and Salads: Genotyping and Antimicrobial Resistance Properties**

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From a clinical and epidemiological perspective, it is important to know which genotypes and antibiotic resistance patterns are present in *H. pylori* strains isolated from salads and vegetables. Therefore, the present investigation was carried out to find this purpose. Three hundred eighty washed and unwashed vegetable samples and fifty commercial and traditional salad samples were collected from Isfahan, Iran. Samples were cultured and those found positive for *H. pylori* were analyzed using PCR. Antimicrobial susceptibility testing was performed using disk diffusion method. Seven out of 50 (14%) salad and 52 out of 380 (13.68%) vegetable samples harbored *H. pylori*. In addition, leek, lettuce, and cabbage were the most commonly contaminated samples (30%). The most prevalent virulence genes were *oipA* (86.44%) and *cagA* (57.625). *Vaca s1a* (37.28%) and *iceA1* (47.45%) were the most prevalent genotypes. Forty different genotypic combinations were recognized. *Sla/cagA+/iceA1/oipA+* (33.89%), *sla/cagA+/iceA2/oipA* (30.50%), and *m1a/cagA+/iceA1/oipA+* (28.81%) were the most prevalent combined genotypes. Bacterial strains had the highest levels of resistance against metronidazole (77.96%), amoxicillin (67.79%), and ampicillin (61.01%). High similarity in the genotyping pattern of *H. pylori* among vegetable and salad samples and human specimens suggests that vegetable and salads may be the sources of the bacteria.

1. Introduction

Vegetables are raised as complete foods. Their high values for minerals and vitamins are undeniable and, in a day, millions of people use the vegetables and salads in their main diet. Therefore, hygienic quality of vegetables and salad has a high importance in public health but sometimes it will be changed and several infections and illnesses will occur. Vegetables are in close contact with soil, animal manure, and even human stool. They are usually irrigated with polluted water. Previous studies showed that soil [1], water [2], animal manure [3, 4], and human stool [5, 6] are the main resources for *Helicobacter pylori* (*H. pylori*). Therefore, vegetables can

easily be contaminated with *H. pylori*. In addition, their cross-contamination in processing stages is irrefutable.

H. pylori is a gram-negative, spiral-shaped bacterium. Its main reservoir is human, particularly the human stomach. It colonizes most of the population, making it one of the most controversial bacteria in the world. *H. pylori* causes peptic ulcer, duodenal ulcer, gastritis, lymphoma, and gastric cancer [7]. According to the reports, the main routes of infection have not been clarified yet [8, 9]. However, it is likely that *H. pylori* infection occurs during childhood or adolescence in both developing and developed countries [8, 9] and its transmission occurs by person to person, either by fecal-oral or oral-oral routes [1]. Nearly 50% of the world population is

estimated to be infected with *H. pylori* [10]. The prevalence of this bacterium among Iranian people is 60–90%, indicating that Iran is a high risk region for *H. pylori* infection [11].

Some of the most important virulence factors such as vacuolating cytotoxin A (*vacA*), cytotoxin associated gene (*cag*), induced by contact with the epithelium antigen (*iceA*), outer inflammatory protein (*oipA*), and urease (*ureC*) play a major role in pathogenicity of *H. pylori* infection [12]. These genes are usually induced by adhesion to and invasion of the gastric epithelial cells [13–15]. Genotyping using these well-known virulence marker genes is considered as one of the best approaches for study of correlations between *H. pylori* isolates from different samples [16, 17]. The *vacA* gene has a mosaic structure comprising allelic variations in the signal (*s*) and mid region (*m*), each having two different alleles (*s1/s2*, *m1/m2*) with different biological activities. Several subregions including *s1a*, *s1b*, and *s1c* and *m1a* and *m1b* have been identified in *s1* and *m1* regions, respectively [18]. Strains carrying the *s1m1* mosaic combination of the gene *vacA* exhibit higher levels of cytotoxic activity than *s1m2* strains, while *s2m2* strains do not secrete the vacuolating cytotoxin [18]. The *iceA* gene has two main allelic variants, *iceA1* and *iceA2*, but their functions are not yet clear. The *cag* pathogenicity island (PAI) has been shown to be involved in inducing ulceration, inflammation, and carcinogenesis [19]. The *cagA* was one of the most common genes in severe cases of peptic ulcer [20]. The *oipA* gene of the *H. pylori* plays an important role in successful colonization of mucosa [21, 22]. The *oipA* gene has the ability to induce interleukin (IL-8) from gastric epithelial cells, as *cagA* and its status have been linked to the discrimination of duodenal ulcer and gastritis [21, 22]. Bacterial urease neutralizes the gastric pH, enabling the colonization of gastric epithelial cells by the bacteria and their motility in the mucus layer [21, 22].

Treatment of diseases caused by *H. pylori* often requires antimicrobial therapy; however, antibiotic-resistant strains of bacteria cause more severe diseases for longer periods of time than their antibiotic-susceptible counterparts. Several studies have shown that antibiotic resistance in *H. pylori* has increased over time [23, 24].

Data on the distribution of genotypes and antibiotic resistance pattern of *H. pylori* strains isolated from vegetable and salad samples are scarce. Therefore, the aim of the present study was genotyping of *H. pylori* strains isolated from vegetable and salad samples and investigating their susceptibility to 13 commonly used antibiotics, as well as investigating seasonal variation in the prevalence of *H. pylori*.

2. Materials and Methods

2.1. Sample Collection and *H. pylori* Identification. A total of 380 washed and unwashed vegetable samples including leek ($n = 20$), radish ($n = 20$), basil ($n = 20$), parsley ($n = 20$), spinach ($n = 20$), lettuce ($n = 20$), cabbage ($n = 20$), carrot ($n = 20$), scallion ($n = 20$), chive ($n = 20$), fenugreek ($n = 20$), coriander ($n = 20$), pepper ($n = 20$), turnip ($n = 20$), beet ($n = 20$), garlic ($n = 20$), maize ($n = 20$), broccoli ($n = 20$), and cucumber ($n = 20$) and 50 commercial

and traditional salad samples were collected from supermarkets and groceries of various parts of Isfahan Province, Iran (Table 2). Samples were collected over a year. Washed vegetables were processed using the high pressure water. All samples were immediately transferred to the Microbiology and Infectious Diseases Research Center of the Islamic Azad University, Shahrekhord Branch, at 4°C. Twenty-five milliliters of each homogenized sample was added to 225 mL of Wilkins-Chalgren anaerobe broth (Oxoid, UK) supplemented with 5% of horse serum and colistin methanesulfonate (30 mg/L), cycloheximide (100 mg/L), nalidixic acid (30 mg/L), trimethoprim (30 mg/L), vancomycin (10 mg/L) and colistin methanesulfonate (30 mg/L), cycloheximide (100 mg/L), nalidixic acid (30 mg/L), trimethoprim (30 mg/L), and vancomycin (10 mg/L) and incubated for 7 days at 37°C with shaking under microaerophilic conditions. Then, 0.1 mL of the enrichment selective broth was plated onto Wilkins-Chalgren anaerobe agar supplemented with 5% of defibrinated horse blood and colistin methanesulfonate (30 mg/L), cycloheximide (100 mg/L), nalidixic acid (30 mg/L), trimethoprim (30 mg/L), and vancomycin (10 mg/L) and incubated for 7 days at 37°C under microaerophilic conditions. For comparison, a reference strain of *H. pylori* (ATCC 43504) was employed.

2.2. Antimicrobial Susceptibility Testing. Pure cultures of *H. pylori* isolates were used for antibiotic susceptibility test. One strain from each *H. pylori*-positive sample was selected for susceptibility tests. Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) supplemented with 5% defibrinated sheep blood and 7% fetal calf serum, according to the Clinical Laboratory Standards Institute [25]. The antimicrobial resistance of *H. pylori* was measured against the widely used antibiotics in cases of *H. pylori* gastric ulcer. The following antimicrobial impregnated disks (HiMedia Laboratories, Mumbai, India) were used: metronidazole (5 μ g), ampicillin (10 u/), clarithromycin (2 μ g), erythromycin (5 μ g), tetracycline (30 μ g), amoxicillin (10 μ g), streptomycin (10 μ g), levofloxacin (5 μ g), rifampin (30 μ g), trimethoprim (25 μ g), cefsulodin (30 μ g), spiramycin (100 μ g), and furazolidone (1 μ g). After incubation at 37°C for 48 h in a microaerophilic atmosphere, the susceptibility of the *H. pylori* to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by CLSI (2012) [26]. The *H. pylori* ATCC 43504 was used as control organisms in antimicrobial susceptibility determination.

2.3. Detection of *Helicobacter pylori* *UreB* Gene Using Polymerase Chain Reaction (PCR). Suspected colonies were identified as *H. pylori* based on the PCR technique. Genomic DNA was extracted from the colonies with typical characters of *H. pylori* using a DNA isolation kit for cells and tissues (Roche Applied Science, Germany, 11814770001) according to the manufacturer's instructions. Set of novel primers for *ureB* gene of the *H. pylori* was designed by the authors. Recorded sequences of the *ureB* gene of the

TABLE 1: Oligonucleotide primers used for genotyping of *Helicobacter pylori* isolated from vegetables and salads in Iran.

Genes names	Primer sequence (5'-3')	Size of product (bp)
<i>ureC</i>	F*: GCTTACTTCAACACTAACGCGC R**: GGATAAGCTTTAGGGGTGTTAGGGG	296
<i>vacA s1a</i>	F: CTCTCGCTTTAGTAGGAGC R: CTGCTTAATGCGCCAAAC	213
<i>vacA s1b</i>	F: AGCGCCATACCGCAAGAG R: CTGCTTAATGCGCCAAAC	187
<i>vacA s1c</i>	F: CTCTCGCTTTAGTGGGGYT R: CTGCTTAATGCGCCAAAC	213
<i>vacA s2</i>	F: GCTAACACGCCAAATGATCC R: CTGCTTAATGCGCCAAAC	199
<i>vacA m1A</i>	F: GGTAAAATGCGGTATGG R: CCATTGGTACCTGTAGAAC	290
<i>vacA m1B</i>	F: GGCCCCAATGCAGTCATGGA R: GCTGTAGTGCCTAAAGAACAT	291
<i>vacA m2</i>	F: GGAGCCCCAGGAAACATTG R: CATAACTAGGCCCTTGCA	352
<i>cagA</i>	F: GATAACAGCCAAGCTTTGAGG R: CTGCAAAAGATTGTTGGCAGA	300
<i>iceA1</i>	F: GTGTTTTAACCAAAGTATC R: CTATAGCCASTYTCTTGCA	247
<i>iceA2</i>	F: GTTGGGTATATCACAAATTAT R: TTRCCCTATTTCTAGTAGGT	229/334
<i>oipA</i>	F: GTTTTGATGCATGGGATTT R: GTGCATCTCTATGGCTTT	401

*F: forward.

**R: reverse.

H. pylori have been gotten from the GenBank Database of the National Center for Biotechnology Information (NCBI) (GenBank: AY714224.1). The CLS sequence viewer software (Version 6/4) has been used for alignments of the *ureB* gene. Forward and reverse primers have been designed based on the protected area in these sequences. Thermodynamic properties of designed primers were studied using the Gene Runner software (Version 3.05). In order to ensure the specificity of designed primers, the Basic Logical Alignment Search Tool (BLAST) service has been used. The forward primer sequence was *UreB*: 5'-CTTAGCGTGGTCCTGCTAC-3' and the reverse primer sequence was *UreB*: 5'-TGGTGGCACACCATAAGCAT-3'. The gene product was 635 bp. PCR reactions were performed in a final volume of 50 μ L containing 5 μ L 10 \times buffer + MgCl₂, 2 mM dNTP, 2-unit Taq DNA polymerase, 100 ng genomic DNA as a template, and 25 picomoles of each primer. PCR was performed using a thermal cycler (Eppendorf Co., Germany) under the following conditions: an initial denaturation for 10 minutes at 94°C; 35 cycles for 1 minute at 94°C, 1 minute at 57°C, 1 minute at 72°C, and a final extension at 72°C for 10 minutes. The PCR products were electrophoresed through 1.5% agarose gels (Fermentas, Germany) containing ethidium bromide. A DNA ladder (Fermentas Co., Germany) was used to detect the molecular weight of observed bands under a UV lamp. All tests were performed in triplicate. Samples inoculated with *H. pylori* were used as positive controls.

2.4. Genotyping of *Helicobacter pylori*. Presence of the *oipA*, *cagA* and the genotypes of *vacA* (*s1a*, *s1b*, *s1c*, *m1a*, *m1b*, and *m2*) and *iceA* (*iceA1* and *iceA2*) alleles were determined by PCR. The primer sequences are shown in Table 1 [17, 20, 26–30].

The PCR was performed in a total volume of 50 μ L containing 1 μ M of each primer, 1 μ L of genomic DNA (approximately 200 ng), 1 mM of dNTPs mix (Invitrogen), 2 mM of MgCl₂, and 0.05 U/ μ L Taq DNA polymerase (Invitrogen). PCR amplifications were performed in an automated thermal cycler (Biometra Co., Germany). The following cycle conditions were used for PCR amplification: for *vacA*: 32 cycles of 45 s at 95°C, 50 s at 64°C, and 70 s at 72°C; for *cagA*: 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; for *iceA*: 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; and, finally, for *oipA*: 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C. All runs included one negative DNA control consisting of PCR grade water and two or more positive controls (26695, J99, SS1, Tx30, 88-23, and 84-183). The amplified products were visualized using ethidium bromide staining after gel electrophoresis of 10 μ L of the final reaction mixture in 1.5% agarose.

2.5. Statistical Analysis. Data was transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), Chi-square test and Fisher's exact two-tailed test analysis were performed and differences were

TABLE 2: Distribution of *Helicobacter pylori* genotypes isolated from washed and unwashed vegetables and commercial and traditional salads in Iran.

Types and numbers of samples	<i>Helicobacter pylori</i> positive (%)			Genotypes (%)								
	S1a	S1b	S1c	vacA S2	M1a	M1b	M2	cagA	iceA	iceA1	iceA2	oipA
Salads												
Traditional (25)	5 (20)	1	1	—	1	2	1	2	4	3	2	5
Commercial (25)	2 (8)	1	—	—	1	1	—	1	2	1	—	2
Total (50)	7 (14)	2 (28.57)	1 (14.28)	—	2 (28.57)	3 (42.85)	1 (14.28)	3 (42.85)	6 (85.71)	4 (57.14)	2 (28.57)	7 (100)
Leek												
Washed (10)	1 (10)	1	—	—	1	1	—	1	—	1	1	1
Unwashed (10)	5 (50)	2	1	—	1	1	—	3	2	2	2	4
Total (20)	6 (30)	3 (50)	1 (16.66)	1 (16.66)	2 (33.33)	2 (33.33)	1 (33.33)	4 (66.66)	2 (33.33)	3 (50)	5 (83.33)	
Radish												
Washed (10)	—	—	—	—	—	—	—	—	—	—	—	—
Unwashed (10)	2 (20)	1	—	—	1	1	—	1	1	1	1	1
Total (20)	2 (10)	1 (50)	—	—	1 (50)	1 (50)	—	1 (50)				
Basil												
Washed (10)	—	—	—	—	—	—	—	—	—	—	—	—
Unwashed (10)	3 (30)	1	1	—	1	1	—	1	2	2	2	3
Total (20)	3 (15)	1 (33.33)	1 (33.33)	—	1 (33.33)	1 (33.33)	1 (33.33)	—	2 (66.66)	2 (66.66)	1 (33.33)	3 (100)
Parsley												
Washed (10)	—	—	—	—	—	—	—	—	—	—	—	—
Unwashed (10)	3 (30)	1	1	—	1	1	—	1	2	2	2	2
Total (20)	3 (15)	1 (33.33)	1 (33.33)	—	1 (33.33)	1 (33.33)	1 (33.33)	—	2 (66.66)	2 (66.66)	1 (33.33)	3 (100)
Spinach												
Washed (10)	1 (10)	1	1	—	1	1	—	1	1	1	1	1
Unwashed (10)	4 (40)	1	1	—	1	1	—	1	2	2	2	3
Total (20)	5 (25)	2 (40)	2 (40)	1 (20)	1 (20)	1 (20)	2 (40)	1 (20)	3 (60)	3 (60)	3 (60)	4 (80)
Lettuce												
Washed (10)	2 (20)	1	—	1	1	—	1	1	1	1	1	2
Unwashed (10)	4 (40)	1	1	—	1	1	—	1	2	2	2	4
Total (20)	6 (30)	2 (33.33)	1 (16.66)	2 (33.33)	1 (16.66)	2 (33.33)	1 (16.66)	3 (50)	3 (50)	3 (50)	3 (50)	6 (100)
Cabbage												
Washed (10)	2 (20)	1	1	—	1	1	—	1	1	1	1	2
Unwashed (10)	4 (40)	2	1	—	1	1	—	1	2	2	2	3
Total (20)	6 (30)	3 (50)	2 (33.33)	1 (16.66)	2 (33.33)	2 (33.33)	1 (33.33)	3 (50)	3 (50)	3 (50)	4 (66.66)	5 (83.33)

TABLE 2: Continued.

Types and numbers of samples	<i>Helicobacter pylori</i> positive (%)			Genotypes (%)								
	Sla	S1b	S1c	vacA	S2	Mla	Mlb	M2	cagA	iceAI	iceA2	oipA
Carrot												
Washed (10)	—	—	—	—	—	—	—	—	—	—	—	—
Unwashed (10)	1(10)	—	—	—	—	—	—	—	—	—	—	—
Total (20)	1(5)	—	—	—	—	—	—	—	—	—	—	—
Fenugreek												
Washed (10)	1(10)	—	—	—	—	—	—	—	—	—	—	—
Unwashed (10)	3(30)	1	—	—	—	—	—	—	—	—	—	—
Total (20)	4(20)	1(25)	1(25)	—	—	1(25)	—	—	—	3(75)	2(50)	1(25)
Coriander												
Washed (10)	1(10)	—	—	—	—	—	—	—	—	1	1	—
Unwashed (10)	3(30)	1	—	—	—	—	—	—	—	1	1	—
Total (20)	4(20)	1(25)	1(25)	—	—	1(25)	1(25)	1(25)	—	2(50)	2(50)	1(25)
Beet												
Washed (10)	1(10)	—	—	—	—	—	—	—	—	—	—	—
Unwashed (10)	4(40)	2	1	—	—	—	—	—	—	2	2	—
Total (20)	5(25)	2(40)	1(20)	—	—	1(20)	1(20)	1(20)	—	2(40)	2(40)	3(60)
Maize												
Washed (10)	—	—	—	—	—	—	—	—	—	—	—	—
Unwashed (10)	1(10)	—	—	—	—	—	—	—	—	—	—	—
Total (20)	1(5)	—	—	—	—	—	—	—	—	—	—	—
Broccoli												
Washed (10)	1(10)	1	—	—	—	—	—	—	—	1	—	—
Unwashed (10)	2(20)	1	—	—	—	—	—	—	—	1	1	—
Total (20)	3(15)	2(66.66)	1(33.33)	—	—	1(33.33)	2(66.66)	—	—	2(66.66)	1(33.33)	1(33.33)
Cucumber												
Washed (10)	—	—	—	—	—	—	—	—	—	—	—	—
Unwashed (10)	3(30)	1	—	—	—	—	—	—	—	1	1	—
Total (20)	3(15)	1(33.33)	1(33.33)	—	—	1(33.33)	1(33.33)	1(33.33)	—	1(33.33)	1(33.33)	1(33.33)
Total (430)	59 (13.72)	22 (37.28)	14 (23.72)	6 (10.16)	15 (25.42)	18 (30.50)	14 (23.72)	15 (25.42)	15 (57.62)	28 (47.45)	25 (42.37)	51 (86.44)

considered significant at values of $P < 0.05$. Distribution of genotypes and antimicrobial resistance properties of *H. pylori* isolated from washed and unwashed vegetables and commercial and traditional salads were statistically analyzed.

3. Results

All of the vegetable and salad samples were examined using the culture and PCR techniques. From 380 vegetable and 50 salad samples, 52 (13.68%) and 7 (14%) were positive for *H. pylori*, respectively (Table 2). There were statistically significant differences in the incidence of bacteria in washed and unwashed vegetables and traditional and commercial salad samples ($P < 0.01$). We found that the leek, lettuce, and cabbage samples had the highest incidence of *H. pylori* (Table 2). There were no positive results for pepper, turnip, garlic, chive, and scallion samples. Genotype *oipA* (86.44%) was the most commonly detected genotype in *H. pylori* isolates, followed by *cagA* (57.625) (Table 2). Genotypes *vacA s1a* (37.28%) and *vacA m1a* (30.50%) regions had the highest incidence in *vacA* genotypes, while *vacA s1c* region (10.16%) had the lowest incidence (Table 2). A significant difference was found in the incidence of *oipA* and other genotypes ($P < 0.05$).

Twenty-five and forty-two percent of *H. pylori* strains harbored both *m1a* and *m2*, while 22.03% harbored both *m1b* and *m2* (Table 3). Frequency of *cagA*, *oipA*, and both *iceA1* and *iceA2* genotypes was 57.62%, 86.44%, and 40.67%, respectively (Table 3).

Forty different genotypic combinations are shown in Table 4. The most commonly detected combined genotypes were *s1a/cagA+/iceA1/oipA+* (33.89%), *s1a/cagA+/iceA2/oipA* (30.50%), *m1a/cagA+/iceA1/oipA+* (28.81%), *m1a/cagA+/iceA2/oipA+* (25.42%), and *s2/cagA+/iceA1/oipA+* (25.42%).

Descriptions of the seasonal profiles of *H. pylori* isolates are shown in Table 5. Samples which were collected in the spring had the highest incidence (71.18%) of *H. pylori*, while those collected in summer had the lowest incidence (3.38%). There were statistically significant differences ($P < 0.01$) in the incidence of bacteria in spring and other seasons.

Distributions of antimicrobial resistance pattern of *H. pylori* strains are shown in Table 6. The highest levels of antibiotic resistance of the *H. pylori* strains isolated from vegetable and salad samples were found against metronidazole (77.96%), followed by amoxicillin (67.79%) and ampicillin (61.01%). Bacterial strains of our study were susceptible to levofloxacin, rifampin, trimethoprim, cefsulodin, and spiramycin. We found statistically significant differences in the incidence of bacterial antibiotic resistance against metronidazole, streptomycin, furazolidone, and rifampin ($P < 0.05$).

4. Discussion

Totally, 13.72% of vegetable and salad samples of our investigation were contaminated with *H. pylori*. High prevalence of *H. pylori* in clinical samples was reported from Scandinavia, Turkey, Japan, Pakistan, South America, and England [31], while low prevalence was reported from Canada [29]. Our

TABLE 3: Distribution of *Helicobacter pylori* genotypes isolated from vegetables and salad samples in Iran.

Genotypes	Prevalence (%)
	<i>vacA</i>
M1as1a	16 (27.11*)
M1as1b	14 (23.72)
M1bs1a	13 (22.03)
M1bs1b	14 (23.72)
M1as1c	4 (6.77)
M1bs1c	2 (3.38)
M2s1a	14 (23.72)
M2s1b	11 (18.64)
M2s1c	3 (5.08)
M2s2	15 (25.42)
M1as2	13 (22.03)
M1bs2	11 (18.64)
M1am2	15 (25.42)
M1bm2	13 (22.03)
	<i>cagA</i>
CagA+	34 (57.62)
CagA-	25 (42.37)
	<i>iceA</i>
IceA1	28 (47.45)
IceA2	25 (42.37)
IceA1 IceA2	24 (40.67)
	<i>oipA</i>
OipA+	51 (86.44)
OipA-	8 (13.55)

* Percentage of positive genes from total 59 positive samples.

work has identified marked seasonality in the incidence of *H. pylori* isolated from vegetable and salad samples. *H. pylori* isolates had the highest incidence in spring season (71.18%). Moshkowitz et al. (1994) [32] reported that the frequency of *H. pylori* infection in dyspeptic patients in Israel is significantly increased in the humid and rainfall months and decreases in the summer, which is similar to our results. Similar seasonal distributions of *H. pylori* were reported previously [33, 34].

Leek, lettuce, and cabbage were the most commonly contaminated samples in our investigation as they are grown in manure rich soil and thus can easily be infected. Differences in amount of activated water (AW), pH, and hygienic conditions during processing of vegetable and salad samples caused high differences in the incidence of *H. pylori* in our study. Also, the role of infected staffs as sources of *H. pylori* infection is so important [11]. The main reason for the high distribution of *H. pylori* in commercial salad samples is the fact that maybe some food safety and quality standards (good agricultural practices (GAPs), good manufacturing practices (GMPs), and the hazard analysis and critical control point (HACCP) system need to be applied and performed in most of the Iranian food units to control growth, proliferation, and survival of bacteria during harvesting, distribution, and storage periods.

TABLE 4: Combined *vacA*, *cagA*, *iceA*, and *oipA* genotypes of *Helicobacter pylori* isolated from salads and vegetables in Iran.

Combined genotypes	Total (59*) (%)
s1a/cagA+/iceA1/oipA+	20 (33.89)
s1b/cagA+/iceA1/oipA+	11 (18.64)
s1c/cagA+/iceA1/oipA+	6 (10.16)
s1a/cagA+/iceA2/oipA+	18 (30.50)
s1b/cagA+/iceA2/oipA+	12 (20.33)
s1c/cagA+/iceA2/oipA+	5 (8.47)
s1a/cagA-/iceA1/oipA+	11 (18.64)
s1b/cagA-/iceA1/oipA+	8 (13.55)
s1c/cagA-/iceA1/oipA+	4 (6.77)
s1a/cagA-/iceA2/oipA+	10 (16.94)
s1b/cagA-/iceA2/oipA+	7 (11.86)
s1c/cagA-/iceA2/oipA+	4 (6.77)
s1a/cagA+/iceA1/oipA-	6 (10.16)
s1b/cagA+/iceA1/oipA-	3 (5.08)
s1c/cagA+/iceA1/oipA-	2 (3.38)
s2/cagA+/iceA1/oipA+	15 (25.42)
s2/cagA+/iceA2/oipA+	14 (23.72)
s2/cagA-/iceA1/oipA+	12 (20.33)
s2/cagA-/iceA2/oipA+	10 (16.94)
s2/cagA-/iceA2/oipA-	6 (10.16)
s2/cagA+/iceA2/oipA-	7 (11.86)
s2/cagA+/iceA1/oipA-	8 (13.55)
m1a/cagA+/iceA1/oipA+	17 (28.81)
m1b/cagA+/iceA1/oipA+	13 (22.03)
m1a/cagA+/iceA2/oipA+	15 (25.42)
m1b/cagA+/iceA2/oipA+	12 (20.33)
m1a/cagA-/iceA1/oipA+	14 (23.72)
m1b/cagA-/iceA1/oipA+	11 (18.64)
m1a/cagA-/iceA2/oipA+	12 (20.33)
m1b/cagA-/iceA2/oipA+	10 (16.94)
m1a/cagA+/iceA1/oipA-	5 (8.47)
m1b/cagA+/iceA2/oipA-	3 (5.08)
m2/cagA+/iceA1/oipA+	14 (23.72)
m2/cagA+/iceA2/oipA+	13 (22.03)
m2/cagA+/iceA2/oipA-	5 (8.47)
m2/cagA+/iceA1/oipA-	6 (10.16)
m2/cagA-/iceA1/oipA+	11 (18.64)
m2/cagA-/iceA2/oipA+	10 (16.94)
m2/cagA-/iceA2/oipA-	3 (5.08)
m2/cagA-/iceA1/oipA-	4 (6.77)

*Total positive samples.

TABLE 5: Seasonal distribution of *Helicobacter pylori* isolated from washed and unwashed vegetables and commercial and traditional salads in Iran.

Types and numbers of positive samples	Seasonal distribution (%)			
	Winter	Summer	Autumn	Spring
Salads				
Traditional (5*)	1 (20)	—	1 (20)	3 (60)
Commercial (2)	—	—	—	2 (100)
Total (7)	1 (14.28)	—	1 (14.28)	5 (71.42)
Vegetables				
Washed (10)	2 (20)	—	1 (10)	7 (70)
Unwashed (42)	6 (14.28)	2 (4.76)	4 (9.52)	30 (71.42)
Total (52)	8 (15.38)	2 (3.84)	5 (9.61)	37 (71.15)
Total				
Vegetables and salads (59)	9 (15.25)	2 (3.38)	6 (10.16)	42 (71.18)

*Numbers of positive samples.

TABLE 6: Distribution of antibiotic resistance properties of *Helicobacter pylori* isolated from salads and vegetables in Iran.

Types and numbers of positive samples	METR5*	AM10	CLRT2	ERT5	TE30	Antibiotic resistance properties (%)						
						AMX10	S10	FZL1	Lev5	Rif30	TRP25	Cef30
Salads												
Traditional (5)	4 (80)	2 (40)	1 (20)	3 (60)	3 (60)	1 (20)	1 (20)	—	—	3 (60)	1 (20)	1 (20)
Commercial (2)	2 (100)	1 (50)	—	1 (50)	1 (50)	—	—	—	—	1 (50)	—	—
Total (7)	6 (85.7)	3 (42.85)	1 (14.28)	4 (57.14)	4 (57.14)	1 (14.28)	1 (14.28)	1 (14.28)	—	4 (57.14)	1 (14.28)	1 (14.28)
Vegetables												
Washed (10)	7 (70)	5 (50)	2 (20)	5 (50)	6 (60)	1 (10)	1 (10)	—	—	5 (50)	2 (20)	1 (10)
Unwashed (42)	33 (78.57)	28 (66.66)	10 (23.80)	11 (26.19)	26 (61.9)	30 (71.42)	7 (16.66)	3 (7.14)	2 (4.76)	26 (61.9)	5 (11.90)	3 (7.14)
Total (52)	40 (76.92)	33 (63.46)	12 (23.07)	13 (25)	31 (59.61)	36 (69.23)	8 (15.38)	8 (15.38)	4 (7.69)	2 (3.84)	31 (59.61)	7 (13.46)
Total	46 (77.96)	36 (61.01)	13 (22.03)	14 (23.72)	35 (59.32)	40 (67.79)	9 (15.25)	9 (15.25)	5 (8.47)	2 (3.38)	35 (59.32)	8 (13.55)
Vegetables and salads (59)	46 (77.96)	36 (61.01)	13 (22.03)	14 (23.72)	35 (59.32)	40 (67.79)	9 (15.25)	9 (15.25)	5 (8.47)	2 (3.38)	35 (59.32)	8 (13.55)

* In this table, METR5 = metronidazole (5 µg/disk); AM10 = ampicillin (10 µg/disk); CLRT2 = clarithromycin (2 µg/disk); ERT5 = erythromycin (5 µg/disk); AMX10 = amoxicillin (10 µg/disk); S10 = streptomycin (10 µg/disk); and FZL1 = furazolidone (1 µg/disk); Rif30 = rifampin (30 µg/disk); TE30 = tetracycline (30 µg/disk); AMX10 = amoxicillin (10 µg/disk); Rif30 = rifampin (30 µg/disk); Lev5 = levofloxacin (5 µg/disk); Lev5 = furazolidone (1 µg/disk); TRP25 = trimethoprim (25 µg/disk); Cef30 = cefsulodin (30 µg/disk); and Sp100 = spiramycin (100 µg/disk).

High incidence of *H. pylori* in uncooked vegetables that had been irrigated with water contaminated with sewage was reported previously [35, 36]. Frequent consumption of raw vegetables was associated with likelihood of *H. pylori* infection [37]. Also, individuals who consume vegetables are more likely to acquire *H. pylori* [38]. Foods with water activity higher than 0.96 and pH from 4.9 to 9.0 (like vegetables) theoretically provide conditions for the survival of *H. pylori* [39].

The most commonly detected virulence genes in *H. pylori* strains of our study were *oipA* (86.44%), *cagA* (57.62%), *iceA1* (47.45%), and *iceA2* (42.37%). High presence of these genes in clinical samples has been reported previously from Japan [40], Turkey [41], Nigeria [42], and the United States [43]. These virulence genes are responsible for cytotoxin production [44], interleukin-8 (IL-8) construction [45, 46], vacuolization and apoptosis in gastric epithelial cells [13, 14], adhesion to gastric epithelial cells, and inflammatory effects [15, 47].

Alleles *vacA s1a* (37.28%) and *iceA1* (47.45%) were the most commonly detected genotypes in *vacA* and *iceA* positive samples of our study, respectively. *VacA m1a/s1* (27.11%), *vacA m2/s2* (25.42%), *vacA m1a/m2* (25.42%), and *iceA1/iceA2* (40.67%) were the most commonly detected genotypes in our study. There were no previously published data about the genotyping of *H. pylori* in vegetables, salads, and even other types of foods. Various genotypes of *vacA* strains were the most commonly detected genotypes in the studies of Linpisarn et al. (2007) (Thailand) [48], López-Vidal et al. (2008) (Mexico) [49], and Rudi et al. (1998) (Germany) [50]. The high presence of *vacA s1a/m2* genotypes has been reported previously from Iran [11] and Germany [50] but far different results have been reported from Thailand [48] and Mexico [49].

Bacterial strains of our study were resistant to the majority of tested antibiotics. We found that bacterial strains exhibited the highest level of resistance to metronidazole (77.96%), amoxicillin (67.79%), ampicillin (61.01%), and tetracycline (59.32%). The high antibiotic resistance to these drugs detected in our study indicates that irregular and unauthorized use of them may have occurred in Iran. Similarly, metronidazole, amoxicillin, ampicillin, and tetracycline resistance profiles have been reported previously [51, 52]. Indian strains of *H. pylori* had the highest antibiotic resistance against metronidazole (77.9%), clarithromycin (44.7%), and amoxicillin (32.8%) [52], which was similar to our results. Bang et al. (2007) [25] found that the *H. pylori* isolates had the high antibiotic resistance to metronidazole (34.7%), clarithromycin (16.7%), and amoxicillin (11.8%). Low antibiotic resistance of *H. pylori* strains against levofloxacin, rifampin, trimethoprim, cefsulodin, and spiramycin may be due to the regular and low prescription of these antibiotics.

H. pylori isolates from African countries like Senegal and Nigeria, Asian countries like India, Taiwan, China, Iran, Egypt, Saudi Arabia, and Thailand, and South American countries like Argentina, Brazil, and Colombia had the

highest antibiotic resistance to metronidazole, followed by clarithromycin, amoxicillin, quinolones, tetracycline, and furazolidone [30], which was similar to our results.

The above data highlight large differences in the prevalence of *H. pylori* in different studies, as well as differences in virulence genes, genotypes, and antibiotic resistance patterns in the clinical samples. This could be related to differences in the type of sample tested (stool, gastric biopsy, saliva, and food), number of samples, method of sampling, experimental methodology, geographical area, antibiotic prescription preference among clinicians, antibiotic availability, and climate differences in the areas where the samples were collected, which would have differed in each study.

5. Conclusions

In conclusion, vegetable and salad samples harbor *H. pylori* similar in genotype of the *vacA*, *cagA*, *oipA*, and *iceA* alleles to isolates recovered from humans. Also, there was a high similarity in the genotyping pattern of *H. pylori* DNA among vegetable and salad samples and human specimens of other investigations suggest that vegetables and salads are the sources of the bacteria and that they entered the human population in a period of time. On the other hand, diversity of *H. pylori* genotypes in vegetable and salad samples with the clinical isolates of other studies suggested that consumption of contaminated vegetables and salads with *H. pylori* strains may be a threat to human health. Our findings should raise awareness about antibiotic resistance in *H. pylori* strains in Iran. Clinicians should exercise caution when prescribing antibiotics, especially during the spring season. Our data showed that conventional ways to wash vegetables cannot reduce their contamination.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Alpha-Melanocyte Stimulating Hormone: An Emerging Anti-Inflammatory Antimicrobial Peptide

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The alpha-melanocyte stimulating hormone (α -MSH) is a neuropeptide belonging to the melanocortin family. It is well known for its anti-inflammatory and antipyretic effects and shares several characteristics with antimicrobial peptides (AMPs). There have been some recent reports about the direct antimicrobial activity of α -MSH against various microbes belonging to both fungal and bacterial pathogens. Similar to α -MSH's anti-inflammatory properties, its C-terminal residues also exhibit antimicrobial activity parallel to that of the entire peptide. This review is focused on the current findings regarding the direct antimicrobial potential and immunomodulatory mechanism of α -MSH and its C-terminal fragments, with particular emphasis on the prospects of α -MSH based peptides as a strong anti-infective agent.

1. Introduction

α -MSH is an endogenous neuropeptide derived from proopiomelanocortin (POMC), a common precursor protein of all melanocortin peptides, which expresses in the pituitary gland [1, 2]. It is primarily a pigmentary hormone of the vertebrates and largely influences immune reactions in the host for controlling inflammation in the brain and peripheral organs [3, 4]. Both *in vitro* and *in vivo* studies have confirmed that, inside the host, α -MSH reduces the concentration of proinflammatory mediators through the induction of cyclic adenosine monophosphate (cAMP) and inhibition of the nuclear factor $\kappa\beta$ (NF- $\kappa\beta$), thus protecting the brain and peripheral organs from inflammatory disorders [5–7].

The presence of the ancestral components of host immunity—AMPs—has been reported across all classes including human, and their immunomodulatory role has been widely implicated in fighting against infection [8–10]. Similar to AMPs, α -MSH is a short peptide, cationic in nature. Although the primary site of α -MSH expression is pituitary gland, its presence in defense cells and several peripheral sites including skin pointed towards its immunoregulatory

properties [11]. These characteristics of α -MSH openly suggest its role in host immunity, specifically innate immunity, and provide a platform to look for its direct antimicrobial properties [12–14]. Over the last decade, a number of independently conducted studies have demonstrated the antimicrobial activity of α -MSH as well as its C-terminals (containing Lys-Pro-Val) against a wide range of microbes including *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus* [15–19].

A major hurdle faced in developing drugs based on natural AMPs is the occurrence of inflammation. Due to this, the development of new anti-infective agents with both antimicrobial and anti-inflammatory properties has got serious attention in recent years. This is where α -MSH comes across as unique compared to other natural AMPs—it is endogenous anti-inflammatory, antipyretic neuropeptide combined with antimicrobial properties [11]. We present here the in-depth analysis of the current evidences supporting the direct antimicrobial and immunomodulatory potential of α -MSH and analogues. This paper is aimed at emphasizing the future perspectives of α -MSH as a therapeutic against microbial infection in humans.

2. Classical AMPs from Humans

It would not be inappropriate to describe AMPs as an ancient evolutionary arsenal deployed by all life forms. They have been instrumental throughout the evolution of multicellular organisms in defending against the ever-evolving microbes [8–10]. AMPs are widely secreted throughout the body, that is, epithelial, epidermal, immune cells, mucosal surfaces, and so forth. Besides being involved in the direct killing of microbes, they also play an indirect role in clearing them through immunomodulatory activities [20, 21].

Human AMPs are broadly grouped into two families: defensins and cathelicidins. Defensins are widely present in vertebrates including primates, rodents, marsupials, and mammals. Generally, they are 29 to 30 amino acid long and up to 5 kDa and contain 6-cysteines, forming 3 disulphide bonds [22]. They are further subgrouped into α -defensins and β -defensins, based on the arrangement of their disulphide bridges. There is also a third class of defensins, found in old-world monkeys, called θ -defensins. In humans, six different α -defensins are present. Four of these were found in the granules of neutrophils and named as human neutrophil peptides (HNP) 1–4 [22, 23].

The other two human α -defensins (HD)—HD5 and HD6—are expressed in the paneth cells of the intestinal tract [24]. The human β -defensins (HBD) are of four types: HBD1–4. HBD1 is expressed constitutively in most tissues, including the epithelial cells and salivary and mammary glands.

The second human AMP family is cathelicidins. Besides humans, cathelicidins are also found in cows, sheep, guinea pigs, rabbits, mice, and primates. The 37 amino acid long; C-terminal peptide of human cathelicidin antimicrobial protein (hCAP18) is called LL-37 because it begins with two leucines. It is a broad spectrum AMP, produced by the epithelial cells, immune cells, and body fluids such as gastric juices and saliva. The expression of LL-37 increases in response to infections and inflammatory conditions of the skin, such as atopic dermatitis and psoriasis [25]. Besides the direct killing of bacteria, LL-37 also clears pathogens by acting as opsonins, leading to the phagocytosis of LL-37 coated bacteria by macrophages [26].

3. Mode of Action and Functions of AMPs

AMPs are the endogenous and integral molecules of the innate immune system and represent the first line of host defense. The cationic and amphiphilic characteristics of AMPs favor their interaction with the anionic microbial membrane, which has differentiating features from the mammalian cell membrane. For example, the bacterial membranes are negative in charge due to the presence of anionic phospholipids (PG, PS, and CL), lipoteichoic acid (LTA), and teichoic acid (TA) in Gram-positive bacteria and lipopolysaccharides (LPS) in Gram-negative bacteria [34]. Another fundamental difference between the microbial and mammalian membranes is that the former have a greater potential difference across the plasma membrane bilayer (-130 mV to -150 mV) compared to the mammalian cell

membranes (-90 mV to -110 mV). This further increases the electronegativity of bacterial membrane [35].

Thus AMPs kill microbes directly by perforation of their membrane through electrostatic interaction [8, 9]. The hydrophilic domain, that is, cationic amino acids of AMPs, binds electrostatically with anionic bacterial membrane and its hydrophobic part helps in penetration in to the lipid part of the bacterial membrane [10]. The interaction of the AMPs with the bacterial membrane happens in the following four steps as described by Shai Matsuzaki and Huang (SMH) model [36–38]. In the first step, the peptides acquire the secondary structure if not preadopted upon binding with membrane in an amphiphilic manner where positive and hydrophobic amino acids are separated in distinct domains and interact with negative heads of the lipids on bacterial membrane [39]. Secondly, these lipids are removed from membrane and thickness of membrane is thus reduced, allowing aggregation of more AMPs at the lipid displaced site of membrane leading to high peptide/lipid ratio. Thirdly, AMPs insert perpendicularly into the membrane through channel or pore formation. In the fourth and last step they perform final bacterial killing either by loss of membrane potential and ionic loss followed by cell death (e.g., magainin and alamethicin) [36] or by travelling across the membrane and targeting other intracellular pathways (e.g., buforin II and mersacidin) of bacterial machinery [40]. In some cases both processes occur simultaneously for the same kind of peptide (e.g., nisin) [41]. To describe peptide insertion and pore formation, three models have been proposed: (1) carpet model, (2) barrel-stave model, and (3) toroidal-pore model [36–38].

In the higher organisms, however, AMPs not only participate in the immediate innate immunity to infections (direct killing), they also establish a connection between innate and adaptive immunity and function as crucial signaling mediators in host immunity and inflammation [20, 42]. On exposure to pathogens, the innate immunity gets triggered through receptor activation. For example, toll-like receptors (TLRs), IL-1R and G-protein coupled receptors (GPCRs), and nod-like receptors (NLRs) are collectively known as pathogen-recognition receptors (PRRs) [21]. They recognize and bind to pathogenic antigens like LPS and endotoxins [23]. The activation of these receptors eventually leads to the stimulation of several genes of immune cells and skin cells (encoded for AMPs). These cells release AMPs to directly encounter the microbes and clear the infection [24].

When the AMPs alone are not sufficient to clear all the microbes, they execute immune modulation through following effector mechanisms, (i) the downregulation of proinflammatory cytokines, (ii) immune cell differentiation and polarization, and (iii) chemoattraction of the immune cells [20, 21, 28, 42, 43]. All these effector mechanisms are interdependent. For instance, defensins stimulate the production of macrophage proinflammatory cytokine to stimulate the migration of antigen-presenting cells (APCs) to the site of infection [35]. It has also been observed that AMPs perform their antimicrobial role by interacting with TLRs in drosophila and its homologues in humans [44]. Moreover, studies have shown that AMPs (i.e., LL-37 and HBD2) have

an anti-inflammatory role in combating various inflammatory diseases such as psoriasis, Crohn's disease, and morbus Kostmann disease [25, 42].

4. Expanding the Repertoire of Human AMPs

Besides defensins and cathelicidins, several other peptides of humans are recognized as important antimicrobial agents. For example, histatins in saliva, thrombin-induced platelet microbicidal protein (tPMP-1) in blood, dermcidin in sweat, hepcidin in liver, lactoferrin in milk, and lipocalin in urinary tract [10]. Moreover, many immunomodulatory peptides, including chemokines, have recently been identified exhibiting direct antimicrobial properties. Such antimicrobial chemokines are termed as kinocidins [20, 21]. Several neuropeptides, such as neuropeptide Y (NPY), and proenkephalin A (PEA), are reported to have antimicrobial properties [45, 46]. In addition, the anti-inflammatory and antipyretic properties of melanocortin peptides and their widespread presence are indicators of their involvement in host defense [47, 48]. A peptide called α -MSH from melanocortin family has been reported for its broad-spectrum antimicrobial effects [14, 49, 50].

The above families of peptides have tremendous antimicrobial potential; however, they are not yet widely recognized as members of host defense peptides [35]. Cathepsin G, human neutrophil elastase, and azurocidin stored in azurophils, collectively called serprocidins, have also been found killing bacteria directly [28]. Table 1 shows the sequence of all major host defense peptides from humans.

5. Melanocortin Peptides and Their Biochemistry

Melanocortin (MC) peptides are derived from proopiomelanocortin (POMC) and include adrenocorticotrophic hormone (ACTH); β -lipoprotein hormone (β -LPH); α -, β -, and γ -MSH; and corticotrophin-like intermediate peptide (CLIP) [3, 4]. In higher vertebrates, the POMC gene is expressed predominantly in the pituitary (intermediate lobe) gland, but its expression has been detected widely in peripheral tissues as well. Melanocortin is an ancient peptide family that has existed since the Paleozoic Era, and the expression of POMC has been recognized in lamprey, an earliest vertebrate [29].

All melanocortins work through interaction with melanocortin receptors (MCRs); thus MC peptides have in common a four amino acid long core sequence—His-Phe-Arg-Trp (HFRW)—which works as both the message sequence and the address sequence and activates the MCRs [30, 31]. MCRs belong to the rhodopsin family of G protein coupled receptors (GPCRs) [32]. There are five MCRs: MC1R, MC2R, MC3R, MC4R, and MC5R. Except MC2R, the other four are present in the brain and can be activated by MSHs or ACTH. MC2R, on the other hand, expresses in adrenal cells, activated only by ACTH and implicated in corticotropin activities [30, 32]. The distribution of MCRs and their agonists, functions, and the signaling system have been illustrated in Table 2. MC1R, MC3R, MC4R, and MC5R take part in

anti-inflammation, thus protecting the brain and peripheral tissues from acute inflammatory injuries [4, 11].

6. Alpha-Melanocyte Stimulating Hormone (α -MSH)

α -MSH is generated as a result of post-translational processing of POMC by serine proteinases called prohormone convertases 1 and 2 (PC1 and PC2). PC1 cleaves POMC at arginine or lysine residue and produces ACTH, γ -MSH, and β -LPH [3]. Thereafter, a second proteinase PC2 cleaves ACTH to give CLIP and α -MSH. β -MSH is produced from the cleavage of β -LPH [4]. α -MSH is a 13 amino acid (AC-S¹Y²S³M⁴E⁵H⁶F⁷R⁸W⁹G¹⁰K¹¹P¹²V¹³-COOH) long, cationic peptide [31]. The N-terminal of α -MSH is acetylated and C-terminal is amidated after post-translational modification. α -MSH is widely secreted from pituitary gland of central nervous system (CNS) to several peripheral cells [4, 30].

Thus, α -MSH performs two types of biological functions based on its (i) hormonal effects such as melanin synthesis, secretion of sebum, regulation of temperature, control of pain, and regulation of behavior involving sex, feeding, and learning and (ii) immunomodulatory effects such as the cure of several inflammatory conditions of brain (meningitis, vasculitis, etc.) and peripheral organs (arthritis, colitis, etc.) and immunosuppression [2, 6, 7, 51]. In addition to this, various studies have supported the protective influence of α -MSH in mouse models of neurodegenerative diseases such as Alzheimer's by improving the memory [47, 52–54].

Until two decades ago this neuropeptide was primarily known as melanogenic hormone; however, later its immunomodulatory role was discovered and its anti-inflammatory effects were implicated in the cure of many inflammatory conditions [55]. The following section describes the in-depth understanding of anti-inflammatory mechanisms of α -MSH.

7. Modulation of Immune Response by α -MSH to Cure Inflammatory Conditions

In mammals, as described above, besides its hormonal effects, α -MSH is also recognized for regulating the level of effector molecules implicated in inflammatory conditions [31, 55, 56]. α -MSH performs anti-inflammatory action by binding to different MCRs, such as MC1R, MCR3, and MCR5 [32]. However, it has specific affinity for MC1R, which primarily signals through the cyclic AMP-protein kinase A (cAMP-PKA) pathway to exert immunomodulatory actions [29, 51]. Inflammation is characterized by increased level of various effector molecules including proinflammatory cytokines (IL-6, IL-1, TNF- α , and IFN- γ), chemokines, and reactive oxygen and nitrogen species [57]. The expression level of α -MSH increases in response to the above effector molecules and thus it modulates immune reactions to reinstate immune homeostasis in both brain and peripheral organs and protect them from inflammatory damages [58–60].

The universal underlying mechanism behind the immunomodulation of α -MSH is the cAMP-PKA signaling

TABLE 1: Major host defense peptides from humans and their sequences.

AMPs/structure	Site of expression	Sequence	References
α -Defensin, HNP1/ β -sheet (3 s-s bonds)	Leukocytes, neutrophils	ACYCRIPACIAGERR YGTCIYQGRLWAFCC C	[21, 22]
α -Defensin, HNP2/ β -sheet (3 s-s bonds)	Leukocytes Neutrophils	CYCRIPACIAGERRY GTCIYQGRLWAFCC	[22, 27]
Human α -defensin, HNP3/ β -sheet (3 s-s bonds)	Leukocytes Neutrophils	DCYCRIPACIAGERR YGTCIYQGRLWAFCC C	[8, 22, 27]
Human α -defensin HNP4/ β -sheet (3 s-s bonds)	Leukocytes Neutrophils	VCSCLVFCRRTELR VGNCLIGGVSFYCC TRV	[22, 28]
Human α -defensin, HD5/ β -sheet (3 s-s bonds)	Intestinal tract, vaginal tract (paneth cells)	ATCYCRHGRCATRE SLSGVCEISGRLYRL CCR	[22, 24]
Human α -defensin, HD6/ β -sheet (3 s-s bonds)	Intestinal tract (paneth cells)	AFTCHCRRSCYSTEY SYGTCTVMGINHRCF CL	[22, 24]
Human β -defensin, HBD1/ β -sheet (3 s-s bonds)	Keratinocytes epithelium and mucous lining, salivary and mammary gland	DHYNCVSSGGQCLY SACPIFTKIQGTCYRG KACCK	[24, 28]
Human β -defensin, HBD2/ β -sheet (3 s-s bonds)	Epithelium, GIT, keratinocytes	TCLKSGAICHCPVFCP RRYKQIGTCGLPGTK CCKKP	[27, 28]
Human β -defensin HBD3/ β -sheet (3 s-s bonds)	GIT, keratinocytes respiratory tract	GIINTLQKYYCRVRG GRCAVLSCPKEEQI GKCSTRGRKCCRRK K	[27, 28]
Human β -defensin HBD4/ β -sheet (3 s-s bonds)	Epithelium, GIT, keratinocytes	MQRLVLLLAWSLLL YQDLPVRSEFELDRI CGYGTARCRKKCRS QEYRIGRCPTNYACC LRKWDESLLNRTKP	[27, 28]
Cathelicidin LL-37/hCAP-18 Linear, α -helical	Epithelium, neutrophils, keratinocytes, monocytes	LLGDFFRKSKKEKIGK EFKRIVQRKDFLRLNL VPRTES	[8, 21, 25]
Histatin-5/histidine rich linear, non- α -helical	Salivary gland	DSHAKRHGYKRKF HEKHHSHRGY	[8]
Lactoferrin linear	Lactoferrin protein	GRRRRSVQWCAVSQ PEATKCFWQWRNMR RVRGPPVSCIKRDSP QCIIQA	[28]
Hepcidin B-sheet, 4 s-s bonds	Liver	DTHFPICIFCCGCCCHR SKCGMCCKT	[8]

pathway, followed by the inhibition of nuclear factor $\kappa\beta$ (NF- $\kappa\beta$) activation, subsequently shutting down all the downstream effector proinflammatory mechanisms [61]. Stimulation of MCIR by α -MSH is induced in the presence of inflammatory signals, that is, proinflammatory cytokines or antigens. Activated MCIR elevates the cAMP level, which causes activation of protein kinase A (PKA) and inhibits $\text{I}\kappa\beta$ kinase (IKK), thus stabilizing the $\text{I}\kappa\beta$ and preventing the translocation of nuclear factor- $\kappa\beta$ (NF- $\kappa\beta$) from cytosol to nucleus [11, 62]. This leads to the following downstream effector mechanisms: (i) downregulation of proinflammatory cytokines and iNOs; (ii) upregulation

of anti-inflammatory/immunosuppressive cytokine (IL-10); and (iii) suppression of chemotaxis. All these reactions eventually result in the inhibition of inflammatory symptoms [63]. However, evidence has shown α -MSH-mediated inhibition of inflammation by involving additional signaling pathways, namely, calcium signaling pathway from MCIR and MC3R and Jak/STAT activation from MC5R [33, 56].

The inflammatory diseases cured by α -MSH can be classified into two categories. The first one comprises inflammatory disorders of the brain including traumatic head injury, cerebral vasospasm like subarachnoid hemorrhage (SAH), multiple sclerosis, meningitis, and brain reperfusion injury

TABLE 2: Melanocortin receptors (MCRs), their agonists, function, signaling system, and site of expression.

MCRs type	Distribution	Function	Agonists	Signaling system	References
MC1R		Pigmentation, regulation of skin physiology, anti-inflammation, pain	α -MSH, β -MSH, γ -MSH and its analogues (MT I, MT II)	cAMP-PKA pathway, Ca++ signaling	[1, 3, 4, 29–32]
MC2R	Adrenal cortex, murine adipocytes, skin, melanoma cells	Adrenal steroids secretion	ACTH	cAMP-PKA pathway	[1, 3, 4]
MC3R	CNS, stomach, kidneys, heart, gut, thymus, placenta	Feeding, energy homeostasis and anti-inflammation	ACTH and γ -MSH	cAMP-PKA, MAPK and IP/Ca++ signaling	[1, 3, 29, 33]
MC4R	CNS	Anti-inflammatory, control of feeding and sexual behaviors, energy homeostasis	α -MSH, ACTH	cAMP-PKA and MAPK	[1, 3, 31, 33]
MC5R	CNS and many peripheral tissues, exocrine glands, spleen, skin, lung, sexual organs, adipose tissues.	Exocrine secretion, lipolysis, regulation of body temperature	ACTH(1–24), α -MSH, MT I & MT II	cAMP-PKA and Jak/STAT phosphorylation pathway	[1, 3, 29, 33]

Mitogen activated protein kinase: (MAPK), Protein kinase A: (PKA), ionositol phosphate: (IP), and cyclic AMP: (cAMP).

[29, 63]. The second category comprises inflammatory disorders of the peripheral organs, including the inflammatory bowel disease (Crohn's disease and ulcerative colitis), arthritis (rheumatoid and gout), systemic inflammation (septic shock syndrome), allergic inflammation of skin, eyes, and lungs, and reperfusion injuries of the gut and heart [29, 64]. Besides, inflammation is also one of the pathologies of several neurodegenerative diseases such as Alzheimer's and others [63, 65]. The pathogenesis of Alzheimer's disease (AD) involves upregulation of several inflammatory cytokines like TNF- α and loss of cholinergic neurons [49, 63]. As mentioned earlier, the production of these cytokines is under the control of NF- $\kappa\beta$ activation, and being a potent inhibitor of NF- $\kappa\beta$ activation, α -MSH limits the progression of AD. Furthermore, very recently, it has been reported that α -MSH administration in mouse models of Alzheimer's disease prevents the GABAergic neuronal loss and thus ameliorates the cognition [47, 52–54].

Besides anti-inflammation, α -MSH/MC1R signaling plays a significant role in immunosuppression in case of allergic reactions [4]. Recently, a study uncovered the mechanism of immune-suppressive behavior of α -MSH in the case of skin inflammatory diseases. It discovered that α -MSH inhibits inflammation and suppresses the immune system in mouse model of psoriasis-like skin inflammation by suppressing the activation and proliferation of the effector T cells through MC1R signaling [60].

Reports have confirmed that the minimum sequence required for anti-inflammatory activity of α -MSH is the C-terminal tripeptide, Lys-Pro-Val (KPV) [62]. Furthermore, a dimer of C-terminal tripeptide of α -MSH (Cys-Lys-Pro-Val)₂—that is, (CKPV)₂—has been found to be a better anti-inflammatory agent than the full-length α -MSH and KPV for

both *in vitro* and *in vivo* models of inflammation [66]. A study conducted by Capsoni et al. [7] has revealed that α -MSH full-length peptide and its C-terminal synthetic derivative (CKPV)₂ can reverse the inflammatory effect of urate crystal formation in gout (a type of arthritis). Moreover, both α -MSH and (CKPV)₂ possess the capacity to prevent crystal-induced chemoattraction of neutrophils [7, 66, 67].

8. Resemblance of α -MSH to AMPs

Similar to AMPs, the anti-inflammatory activity and occurrence of α -MSH and its receptors in defense cells such as keratinocytes, lymphocytes, and phagocytes point towards its involvement in the clearance of infection [4]. Besides, several features of α -MSH overlap with AMPs [12]. Some of these are as follows. (i) α -MSH is a linear short peptide containing thirteen amino acids. (ii) It is positively charged and adopts the alpha helical secondary structure in bioactive form. (iii) It is a very ancient peptide that has existed since the Paleozoic Era when innate immunity was the only defense available to organisms [29]. (iv) It has a conserved nature from lower to higher vertebrates. (v) It expresses in defense organs such as skin. (vi) It is an endogenous peptide, which mitigates inflammation by immunomodulation [19, 33, 55]. (vii) The current evidences suggest that, like AMPs, α -MSH also exerts antimicrobial activity through membrane permeabilization [16]. (viii) It shows broad-spectrum antimicrobial activity against several *Candida* species and different bacterial genera including both Gram-positive and Gram-negative groups [14–16]. (ix) Like other AMPs, it works in synergy with the host immune system in order to fight against microbes as suggested by experiments that α -MSH enhances the killing

activity of neutrophils [14]. (x) It acts synergistically with other conventional antibiotics and enhances their antimicrobial efficacy (this characteristic of α -MSH could be very useful against antibiotic resistant bacteria). (xi) Unlike defensins, which are a major class of human AMPs, α -MSH is salt tolerant and its antibacterial activity is intact even in the presence of physiological concentration of NaCl, MgCl₂, and CaCl₂ [18]. (xii) Most importantly, this neuropeptide has not shown any cytotoxicity or hemolytic activity in *in vitro* assay (the cytotoxicity has been a big obstacle in the way of clinical development of many AMPs) [68]. All the above characteristics of α -MSH approve its strong antimicrobial nature and it would not be inappropriate to include it in the category of AMPs of humans.

9. Direct Antimicrobial Activity of α -MSH

α -MSH is a natural guard against prolonged acute-phase reactions such as inflammation and could be a unique, potential antimicrobial agent [7, 12]. Considering the increasing tolerability of bacterial and fungal pathogens towards the existing antimicrobials and the paucity of new tools to fight against them, researchers are now exploring the antimicrobial role of various immunomodulatory peptides (also known as host defense peptides (HDPs)) [14, 31]. It is well evident from previous research that α -MSH has the potential to cure various inflammatory diseases and neurodegenerative disorders [54, 56, 60]. Moreover, its role in tuning the host immune reactions has extensively been explored in various ailments including infections [1, 64].

A decade ago, Cutuli and group reported the antimicrobial activity of α -MSH against *C. albicans*, *S. aureus*, and *E. coli*. They found that the peptide inhibited the formation of the germ tube and increased the level of cAMP in *C. albicans*, similar to its role in vertebrates. This group also reported that its carboxy-terminal tripeptide (KPV) and 6–13-amino-acid region (HFRWGKPV) had antimicrobial potential [14]. This shows that the essential anti-inflammatory sequence (KPV) is also essential for its direct antimicrobial efficacy. Later in 2003, Grieco et al. reported that its synthetic analogue NDP- α -MSH(6–13) had better candidacidal effect than native α -MSH [50]. Masman et al. [69] showed that the core message sequence (HFRW) of α -MSH possesses strong antifungal activities against *Cryptococcus neoformans*, and the antifungal activity appears to be closely related to the full-length peptide [69]. Similarly, the anticandidal activity of (CKPV)₂ has been demonstrated in *Candida vaginitis*, both *in vitro* and *in vivo* [17, 19]. Its role in the cure of HIV patients has also been reported [4]. Further, Charnley et al. in 2008 confirmed similar antibacterial activity of C-terminal tripeptide of α -MSH (i.e., KPV and KP-D-V) against *E. coli* [15]. Recent studies have highlighted the antimicrobial activity of α -MSH against both planktonic and biofilm phenotype of *S. aureus* strains irrespective of their susceptibility to methicillin [16, 70].

Subsequently, the report emerged claiming that the C-terminal peptide region (KPV) of α -MSH is requisite for its antistaphylococcal effect, and the fragments of α -MSH

devoid of KPV could not show any substantial antimicrobial activity [18]. It is interesting to note that the antimicrobial activity of α -MSH and its C-terminal fragments— α -MSH(6–13) (HFRWGKPV) and α -MSH(11–13) (KPV)—remains intact even in the presence of salts at their physiological concentrations, namely, NaCl (150 mM), CaCl₂ (2 mM), and MgCl₂ (1 mM) [18]. More recently, it has been suggested that α -MSH acts synergistically with antibiotics belonging to different classes. The *in vitro* pairing of α -MSH with gentamicin (GM), ciprofloxacin (CF), and tetracycline (TC) in a study killed the MRSA clinical strain, which was found to be strongly resistant to these antibiotics [68].

Although the antimicrobial activity of α -MSH is intact in the presence of blood biometrics (plasma, serum, and whole blood) and physiological salts, the peptide is not active in the presence of culture medium that is routinely used in laboratory practices [14]. To solve this obstacle, two α -MSH based antimicrobial analogues were derived by replacing Gly-10 in ([Dnal(2')-7-Phe12]-MSH-(6–13) with unnatural amino acids 2-aminoindane-2-carboxylic acid (Aic) and L-cyclohexylalanine (Cha), respectively. These had substantial killing against Gram-positive and Gram-negative bacteria and the *Candida* species in the presence of culture media [71].

The antibacterial efficacy of α -MSH was evaluated in mice using the intravenous staph infection model and the skin infection model. Interestingly, the *in vivo* results revealed that animals treated with α -MSH showed >3 log reduction in kidney bacterial counts and ≥2 log reduction in the heart, liver, spleen, and lungs. Additionally, rapid healing was observed in wounds of mice infected with *S. aureus* that were treated with α -MSH (M. Singh, PhD thesis, Jawaharlal Nehru University, New Delhi, India, 2013). In this connection, it is very important to mention that α -MSH has negligible *in vitro* hemolytic and cytotoxic effects at concentrations well above the dose required for its antibacterial effect [68].

10. Mechanism of Antimicrobial Activity of α -MSH

As this peptide shares many properties with cationic AMPs, it is more likely to target the bacterial membrane through electrostatic interaction, leading to membrane damage and, eventually, cell death [8, 12, 27, 72]. However, the biological functions of α -MSH such as melanogenesis and immunomodulation in host cells are executed through the MC1R-cAMP signaling pathway [30]. Therefore, the involvement of MC1R in the antimicrobial mechanism might be another possibility. The first report by Cutuli et al. [14] clearly demonstrated that the candidacidal effect of α -MSH was mediated through the induction of cyclic adenosine monophosphate (cAMP), as they could not observe any linear relation between the timing of *Candida* killing and membrane leakage. They showed that a two-hour treatment of *C. albicans* with α -MSH greatly reduced the colony forming unit (CFU) but not the propidium iodide incorporation in the cells. However, they did observe the leakage at later time points. Cutuli et al. also observed that α -MSH increased the production of cAMP in *C. albicans* while the adenylyl cyclase inhibitor

ddAdo partly reversed the candidacidal effect of α -MSH [14]. Their study also showed that the cAMP inducer, forskolin, alone caused the reduction of *C. albicans* CFU which was similar to that exerted by α -MSH. Therefore, they explained that the *Candida* cell death due to α -MSH exposure was caused by cAMP induction, a receptor mediated mechanism, and it appeared that membrane disruption was perhaps the consequence rather than the cause of *C. albicans* death. This MCIR-cAMP signaling-mediated α -MSH killing of *C. albicans* was further supported by another study where an analogue of α -MSH, [D-Nal-7,Phe-12]-MSH(6-13), exhibited an enormous increase in antimicrobial potency against *C. albicans*.

As DNal-7 is known to increase the affinity of α -MSH for MCIR, increased activity of melanocortins may be linked to the receptor-mediated mechanism [50]. Further, similar to the parent peptide, the dimer of C-terminal tripeptide of α -MSH (CKPV)₂ also killed *C. albicans* in a rat *Candida vaginitis* model by activating the MCIR, subsequent induction of cAMP, and M2 polarization of macrophages (anti-inflammatory action) [19]. However, other studies using flow cytometry and spectrofluorimetry demonstrated that α -MSH and its C-terminal tripeptide containing fragments and α -MSH(6-13) and α -MSH(11-13) exert their staphylocidal activity through bacterial membrane depolarization followed by membrane permeabilization [18]. Electron microscopic images of *S. aureus* exposed with α -MSH and its C-terminal peptide demonstrated remarkable morphological changes including altered cell surface, incipient hole marks, rupture lines, and leakage of cell materials [18].

It appears from all the above evidence that the bacterial membrane is the major target for staphylococcal activity of α -MSH and related peptides. However, other targets could not be ruled out either, particularly due to the fact that the killing by α -MSH based peptides was very rapid, while substantial membrane disruption happened at later time points [18]. These observations suggest that either (i) the membrane damage is a secondary effect of α -MSH based peptide exposure and they target bacterial components other than the membranes, such as nucleic acids; or (ii) it is an important enzymatic process that leads to cell death and eventually causes membrane damage, or the occurrence of all these processes simultaneously [18]. It has been reported that a given host-defense antimicrobial peptide may use more than one mechanism for its microbicidal activity [21]. Subsequent work by Singh et al. demonstrated that α -MSH induced the inhibition of DNA replication and protein synthesis of *S. aureus* directly or indirectly with little effect on RNA synthesis [68].

As already described, the pronounced synergistic relation of α -MSH in the case of CF, GM, and TC may be due to a mechanistic analogy between these antibiotics and α -MSH. (CF inhibits DNA replication and GM and TC primarily decline protein synthesis.) The common killing mechanisms—either inhibition of protein synthesis or inhibition of DNA synthesis—along with other known or unknown mechanisms, such as the membrane-damaging ability of the peptide, make the combination of GM or TC or CF with α -MSH synergistic [68]. A scant research is done exploring

the relation between cationicity of α -MSH and its antimicrobial efficacy. In this connection, Grieco et al. [50] have reported that the replacement of positively charged amino acid Lys at position 11 of α -MSH with neutral Ala diminished the anticandidal activity of α -MSH(6-13) by 50% [50]. However, Charnley et al. [15] showed that the cationic charge on the lysine residue is not required for the bactericidal activity of α -MSH(11-13). The effect of whole α -MSH cationicity on its antimicrobial efficacy is yet to be understood clearly.

11. Conclusions

In this postantibiotic era, AMPs have already created huge hopes for their host defense mechanism and several of these peptides find ways into medical practice although the numbers are less than expected. The development of AMPs as therapeutic alternatives to combat the resistant pathogenic microbes is facing problems, majorly due to the enhanced inflammatory reactions associated with them and their potential for toxicity. α -MSH overcomes both of these issues being anti-inflammatory and nontoxic. This endogenous neuropeptide was initially characterized as a pigment producing peptide and later received serious attention due to its potent protective and anti-inflammatory activity. α -MSH performs these actions by binding to centrally expressed melanocortin receptors, which subsequently coordinate numerous anti-inflammatory pathways leading to shutting down all the downstream effector proinflammatory mechanisms. Besides anti-inflammation, it also exhibited immunosuppression in case of skin-inflammatory diseases like psoriasis. The striking resemblance of this anti-inflammatory neuropeptide with cationic AMPs compelled the scientists to further explore its antimicrobial efficacy. Indeed, α -MSH appeared to possess potent antimicrobial activity against pathogens from different classes like *C. albicans*, *S. aureus*, *E. coli*, and more. It also adopts variable approaches to kill different microbes. For instance, it kills fungal cells through the induction of cAMP and bacterial cells by damaging the membrane [14, 18]. The C-terminal region (KPV) of α -MSH demands special attention for several reasons. It exhibits *in vitro* and *in vivo* anti-inflammatory activity similar to that of parent peptide without melanotropic effect. This observation removes the main obstacle in developing α -MSH based peptides as therapeutics, which is nothing, but its pigmentary effects and KPV are devoid of that. Moreover, this essential anti-inflammatory sequence, that is, C-terminal tripeptide (KPV) of α -MSH, is also essential for its direct antimicrobial efficacy. Therefore, this short molecule KPV appears to have tremendous potential to be developed as therapeutic agent as it is more suitable for clinical use and demands further research. The dimer of this short peptide, that is, (CKPV)₂ being both anti-inflammatory and antimicrobial, is already in clinical trial and will definitely make its way to enter into medical practice in near future.

The pleiotropic effects of α -MSH and its C-terminal peptides, including their anti-inflammatory, immunosuppressive, antipyretic, and antimicrobial activities, are unique. These endogenous properties make them the most promising antimicrobial host defense peptides. More work is however

needed to bring these peptides from the lab to clinic. First, a deeper corelation is required to be established between its anti-inflammatory and anti-infective reactions through the *in vivo* models of infections. Second, further biophysical studies are required to design a potent α -MSH based AMP with enhanced killing and immunomodulatory activities. Third, the possibility of resistance developing against this peptide needs to be ruled out. Fourth, overall safety profile of these peptides particularly with α -MSH fragments requires to be vigorously examined.

In conclusion, α -MSH, its analogues, and related C-terminal tripeptide with broad-spectrum antimicrobial activity combined with immunomodulating effects and no cytotoxicity could emerge as excellent therapeutic agents against resistant pathogens.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Recombinant Lysostaphin Protects Mice from Methicillin-Resistant *Staphylococcus aureus* Pneumonia

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The advent of methicillin-resistant *Staphylococcus aureus* (MRSA) and the frequent and excessive abuse of ventilators have made MRSA pneumonia an inordinate threat to human health. Appropriate antibacterial therapies are crucial, including the use of lysostaphin as an alternative to antibiotics. To explore the potential use of lysostaphin as a therapeutic agent for MRSA pneumonia, mice were intranasally infected with MRSA and then treated with recombinant lysostaphin (rLys; 45 mg/kg in the high-dose group and 1 mg/kg in the low-dose group) (0.33 mg/mL, 15 mg/mL), vancomycin (120 mg/kg) (40 mg/mL), or phosphate-buffered saline (PBS, negative control) 4 h after infection. Therapeutic efficacy was assessed by mouse survival, lung histopathology, bacterial density in the lungs, bodyweight, lung weight, temperature, white blood cells counts, lymphocytes counts, granulocytes counts, and monocytes counts. The mice treated with rLys showed lower mortality, less lung parenchymal damage, and lower bacterial density at metastatic tissue sites than mice treated with PBS or vancomycin. The overall mortality was 100%, 60%, 40%, and 60% for the control, vancomycin, high-dose rLys, and low-dose rLys groups, respectively. These findings indicate that, as a therapeutic agent for MRSA pneumonia, lysostaphin exerts profound protective effects in mice against the morbidity and mortality associated with *S. aureus* pneumonia.

1. Introduction

Staphylococcus aureus is one of the most common human pathogens. Up to 20–30% of carriers are persistently and asymptotically colonized and 50–60% are intermittently colonized [1]. *Staphylococcus aureus* causes many skin and soft-tissue infections and invasive diseases such as sepsis, endocarditis, pneumonia, and osteomyelitis [2]. These infections are complex to treat because this bacterial species can become resistant to antibiotics. At present, methicillin-resistant *S. aureus* (MRSA) is one of the most commonly identified antibiotic-resistant pathogens in many parts of

the world. Moreover, MRSA infection rates have increased exponentially worldwide over the past few decades. Most of these infections, including sepsis and pneumonia, are often characterized by fulminant onset, rapid progression, and in a subset of patients, a fatal outcome [3]. Among these invasive infections, necrotizing *S. aureus* pneumonia has emerged as one of the most lethal [4, 5]. The reduced efficacy of vancomycin and linezolid against MRSA has increased the threat of incurable staphylococcal infections [6].

The proportion of MRSA exceeds 10% in the 24 participant countries within the European Antimicrobial Resistance Surveillance System (EARSS) [7]. Moreover, accumulating

TABLE 1: Bacterial antibiotic susceptibility testing of MRSA strain MRSA-117.

Antibiotic	MIC ($\mu\text{g/mL}$)	Sensitivity
Amoxicillin/CA		R
Gentamicin	$>=16$	R
Imipenem		R
Oxacillin		R
G Penicillin-G	$>=0.5$	R
Rifampin	$>=32$	R
Tetracycline	$>=16$	R
SMZCO	40	S*
Vancomycin	$<=0.5$	S*
Levofloxacin	$>=8$	R
Azithromycin		R
Ampicillin/sulbactam		R
Clarithromycin		R
Quinupristin/dalfopristin	0.5	S*
Nitrofurantoin	32	S*
Linezolid	2	S*
Moxifloxacin	$>=8$	R
Tigecycline	$<=0.12$	S*

S: sensitive; R: resistant; * very sensitive.

data indicate that MRSA infections are associated with a worse prognosis than methicillin-susceptible *S. aureus* infections [8–11]. Severe healthcare-associated MRSA infections, including bacteremia, hospital-acquired pneumonia, and ventilator-associated pneumonia, are associated with a particularly high risk of mortality and complications. The optimal therapy for these infections remains a therapeutic challenge.

Lysostaphin is a 27 kDa peptidase produced by *Staphylococcus simulans*, which was isolated in 1964 by Schindler and Schuhardt [12–14]. Lysostaphin specifically cleaves the pentaglycine cross-links unique to the cell wall of *Staphylococci* and lyses cells in all metabolic states (growing, resting). Because *Staphylococci* are highly resistant to lysis with standard agents, such as lysozyme or detergents, lysostaphin has been widely used in research laboratories as a staphylocolytic agent. Here, we assessed the therapeutic efficacy of lysostaphin against infection with a clinical MRSA isolate in an animal model and compared its antibacterial efficacy with that of vancomycin.

2. Materials and Methods

2.1. *Staphylococcus aureus* Isolate. The MRSA isolate strain MRSA-117 used in this study was isolated from the Affiliated Hospital, Academy of Military Medical Sciences (China). The isolate selected was recovered from the sputum of a 72-year-old male patient with pneumonia. MRSA-117 was shown to be resistant to several antibiotics (Table 1).

2.2. Production and Purification of Lysostaphin. pQE30-lysostaphin was constructed by subcloning a gene encoding

lysostaphin into the pQE30 vector (Invitrogen, China). The protein-coding sequence of lysostaphin was obtained from the National Center for Biotechnology Information (GenBank accession: YP_003505772). The modified lysostaphin gene only contains the functional genes but not signal genes (see Table 2 about the modified lysostaphin gene sequence), and the modified lysostaphin gene was inserted into the pQE30 vector using conventional cloning techniques, with restriction enzymes *Bam*HI and *Hind*III. The resulting lysostaphin-expressing plasmid was designated pQE30-lysostaphin. *Escherichia coli* M15 cells transformed with pQE30-lysostaphin were used as the production host for lysostaphin. Actually, the active lysostaphin protein could be expressed by only part of the whole lysostaphin gene, which was synthesized by Invitrogen, and the sequence information was in the Table 2 (5'-*Bam*HI, 3'-*Hind*III). The expression of lysostaphin was induced with 1 mmol/L Isopropyl β -D-1-Thiogalactopyranoside (IPTG) at an optical density at 600 nm (OD₆₀₀) of 0.6 and the induced bacterial cells were then incubated for an additional 8 h at 37°C with shaking at 200 rpm. The bacterial cells were recovered by centrifugation (6000 × g for 20 min) and the resulting cell pellet was resuspended in lysis buffer (50 mmol/L NaH₂PO₄ containing 300 mmol/L NaCl, pH 8.0) and disrupted with a conventional ultrasonic treatment for 30 min (2 s pulses with 2 s rest intervals between pulses). Following centrifugation (5000 × g for 30 min), the supernatant was recovered and subjected to two-step chromatography that included ion-exchange chromatography (SP Sepharose Fast Flow column; GE Healthcare, Sweden) and hydrophobic-interaction chromatography (Toyopearl PPG-600M column; Tosoh Bioscience, Japan).

The endotoxin unit of recombinant lysostaphin (rLys) was determined to be less than 1.0 international endotoxin units (EU)/mL by the clinical laboratory of the Affiliated Hospital, Academy of Military Medical Sciences (Table 3) using both micro-Kjeldahl method and Micro-Ultraviolet Spectrophotometer. And the purity of the rLys was shown to be 90%.

2.3. In Vitro Antibacterial Activity Test. The *in vitro* antibacterial activity of the rLys against MRSA was investigated with the double AGAR plate method. Various concentrations of lysostaphin were dropped onto the culture of double AGAR plate. The bacteria were allowed to grow for 8 h after treatment with lysostaphin, and the plates were then examined to determine whether the bacterial growth was inhibited by lysostaphin.

2.4. MRSA Infection and Lysostaphin Treatment in a Mouse Model. To prepare an animal inoculum, a frozen stock of MRSA-117 was subcultured on trypticase soy agar and cultured overnight at 37°C. Trypticase soy broth (TSB; 5 mL) was inoculated with a single colony and was cultured overnight at 37°C with shaking at 200 rpm. After 100-fold dilution, the overnight culture was grown in fresh TSB and incubated for about 3 h 37°C at 200 rpm (OD₆₀₀ = 1.0). The bacteria were centrifuged at 10 000 × g for 10 min, washed, and resuspended in sterile phosphate-buffered saline (PBS). This process was

TABLE 2: The modified lysostaphin gene sequence.

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1 ggatccgctg caacacatga acattcagca caatgggtga ataattacaa aaaaggatat
61 ggttacggtc ttatccatt aggtataat ggccgtatgc actacggagt tgatttttt
121 atgaatattg gaacaccatg aaaagctt tcaagcgaa aaatagttga agctgggtgg
181 agtaattacg gaggaggtaa tcaaataagg ctattgaaa atgatggagt gcatagacaa
241 tggtatatgc atctaagtaa atataatgtt aaagttaggg attatgtcaa agctggtcaa
301 ataatcggtt ggtctggaaag cactggttat tctacagcac cacattaca cttccaaaga
361 atggtaatt catttcaaa ttcaactgcc caagatccaa tgccttctt aaagagccaa
421 ggatggaa aagcaggatgg tacagtaact ccaacgccc atacagggtt gaaaacaac
481 aataatggca cactatataa atcagatgtca gctagcttca cacctaatac agatataata
541 acaagaacga ctggccatt tagaagcatg ccgcgtcgag ggtcttaaa agcaggctaa
601 acaatttattt atgatgaatg gatgaaaca gacggcatg ttgggtttagg ttatacaggt
661 aacagtggcc aacgtatttta ctgcctgtt agaacatgga ataatctac taatactta
721 ggttcttt gggactaaatgaaag ctt

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The full lysostaphin gene sequence is 1359 bp, and the modified lysostaphin gene sequence in this work is 753 bp.

TABLE 3: Endotoxin detection in the samples to be used for treatment.

Samples	Result (EU/mL)
O	0.68
H	0.76
L	0.72

O: PBS used for the negative control group; H: rLys (45 mg/kg) used for the high-dose group; L: rLys (1 mg/kg) used for the low-dose group. Negative results: <1 EU/mL; suspicious results: 1-2 EU/mL; positive results: ≥2 EU/mL.

repeated twice, and the bacterial suspension was adjusted to a final density of 1×10^{10} colony-forming units (CFU)/mL (6×10^8 CFU per 60 μ L).

Six-week-old specific-pathogen-free (SPF) female BALB/c mice were obtained from the Experimental Animal Center of the Academy of Military Medical Sciences and maintained in a biosafety level 2 facility. The animal experiments were conducted in accordance with the regulations for laboratory animals of the Ministry of Science and Technology. The mice were immunosuppressed with 200 mg/kg cyclophosphamide (CTX, Baxter Oncology GmbH, Germany) injected intraperitoneally for two consecutive days before infection. The mice were then anesthetized with sodium pentobarbital, hung in an upright position, and inoculated intranasally with 60 μ L of MRSA-117 suspension (6×10^8 CFU). After inoculation, the animals were held upright for 30 s. Four hours after infection with MRSA-117, the mice were anesthetized again with sodium pentobarbital and intranasally administered 60 μ L of rLys (two dosage groups: 45 mg/kg and 1 mg/kg), vancomycin (VAN; 120 mg/kg), and PBS. The intrarectal temperature of the infected mice was monitored with an electronic thermometer (MC-246, Omron, Japan).

To determine the pathological correlations of staphylococcal pneumonia, the weight, illness condition and mortality of the infected mice were recorded daily for 30 days after

infection. And the infected mice were euthanized with cervical dislocation under anesthesia, their lungs were weighed and homogenized to calculate the bacterial burden by the double AGAR plate method.

2.5. Mouse White Blood Cell (WBC) Counts. Peripheral blood samples obtained from the tails of the mice were collected in heparin-coated quantitative blood collection tubes. The samples were analyzed with an M16 Medonic automated cell-counting instrument (Medonic, Sweden). The results are presented as mean values \pm standard deviations.

2.6. Histology. After the mice were killed, their lungs were excised and weighed. They were then washed in PBS and inflated with 4% buffered formalin, sequentially infiltrated with increasing concentrations of ethanol and xylene, and embedded in paraffin. The tissues were then sectioned, stained with hematoxylin and eosin, and visualized with microscopy.

2.7. Statistical Analysis. All data are given as mean values and standard deviations. Survival curves were created using the Kaplan-Meier method and compared with a log-rank (Mantel-Cox) test. Weight loss, temperature loss, and blood cell data were compared with student's *t*-test in the Origin version 8.0 software. A value of $P < 0.05$ was considered to indicate a significant difference.

2.8. Ethical Approval. All animal work was approved by the Animal Ethics Committee of the Beijing Institute of Microbiology and Epidemiology (permit number: SCXK-(JUN) 2007-004).

3. Results

3.1. Production and Purification of Lysostaphin. The lysostaphin gene was inserted into the pQE30 vector using conventional cloning techniques with the restriction enzymes

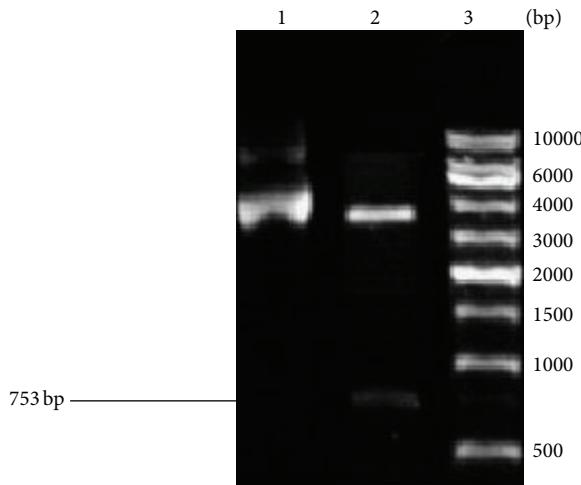


FIGURE 1: Restriction enzyme digestion of the constructed plasmid pQE30-lysostaphin. Line 1: plasmid pQE30 (3461 bp); line 2: plasmid pQE30-lysostaphin digested with *Bam*HI and *Hind*III, producing fragments of 3461 bp (vector plasmid pQE30) and 753 bp (*Lys* gene), respectively; line 3: DNA molecular weight marker.

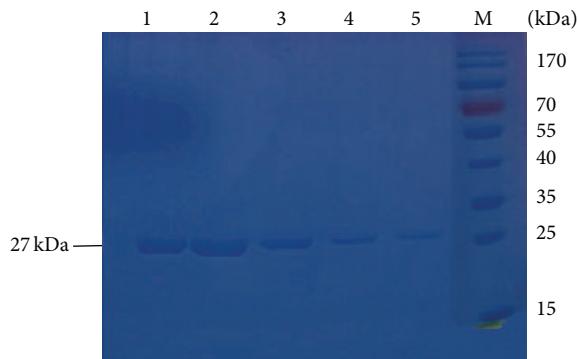


FIGURE 2: SDS-PAGE analysis of the purified recombinant protein. M: protein molecular weight marker; lines 1–5: five different purified His-rLys protein samples.

*Bam*HI and *Hind*III (Figure 1), and the rLys was successfully expressed in a soluble form, as demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Figure 2). rLys was expressed with a yield of 90.7 mg/L of culture.

3.2. In Vitro Antibacterial Properties of rLys. Recombinant lysostaphin was dropped onto a plate inoculated with MRSA-117 when the bacterial lawn formed; a clear circular “halo” (zone of inhibition) appeared around the area upon which the lysostaphin had been dropped, indicating that rLys has an antimicrobial effect against MRSA strain MRSA-117 *in vitro* (Figure 3).

3.3. Therapeutic Effects of Lysostaphin in the Mouse Challenge Model. Four groups of animals were inoculated with MRSA-117 in this study. rLys (high and low doses), VAN, or PBS (the negative control) was administered 4 h after inoculation.

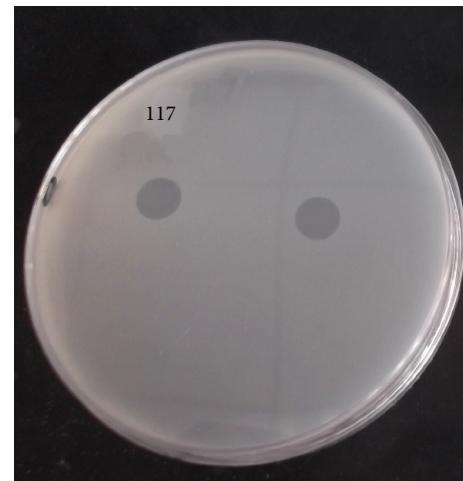


FIGURE 3: *In vitro* activity of rLys. rLys was dropped onto a plate containing MRSA-117, which was then incubated at 37°C for 8 h. The left one is 1 μL 1 mg/kg rLys and the right one is 1 μL 45 mg/kg rLys; both kinds of rLys are active enough to lyse the bacteria *in vitro*.

After their recovery from anesthesia, all the inoculated animals appeared ill within 2 h, with rapid labored breathing. All the mice curled up with one another and were listless, drinking, and eating little.

The control animals treated with PBS all died within 96 h. The animals treated with rLys or VAN lost less weight than the negative control animals. Mice that received 45 mg/kg rLys lost 24% of their original bodyweight on average, whereas those given 120 mg/kg VAN or 1 mg/kg rLys lost 34% and 28% of their original bodyweights, respectively (Figure 4(a)). The mice in the high-rLys group were less hypothermic and recovered more rapidly than the animals in the negative control group and the other two treatment groups ($P < 0.05$; Figure 4(b)). A histochemical analysis showed that only animals in the high-rLys group were significantly protected from pulmonary edema 96 h after infection ($P < 0.05$), and the protective effects on mice treated with 1 mg/kg rLys or VAN were less obvious than the effects on those treated with 45 mg rLys (Figure 4(c)).

The bacterial burden in the lungs was quantified to assess the influence of rLys on MRSA survival within the mouse lungs. The bacteria content in the lungs of mice treated with 45 mg/kg rLys was significantly lower than those in the PBS group at 24, 48, 72, and 96 h after infection ($P < 0.001$; Figure 4(d)).

To determine whether rLys affects the immune cells, we analyzed the WBC in the peripheral blood of mice receiving the high and low doses of rLys separately. WBC counts were made at early time points, and the mean values and standard deviations of each group are presented in Figure 5(a). The results showed a significant reduction in the WBC counts after the injection of CTX, which indicated that the mice were immunosuppressed by CTX. After the treatments, the groups receiving VAN or low-dose rLys showed a persistent marked increase in WBCs, which were almost restored to the normal range on day 5 after bacterial challenge, whereas the WBC

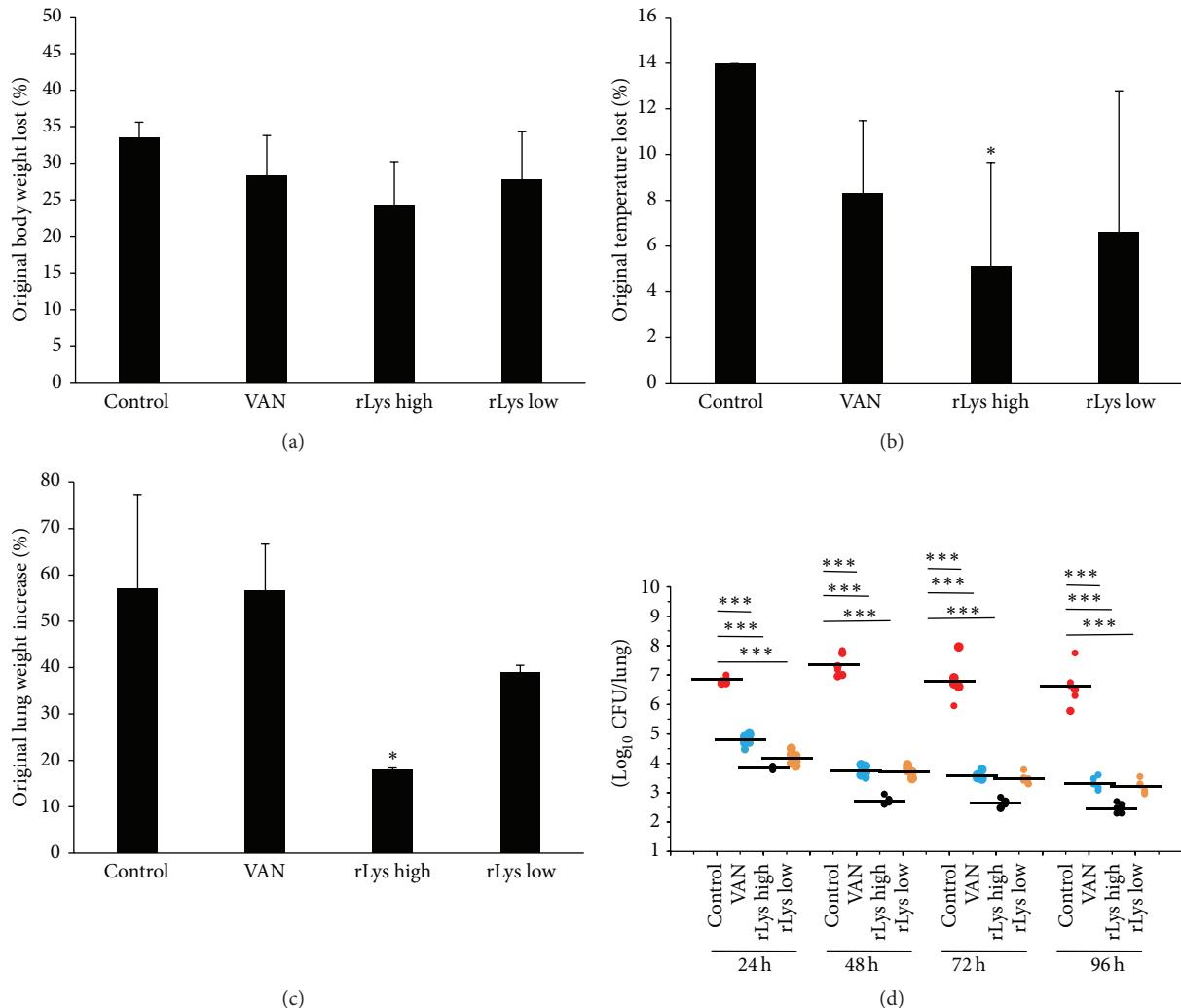


FIGURE 4: MRSA-infected animals treated with PBS, VAN, high-concentration rLys, or low-concentration rLys. (a) Weight loss in MRSA-infected animals. Mice were weighed 96 h after infection. The mouse bodyweight loss is represented as mean (\pm SD) of original bodyweight lost (* $P < 0.05$, Student's *t*-test). (b) Intrarectal temperature was monitored at different time points and the change in temperature between 0 and 96 h (or death) is plotted. (c) Increase in lung weight, represented by the mean (\pm SD) of original lung weight increase (* $P < 0.05$, Student's *t*-test). (d) Viable bacterial counts (\log_{10} CFU/lung) in the lung homogenates of MRSA-infected mice killed at different time intervals after treatment. Values are expressed as means (\pm SD) of the original data, $n = 5$. Statistical significance was determined by one-way analysis of variance with the Bonferroni test (* $P < 0.05$, *** $P < 0.001$).

counts in the mice treated with high-dose rLys were even higher, increasing above the normal range. No differences were observed in the WBC nadirs of the treated mice and the control mice. All the treated groups had recovered normal WBC counts by day 5 after infection (Figure 5(b)). Further analysis of the blood cell counts suggested that the reduced WBC counts in the immunosuppressed animals were mainly attributable to a severe reduction in lymphocytes, from an average of $6.1 \times 10^9/L$ to $4.5 \times 10^8/L$ (Figure 5(c)). However, the increased WBC counts in the VAN- and rLys-treated mice resulted from substantial increases in granulocytes and monocytes (Figures 5(c) and 5(d)).

To evaluate the impact of the rLys treatment on the pathological manifestations of lung injury, we performed a histopathological analysis 96 h after infection of the lungs

from mice treated with VAN, high- and low-dose rLys, or PBS (control). Gross inspection indicated that the lung tissues of the infected mice were crimson and had a tight texture. Following treatment with rLys, the lung tissues of the infected mice were light pink, whereas the lungs of the VAN-treated mice showed multifocal inflammatory cell infiltration (Figures 6(a)–6(d)). There was significant accumulation of inflammatory cells (dark blue or purple) in the alveolar spaces after MRSA infection, as shown in Figures 6(e)–6(h). Treatment with high-dose rLys resulted in a marked alleviation of pulmonary inflammation, as indicated by the lower accumulation of cellular infiltrates in the alveolar spaces.

There was evidence of reduced acute inflammation and injury in the lungs of the MRSA-infected mice treated with rLys, especially in the high-dose rLys group, compared with

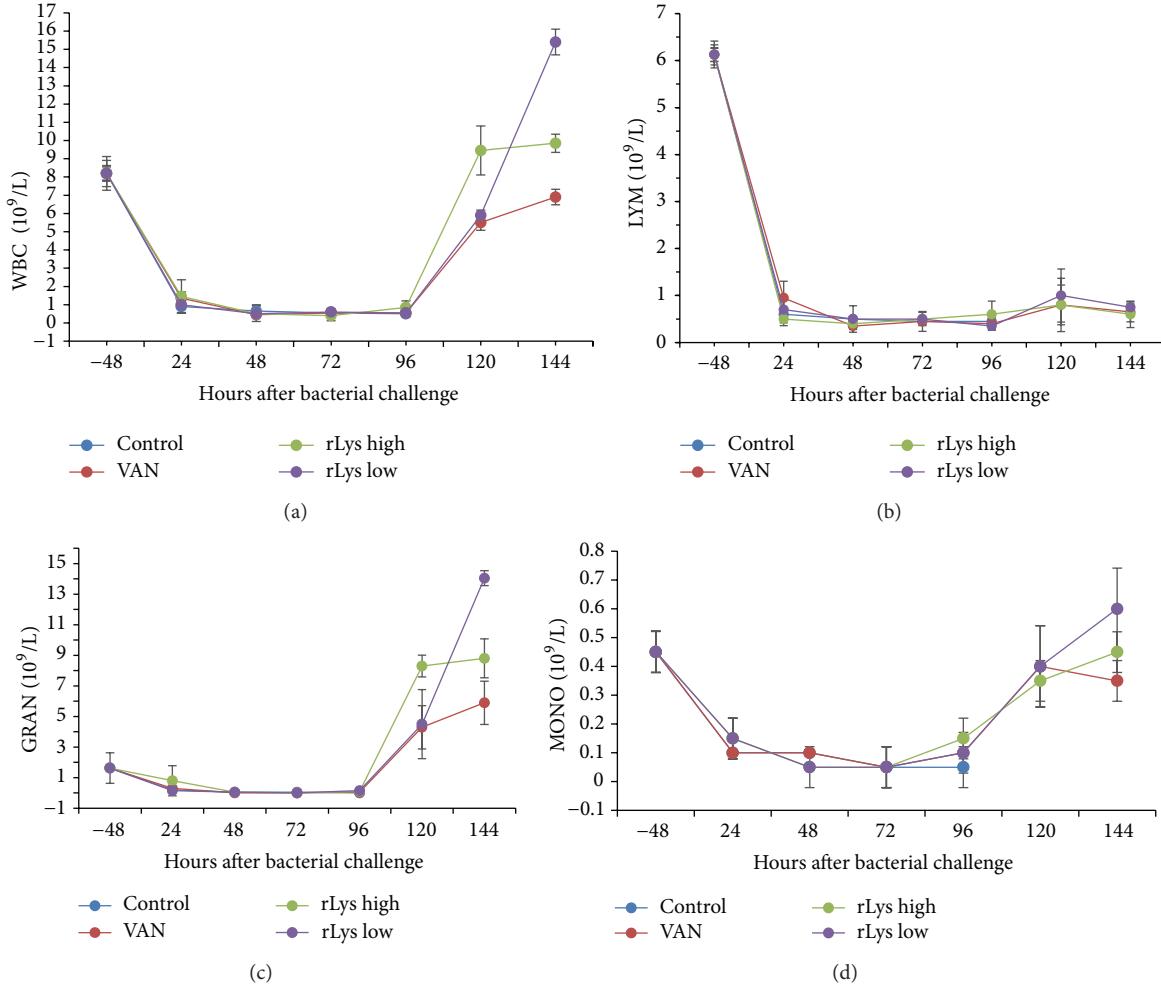


FIGURE 5: Blood cell analysis in mice at different time intervals after treatment ($n = 5$). (a) White blood cell counts; (b) lymphocyte counts; (c) granulocyte counts; (d) monocyte counts.

those treated with either PBS or VAN (Figure 6). Hematoxylin-eosin staining of the tissues seldom revealed bacteria in the VAN- or rLys-treated mice (Figures 6(f)–6(h)).

On day 1 after MRSA infections, the mice in the control group (treated with PBS) began to die, and on day 2, the mice in the low-dose rLys treatment group began to die, whereas mice in the other treatment groups (VAN and high-dose rLys) began to die on day 3. There was no death in the blank control group (uninfected) or in the immunocompetent control group (MRSA infected). At the end of the experiment, all the mice in the PBS-treated group had died. Four, six, and three mice survived in the VAN group, high-dose rLys group, and low-dose rLys group, respectively, and the survival rates in each group were 40%, 60%, and 30%, respectively. The mean survival time of each treatment group differed significantly from that of the PBS control group ($P < 0.01$; Table 4). The mice in the treatment groups had longer median survival times and lower death rates than those in the control group. The survival times of the being infected and eventually dead animals in the lysostaphin treatment group were longer than those in the control group or the VAN treatment group

($P < 0.05$). This indicates that lysostaphin delayed death and even protected the animals from death after infection (Table 4).

The clinical consequences of intranasal PBS, rLys (45 mg/kg), rLys (1 mg/kg), or VAN treatment were studied in the MRSA-infected mice. The overall survival rate in the control group (PBS; 0% survival rate) was lower than that in the animal groups treated with VAN (40% survival rate), high-dose rLys (60% survival rate), or low-dose rLys (40% survival rate), and these differences were statistically significant ($P < 0.01$). The mice in the control group began to die from day 1 after infection. On day 5, mortality was 100%, 40%, 30%, and 20% for the control, VAN, low-dose rLys, and high-dose rLys groups, respectively. Mice treated with high-dose rLys had increased life spans compared with those of the mice in the control, VAN, and low-dose rLys groups. The survival curve for the high-dose rLys group differed significantly from that of the control group ($P < 0.01$). When the dose was reduced to 1 mg/kg, the survival curve was also markedly different from the control ($P < 0.01$). Compared with the mice treated with PBS, the animals treated with

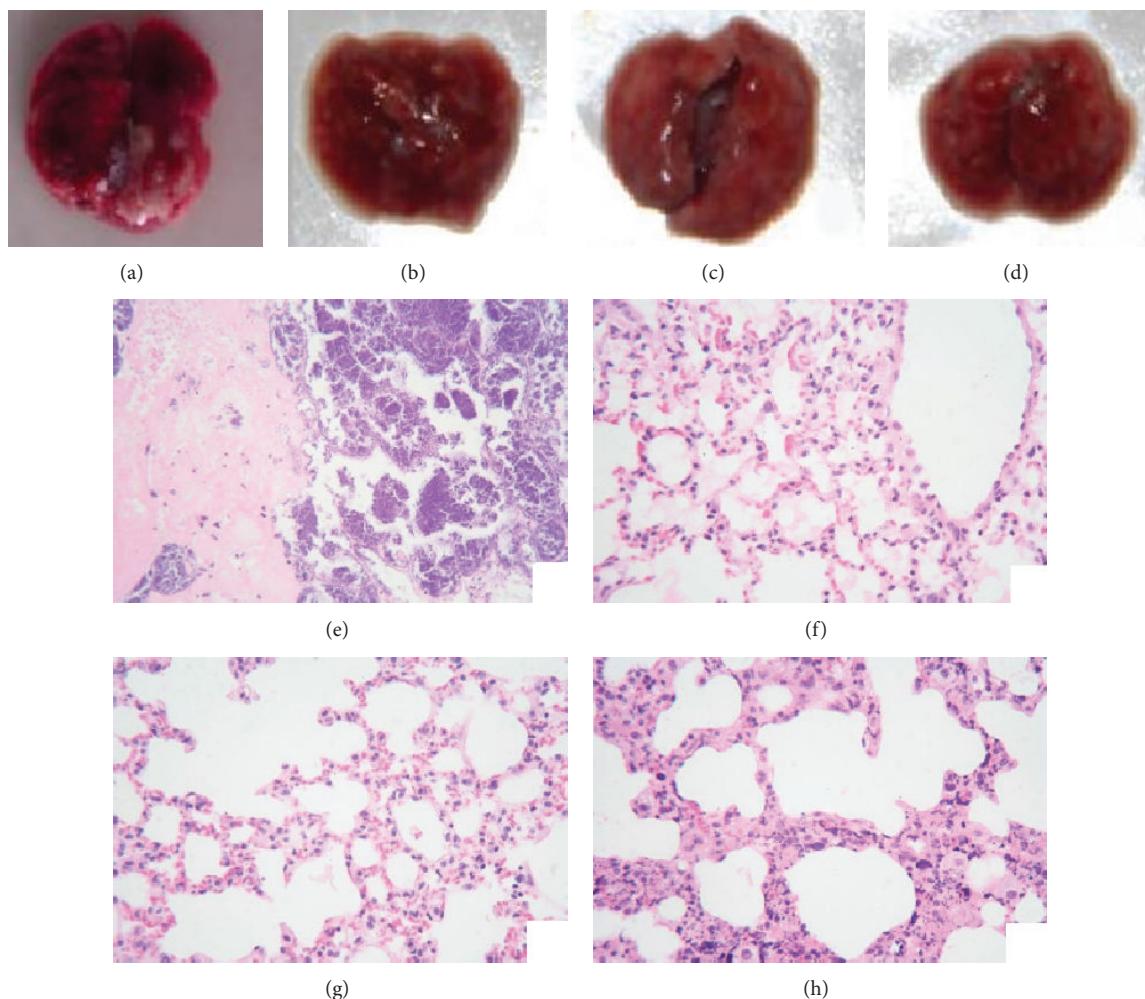


FIGURE 6: Histopathology of the lungs of untreated (PBS) and treated (VAN or rLys) animals ($n = 5$). (a)–(d) Gross histopathology of the lungs. (e)–(h) Histopathology of the lung tissues. (a) and (e) Control mice treated with PBS. Infection with MRSA showing typical necrotizing pneumonia with multifocal bacterial colonies, complete destruction of the alveolar architecture, hemorrhage, and perivascular growth of *S. aureus*. Presence of acute pneumonia with neutrophils in the distal bronchioles and alveolar spaces is also evident. Suppurative inflammation is present in the interstitium and at perivascular locations. (b) and (f) Lungs from mice infected with MRSA after treatment with VAN. This panel demonstrates the reduced acute inflammation, although it is still apparent as neutrophils and neutrophil debris throughout the lung interstitium, with congestion and intra-alveolar fluid. (c) and (g) Lungs from mice treated with 45 mg/kg rLys. This panel shows the reduced acute inflammation; the lung tissue is similar to normal lung tissue. (d) and (h) Lungs from mice treated with 1 mg/kg rLys. This panel shows the reduced acute inflammation, although inflammation is still evident as neutrophils and neutrophil debris. (e)–(h) Tissues were stained with hematoxylin and eosin (original magnification, $\times 400$).

TABLE 4: Survival times of mice.

Group	Time of death (days)		Number of survival animals being infected	Survival (%)	Median survival time of being infected and eventually dead animals (days)	The mean survival time of being infected and eventually dead animals (days)	The mean survival time of being infected and eventually dead animals (days)
	Begin	The final					
Control	1	5	0	0	3	3.2 ± 1.1	3.2 ± 1.1
VAN	3	18	4	40	4	$15.8 \pm 13.0^{**}$	6.3 ± 5.8
rLys (45 mg/kg)	3	25	6	60	11	$23.0 \pm 11.0^{***}$	$12.5 \pm 10.8^*$
rLys (1 mg/kg)	3	25	4	40	7	$18.4 \pm 12.0^{***}$	$10.7 \pm 9.0^*$

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control group.

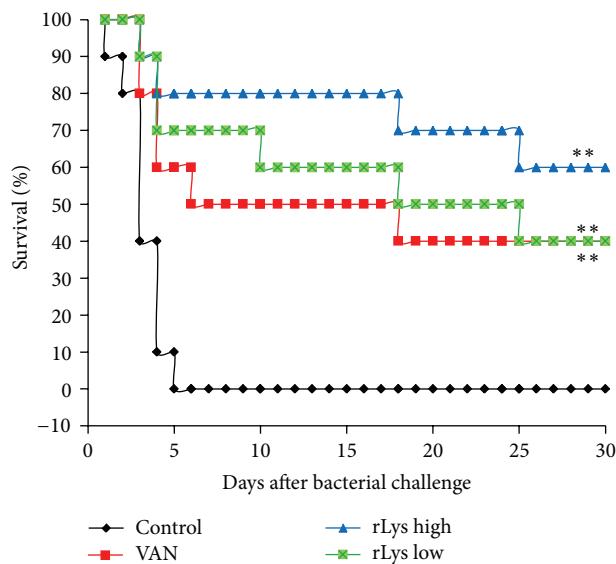


FIGURE 7: Survival of mice treated with intranasal PBS, rLys (45 mg/kg), rLys (1 mg/kg), or VAN. Each group was monitored for 30 days, and the results are shown as a Kaplan-Meier plot, $n = 10$. Statistical significance was determined with one-way analysis of variance and the Bonferroni test ($*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

VAN or rLys exhibited significantly better survival, free from severe pneumonia. The mice in the high-dose rLys group were best protected from death compared with the other groups (Figure 7).

4. Discussion

The frequency of pneumonia caused by hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) is increasing. CA-MRSA pneumonia is associated with an influenza-like illness, often occurs in young healthy individuals, and results in an acute infection with a stormy course, numerous complications, and high mortality rates. HA-MRSA pneumonia is a frequently fatal illness that occurs in older, debilitated patients, especially those receiving ventilator support. Most cases of MRSA pneumonia are caused by HA-MRSA. With the continuing increase in antibiotic resistance and the decline in the discovery of new antibiotics, we are now entering the “postantibiotic era,” with limited treatment options available for many bacterial infections, including MRSA [15].

The intranasal inoculation of mice with MRSA isolates caused illness with reproducible clinical and pathological features. However histopathological analysis of lung tissues revealed necrotizing pneumonia resembling that documented in postmortem tissues from patients [16] and was characterized by the obliteration of the alveolar architecture, the perivasculär accumulation of *S. aureus*, and hemorrhage. The presence of pulmonary hemorrhage is noteworthy because hemorrhage has recently been identified as an important risk factor in predicting mortality in patients with

MRSA necrotizing pneumonia [17]. A high level of suspicion, aggressive diagnostic measures, and the rapid application of an effective therapy are essential if we are to improve the mortality rates for these diseases.

Recent studies have shown that MRSA is resistant to almost all the β -lactam antimicrobial agents (ceftazidime is an exception) in the present market and is also resistant to 80% of common antimicrobial agents such as gentamicin and the macrolides. Therefore, vancomycin and linezolid are currently recommended for the treatment of clinical MRSA pneumonia [18], but these treatments have been disappointing. With the widespread use of vancomycin, the drift in its minimal inhibitory concentration (MIC) for MRSA has been high, and large numbers of MRSA strains that are resistant to vancomycin have appeared [19, 20]. The significant increase in vancomycin MIC values not only prolongs hospitalization times, but also leads to significant increases in mortality. The sensitivity of MRSA to linezolid has declined, and long-term medication with linezolid (>14 days) can cause adverse events, such as thrombocytopenia [21].

Therefore, rLys may be a better option for the treatment of MRSA pneumonia. Our animal experiments show that lysostaphin can reduce the bodyweight loss and decrease in body temperature associated with MRSA pneumonia in the mouse, indicating that lysostaphin promotes the recovery of the animal's bodyweight and body temperature. Lysostaphin also reduced the number of bacteria in the lungs through its direct bactericidal activity, thereby reducing lung inflammation and reducing the weight increase in the infected lung that occurs during inflammation. All these observations were confirmed by hematoxylin-eosin staining of the mouse lung tissues.

Mouse blood leukocyte counts reflect one aspect of their immune function. The numbers of mouse leukocytes (WBC, lymphocytes, granulocytes, and monocytes) decreased significantly after the injection of the immunosuppressant CTX and continued to decline until death if not treated appropriately. However, these leukocyte numbers began to increase gradually in the mice treated with VAN or rLys and recovered faster in the lysostaphin-treated mice than in the VAN-treated mice. The survival curves showed that the mice in the control group began to die on day 1 after infection, and most deaths occurred 72–96 h after infection. rLys treatment delayed the death of the MRSA-infected mice and increased the overall survival rate of the infected animals. Accordingly, the median survival time and the average survival time of the animals were also improved by treatment with lysostaphin.

By increasing the body weights and temperatures of the mice, lysostaphin treatment enhanced their metabolism, promoting the formation of immune cells and improving the body's defenses, so that the mice could recover rapidly, with greater alleviation of the MRSA pneumonia symptoms, a lower incidence of death, and longer survival times.

Although a few studies have shown lysostaphin to be an effective agent for the treatment of experimental MRSA keratitis and endophthalmitis [22], there have been concerns regarding enzyme degradation and the immunogenicity of lysostaphin in terms of its safety and efficacy. Lysostaphin treatment for MRSA pneumonia has not been studied until

now. Here, we explored the treatment of MRSA pneumonia with lysostaphin in mice, and our findings may clarify how lysostaphin protects mice against MRSA infection. Further studies of its efficacy in treating both HA-MRSA pneumonia and CA-MRSA pneumonia are essential.

Several studies [23] have demonstrated that lysostaphin is a novel antistaphylococcal agent for the treatment of *S. aureus* infections. However, there are certain limitations to its use. For instance, a mechanism of resistance to lysostaphin was identified that involves mutations affecting *femA*, which encodes the protein responsible for the addition of the second and third glycines to pentaglycine cross-bridges. Mutations affecting *femA* renders this protein nonfunctional, resulting in monoglycine cross-bridges rather than pentaglycine bridges [24–29], and this causes *S. aureus* cells to be either partially or completely resistant to lysostaphin.

Lysostaphin is unique among antistaphylococcal agents in that it kills bacteria, whether active or resting, capsulated or noncapsulated, and it is thus capable of killing a large number of organisms in the genus *Staphylococcus* because of the existence of glycine-glycine bonds [30]. More significantly, because the effectiveness of this enzyme against MRSA has been demonstrated *in vivo*, lysostaphin might prove to be useful in the treatment of MRSA infections alone or in combination with antibiotics. Ultimately, it could have potential use in the treatment and prevention of many resistant staphylococcal infections.

In summary, rLys displayed better antibacterial activity against MRSA than VAN *in vitro* and *in vivo*. Its unique specificity, low toxicity, and increasing stability mean that rLys might be a potential agent for the treatment of various *S. aureus* infections in humans. However, more understanding of the structural and functional properties of lysostaphin is required to standardize drug formulations containing lysostaphin either alone or in combination with other antibiotics for use against MRSA and other antibiotic-resistant *S. aureus*.

Ethical Approval

All animal work was approved by the Animal Ethics Committee of the Beijing Institute of Microbiology and Epidemiology (permit number: SCXK-(JUN) 2007-004).

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Chen Chen and Huahao Fan contributed equally to this work.

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Research Article

The Bacterial Contamination of Allogeneic Bone and Emergence of Multidrug-Resistant Bacteria in Tissue Bank

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Present study was carried out for the microbiological evaluation of allogeneic bone processed from femoral heads. A total 60 bacterial isolates comprising five different species including *Streptococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Bacillus* spp., and *Pseudomonas* spp. were characterized based on their cultural and biochemical characteristics. Average bioburden was ranged from 5.7×10^1 to 3.9×10^4 cfu/gm. The majority (81.7%) of the microbial contaminants were detected as Gram positive with the predominant organism being skin commensal coagulase negative Staphylococci (43.3%). Antimicrobial resistance was evaluated by the activities of 14 broad and narrow spectrum antibiotic discs. Comparing the overall pattern, marked resistance was noted against Penicillin and Amoxicillin 100% (60/60). The most effective single antibiotics were Gentamicin, Tobramycin, and Ofloxacin which were bactericidal against 100% (60/60) isolates. Multidrug resistance (MDR) was confirmed in 70% (42/60) of the samples. Among them, the most prevalent antibiotic types were Penicillin, Amoxicillin, Oxacillin, Polymyxin, and Cefpodoxime (80% of total MDR). The study results revealed higher contamination rate on bone allografts and recommend the implementation of good tissue banking practices during tissue procurement, processing, and storage in order to minimize the chances of contamination.

1. Introduction

Human bone is the second most transplanted tissue after blood which has the unique ability to heal itself perfectly. It is estimated that more than 2.2 million bone grafting procedure annually take place worldwide in order to revise skeletal defects by replacement or augmentation [1]. In addition, bone grafts are also used to repair the defects in bone caused by birth defects, maxillofacial defects, traumatic injury, infections or en bloc resection of malignant tumours and in reinforcement of host bone prior to implantation of prosthesis [2–6].

Disease transmission and bacterial contamination are always a risk in allograft transplantation [7]. Thorough donor screening for the presence of transmissible diseases, bacterial testing, and aseptic processing practices can substantially reduce the risk but do not completely eliminate all the possible microbial contaminants from allograft [8]. So, for

the safety of allogeneic tissue grafts, complete eradication of microorganisms is essential.

The risk of infectious disease transmission emphasizes the need of appropriate sterilization technique in tissue banking practice [9]. But the alteration in the biomechanical properties of particular tissues made it obvious that all forms of sterilization technique are not applicable [10]. Antibiotics have for long time been used to control infectious diseases. Even potentially fatal infections are now curable with the courses of antibiotics. But one of the alarming matters is that despite the development of new antibiotics with novel mechanism of action, it has become difficult to control the local bacterial prevalence and emergence of infectious diseases due to their resistance to the common antibiotics. Bacteria can defend themselves from the action of antibiotics by producing various metabolites which either degrade antibiotics or help bacteria to survive by various mechanisms.

2. Materials and Methods

2.1. Tissue Sample Collection. Tissue samples were collected through the donation of femoral heads removed during hip replacement, hemiarthroplasty, and traumatic limb amputation surgery from eleven hospitals of the Dhaka city including BDM Hospital, Center for Rehabilitation of Paralyzed Hospital, National Institute of Traumatology, Orthopaedic and Rehabilitation Hospital, Bangabandhu Sheikh Mujib Medical University Hospital, Ibn Sina Hospital, Bangladesh Medical College Hospital, Islami Bank Hospital, Central Hospital, Shikdar Medical college Hospital, and Al-Markajul Hospital and Trauma Center.

2.2. Tissue Donor Identification and Screening. All the tissues were collected by the written consent of the donor or next of kin by following “Human Organ/Tissue Donation and Transplantation Act” that has been passed by the National Parliament of the People’s Republic of Bangladesh. The ages of donors were ranged from 40 to 75 years and all the donors were prescreened for the presence of transmissible diseases (e.g., HIV, HBV, and VDRL).

2.3. Initial Laboratory Processing and Bioburden Estimation. Fresh bones were collected under aseptic/sterile condition. During collection each container was labeled with donor ID and hospital registration number and kept at freezer (below -20°C). The plastic container with bone is placed in a cool box and transported immediately to the tissue banking laboratory. In the tissue banking laboratory the bones were preserved in freezer at -40°C . For the isolation, tissue samples were weighed by digital balance and taken into a sterile beaker containing 150 mL sterile normal saline and/or sterile distilled water. After using the orbital shaker the beaker containing the sample was gently shaken. 10 mL of suspension was taken by sterile pipette, which was sterilized by a sterilizer (at 180°C for 1 hour) into a test tube from the beaker. Then the sample was serially diluted up to 10^{-4} . If discrete colonies were not detected in 10^{-4} dilution, further dilutions were prepared and the tests were then repeated. All the plates were incubated at 37°C for 24 hours. The bacterial colonies were counted after 24–72 hours.

2.4. Cultural Characterization and Biochemical Studies of Microbial Contaminants. The bacterial isolates, obtained from the selective and differential media, were characterized on the basis of their morphology (size, shape, and arrangement) by following Gram staining procedure. Cultural characteristics of the bacterial isolates were studied after 24–48 hours of incubation using freshly prepared reagents. According to Bargey’s Manual of Determinative Bacteriology [11], several biochemical tests were performed to identify the biochemical characteristics of the bacterial isolates. The tests were Oxidase test, Catalase test, Indole production test, Methyl Red test, Voges-Proskauer test, Urease test, Citrate utilization test, Triple Sugar Iron test, and Carbohydrate (Lactose, Sucrose, and Dextrose) fermentation tests.

2.5. Antimicrobial Susceptibility Testing. Total 60 bacterial isolates were selected for antibiotic susceptibility test by Kirby-Bauer disc diffusion method described by Bauer et al. [12] using 14 broad and narrow spectrum antibiotic discs. Muller-Hinton agar plates were used to determine the antibiotic susceptibility of the bacterial isolates. A 0.5 McFarland was used as a standard tool to maintain the perfect turbidity. After swabbing with the bacterial suspension, antibiotic disks were placed aseptically over the inoculated media surface and at the same time spatial arrangement was maintained by means of sterile needle within a distance of 5 mm. Then the plates were incubated for 24 hours at 37°C . After the completion of incubation period, the plates were examined and the diameters of the clear zones were measured by a ruler in mm. The zone diameters were translated into susceptible (S), intermediate (I), and resistant (R) categories according to the National Committee for Clinical Laboratory Standards (NCCLS) [13].

3. Results

3.1. Determination of Bioburden in Bone. Microbial evaluation of bone allograft was carried out. A total 60 bacterial isolates obtained from 4 different batches of allograft processing. The bioburden varied from 0.57 to 3.94 Log cfu/gm. Maximum count was recorded for the first batch of processing, ranged from 3.23 to 3.94 Log cfu/gm. The lowest microbial levels from 0.93 to 1.92 Log cfu/gm were observed for the fourth batch. Microbial load of bone allografts from different batches of processing is presented in Figure 1.

3.2. Characterization of Bacterial Isolates. Characterization of the bacterial isolates was performed based on their colony morphology. According to the Gram staining, majority (81.7%) of the microbial contaminants found as Gram positive, in which 67.8% were Gram positive cocci. The second most frequently isolated group was Gram positive bacilli as 13.9%. On the contrary, 18.3% of the microbial contaminants were Gram negative rods. No fungi or yeast were found. Types of microbial contaminants are presented in Figure 2.

3.3. Physiological and Biochemical Studies of the Bacterial Isolates. Several physicochemical tests were performed to identify the selected bacterial isolates up to genus level (Table 1). Based on the physicochemical characteristics, Twenty-one Gram positive cocci (B1, B5, B7, B14, B17, B19, B31, B32, B33, B34, B35, B39, B41, B42, B44, B45, B48, B50, B52, B58, and B59) were identified as *Staphylococcus* spp. and twelve Gram positive cocci (B3, B10, B11, B15, B21, B22, B26, B28, B29, B49, B53, and B55) were identified as *Streptococcus* spp. On the other hand, sixteen isolates of Gram positive rods (B2, B8, B16, B20, B25, B24, B30, B36, B38, B40, B43, B46, B51, B54, B60, and B63) were identified as *Bacillus* spp. Among the eleven Gram negative rods, eight of the bacterial isolates were *Pseudomonas* spp. (B4, B12, B18, B23, B27, B47, B56, and B57) and only three of the isolates were *Klebsiella* spp. (B6, B9, and B13).

TABLE 1: Summary of the biochemical tests of bacterial isolates.

Oxidase test	Motility test	IMViC test				TSI test			Suspected organism
		Indole	MR	VP	Citrate utilization	Slant	Butt	Gas production	
(-)ve	(-)ve	(-)ve	(+)ve	(+)ve	(-)ve	R	Y	(+)ve	<i>Staphylococcus</i>
(-)ve	(-)ve	(-)ve	(+)ve	(-)ve	(-)ve	R	Y	(+)ve	<i>Streptococcus</i>
(-)ve	(+)ve	(-)ve	(-)ve	(+)ve	(-)ve	R	Y	(+)ve	<i>Bacillus</i>
(+)ve	(+)ve	(-)ve	(-)ve	(-)ve	(+)ve	R	R	(-)ve	<i>Pseudomonas</i>
(-)ve	(-)ve	(-)ve	(-)ve	(+)ve	(+)ve	Y	Y	(+)ve	<i>Klebsiella</i>

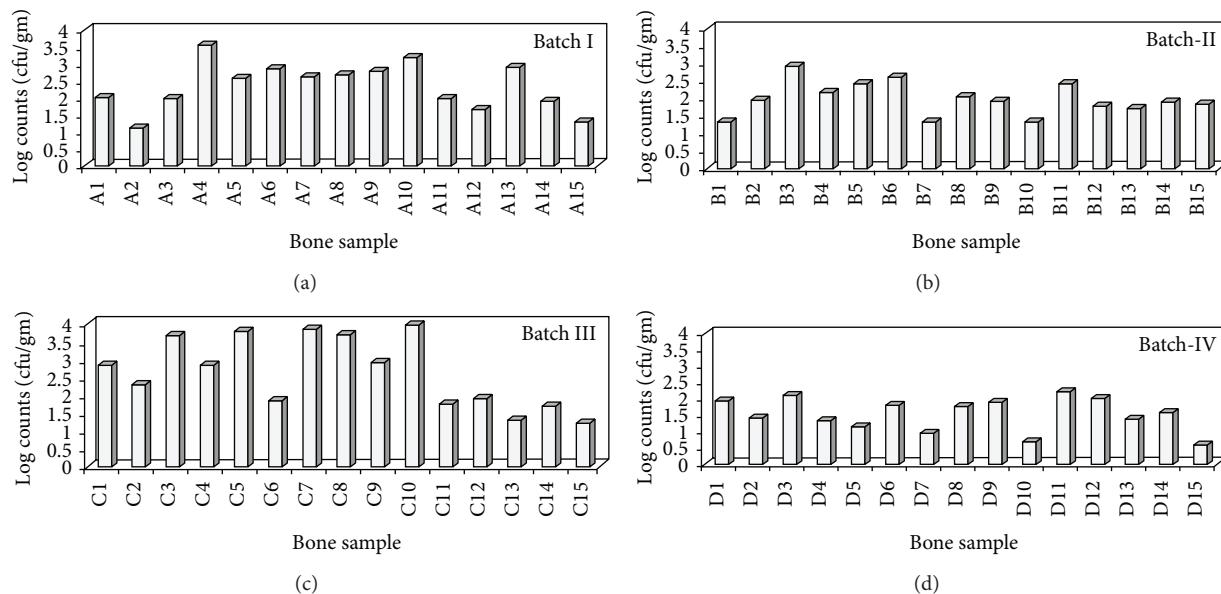


FIGURE 1: Microbial load of bone allografts from different batches of processing.

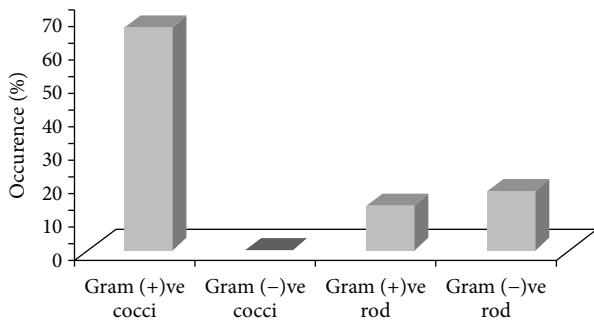


FIGURE 2: Types of microbial contaminants enumerated from bone.

3.4. Antibiogram Profile of the Bacterial Isolates. The bacterial isolates ($n = 60$) were subjected to antibiotic susceptibility test against 14 antibiotics from different groups including, Penicillin (P), Oxacillin (OX), Gentamicin (G), Erythromycin (E), Clindamycin (DA), Tobramycin (TOB), Ofloxacin (OXF), Polymyxin (PB), Azithromycin (AZM), Levofloxacin (LEV), Imipenem (IPM), Cefpodoxime (CPD), Amoxicillin (AML), and Meropenem (MEM) (Table 2). These antibiotics were selected upon the consideration of two facts: which antibiotics are the commonly prescribed by the

physicians and which antibiotics are susceptible against bone contaminants. Disc diffusion method was used to frequently observe the antibiotic effects among the strains.

Among the 14 drugs, Penicillin and Amoxicillin were 100% ($n = 60$) resistant. On the contrary, Gentamicin, Tobramycin, and Ofloxacin were 100% sensitive. Apart from this, other drugs showed different level of resistance such as Oxacillin (80%), Polymyxin (70%), Cefpodoxime (60%), Imipenem (45%), Meropenem (40%), and Erythromycin (30%). Individual resistance and sensitivity pattern of the bacterial isolates is presented below (Figure 3).

Among the 60 bacterial isolates, 70% ($n = 42$) were multidrug resistant (MDR). The highest prevalent antibiotic resistance pattern was P, AML, OX, PB, CPD, IPM, E, MEM, and DA showed by bacterial isolates of batch-I. On the other hand, the lowest prevalent antibiotic resistance pattern was shown by batch-III as P, AML, OX, PB, and IPM (Table 3).

4. Discussion

The primary focus of our study was to determine the bioburden level of allogeneic bone. Study results showed that most of the samples were contaminated with Gram positive cocci specifically coagulase negative *Staphylococci*. Cultures were also positive for *Streptococcus* spp., *Pseudomonas* spp.,

TABLE 2: Antimicrobial susceptibility pattern of the bacterial isolates from bone allograft.

Antibiotics	Total number resistant/total number tested	% Resistance	Antibiotics	Total number resistant/total number tested	% Resistance
Oxacillin	48/60	80	Gentamicin	0/60	0
Imipenem	27/60	45	Polymyxin	42/60	70
Erythromycin	18/60	30	Ofloxacin	0/60	0
Penicillin	60/60	100	Meropenem	24/60	40
Clindamycin	6/60	10	Levofloxacin	6/60	10
Tobramycin	0/60	0	Azithromycin	12/60	20
Amoxicillin	60/60	100	Cefpodoxine	36/60	60

TABLE 3: MDR pattern of different bacterial isolates.

Total number of isolates	Resistance patterns	Multidrug resistance (MDR)
Batch-I (15)	P, AML, OX, PB, CPD, IPM, E, MEM, DA	(+)
Batch-II (15)	P, AML, OX, PB, CPD, E, AZM, LEV	(+)
Batch-III (15)	P, AML, OX, PB, IPM	(+)
Batch-IV (15)	P, AML, OX, PB, MEM, CPD, DA	(+)

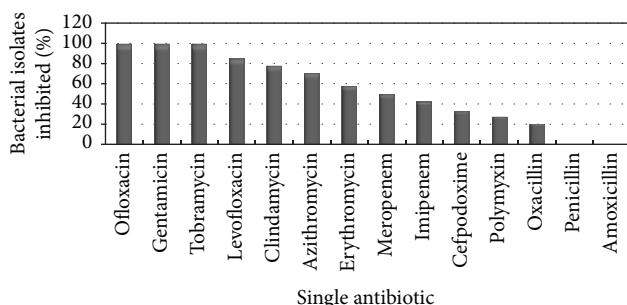


FIGURE 3: Percentages of antimicrobial resistance on bacterial isolates.

Bacillus spp., and *Klebsiella* spp., respectively. The bacterial isolates found in our study are comparable with the previous reported studies. According to Saegeman et al. [14] in 36–38% cases infection of cadaveric bone and soft tissue allograft occurs due to coagulase negative Staphylococci especially that *Staphylococcus epidermidis* is the causative agent of disease. Deijkers et al. [15] analysed the bacterial contamination of bone allograft under aseptic operating condition and divided the organisms into low and high pathogenicity in which they considered organisms of low pathogenicity to be skin commensals and microorganisms of high pathogenicity were thought to be originated from endogenous sources in the donor, which more likely to cause infection in the recipient. Though *Streptococcus* spp. are not usually associated with graft infections, a survey study of tissue bank conducted by Vangsness et al. [16] reported about the invasive bacterial disease in which a 17-year-old male was found to be infected with *Streptococcus pyogenes* after reconstructive knee surgery. Ibrahim et al. [17] also reported that twelve of their bone

allografts were contaminated with streptococci. Emergence of *Bacillus subtilis* and *Micrococcus* spp. was also summarized by many authors [18, 19]. Besides bacterial contaminations, environmental exposure, underlying diseases, and host defense mechanism can also contribute to the graft contamination in ratio between 2 and 5% [20].

We think that disease transmission can occur mainly in two ways: either through an infected donor or during tissue procurement, processing, even at the time of surgery in the operating theatre, as it has already been reported with surgical needles and suckers [21]. Bacterial transmission might be occurring from infected donor to recipient (tuberculosis and syphilis) or through viral transmission from infected donor (HIV and Hepatitis) or through bacterial contamination during procurement, processing, and storage of the bone allograft [22].

In order to avoid infection or diminish its incidences in bone allograft, strategies like careful donor selection, aseptic processing, proper use of disinfectants, and application of sterilization procedure with bacterial cultures need to be taken [23]. Even all the procedures are followed carefully, but what should be done if the culture from an implanted allograft is positive. The perioperative administration of systemic antibiotics is the choice to limit the infection which can occur after graft implant. This method is highly effective against bacteria while the effectiveness is depending on the constituents of antibiotics [24]. One of the feared complications is that, in our study, most of the bacterial isolates enumerated from bone showed multidrug resistance (more than one antibiotic) to the supplied antibiotics, as an explanation of such resistance might be the subsequent external contamination of the allograft. To prevent the endovascular graft infections, antibiotics are recommended to be used in the initial postoperative stage of bacterial seeding [25].

5. Conclusion

Bone allografts were found to be contaminated and about 80% of the contaminants were Gram positive. Study results also revealed the growing antimicrobial resistance of pathogens associated with the bone allografts. To minimize the contamination rate and to reduce the risk of dissemination of antibiotic resistant bacteria through the tissue allografts, it is suggested to use aseptic techniques in all the steps of allograft procurement, processing, and storage.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Reversal of Ampicillin Resistance in MRSA via Inhibition of Penicillin-Binding Protein 2a by *Acalypha wilkesiana*

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The inhibitory activity of a semipure fraction from the plant, *Acalypha wilkesiana* assigned as 9EA-FC-B, alone and in combination with ampicillin, was studied against methicillin-resistant *Staphylococcus aureus* (MRSA). In addition, effects of the combination treatment on PBP2a expression were investigated. Microdilution assay was used to determine the minimal inhibitory concentrations (MIC). Synergistic effects of 9EA-FC-B with ampicillin were determined using the fractional inhibitory concentration (FIC) index and kinetic growth curve assay. Western blot experiments were carried out to study the PBP2a expression in treated MRSA cultures. The results showed a synergistic effect between ampicillin and 9EA-FC-B treatment with the lowest FIC index of 0.19 (synergism ≤ 0.5). The presence of 9EA-FC-B reduced the MIC of ampicillin from 50 to $1.56 \mu\text{g mL}^{-1}$. When ampicillin and 9EA-FC-B were combined at subinhibitory level, the kinetic growth curves were suppressed. The antibacterial effect of 9EA-FC-B and ampicillin was shown to be synergistic. The synergism is due to the ability of 9EA-FC-B to suppress the activity of PBP2a, thus restoring the susceptibility of MRSA to ampicillin. Corilagin was postulated to be the constituent responsible for the synergistic activity showed by 9EA-FC-B.

1. Introduction

Infection caused by methicillin-resistance *Staphylococcus aureus* (MRSA) is a world-wide health problem. These infections are predominantly observed among immunocompromised patients in hospitals. However, in recent years, there have been an increasing number of fatalities, failed treatment, and healthcare costs. Progressive escalation of antibiotic resistance in MRSA has resulted in a limited option for treatment [1]. Thus, there is an immediate need for alternative therapies to control the spread of illness caused by MRSA.

A key strategy in combating a resistant microorganism is to suppress its resistance factor. There are at least two mechanisms that *Staphylococci* can evade beta-lactam toxicity which are by synthesizing the penicillin-binding protein 2a (PBP2a) and β -lactamases [2]. In normal circumstances, *Staphylococcus aureus* strains produce penicillin-binding proteins (PBPs) for synthesis of bacterial cell wall. However, when exposed to lethal concentration of beta-lactams antibiotics, resistant *S. aureus* strains produce PBP2a, which has unusually low

binding affinity to beta-lactams [3]. PBP2a, in turn, replaces the function of normal PBPs (blocked by beta-lactams) in the resistant strains for cell wall biosynthesis [4]. This confers MRSA resistance to the entire beta-lactam family. Hence, suppression of PBP2a production is a promising approach to overcome MRSA's resistance. By doing so, there is a possibility to restore the susceptibility of MRSA to beta-lactam antibiotics [5].

It has been observed that extracts, fractions, or metabolites of plant origin are able to inhibit production of PBP2a when they are used in combination with current available antibiotics. In a review article [6], combinations of drugs such as beta-lactams and beta-lactamase inhibitors of plant origin have been shown to exhibit synergistic activities against antibiotic resistant microorganisms. The article also highlights plants as a source of small molecule antibiotics and synergism observed in natural products with antibiotics against growth of bacteria, fungus, and mycobacteria. Using combination treatment as a strategy to tackle resistant microorganism has also demonstrated several successes at

experimental level. For an example, combination of corilagin from the plant, *Arctostaphylos uva-ursi*, with oxacillin successfully inhibited production of PBP2a in MRSA. The MIC of oxacillin and other tested beta-lactams was reduced between 100-fold and 2000-fold, strongly suggesting that combination treatment can potentially be an alternative method to combat virulence of MRSA [7].

A. wilkesiana is a medicinal plant which has widely been utilized for treating bacterial and fungal infections [8]. In some cases, the plant is also used to treat malaria, gastrointestinal problems [9], and potentially cancers [10, 11]. Previously, we found anti-MRSA and other antibacterial activities in the ethyl acetate and ethanol extracts of *A. wilkesiana* [12]. In continuation to our earlier findings, we have now embarked to further investigate the effects of the semipure extracts of *A. wilkesiana* in the reversal of ampicillin resistance in MRSA.

2. Methods

2.1. Plant Extraction. The plant material was collected from Broga, Selangor, Malaysia (September, 2010). Voucher sample is deposited in the herbarium of Faculty of Science, University of Nottingham Malaysia Campus, and assigned as UNMC 9. The dried plant material (3.6 kg) consisting of the whole plant was subjected to sequential extraction using *n*-hexane, followed by ethyl acetate and finally 95% ethanol [13, 14].

2.2. Isolation of Bioactive Fraction 9EA-FC-B. The ethyl acetate extract of *A. wilkesiana* (9EA) was fractionated by using vacuum liquid chromatography (silica gel). The solvent system used for elution was *n*-hexane (He) with increasing amount of chloroform (CHCl_3) and CHCl_3 with increasing amount of methanol (MeOH) [He/ CHCl_3 (1:1) \rightarrow CHCl_3 \rightarrow $\text{CHCl}_3/\text{MeOH}$ (97:3 v/v) \rightarrow $\text{CHCl}_3/\text{MeOH}$ (95:5 v/v) \rightarrow $\text{CHCl}_3/\text{MeOH}$ (93:7 v/v) \rightarrow $\text{CHCl}_3/\text{MeOH}$ (90:10 v/v) \rightarrow $\text{CHCl}_3/\text{MeOH}$ (85:15 v/v)]. Fractions obtained were further fractionated via preparative centrifugal thin layer chromatography (silica gel) using a similar solvent system. The semipurified fractions were then tested for anti-MRSA activity.

2.3. Microorganism and Growth Conditions. Methicillin sensitive *S. aureus* ATCC 11632 (MSSA) was grown in tryptic soy broth (TSB) (Hi-Media, India) at 37°C for 24 h with a shaking mode of 220 rpm. Aliquot from this suspension was streaked on tryptic soy agar (TSA) (Hi-Media, India) and incubated at 37°C for another 24 h. Two to four single colonies from the TSA plate were inoculated in 10 mL of Muller Hinton broth (MHB) (Hi-Media, India) and allowed to grow at 37°C until they reached exponential stage ($2 \times 10^8 \text{ CFU mL}^{-1}$). The suspension was then used for microbroth dilution assay. MRSA ATCC 43300 was grown similarly except all the media used was supplemented with 2% sodium chloride (NaCl) (Merck, Germany), and incubation temperature was at 35°C. Bacterial stocks were kept at -80°C in TSB added with 10% (v/v) glycerol (Sigma, USA).

2.4. Test Samples. The crude ethyl acetate extract of *A. wilkesiana*, 9EA, and a bioactive fraction derived from it 9EA-FC-B (identified from previous experiments) were dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) at stock concentration of 100 mg mL^{-1} . Further dilution was carried out using media, and the final concentration of DMSO in the media did not exceed 1%. Our earlier study has reported the lack of solvent (DMSO) effect in the test samples [14]. Antibiotics for susceptibility testing were prepared at 10 mg mL^{-1} in sterile distilled water. Tested antibiotics were ampicillin (Amresco, USA), oxacillin (Discovery Fine Chemicals, UK), and methicillin (Sigma, USA).

2.5. Determination of MIC. MICs of antibiotics, crude extract 9EA, and the active fraction 9EA-FC-B against MRSA and MSSA were determined via microdilution assay with a 96-well plate. Test samples were tested in a twofold serial dilution. Antibiotics were tested with concentrations ranging from 0.19 to 100 $\mu\text{g mL}^{-1}$ and plant extract samples from 0.09 to 12 mg mL^{-1} .

MSSA and MRSA broth cultures were grown in MHB and MHB + 2% NaCl, respectively, until an exponential stage ($2 \times 10^8 \text{ CFU mL}^{-1}$) was reached (see Section 2.3). These broth cultures were diluted to correspond to final inoculums of $5 \times 10^5 \text{ CFU mL}^{-1}$ upon inoculation into each well containing twofold serial dilutions of test sample. Media used in the assay were MHB for MSSA and MHB + 2% NaCl for MRSA. In the final incubation step, plates were incubated for 24 h at 37°C for MSSA and at 35°C for MRSA. General guidelines for this experiment were obtained from Clinical and Laboratory Standards Institute 2007 [15] with recommendations adapted from several other studies [16–18].

2.6. Synergistic Studies

2.6.1. Growth Curves Assay. MRSA was grown in a 96-well plate in the presence of following subinhibitory concentrations of 9EA-FC-B; $1/4 \times \text{MIC}$ (0.75 mg mL^{-1}), $1/8 \times \text{MIC}$ (0.38 mg mL^{-1}), and $1/16 \times \text{MIC}$ (0.19 mg mL^{-1}) in combination with subinhibitory concentrations of ampicillin ranging from $1/2 \times \text{MIC}$ to $1/64 \times \text{MIC}$ (25 to 0.78 $\mu\text{g mL}^{-1}$) under aerobic condition. Cell growth was monitored by reading optical density (OD) values at 600 nm at indicated time points for 24 h. Reading was monitored by using Varioskan Flash Multimode Reader (Thermo Scientific, USA).

2.6.2. FIC Index Interpretation. FIC index for the combination treatments in synergy growth curves assay was calculated. The formula used was FIC ampicillin = MIC of ampicillin in combination/MIC ampicillin alone, FIC plant extract = MIC of plant extract in combination/MIC of plant extract alone, and FIC index = FIC ampicillin + FIC plant extract. The combination was defined as synergy if the FIC index was ≤ 0.5 , indifference was defined > 0.5 but ≤ 4.0 , and antagonism was defined as when the FIC index was > 4 [19].

2.7. Protein Extraction. MRSA was grown in MHB + 2% NaCl in the presence of subinhibitory concentrations of 9EA-FC-B until late exponential phase. The bacterial lysates were prepared in an extraction buffer containing Tris and EDTA, and culture supernatants were harvested via centrifugation 4340 g at 4°C for 10 min. The collected pellets were then treated with 150 mg mL⁻¹ lysozyme, DNase, and protein inhibitors cocktail before being subjected to 2 h incubation at 37°C. To enhance cell disruption, 15-minute sonication in ice-bucket was done. Following 15 min of centrifugation at 13 850 g, the pellets were obtained as the insoluble protein extracts that were harvested in elution buffer containing Tris, urea, and sodium dihydrogen phosphate. Protein concentrations were measured using Pierce 660 nm protein assay.

2.8. SDS-PAGE and Western Blot Assay. Extracted protein (3 µg mL⁻¹) was stained with 4X lithium dodecyl sulphate (LDS) sample buffer and subjected to sodium dodecyl sulfate (SDS)—polyacrylamide (12%) gel electrophoresis run at 120 V. Upon completion, the gel was stained in Coomassie Blue staining solution until a clear background was obtained for scanning with GS-800 calibrated densitometer (Bio-Rad, USA). In western blot analyses, electrophoresed gels were transferred to BioTrace NT nitrocellulose transfer membrane (Pall, USA). Membranes were incubated overnight at 4°C in gelatin from cold water fish skin (blocking agent) (Sigma, USA). The production of PBP2a from MRSA was detected by probing the membranes with mouse anti-PBP2a primary antibody (Denka Seiken, Japan) and antglyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Thermo Scientific, USA) with a dilution factor of 1:10000. The same membranes were hybridized with anti-mouse horseradish peroxidase-linked secondary antibody (Abcam, UK) diluted to 1:10000 to facilitate colorimetric detection with 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Nacalai Tesque, Japan). Assay response was recorded using GS-800 calibrated densitometer (Bio-Rad, USA). Densitometric quantification of western blot images was done using Image J 1.38 programme (Windows version of NIH Image). Results were scored in percentage of expression (%) normalized to GAPDH control.

3. Results

3.1. Anti-MRSA Activity of Antibiotics and *A. wilkesiana* Extract. The MIC values of ampicillin, crude extract of *A. wilkesiana* 9EA, and fraction 9EA-FC-B for MRSA ATCC 43300 and MSSA 11632 are presented in Table 1. The MIC value of ampicillin against MRSA notably confirmed the resistance of the studied strain, while MIC value of ampicillin against MSSA revealed the susceptibility of this strain to the antibiotic. Crude extract 9EA demonstrated antibacterial activity against both the resistant and sensitive strains tested with a lower MIC value observed for MSSA. The anti-MRSA activity of fraction 9EA-FC-B was fourfold more effective than crude extract 9EA.

TABLE 1: MIC values of ampicillin, crude extract 9EA, and fraction 9EA-FC-B against MRSA and MSSA.

Strain	Ampicillin (µg mL ⁻¹)	9EA (mg mL ⁻¹)	9EA-FC-B (mg mL ⁻¹)
MRSA	50	12	3
MSSA	6.25	6	3

Values represent triplicates of three independent experiments.

3.2. Synergistic Effects of 9EA-FC-B with Ampicillin on MRSA Growth Curves. The synergistic effects of 9EA-FC-B with ampicillin at subinhibitory concentrations on the growth of MRSA are shown in Figure 1. The growth inhibitory effects shown by both ampicillin and 9EA-FC-B on MRSA are concentration dependent, while the curve for the untreated MRSA culture (control) showed an exponential growth. Suppression of MRSA growth was detected when the MRSA cultures were treated with 9EA-FC-B alone at 1/4 × MIC, 1/8 × MIC and 1/16 × MIC (not shown in graph). The growth curves suggested enhanced growth inhibitory effects when MRSA was treated with ampicillin at subinhibitory concentrations in the presence of 9EA-FC-B also at subinhibitory concentrations. The inhibitory effect was observed at as low as 1/32 × MIC ampicillin in the presence of 1/4 × MIC 9EA-FC-B. Similar inhibitory effect was also achieved in the presence of 1/8 × MIC of 9EA-FC-B in combination with 1/16 × MIC ampicillin.

3.3. Synergistic Effects of 9EA-FC-B with Ampicillin Based on FIC Index. The FIC indices for the tested combinations are presented in Table 2. Synergistic effects were observed when 9EA-FC-B was introduced in the treatment at 1/4 × MIC, 1/8 × MIC, and 1/16 × MIC with ampicillin at subinhibitory concentrations (1/4 to 1/32 × MIC). In the presence of 1/4 × MIC 9EA-FC-B, synergistic effects were observed for the widest range of subinhibitory concentrations of ampicillin, and the range gradually decreased as the subinhibitory concentration of 9EA-FC-B was lowered.

Analysis of the FIC indices revealed the new MIC values of ampicillin in the presence of 9EA-FC-B at subinhibitory level (Table 3). 9EA-FC-B at subinhibitory concentrations has enhanced the activity of ampicillin by up to 32-fold against MRSA; for example, MIC of ampicillin alone is 50 µg mL⁻¹, while the MIC of ampicillin in the presence of 9EA-FC-B at 1/4 × MIC is 1.56 µg mL⁻¹. It is notable that the MIC values of ampicillin (for MRSA) in the presence of 1/4 × MIC 9EA-FC-B and 1/8 × MIC 9EA-FC-B are lower than the MIC value of ampicillin for MSSA, that is, 6.25 µg mL⁻¹.

3.4. Expression of PBP2a. Expression of PBP2a was detected at 76 kDa. The percentage of PBP2a expression in tested treatments is summarized in Figure 2. GAPDH which served as an internal control was detected in all treatments (results not shown). The presence of PBP2a band was detected for MRSA cultures that were grown at subinhibitory concentrations of ampicillin (1/16 × MIC and 1/32 × MIC) in western blot

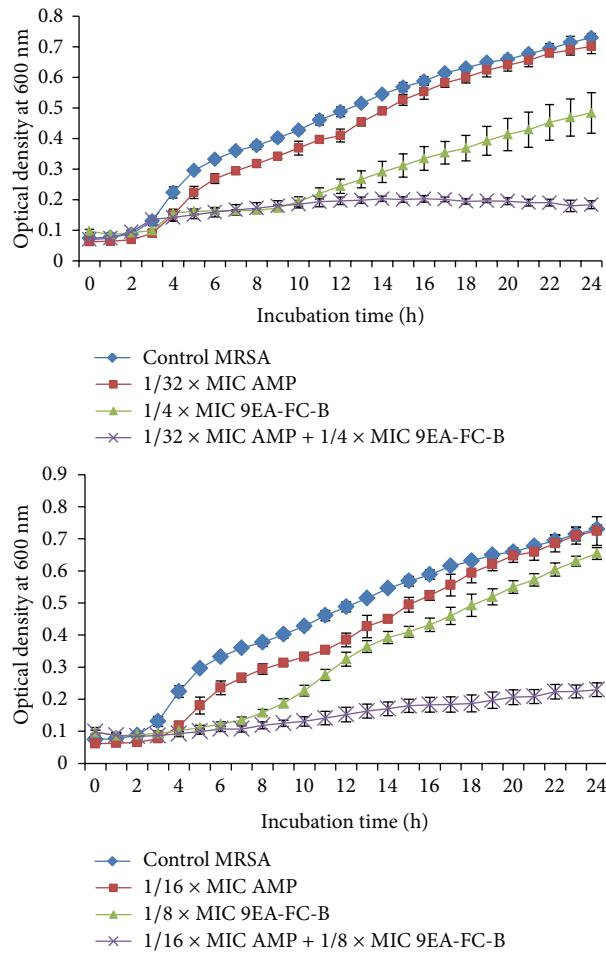


FIGURE 1: Effects of subinhibitory concentration of ampicillin alone, 9EA-FC-B alone and combination treatment on the growth of MRSA. Cell growth was measured by using OD at 600 nm at indicated time points. The curves represent triplicates of three independent experiments. Error bars show the standard deviation (AMP = ampicillin, MIC = minimum inhibitory concentrations).

TABLE 2: FIC indices of some combinations of ampicillin and 9EA-FC-B for MRSA.

Ampicillin ($\mu\text{g mL}^{-1}$)	9EA-FC-B (mg mL^{-1})		
	1/4 × MIC (0.75)	1/8 × MIC (0.38)	1/16 × MIC (0.19)
1/2 × MIC (25)	0.75	0.65	0.56
1/4 × MIC (12.5)	0.43	0.36	0.31
1/8 × MIC (6.25)	0.38	0.25	—
1/16 × MIC (3.125)	0.31	0.19	—
1/32 × MIC (1.563)	0.28	—	—
1/64 × MIC (0.781)	—	—	—

Values represent triplicates of three independent experiments. Index interpretation: ≤ 0.5 = synergy, > 0.5 but ≤ 4.0 = indifference, and > 4 = antagonism. (MIC = minimum inhibitory concentrations; — = no activity.)

experiment. Both western blot and quantitative densitometric analysis showed that these cultures have higher expression of PBP2a compared to the untreated MRSA culture (control). Inhibition of PBP2a expression was observed when MRSA cultures were exposed to subinhibitory concentrations of 9EA-FC-B, where no PBP2a band was seen at $1/4 \times$ MIC 9EA-FC-B and only a very low expression (3.9%) was detected at $1/8 \times$ MIC 9EA-FC-B. Likewise, the MRSA culture did not

show the presence of PBP2a when grown in the presence of $1/32 \times$ MIC ampicillin + $1/4 \times$ MIC 9EA-FC-B.

4. Discussion

The scope of the study is to explore the potential of using active plant extracts or fractions to combat resistance in

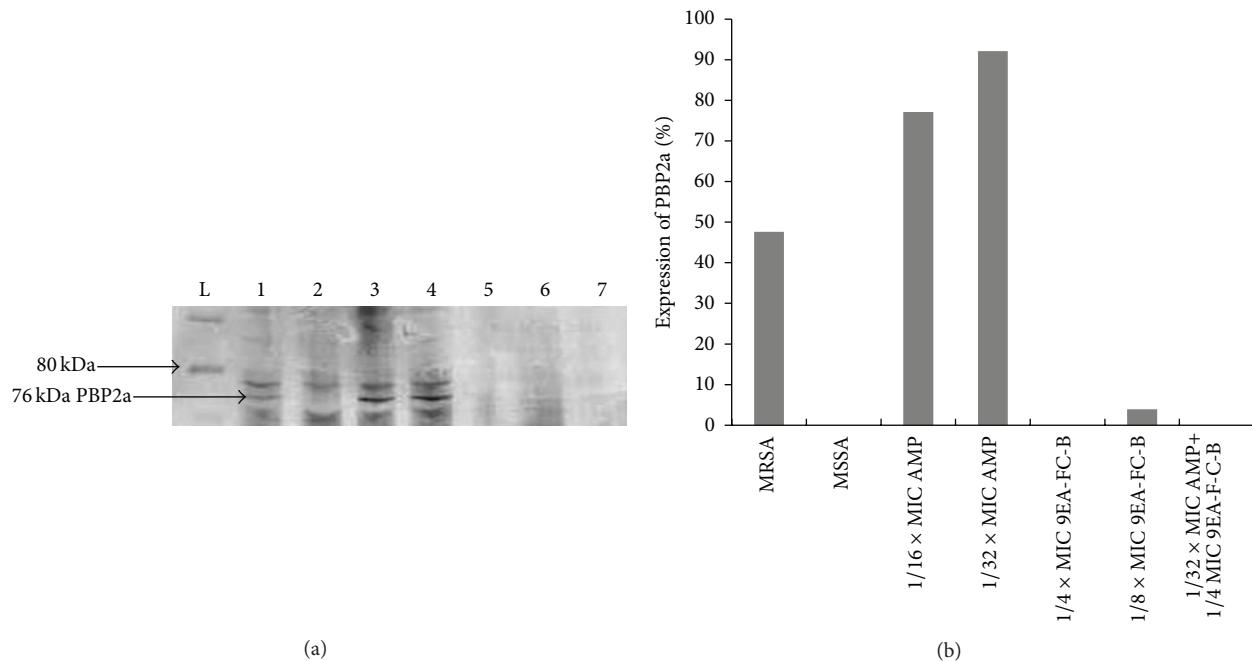


FIGURE 2: Expression of penicillin-binding protein 2a (PBP2a) of MRSA cultures grown in the presence of subinhibitory concentrations of 9EA-FC-B and ampicillin. (a) Western blot image, lane L; molecular mass markers, 1: control MRSA, 2: control MSSA, 3: 1/16 × MIC AMP, 4: 1/32 × MIC AMP, 5: 1/4 × MIC 9EA-FC-B, 6: 1/8 × MIC 9EA-FC-B, 7: 1/32 × MIC AMP + 1/4 × MIC 9EA-FC-B. (b) Quantitative densitometric analysis of PBP2a expression of MRSA cultures grown in the presence of ampicillin alone, 9EA-FC-B alone, and in combinations, normalized to GAPDH loading control. (AMP = ampicillin; MIC = minimum inhibitory concentrations).

TABLE 3: MIC values of ampicillin in combination with subinhibitory concentrations of 9EA-FC-B for MRSA.

Treatment	MIC ($\mu\text{g mL}^{-1}$) of ampicillin
Ampicillin alone	50
With 9EA-FC-B at 1/4 × MIC	1.56
With 9EA-FC-B at 1/8 × MIC	3.13
With 9EA-FC-B at 1/16 × MIC	12.5

Values represent triplicates of three independent experiments (MIC = minimum inhibitory concentrations).

MRSA. For the first time, in 2006, U.S. Food and Drug Administration (FDA 2006) has approved a special green tea extract containing a proprietary mixture of phytochemicals (the active ingredient listed as Polyphenon E) [20], as a prescription drug for the topical (external) treatment of genital warts caused by the human papilloma virus (HPV). This example reinforces the efforts to study medicinal plant extracts for possible application in clinical practice. The present result established the antimicrobial activity of fraction 9EA-FC-B obtained from the ethyl acetate crude extract of *A. wilkesiana*. It also demonstrated synergism between the fraction 9EA-FC-B and ampicillin in overcoming resistance of MRSA by inhibiting production of PBP2a. In the presence of subinhibitory concentrations of 9EA-FC-B, the MIC of ampicillin was reduced by as much as 32-fold, from $50 \mu\text{g mL}^{-1}$ to $1.56 \mu\text{g mL}^{-1}$, indicating that MRSA became more sensitive to ampicillin when fraction 9EA-FC-B was

introduced in the treatment. Based on these findings, we predict that the active constituents from fraction 9EA-FC-B may potentially be used for combating MRSA's virulence. Several researches have exploited the synergistic effects of natural products for drug development [21–23]. The most apt example that is closely related to the present study is the synergistic effects of corilagin and tellimagrandin I in combination with beta-lactam antibiotics on antibacterial activity against MRSA by inactivation of PBP2a [24].

Results of bacterial growth curve experiment indicated that combination of 9EA-FC-B with ampicillin (both agents at subinhibitory concentrations) distinctly suppressed the growth of MRSA in contrast to MRSA cultures that were treated with either 9EA-FC-B or ampicillin alone. Generally, MIC of ampicillin reduced when subinhibitory concentration of 9EA-FC-B increased in the combination treatment. From the kinetic growth curves, we were able to deduce that the antimicrobial action of the ampicillin and 9EA-FC-B combination was observed at the beginning of the exponential phase. A very minimal bacterial growth was seen with increase in incubation hours. Instead of growing rapidly during the exponential stage, the graph portrayed low growth of bacterial cells (approximately fourfold lower) with extended lag phase compared to the untreated MRSA (control). An extended lag phase was also detected for MRSA treated with 9EA-FC-B alone. Lag phase is the particular stage when bacteria equilibrate to adapt to the new environment by undergoing macromolecular repair and synthesis of cellular growth through DNA replications [25]. Hence, we deduced

that a lengthy lag phase observed in our experiment is due to the inhibition of DNA replications that delays the cellular growth process. The prolonged lag phase of MRSA observed in this experiment is reminiscent of the action of fluoroquinolones that caused inhibition of DNA replication in MRSA, leading to a longer lag phase [26]. A potent antimicrobial action was also identified at the exponential phase in which bacterial cells were prevented from growing rapidly in the presence of 9EA-FC-B alone and in combination with ampicillin. This phenomenon showed probable interference in cell division which involves multiple rounds of DNA synthesis that are controlled by a variety of gene regulatory processes [27, 28]. The plausible mechanism of action of 9EA-FC-B observed at the exponential phase is therefore associated with the interruption of cell division that causes membrane derangements and failure in membrane functions.

As for interpretation of the FIC indices, lower indices indicate better synergism [19]. Based on the FIC indices obtained, eight out of the 18 combinations tested showed synergistic effects. The presence of 9EA-FC-B significantly enhanced the potency of ampicillin by up to 32-fold (MIC reduced from $50 \mu\text{g mL}^{-1}$, in the absence of 9EA-FC-B, to $1.56 \mu\text{g mL}^{-1}$, in the presence of $1/4 \times \text{MIC}$ of 9EA-FC-B) for MRSA.

Ampicillin is a beta-lactam antibiotic that is designed to inhibit PBPs involved in late stage of peptidoglycan biosynthesis. Interference with peptidoglycan biosynthesis causes deformities in the bacterial cell wall and eventually leads to cell death due to high internal osmotic pressure. Nearly, all bacteria can be inhibited by interfering in mechanism of peptidoglycan synthesis [29]. Nevertheless, targeting this mechanism is no longer effective due to the production PBP2a in MRSA. The blocking of normal PBPs by beta-lactams did not exert effects on peptidoglycan or cell wall synthesis, because PBP2a replaces their function and ensures normal formation of cell wall in presence of lethal concentration of beta-lactam drugs [30]. However, in this study, we experimentally demonstrated restoration of ampicillin's antimicrobial activity by the addition of 9EA-FC-B at subinhibitory level.

The synergistic activity observed between 9EA-FC-B and ampicillin against MRSA was shown to be associated with inhibition of PBP2a. PBP2a is an inducible protein that regulates methicillin resistance. Its expression is heterogeneous in nature amidst level of resistance differing to the beta-lactam being used [31]. The *mecA* gene complex which encodes for this protein encompasses the regulatory genes, *mecI* and *mecR*. Interaction of beta-lactam antibiotics with these regulatory genes eventually allows expression of *mecA* in terms of production of PBP2a [32–34]. As such, the occurrence of intense PBP2a bands in MRSA cultures grown in low concentrations of ampicillin compared to the control culture in western blot experiment suggested the induction of *mecA* gene transcription. In contrast, no PBP2a bands were detected in MRSA cultures that were exposed to $1/4 \times \text{MIC}$ of 9EA-FC-B, while PBP2a is only very mildly expressed when the concentration of 9EA-FC-B was lowered to $1/8 \times \text{MIC}$. This suggests that 9EA-FC-B can either inhibit the production of PBP2a or directly inactivate PBP2a. This

suggestion is in agreement with the observation that 9EA-FC-B enhanced the ampicillin susceptibility of MRSA. In 2004, Shiota et al. [24] reported the antibacterial effect of corilagin and tellimagrandin I (isolated from *Arctostaphylos uva-ursi* and *Rosa canina*, resp.) against MRSA. The two polyphenolic compounds had exceptionally reduced MICs of beta-lactams for MRSA via inactivation of PBP2a. We believe that corilagin (a tannin) is also present in the test fraction 9EA-FC-B, since corilagin was previously isolated and identified by our colleagues from the same source of plant materials [35]. This is further supported by phytochemical testing that showed the presence of tannins in 9EA-FC-B (data not shown). Although Shimizu et al. [7] previously reported that corilagin enhanced antibacterial activity of various beta-lactams by 100- to 2000-fold against MRSA, 9EA-FC-B was only shown to enhance the activity of ampicillin by up to 32-fold in the present study. The stark difference in antibacterial enhancements can readily be rationalized by the fact that 9EA-FC-B is a semipure fraction that possibly contains only a small amount of corilagin in addition to many other secondary metabolites that were assumed to have negligible effect on the antibacterial activity observed.

5. Conclusion

From the results reported in this study, we found that the antibacterial effect of the fraction 9EA-FC-B obtained from *A. wilkesiana* and ampicillin is synergistic. The synergism is due the ability of 9EA-FC-B to suppress the production of PBP2a or directly inactivate it, leading to the restoration of the susceptibility of MRSA to ampicillin.

Conflict of Interests

The authors declared no conflict of interests.

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Research Article

Experimental Protection of Diabetic Mice against Lethal *P. aeruginosa* Infection by Bacteriophage

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The emergence of antibiotic-resistant bacterial strains has become a global crisis and is vulnerable for the exploration of alternative antibacterial therapies. The present study emphasizes the use of bacteriophage for the treatment of multidrug resistant *P. aeruginosa*. *P. aeruginosa* was used to induce septicemia in streptozotocin (STZ) induced diabetic and nondiabetic mice by intraperitoneal (i.p.) injection of 3×10^8 CFU, resulting in a fatal bacteremia within 48 hrs. A single i.p. injection of 3×10^9 PFU phage GNCP showed efficient protection in both diabetic (90%) and nondiabetic (100%) bacteremic mice. It was further noted that the protection rate was reduced in diabetic mice when phage GNCP was administered after 4 h and 6 h of lethal bacterial challenge. In contrast, nondiabetic bacteremic mice were rescued even when treatment was delayed up to 20 h after lethal bacterial challenge. Evaluation of results confirmed that a single intraperitoneal injection of the phage dose (3×10^9 PFU/mL) was more effective than the multiple doses of imipenem. These results uphold the efficacy of phage therapy against pernicious *P. aeruginosa* infections, especially in cases of immunocompromised host.

1. Introduction

After decades of extensive use of antibiotics in the treatment of infectious diseases caused by pathogenic bacteria, the emergence of multidrug resistant bacterial strains combined with a slowdown in the discovery of new classes of antibiotics is currently viewed as a major public health concern. *Pseudomonas aeruginosa* is the predominant pathogen that causes severe nosocomial diseases such as septicemia, pneumonia, and urinary tract infection in immunocompromised individuals [1]. *P. aeruginosa* has now become a major cause of nosocomial infections due to its remarkable propensity to rapidly acquire resistance determinants to a wide range of antibacterial agents [2]. Diabetes mellitus affects several aspects of the immune system. Functional properties of

polymorphonuclear leukocytes, monocytes, and lymphocytes such as adherence, chemotaxis, and phagocytosis are depressed in patients with diabetes [3]. Other alterations in the immune system may include reduced cell-mediated immune responses, impaired pulmonary macrophage function, and abnormal delayed type hypersensitivity responses. The risk of recurrence of such infections is also higher in diabetic patients. Some specific types of infections also occur predominantly in diabetic patients (malignant otitis externa, rhinocerebral mucormycosis, emphysematous pyelonephritis and cholecystitis, and Fournier's gangrene) [4]. The prevalence of *P. aeruginosa* in the impaired immune system candidates leads to the complications of secondary infections. Infections of *P. aeruginosa* in diabetic patients are bacteremia, sepsis, emphysematous pyelonephritis, skin

and soft tissue infections, and more frequent in malignant otitis external disease. Of note *P. aeruginosa* has a greater ability to develop resistance to virtually any antibiotic to which it is exposed, because of multiple resistance mechanisms that can be present within the pathogen. Therefore, the development of the alternative antibacterial approach is necessary for the treatment of a broad array of antibiotic-resistant infectious diseases. Bacteriophages or phages are viruses that specifically infect and lyse bacteria. A method of using phage for the treatment of bacterial infectious disease is called bacteriophage therapy or phage therapy. Recently, phage therapy has gained an increasing attention because it has many advantages over chemotherapy. Phages have high specificity for their target bacteria, indicating that they do not harm the normal intestinal microflora. Phages are effective against multidrug resistant pathogenic bacteria because the mechanisms by which they induce bacteriolysis differ completely from those of antibiotics. Moreover, phage has self-limitation, meaning that the number of phages remains at very low level after killing the target bacteria [5].

The following study, was employed to examine the efficacy of phage therapy in abrogating fatal *P. aeruginosa* infection in diabetic and nondiabetic mice models.

2. Materials and Methods

2.1. Bacterial Strains. *P. aeruginosa* strains were isolated from diabetic patients using BHI agar, and positive isolates were selected for future studies. Standard strain *P. aeruginosa* ATCC 27853 was used as a control.

2.2. Bacteriophage Isolation and Purification. *P. aeruginosa* strains were used as hosts to isolate specific phage from raw sewage. Phage isolations were accomplished by adding salt (58 g of NaCl) to 1 liter of sewage, followed by centrifugation at 12,000 rpm for 10 min. The supernatant was decanted into a separate sterile container and mixed with polyethylene glycol (PEG 8.00) to provide a final PEG concentration of 10% (w/v). The PEG containing supernatant was precipitated overnight at 4°C and centrifuged at 14,000 rpm for 20 min. The resulting precipitate was dissolved in 5 mL of phage dilution buffer (SM) and extracted once with an equal volume of chloroform. An aliquot (200 μ L) of this processed sewage was mixed with 100 μ L of an overnight culture of *P. aeruginosa* strain, incubated at 37°C for 20 min, mixed with 2 mL of molten top agar (0.8% agarose) at 50°C, and poured onto bacteriophage isolation agar plates (1.6% agar). Plates were incubated overnight at 37°C. Phage plaques were harvested from the plate, and single plaques were purified thrice on host strains.

2.3. Large Scale Amplification and Purification of Phage Particles. Phage was purified according to the procedure described by Biswas et al. (2002). *P. aeruginosa* host strains were suspended at 2×10^8 cells/mL in 20 mL LB medium and were exposed to the crude preparation of GNCP phage and vigorously shaken for 4–5 h at 37°C, resulting in the complete lysis of bacteria. Later on treatment with 1% chloroform at

37°C for 10 min, followed by treatment with 1 μ g/mL DNase I and 1 μ g/mL RNase A for 30 min at 37°C, the culture fluid was centrifuged at 10,000 rpm for 10 min at 4°C to remove cell debris. PEG/NaCl was added to the supernatant to a final concentration of 1/6 (v/v) and was kept overnight at 4°C. The resultant precipitate containing the phage particles was collected by centrifugation at 10,000 rpm for 20 min at 4°C and resuspended in 500 μ L of SM buffer. The phage was collected and dialysed against 10 mM saline that contained 50 mM Tris-Chloride (pH 8.0) and 10 mM MgCl₂ for 2 h at 4°C until used. The samples were approximately diluted with LB just before use. The titers (for infections) (PFU/mL) of purified samples were determined by inoculating them into *P. aeruginosa* strain [6].

2.4. Characterization of Phage

2.4.1. Transmission Electron Microscopy. To observe the morphology, transmission electron microscopy (TEM) was performed by the modified method [6]. A drop of purified phage suspension was applied to former carbon-coated copper grid for five min. The suspension was removed with a pipette and negatively stained with 2% uranyl acetate (TAAB Laboratory, UK). After ten minutes the grids were examined in a Tecnai Biotwin (Philips), transmission electron microscope (The Netherlands).

2.4.2. Bacteriophage DNA and Restriction Enzyme Analysis. Purified phage particles (2×10^8 PFU/mL) were treated with 1 μ g of DNase I and RNase A (Bangalore Genei, Bangalore, India) at 37°C for 1/2 h. To the mixture, proteinase K (Bangalore Genei, Bangalore, India) and SDS were added at a final concentration of 0.05 mg/mL and 0.5%, respectively, and incubated at 56°C. After 1 h of incubation, an equal volume of phenol : chloroform was added to remove proteinaceous material. The extraction was repeated thrice with phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The nucleic acid was precipitated with chilled ethanol and suspended in 20 μ L of TE buffer (10 mM Tris-HCl, pH 7.0, 1.0 mM EDTA, pH 7.0) according to standard procedure.

Restriction enzyme digestion of the isolated phage DNA was carried out following the instructions supplied by manufacturers. Hind III, Eco RI, and Bam HI were added to the purified bacteriophage DNA.

2.5. Experimental Animals. Specific pathogen free, colony bred, virgin adult Swiss mice (Wistar strain) of both sexes with the commendation of the Institute Animals Ethics Committee (Reg. number 346/CPSCEA) of Luqman Pharmacy college, Gulbarga, Karnataka, India, were obtained. Animals were fed standard pellet and water ad libitum. Each group of mice received an intraperitoneal injection of 60, 120, 150, and 180 mg of STZ (kg^{-1} of body weight). Control mice received citrate buffer (pH 4.5) alone. Blood glucose levels were monitored over the stabilization period of 10 days following administration of STZ by blood glucose monitors drawing blood from the tail vein [7].

2.6. Experimental Induction of *P. aeruginosa* Bacteremia in Diabetic and Nondiabetic Mice. For each infection experiment, 6- to 8-week-old diabetic and nondiabetic mice were divided into two groups, and each group was given inocula of various sizes. The infecting bacteria were prepared by growing *P. aeruginosa* in LB broth medium, at 37°C, and were centrifuged at 8000 rpm for 5 min. The cell pellet was washed with normal saline (0.9 gm in 100 mL distilled water), centrifuged again under the same conditions, and finally resuspended in 10 mL saline. After appropriate dilution, turbidity at 600 nm was measured to determine bacterial cell numbers. To find the minimal lethal dose (MLD), serial dilutions of *P. aeruginosa* (10^7 , 10^8 , and 10^9 CFU/mL) were injected intraperitoneally (i.p.) into both diabetic and nondiabetic mice in 100 μ L aliquots. After infection, mice were kept under standard laboratory conditions with free access to food and water. Six mice were used for each dose; the survival rates of nondiabetic and diabetic mice were then measured at 2 days after infection [8]. Mice inoculated with *P. aeruginosa* were observed for several clinical signs, including ruffled fur, hunchback moribund, and partially closed eyes.

2.7. Imipenem Treatment in *P. aeruginosa* Infected Mice. Followed by intraperitoneal injection of *P. aeruginosa*, both diabetic and nondiabetic mice were randomly separated into four groups. The first two diabetic and nondiabetic groups received no antibiotic; the remaining groups received intraperitoneal injections of imipenem [$30\text{ mg (kg}^{-1}\text{ of body weight)}$] after 20 min of pathogen administration. An antibiotic was given once daily for 5 days. The mice were observed for 30 days after the completion of treatment to determine the effect of antibiotics on infected diabetic and nondiabetic mice [9].

2.8. Treatment of Diabetic and Nondiabetic Bacteremic Mice with Phage. Ability of phage GNCP to rescue mice was seen in two groups using diabetic and nondiabetic *P. aeruginosa* DPA-12 bacteremic mice model. The effect of phage dose was studied in the six groups of mice (six mice in each group) and was challenged by intraperitoneal injection of *P. aeruginosa* DPA-12. Each of these groups was treated with a single injection of GNCP administered i.p. 20 min after the bacterial challenge at 3×10^{10} , 3×10^9 , 3×10^8 , 3×10^7 , 3×10^6 , and 0 PFU. The state of the death of the mice was monitored for 30 days.

The result on the outcome of delaying treatment for various periods was also monitored. In the delayed-treatment study, treatment was initiated at 0, 1, 2, 3, 4, and 6 h after bacterial challenge with the MLD. The state or health of diabetic and nondiabetic bacteremic mice was monitored for 30 days.

2.9. Chronic Studies on STZ Induced Diabetic Mice. Chronic study was carried out in normal mice and streptozotocin induced diabetic mice for 21 days. The blood glucose levels of the animals were checked after 18 hrs of fasting and were considered as a 0 day reading. The phage 3×10^9 dose was given daily to the animal for 21 days. Glipizide was used as

a standard control. The blood levels were checked at 0-, 7-, 15-, and 21-day period. Blood glucose levels were monitored by drawing blood from the tail vein [7].

2.10. Determination of Immunologic Response to Phage GNCP in Mice. Indirect enzyme-linked immunosorbent assay was used to check phage GNCP-specific immunogenicity for IgG and IgM antibody titers in sera of diabetic and nondiabetic mice as described [6].

2.11. Histopathological Studies. With the intention to know the histological changes during the course of therapy by using bacteriophage as immunogens in mice, the careful gross examination of spleen, liver, kidney, and lung was done and dissected out. Samples preserved in 10% formalin were dehydrated in an ascending series of alcohol (70–100%). The tissue was embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin [10].

3. Results

3.1. Isolation and Characterization of Bacteriophage. The phage isolated in the present study was found to form plaques on *P. aeruginosa* clinical isolate. A total of 12 *P. aeruginosa* isolates from diabetic samples were used for isolation of lytic bacteriophage. The isolated phage was found to form plaques on four imipenem susceptible clinical isolates and also inhibited bacterial growth of two imipenem resistant strains including DPA 12. The phage GNCP showed it was able to multiply very rapidly on a *P. aeruginosa* DPA-12 culture, approximately reaching counts of 10^8 PFU/mL within 2 h at 37°C. Electron microscopy revealed that phage GNCP has an icosahedral-shaped head, approximately 50 nm, and a nonrigid tail. Based on the morphology, the phage is tentatively placed in the Siphoviridae family (Ackermann, 2001). The phage DNA was 21 Kb in size. Of the four restriction enzymes (Hind III, EcoRI, BamHI, and Sma I) which were tried on bacteriophage, EcoRI was found to produce the clearest pattern of bands (data not shown).

3.2. Diabetes Induction in Experimental Mice. Each group of mice received an intraperitoneal injection of 60, 120, 150, and 180 mg of STZ (kg^{-1} of body weight), while control mice received citrate buffer alone. Mice given multiple doses of STZ [$150\text{ mg (kg}^{-1}\text{ body weight)}$] showed marked and persistent hyperglycemia. Some mice received 180 mg of STZ (kg^{-1} body weight) and died within 15 days, but almost all mice given 150 mg of STZ (kg^{-1} body weight) were alive for more than 2 months. Although diabetic mice exhibited glycosuria, they developed a ruffled thinner appearance and consumed more water per day than the untreated group. Mice with fasting blood glucose levels $>250\text{ mg/mL}$ were defined as diabetic mice and used as STZ induced mice model in the following experiments.

3.3. Selection of *P. aeruginosa* Strain to Induce Experimental Bacteremia in Diabetic and Nondiabetic Mice. Among 12

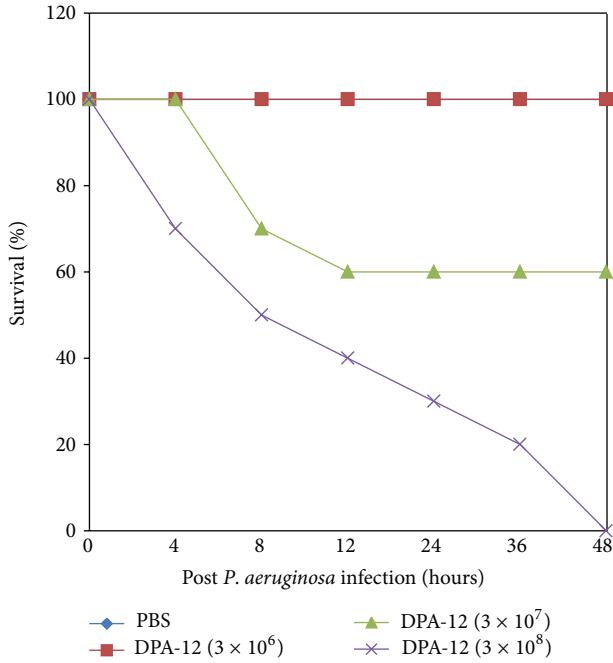


FIGURE 1: Determination of the MLD of *P. aeruginosa* (DPA-12) in nondiabetic mice. 25 in each group were infected i.p. with serially diluted suspensions of *P. aeruginosa*. The percentage of survival was determined up to 48 h.

clinical isolates of *P. aeruginosa*, DPA-12 was selected on the basis of multidrug resistance including imipenem and its susceptibility for phage GNCP lytic multiplication. Hence, *P. aeruginosa* DPA-12 was selected for induction of bacteremia in diabetic and nondiabetic mice.

3.4. Induction of Experimental Bacteremia in Diabetic and Nondiabetic Mouse Model with *P. aeruginosa* DPA-12. The lethal dose of *P. aeruginosa* in mice was determined by injecting both diabetic and nondiabetic mice with varying numbers of *P. aeruginosa* DPA-12, ranging from 3×10^4 to 3×10^9 cells per dose. Intraperitoneal injections of 3×10^4 to 3×10^6 DPA-12 did not reduce the survival rate of nondiabetic mice whereas 10% diabetic mice died during the subsequent 7-day observation period. In contrast, injections of *P. aeruginosa* DPA-12 of 3×10^7 cells showed survival rate of 60% in nondiabetic and 40% in diabetic mice within 48 h, whereas 3×10^8 CFU/mL of *P. aeruginosa* DPA-12 showed 100% lethal effect within 48 h of injection in both groups of animals (Figures 1 and 2); therefore this dose 3×10^8 CFU/mL was considered to be optimal and fixed throughout the experiment.

3.5. Efficacy of Phage against Lethal Bacteremia in Diabetic and Nondiabetic Mice. Administration of a single dose of purified phage GNCP of 3×10^6 to 3×10^8 PFU protected up to 90% of diabetic mice and 90% of nondiabetic mice from DPA-12-induced lethal bacteremia. Phage dose of 3×10^9 PFU/mL significantly rescued 90% of diabetic and 100%

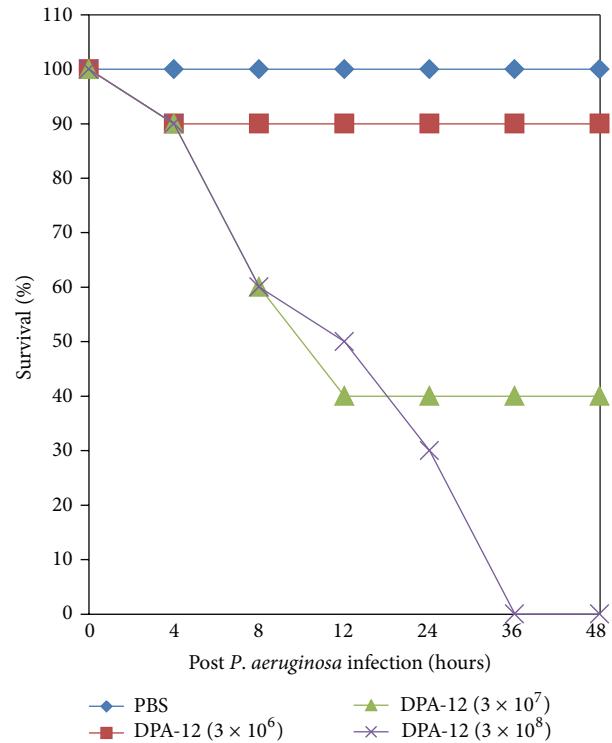


FIGURE 2: Determination of the MLD of *P. aeruginosa* (DPA-12) in diabetic mice. 25 in each group were infected i.p. with serially diluted suspensions of *P. aeruginosa*. The percentage of survival was determined up to 48 h.

of nondiabetic mice from lethal bacteremia. All live mice remained healthy for an additional 30 days of observation. At this point the experiment was terminated. The phage dose effect on the state of health of the infected animals was clearly visible. Administration of a high dose (3×10^9) of phage GNCP alone to experimental mice did not affect their physical condition or survival during the one-month period of observation (Figures 3(a) and 3(b)).

3.6. Phage Treatment Compared with Imipenem Treatment in Diabetic and Nondiabetic Bacteremic Mice. On comparing the protective efficacy of phage therapy with chemotherapeutic treatment of diabetic and nondiabetic bacteremic mice, a single i.p. dose of imipenem [$(30 \text{ mg} (\text{kg}^{-1} \text{ body weight})]$] showed 20% protection of diabetic bacteremic mice, whereas three consecutive injections of imipenem protected 40% of diabetic mice from *P. aeruginosa* DPA-12 bacteremia (Figure 3(a)). However, a single dose of imipenem treatment rescued 20% of nondiabetic bacteremic mice as shown in Figure 3(b). The administration of a single dose of phage GNCP of 3×10^9 PFU rescued 90% and 100% of diabetic and nondiabetic mice from *P. aeruginosa* bacteremia, respectively. The experimentally protected mice were healthy and active for the subsequent 7-day observation period. There were no survivors among untreated mice (in both groups) 2 days after intraperitoneal injection of 3×10^8 CFU cells of *P. aeruginosa* DPA-12 ($P > 0.02$).

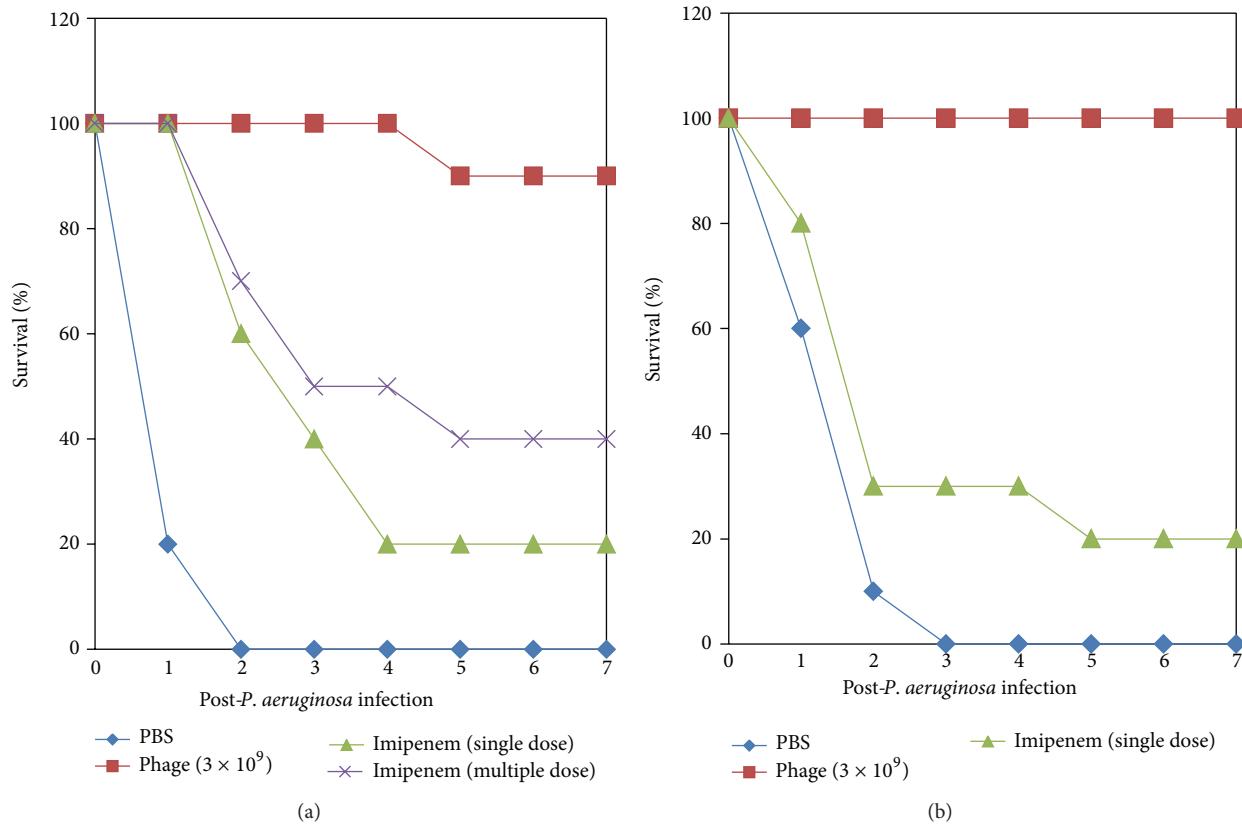


FIGURE 3: (a) The protection efficacy of phage therapy with imipenem (single and multiple dose) treatment of diabetic bacteremic mice. The percentage of survival was determined up to 7 days following i.p. challenge. (b) The protection efficacy of phage therapy with imipenem (single dose) treatment of nondiabetic bacteremic mice. The percentage of survival was determined up to 7 days following i.p. challenge.

3.7. Effect of Delay in Treatment on the Ability of the Phage GNCP to Protect Diabetic and Nondiabetic Mice from *P. aeruginosa* DPA-12 Bacteremia. After induction of bacteremia in diabetic and nondiabetic mice with a lethal dose of *P. aeruginosa* DPA-12, phage therapy was applied through a single injection of 3×10^9 PFU of phage GNCP at various intervals thereafter, ranging from 4 to 20 h. The experiments revealed that a single injection of phage (3×10^9 PFU) can rescue 90% and 100% of diabetic and nondiabetic bacteremic mice, respectively, even when treatment is delayed for 4 h after i.p. injection of lethal dose of *P. aeruginosa* DPA-12 of bacterial challenge (Figure 4). A delay in phage administration by 6 and 8 h led to decreased protection rates, 80% and 60%, respectively ($P > 0.03$), in diabetic bacteremic mice. In comparison, nondiabetic bacteremic mice were rescued 90%, 90%, and 40%, when the treatment was delayed up to 6, 8, and 16 h, respectively, after lethal dose of bacterial infections ($P > 0.05$). When treatment was delayed beyond 20 h, only 10% of nondiabetic bacteremic mice were rescued; however, at the same time, phage GNCP completely failed to protect diabetic bacteremic mice from severe morbidity.

3.8. Chronic Studies on STZ Induced Diabetic Mice. Chronic studies of phage for a period of 21 days were carried out on STZ induced mice; results are shown in Table 1.

Phage dose of 3×10^9 PFU/mL significantly reduced to 95.19 ± 4.15 mg/dL from 121.97 ± 4.15 mg/dL. However, at day 7 and day 15 it reduced moderately to 161.21 ± 4.25 mg/dL and 121.97 ± 4.15 mg/dL, respectively, as compared with the diabetic group.

The group treated with standard drug, glipizide, showed a maximum reduction in diabetic blood glucose levels. Significant reduction to 189.080 ± 8.610 mg/dL and 128.87 ± 9.83 mg/dL was noted on the 7th and 15th days of treatment, respectively, while further reduction to 105.79 ± 5.9 blood glucose level was noted on the 21st day of treatment when compared to the diabetic control group.

3.9. The Immune Response to Phage in Diabetic and Nondiabetic Mice. After 28 days of single dose injection of phage GNCP in diabetic and nondiabetic mice, titers of IgG and IgM against the phage increased above the background by 500-fold and 100-fold, respectively, in both groups. No substantial deviation was found between diabetic and nondiabetic IgG and IgM titers against phage GNCP. No anaphylactic reactions, changes in core body temperature, or other adverse events were observed in the two groups.

3.10. Histopathological Studies. The results of the bacteriophage therapy were also confirmed on the basis of

TABLE 1: Chronic study of phage (3×10^9) on blood glucose level in STZ induced diabetic mice.

Groups	Blood glucose level mg/dL			
	0	7	15	21
Control +ve (diabetic normal)	240.20 ± 5.167	273.018 ± 6.197	286.9 ± 619	262.29 ± 6.46
Standard glipizide 10 mg/kg	258.00 ± 4.496	$189.080 \pm 8.610^{**}$	$128.87 \pm 9.83^{**}$	$105.79 \pm 5.9^{**}$
Phage (3×10^9)	263.081 ± 6.679	$161.21 \pm 4.25^{**}$	$121.97 \pm 4.15^{**}$	$95.19 \pm 4.15^{**}$

Values are mean \pm SEM; $n = 6$ * $P < 0.05$, ** $P < 0.01$.

* Indicates level of significance.

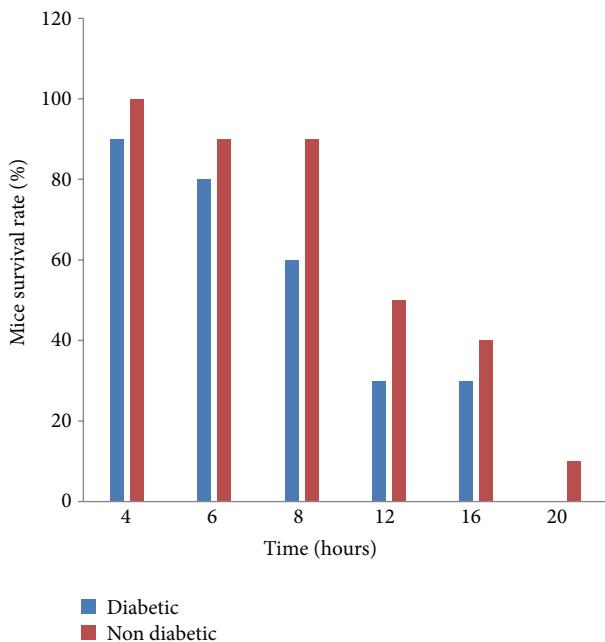


FIGURE 4: Delayed phage treatment of diabetic and nondiabetic bacteremic mice. A single i.p. injection of 3×10^9 PFU was administered to bacteremic mice at the indicated times after bacterial challenge. Delayed phage administration rescued significantly higher numbers of nondiabetic bacteremic mice than diabetic bacteremic mice.

histopathological examination of the vital organs: liver, kidney, lung, and spleen.

Histological examination of the normal structure of the kidney of control is illustrated in Figure 5(d). Microscopic examination of the kidney in diseased mice infected with *P. aeruginosa* (Figure 5(c)) revealed some degenerative changes such as prominent internal hemorrhage and atrophied and vacuolated convoluted tubules. Intertubular alterations included the occurrence of collapsed glomeruli and congested glomerular capillaries. Imipenem antibiotic treated mice group (Figure 5(b)) showed focal areas of necrosis, hypertrophied glomeruli, and nuclear degeneration. In the group treated with phage GNCP showed the normal architecture with glomeruli and tubules lined by epithelium with eosinophilic cytoplasm; this showed to be a sign of recovery (Figure 5(a)).

Histological examination of the normal structure of the spleen of mice in Control group receiving PBS was surround by thick fibrous connective tissue capsule with some myofibroblasts and a covering mesothelium (Figure 6(d)).

Internally, thick connective tissue trabeculae bear branches of the splenic artery and veins, with normal white and red pulps. The group of mice that received a lethal dose of *P. aeruginosa* (Figure 6(c)) was represented by internal haemorrhage, complete loss of lymphoid follicular structure, and increased number of megakaryocytes. The imipenem treated group of infected mice revealed thickened splenic capsule with subcapsular dark pigments possibly hemosiderin (Figure 6(b)). The group of infected mice treated with phage showed little expansion of red pulp, and restricted white pulps were detected. Vacuolation was also observed. The appearance of degeneration was common all over the spleen tissue with some of the improvement in phage treated mice (Figure 6(a)).

Histological examination of the group showed normal architecture with occasional dilated central vein with feathery degeneration of hepatocytes (Figure 7(d)). The section of diseased mice showed degeneration of hepatocytes with focal areas of hemorrhages (Figure 7(c)). The segment of the liver from the antibiotic treated mice showed eccentrically placed nuclei with vacuolated cytoplasm and few of hepatocytes showing feathery degeneration of hepatocytes with focal areas of hemorrhage (Figure 7(b)). The phage treated mice group showed the normal architecture with little quantity of regeneration of focal areas of hemorrhages (Figure 7(a)).

Histological examination of the normal structure of the lung from control group section studied shows aerated alveolar spaces and few dilated and congested blood vessels (Figure 8(d)). Microscopy examination of the lung in diseased mice infected with *P. aeruginosa* (Figure 8(c)) revealed significant inflammatory lung disease; many medium and small airways obstructed with beads and localized cell inflammation were observed. The beads were still visible in some sections, suggesting that the infection was sustained antibiotic treated group of mice Figure 8(b). In the group treated with phage GNCP (Figure 8(a)) showed the normal architecture with aerated alveolar spaces and few dilated and congested blood vessels with no signs of damaged.

4. Discussion

P. aeruginosa is an important opportunistic pathogen that causes chronic infections in the lungs of patients with the genetic disease cystic fibrosis and acute infections such as severe skin infections in cases of burns and urinary tract infections in immunocompromised individuals [11]. *P. aeruginosa* is the one among the dominant pathogen, particularly

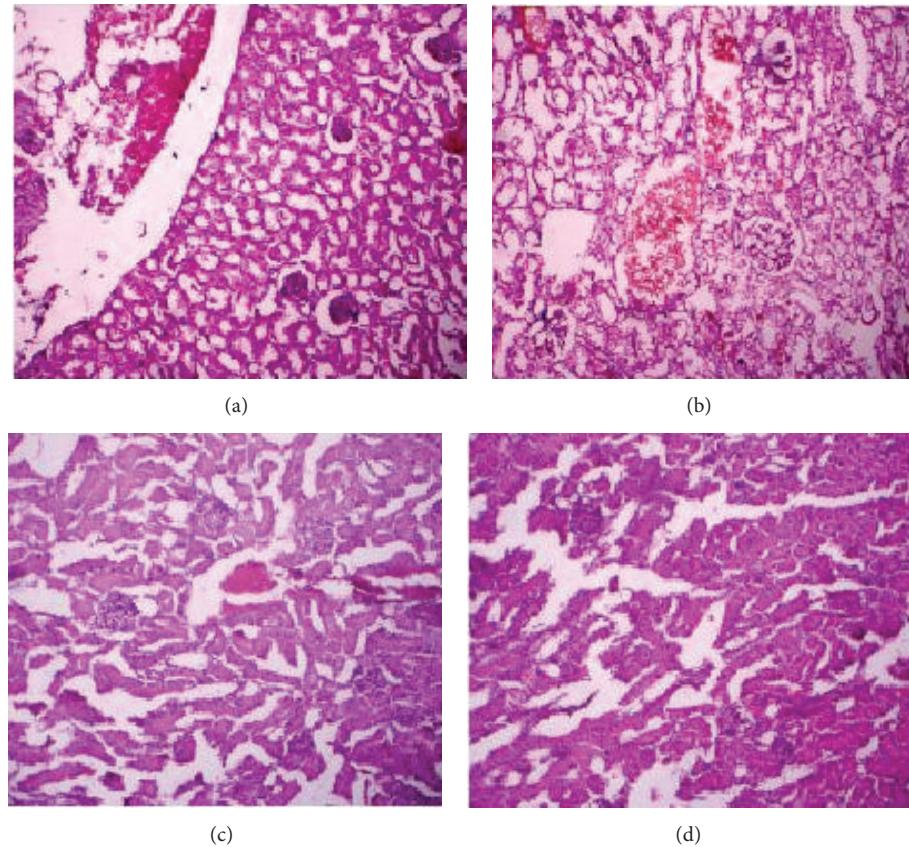


FIGURE 5: Histopathology of the kidney. Sections of hematoxylin and eosin-stained kidney ($\times 500$ magnification) are shown. (a) Diseased mice treated with phage GNCP. (b) Diseased mice treated with imipenem. (c) Diseased mice infected with lethal dose. (d) Control group of mice received PBS.

in diabetic patients, causing a range of diseases like foot infections, pneumonia, postoperative infections, UTI, and invasive external otitis [12].

To deduce the severity of infection due to *P. aeruginosa* in the immunocompromised patients, an experimental model of STZ induced diabetic mice with bacteremia was developed. Experimental diabetes was induced by multiple doses of STZ [$150 \text{ mg} (\text{kg}^{-1} \text{ body weight})$] in a mouse model. Animals with blood glucose levels $>250 \text{ mg/dL}$ were considered a diabetic model. The STZ diabetic model can serve as a better model since diabetes may be initiated at a younger age when all animals have reached maturity and with negligible weight loss [13, 14].

In this study, 12 *P. aeruginosa* isolates were isolated from 120 diabetic clinical samples, and most of them were multidrug resistant including imipenem. DPA-12 was selected for further studies based on its susceptibility to phage and drug resistance.

Experimental induction of bacteremia with *P. aeruginosa* DPA-12 was exacerbated in diabetic mice compared with nondiabetic mice. 100% mortality was observed in both groups. However, 100% mortality occurred in diabetic mice within 36 h, in contrast to 100% in nondiabetic mice at 48 h. The severe morbidity and higher mortality rate in diabetic bacteremic mice than in nondiabetic bacteremic

mice suggested that diabetic animals are prone to bacterial infections, including *P. aeruginosa* [15, 16].

The effect of imipenem on *P. aeruginosa* bacteremic diabetic and nondiabetic mice revealed more greatly enhanced clearance of *P. aeruginosa* from the 20% of nondiabetic mice than from diabetic bacteremic mice. However, mice treated with multiple imipenem injections showed a survival rate, suggesting that the failure or poor efficacy of imipenem treatment in diabetic mice correlates with the fact that diabetic patients with bacterial infections need to undergo longer antibiotic treatment [17]. The present study provides the first experimental evidence that administration of imipenem is poorly protective of diabetic bacteremic mice compared to nondiabetic bacteremic mice under conditions of *P. aeruginosa* lethal bacteremia used in our work.

Although antibiotics have historically been successful for the treatment of wound infections, the emergence of MDR bacteria and the failure of drug discovery programs over the last 10 years to provide new broad spectrum antibiotics with truly novel modes of action pose a major threat to public health worldwide. In this light, the western critical skepticism towards phage therapy was once more accompanied by a renewed interest and reappraisal of the beginning of the 21st century [18]. The innocuous nature of phage was demonstrated by adding high-titer T4 phage stock to the drinking

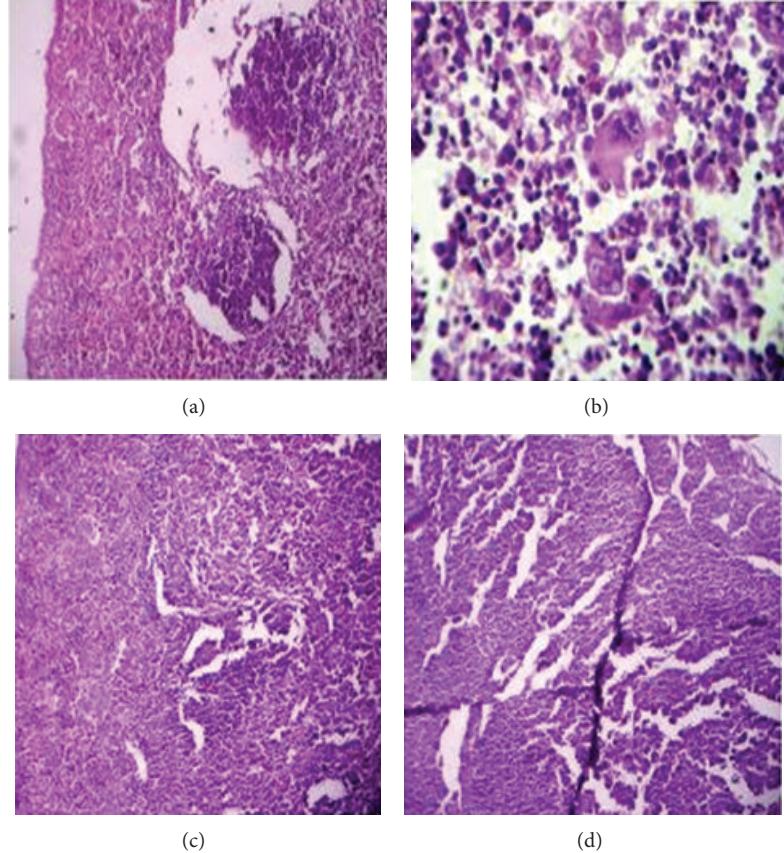


FIGURE 6: Histopathology of the spleen. Sections of hematoxylin and eosin-stained spleen ($\times 500$ magnification) are shown. (a) Diseased mice treated with phage GNCP. (b) Diseased mice treated with imipenem. (c) Diseased mice infected with lethal dose. (d) Control group of mice received PBS.

water of human volunteers [19]. Various research groups performed controlled animal experiments and reported the effectiveness of phage therapy for treatment of vancomycin-resistant *Enterococcus faecium* [6] and methicillin-resistant *Staphylococcus aureus* infections [20].

Many research groups turned their attention to the phage treatment of *P. aeruginosa* infections in mice, guinea pigs, and pet dogs [21]. A report on the treatment of single cases of human burns, wounds indicates that bacteriophage multiplication is associated with clinical improvement.

A first randomized, double-blind, and placebo-controlled phase I/II clinical trial was executed on 24 patients suffering from chronic otitis caused by MDR *P. aeruginosa*, showing efficacy and safety in the treatment of this infection [22]. An important step was recently taken by the detailed description of a quality-controlled small-scale production of a bacteriophage preparation, leading to a safety trial in burn wound patients at the Burn Centre of the Queen Astrid Military Hospital in Brussels, Belgium [23]. Nevertheless, a crucial condition towards practical application of phage therapy will be the availability of a large library of well-characterized phage, subjected to in-depth genomic analysis to confirm the absence of undesired genes [24].

Hence, in the present work, an observational evaluation of the healing potential of newly isolated bacteriophage in

diabetic and nondiabetic mice was performed. The phage isolated was found to form plaques on three MDR *P. aeruginosa* clinical isolates. *In vitro* characterization of phage showed it was able to multiply very rapidly on a *P. aeruginosa* culture DPA-12, reaching counts of 10^8 PFU/mL within 2 h at 37°C . Electron microscopy revealed that phage GNCP has an icosahedral head, approximately 50 nm, and a nonrigid tail. Based on the morphology the phage is tentatively placed in the Siphoviridae family [25]. The phage DNA was 21kb length and was found resistant with several restriction endonucleases.

The i.p. administration of purified phage GNCP rescued both diabetic and nondiabetic mice from *P. aeruginosa* lethal bacteremia. A single i.p. injection of 3×10^9 PFU phage GNCP showed efficient protection in both diabetic (90%) and nondiabetic (100%) bacteremic mice (Figures 3(a) and 3(b)). The combination of diabetic complications and the heavy bacterial load might have restricted phage GNCP activity to 90% protection in diabetic bacteremic mice. The same effectiveness of phage was attained even when treatment was delayed up to 4 h in both diabetic and nondiabetic bacteremic mice, whereas the protection rate was reduced in diabetic mice when phage GNCP was administered after 4 and 6 h of lethal bacterial challenge. In contrast, nondiabetic bacteremic mice were rescued even when treatment was delayed up to 20 h after lethal bacterial challenge.

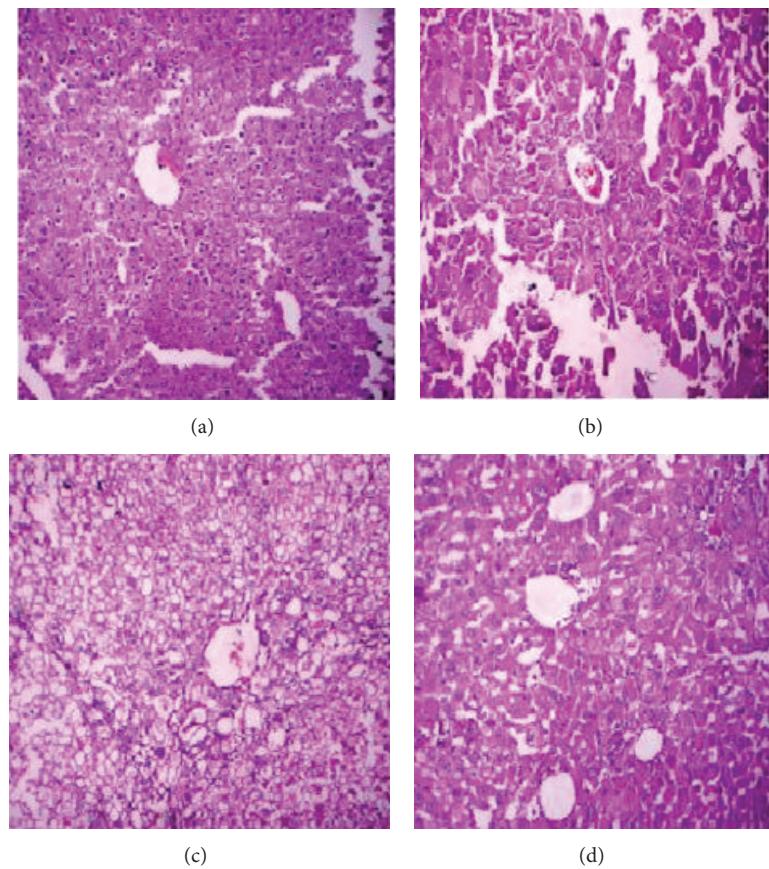


FIGURE 7: Histopathology of the liver. Sections of hematoxylin and eosin-stained liver ($\times 500$ magnification) are shown. (a) Diseased mice treated with phage GNCP. (b) Diseased mice treated with imipenem. (c) Diseased mice infected with lethal dose. (d) Control group of mice received PBS.

These experiments demonstrate a powerful curative effect of phage on IMP R-Pa bacteremia in our mouse model. The survival rate between phage treated and the control groups is statistically significantly different. The survival rate of bacteremic mice by delaying treatment was reduced, suggesting that stressed animals are more sensitive to various factors; in this case either the phage itself or trace amounts of endotoxins and exotoxins are present in the phage preparations. Healthy animals did not display apparent reactions to these factors, as evidenced by the lack of any adverse effects in the control groups inoculated with a high dose of the phage preparation.

From the results of chronic study on STZ induced diabetes in mice on 21st, it was found that the phage dose (3×10^9 PFU/mL) has reduced the blood glucose levels from 263.81 ± 6.679 mg/dL to almost normal levels of 95.19 ± 4.15 mg/dL which was more significant compared to standard glipizide (105.79 ± 5.9 blood glucose level) than compared to the diabetic control group.

The most significant finding of this work lay in the comparison of the outcome of treatment of diabetic and non-diabetic bacteremic mice with imipenem and phage GNCP. Evaluation of results confirmed that a single intraperitoneal injection of the phage dose was more efficacious than the multiple doses of imipenem. The reduction in bacterial load was reflected in the lower morbidity and mortality observed

in the phage-GNCP-treated group. These results also agree with earlier studies which showed a marked difference in the effect of phage therapy was observed in groups treated with bacteriophage compared to antibiotics [26–28]. It is well known that drugs are catabolized and removed from the body (half life span), whereas phage keeps on multiplying until all host bacteria are followed in the circulation and killed like guided targets. It has been reported that a small population of mutant I phage survived in the circulation, with a concomitant alteration to major head protein E [8]. Therefore, the authors postulated that such “serially passed” immune-escape mutants may facilitate improvements in the therapeutic efficacy of phage.

The present study showed that phage GNCP induces an immune response in both diabetic and nondiabetic mice; the levels of IgG (500-fold) and IgM (100-fold) in both diabetic and nondiabetic mice revealed that the immune response to phage GNCP was not associated with anaphylaxis or other adverse immunological reactions. Similar findings were reported in the treatment of *P. aeruginosa* cystic fibrosis strains [29]. Histological analysis confirmed that the organ damage in the treated group was less severe than in the antibiotic and untreated animals. Bacteria were detected causing severe impairment in most critical organs, especially the liver and spleen in the mice treated with antibiotic and untreated

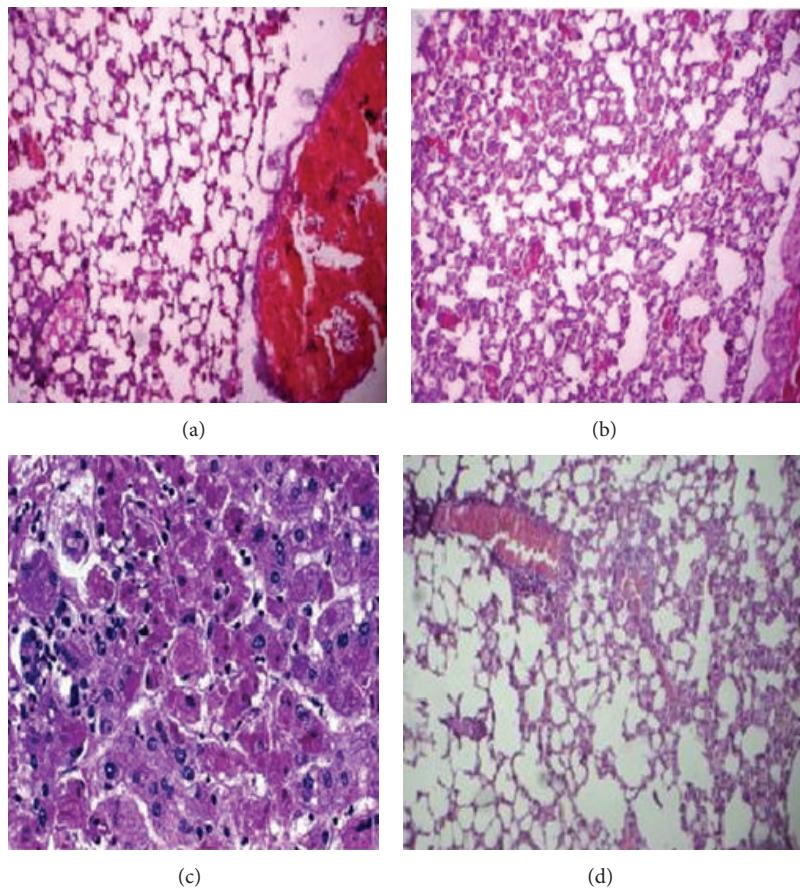


FIGURE 8: Histopathology of the lung. Sections of hematoxylin and eosin-stained lung ($\times 500$ magnification) are shown. (a) Diseased mice treated with phage GNCP. (b) Diseased mice treated with imipenem. (c) Diseased mice infected with lethal dose. (d) Control group of mice received PBS.

animals. In contrast, the bacteriophage treated mice showed moderate or mild impairment of vital organs observed. No alterations were observed in uninfected animals. These observations were consistent with the fact that bacteriophage targets extracellular bacteria and also the role of phagocytosis in bacterial removal [30].

Based on our observations of this study, phage therapy is used as an alternative therapy for the patients not responding to multidrug resistance regime.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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