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The Antimicrobial Resistance Plasmid Mobilome of Salmonella enterica and Related Enteric Species and Factors that Influence the Transfer Efficiency.

Suad Algarni
University of Arkansas, Fayetteville

The Antimicrobial Resistance Plasmid Mobilome of *Salmonella enterica* and Related Enteric
Species and Factors that Influence the Transfer Efficiency

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by

Suad Algarni
King Abdulaziz University
Bachelor of Science Zoology, 2004
Quinnipiac University
Master of Health Sciences, Medical Lboratory Sciences, 2013

December 2022
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Young Min Kwon, Ph.D.
Dissertation Director

_____
Steven L. Foley, Ph.D.
Committee Member

_____
Steven C. Ricke, Ph.D.
Committee Member

_____
Douglas D. Rhoads, Ph.D.
Committee Member

ABSTRACT

The dynamic distribution of antimicrobial resistance genes in *Salmonella enterica* is considered a public health risk. *S. enterica* is one of the most important etiological agents of foodborne illness and has a critical impact on global human health. In *S. enterica* and related species, mobile genetic elements (MGEs) serve as primary vehicles for the dissemination of antibiotic resistance genes in the bacterial evolution. This dissemination can be impacted by different selective pressures that leads to diverse antibiotic response phenotypes. This project focusses on the dynamics of antimicrobial resistance genes, particularly exploring the transfer efficiency of multidrug resistance plasmids in *S. enterica* using a combination of *in silico* and *in vitro* techniques. Plasmid physiology is part of an essential genetic engineering tool has mediated the dynamic transfer and spread of antimicrobial resistance in *Salmonella* isolates. Several phenotypic and molecular techniques were selected in these studies to better understand the molecular biology and epidemiology of resistance plasmids. These methods included whole genome sequencing (WGS) analyses, replicon (incompatibility; Inc) typing and conjugation studies of plasmids from *S. enterica* and related species. Multiple *in silico* analyses were used to evaluate WGS and plasmid sequencing data to examine the distribution of MGEs, AMR, biocide, disinfectant, and heavy metal resistance genes across the different plasmid types and to develop a plasmid characterization database. The database supported the development of the computational algorithms to process the WGS data from *S. enterica* isolates and is very useful for identifying the plasmid Inc types and their specific conjugal transfer systems, which are valuable to study the diversity and dissemination of plasmids associated with AMR in *S. enterica* and other Enterobacteriaceae. *In vitro* studies explored the impact of different antimicrobial exposures on conjugal transfer potential of plasmids. For some *S. enterica* strains, exposure to

different concentrations of tetracycline or chloramphenicol led to differences in the efficiency of AMR plasmid transfer. These data help to better understand the distribution of resistance genes and provide a useful method for a comprehensive molecular studies of plasmid transfer dynamics. The impact of these findings clarifies the role of the conjugation dynamics in the dissemination of antimicrobial resistance in *S. enterica* and related species, which can potentially impact the transfer of resistance genes within the gastrointestinal microbiome. Further studies are required to identify the underlying genetic mechanisms for *Salmonella* resistance plasmid transfer using approaches like RNA-sequencing methods to extend the understanding of the regulation of genetic pathways during conjugal transfer. Overall, the research study expands our knowledge of plasmid transfer dynamic and the provides tools that can be used to better understand AMR gene transfer among *Salmonella enterica* isolates which can have an impact in clinical and diagnostic laboratories as well as in epidemiological surveillance.

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DEDICATION

I dedicate all my work to my valuable inspiration, my beautiful, brilliant, and charming mother Mrs. Asia Algarni. My father Mr. Mohammed Algarni, your support and belief in me in the project provided it sturdy roots to grow from and therefore I am very grateful!

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GENERAL INTRODUCTION

Foodborne pathogens, such as *Salmonella enterica* and their related species, are considered a global public health risk. It has been widely shown that *S. enterica* strains can develop resistance to multiple antimicrobial drugs and can facilitate the spread of antimicrobial resistance (AMR) genes through enteric bacterial populations. To further understand AMR dynamics, phenotypic and molecular techniques have been developed to conduct the molecular biology and epidemiological assessments of plasmids.

The dynamics of AMR genes and the mobilome, the collection of mobile genetic elements (MGEs) associated with AMR, could provide insights for identification and verification of emerging multidrug resistance phenotypes in bacterial populations. Additionally, the AMR mobilome facilitates the dissemination of resistance genes encoded in MGEs including plasmids, transposons (Tns), and insertion sequences (ISs). The diversity and distribution of AMR genes and MGEs across the different plasmid replicon types in enteric bacterial pathogens has been demonstrated to be a concern and area for further study.

Technological and bioinformatics advancement in *in silico*-prediction approaches showed the diverse population of resistance genes are prevalent through several plasmid replicon or incompatibility (Inc) types that allow the dissemination of AMR across enteric bacteria. These findings highlighted the value of the assessment of AMR genes and MGEs associated with plasmids to help bolster molecular epidemiology and identify potential ways to improve public health outcomes. One of the drivers of the potential dissemination of AMR is the selective pressure for resistance spread following antimicrobial exposure to drugs such as tetracycline and chloramphenicol. Efforts to quantify how antimicrobial agent exposure can impact the transfer

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of AMR among some *S. enterica* isolates and will provide data on potential improved antibiotic use strategies to minimize resistance spread.

Approaches to assess the conjugation mechanism of *S. enterica* isolates using whole genome sequencing (WGS) is increasingly important, especially as costs continue to drop and analyses techniques improve, as an effective tool to characterize plasmids and AMR dissemination in *Salmonella*. An initial assessment of WGS analyses tools available for plasmids found what was available was limited, with most directed at plasmid identification/classification. There was a gap in tools available to understand the functionality of the genes carried on plasmids, such as the transfer-associated gene sequences that facilitate plasmid transfer among bacteria strains. These tools may help to understand the distribution of plasmids across bacterial populations and augment other available WGS analyses tools for AMR gene identification. Since there was a demonstrated need, databases were developed for the plasmid transfer factor assessment and plasmid transfer factor comparison tools to enhance the evaluation and comparison of plasmids from *S. enterica* and other enteric bacteria.

The data and approaches provided in this dissertation provide valuable information on the AMR mobilome, the distribution of multiple resistance elements across different plasmid types and tools for the analyses of the transfer potential of plasmids in *S. enterica* and related species. These analyses provide valuable resources to carryout experiments quantifying the impact of antimicrobial exposure on the dissemination of resistance plasmids and will assist solving the causes behind the evolution and dissemination of AMR plasmids and improve the understanding of plasmid epidemiology.

CHAPTER ONE

REVIEW PAPER

The Dynamics of the Antimicrobial Resistance Mobilome of *Salmonella enterica* and Related Enteric Bacteria

Suad Algarni[1,2], Steven C. Ricke[3], Steven L. Foley[1,2], and Jing Han[1]

[1]Division of Microbiology, FDA National Center for Toxicological Research, Jefferson, Arkansas, 72079

[2]Cellular and Molecular Biology Graduate Program, University of Arkansas, Fayetteville, Arkansas, 72704

[3]Meat Science and Animal Biologics Discovery Program, Dept. of Animal and Dairy Sciences, University of Wisconsin, Madison, WI 53706

*Corresponding Authors: Dr. Jing Han, Mailing Address: National Center of Toxicological Research, Food and Drug Administration, 3900 NCTR Rd, Jefferson, AR 7209. Phone: (870) 543-7390

Running Title: antimicrobial resistance mobilome of *Salmonella enterica*

Abstract

The foodborne pathogen *Salmonella enterica* is considered a global public health risk. *S. enterica* isolates can develop resistance to several antimicrobial drugs due to the rapid spread of antimicrobial resistance (AMR) genes, thus increasing the impact on hospitalization and treatment costs, as well as the healthcare system. Mobile genetic elements (MGEs) play key roles in the dissemination of AMR genes in *S. enterica* isolates. Multiple phenotypic and molecular techniques have been utilized to better understand the biology and epidemiology of plasmids including DNA sequence analyses, whole genome sequencing, incompatibility typing, and conjugation studies of plasmids from *S. enterica* and related species. Focusing on the dynamics of AMR genes is critical for identification and verification of emerging multidrug resistance. The aim of this review is to highlight the updated knowledge of AMR genes in the mobilome of *Salmonella* and related enteric bacteria. The mobilome is a term defined as all mobile genetic elements, including plasmids, transposons, insertion sequences, gene cassettes, integrons, and resistance islands, that contribute to the potential spread of genes in an organism, including *S. enterica* isolates and related species, which are the focus of this review.

1.1. Introduction to *Salmonella enterica:*

Salmonella enterica is a facultative intracellular microorganism that is one of the more important etiological agents of foodborne illnesses and has a significant impact on global human health. *Salmonella* can be classified, based on the acquisition of *Salmonella* pathogenicity islands (SPIs), into two species: *S. enterica* and *S. bongori* [Majowicz et al., 2010; Moreno Switt et al., 2012]. Annually, more than 90 million human cases of *Salmonella* gastroenteritis are estimated globally [Mather et al., 2013]. S*almonella* infections cost an estimated $3 billion per year in both the European Union and in the United States (U.S.). There are around 1.03 million infections and 400 deaths annually in the U.S. due to mortality, disability, medical and productivity costs, resulting in the loss of about 16,782 quality-adjusted life years [Foley et al., 2013; Hoffmann et al., 2012; Mather et al., 2013].

Salmonella isolates can be differentiated into more than 2,600 serotypes based on their surface antigen profiles. The most common serotypes associated with human infections in the U.S. include Typhimurium, Enteritidis, Newport, Javiana, and Heidelberg [Foley et al., 2013; Foley and Lynne et al., 2008]. These serotypes have shown the ability to infect multiple host species while other serotypes have a narrow host range; for example, *S.* Typhi and *S.* Paratyphi are human-associated serotypes that typically are not associated with foodborne transmission [Foley et al., 2013; Lynne et al., 2009]. According to data from the U.S. Centers for Disease Control and Prevention (CDC) Food Net Program, greater than half of the human infections involving narrow host range serotypes caused invasive infection, rather than gastroenteritis, which leads to more severe disease outcomes [Foley et al., 2013; Vugia et al., 2004].

Over the past several decades, the incidence of multidrug-resistant (MDR) *Salmonella* infections has exhibited a steady rise in many regions including Europe and North America

[Helms et al., 2005] and they have been associated with a number of outbreaks in the U.S. [Nair et al., 2018]. Several studies have indicated that *S. enterica* resistance to antimicrobial drugs is correlated with an increased need for hospitalization, higher risk of invasive illness and deaths, as well as increased treatment costs [Helms et al., 2005; Mather et al., 2013]. Based on epidemiological data, the CDC estimates the incidence of *Salmonella* infections per 100,000 population ranged from 11 to 15 during the years of 1996 to 2011 [Boore et al., 2015]. There were 91,408 confirmed clinical cases of *Salmonella* foodborne illnesses in the European Union in 2014 [Pornsukarom et al., 2018].

1.2. The rise of AMR:

Over the past several decades, antimicrobial agents have been a cornerstone of modern medicine and used extensively in animal production, veterinary medicine and industrial production [Van Schaik et al., 2015]. Antimicrobial therapies target specific aspects of bacterial physiology which selectively impacts the pathogens, while having less deleterious effects on the vertebrate host, but potentially can impact the commensal microbiome of the host [Jernberg et al., 2010]. Antimicrobial agents are categorized based on multiple features involving their structure, ranges of target organisms (spectrum of activity) and mode of action. Most act by specifically binding to their targets, which plays a vital role in bacterial growth and survival, thereby preventing the physiological function of these targets and becoming lethal to the bacterial cells or inhibitory to cell growth [Lambert et al., 2005] The efficacy of an antimicrobial is related largely to its mechanisms of action, which include: (i) the inhibition of cell wall biosynthesis (penicillins and other β-lactams); (ii) protein synthesis inhibition by the targeting of the 16S rRNA (A-site) of the 30S ribosomal subunits (tetracyclines and aminoglycosides) or

prevention of the 50S ribosomal subunit function (macrolides and chloramphenicol); (iii)

inhibition of nucleic acid biosynthesis including inhibition of RNA transcription (rifampicin) or

inhibition of DNA synthesis (quinolones and fluoroquinolones); (iv) inhibition of the metabolic

pathways (including folic acid analogs such as sulfonamides and trimethoprim); and (v) by

damaging the bacterial cell membrane structure (polymyxins) [Bockstael and Van Aerschot,

2009; Sultan et al., 2018; Figure 1. As a result of their therapeutic use, antimicrobial agents have

led to increased human life expectancy and decreased human morbidity and mortality [Van Hoek

et al., 2011].
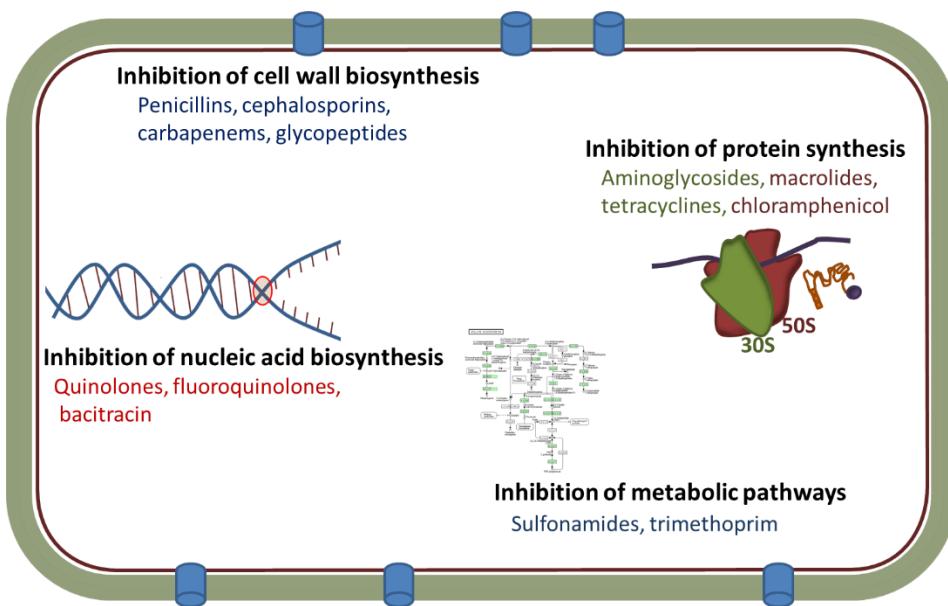
Figure 1. Mechanisms of actions and targets of different antimicrobial agents used in human
and/or veterinary medicine.

However, due to overuse or misuse of the antimicrobials, AMR (a situation in which the

drugs are unable to inhibit bacterial growth and lose the ability to be used for treating a bacterial

infection) has emerged worldwide, and has become a major public health concern [Blair et al.,

2015; Ventola et al., 2015; Zaman et al., 2017]. In the U.S., MDR bacteria infections have led to increased mortality and a strained health care system, with an estimated economic impact of over $20 billion associated with more than two-million infections and approximately 23,000 deaths each year [Blair et al., 2015].

The mechanisms of acquired resistance to antimicrobial agents are broadly classified into three categories: (i) use of energy-dependent efflux pumps to extrude the antimicrobials; (ii) production of hydrolytic or modifying enzymes to inactivate antimicrobials; and (iii) modifying antimicrobial targets [Blanco et al., 2016; Reygaert et al., 2018]; Figure 2. In addition, many Gram-negative bacteria are naturally resistant to some larger antimicrobials, such as vancomycin, due to cell membrane characteristics that prevent entry into the cell and subsequent effectiveness.

Efflux pumps are bacterial transport proteins that are encoded by genes located on the bacterial chromosome and/or plasmids. The efflux pumps function primarily to extrude substrates (antimicrobials) from the cellular interior to the external environment, therefore imparting the efflux pump-expressing bacteria with an AMR phenotype. While some efflux pumps are expressed constitutively, others are induced under certain environmental stimuli or when a suitable substrate is present [Blanco et al., 2016]. Many of these efflux pumps can transport a large variety of compounds (MDR efflux pumps), resulting in the acquisition of MDR by bacteria. In order to transport substrates against a concentration gradient, these efflux pumps are energy-dependent. Based on the type of the energy used, the efflux pumps are broadly classified into two categories, the primary efflux pumps which utilize energy from active hydrolysis of ATP and the secondary efflux pumps which derive energy from chemical gradients formed by either protons or ions such as sodium [Sharma et al., 2019].
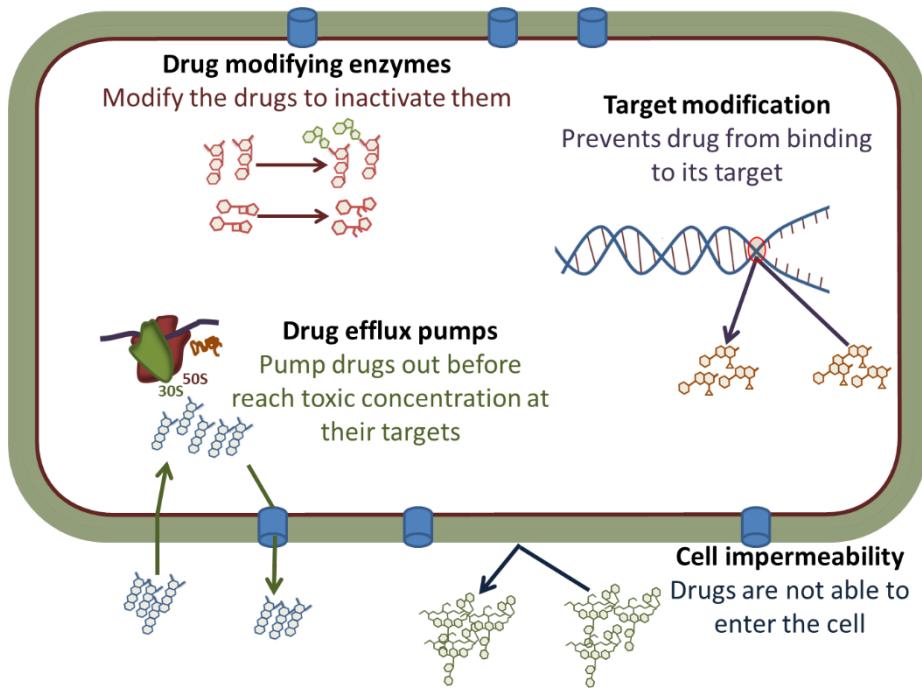
Figure 2. Antimicrobial resistance mechanisms observed in *Salmonella*. The different mechanisms are described in detail in the body of the manuscript. Briefly, drugs such as many aminoglycosides can be modified by the addition of functional groups, while others such as β-lactam antibiotics can be cleaved by enzymes, thus modifying their structures and inhibiting function. Target modification, such as can occur in metabolic pathways or in the nucleic acid replication machinery, can limit the ability of antimicrobials to bind to their targets to prevent efficacy. A number of other drugs are impacted by drug efflux pumps that prevent drugs such as tetracyclines and chloramphenicol from reaching their targets, such as the ribosomes, in a high enough concentration which limits their effectiveness. Other drugs, such as vancomycin, are too large to enter through the Gram-negative cell wall and *Salmonella* are intrinsically resistant due to inability to enter the cell.

In prokaryotes, five major families of efflux pumps have been described: (i) ATP binding cassette (ABC), which are primary active transporters; (ii) small MDR family; (iii) multidrug and toxin extrusion (MATE) family; (iv) major facilitator superfamily (MFS); and (v) resistance

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nodulation cell division (RND) family, which are all secondary active transporters [Sharma et al., 2019]. Based on sequence similarity, the nine functional drug efflux pumps identified in *S. enterica* (AcrAB, AcrD, AcrEF, MdtABC, MdsAB, EmrAB, MdfA, MdtK, and MacAB) either belong to the MFS family (EmrAB and MdfA), the RND family (AcrAB, AcrD, AcrEF, MdtABC, and MdsAB), the MATE family (MdtK); or the ABC family (MacAB), respectively [Horiyama et al., 2010]. Among these efflux pumps, the AcrAB-TolC efflux pump (a tripartite complex consisting of a periplasmic membrane fusion protein AcrA, a cytoplasmic membrane transporter protein AcrB, and an outer membrane channel TolC) is the most effective in causing MDR *Salmonella* and has been shown to directly contribute to resistance to fluoroquinolones, chloramphenicol, and tetracyclines in *Salmonella* [Horiyama et al., 2010; Piddock et al., 2006; Shen et al., 2017]. The OqxAB efflux pump also mediates MDR in various bacteria, including *Salmonella* [Aljahdali et al., 2019]. It belongs to the RND family and significantly contributes to reduced susceptibility to olaquindox, nalidixic acid, tigecycline, nitrofurantoin, chloramphenicol, and facilitates the development of high-level fluoroquinolone resistance [Aljahdali et al., 2019; Li et al., 2013]. The *oqxAB* gene is located either on the chromosomal DNA of *Salmonella* [Wong and Chen et al., 2013] or on plasmids with other AMR genes [Li et al., 2013; Wong et al., 2016]. The carriage of *oqxAB* on transferable plasmids would facilitate its transmission via horizontal gene transfer and the emergence of MDR strains [Aljahdali et al., 2019; Wong et al., 2016]. Plasmids from the incompatibility group (Inc) HI2 have been shown to play a pivotal role in dissemination of *oqxAB* in *Salmonella* spp. [Wong et al., 2016].

The resistance to antimicrobials that target the ribosomal subunits interferes with the ability to bind to the ribosome. This binding interference is due to ribosomal mutation (aminoglycosides and oxazolidinones), ribosomal subunit methylation (aminoglycosides,

macrolides, oxazolidinones, and streptogramins) most commonly involving *erm* genes, or ribosomal protection (tetracyclines) [Reygaert et al., 2018]. The tetracycline family of antimicrobials inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) and is widely used because it offers a broad spectrum of activity against Gram-positive and Gram-negative bacteria. However, the increasing incidence of resistance to tetracyclines in *Salmonella* spp. of human and animal origins has been reported worldwide. In addition to efflux pump-mediated resistance mechanisms, resistance to tetracyclines can involve ribosomal protection proteins (RPPs), which are a group of cytoplasmic proteins that can bind to the ribosome, resulting in the release of tetracycline from the ribosome, enabling protein synthesis to proceed [Warburton et al., 2016]. To date, 12 tetracycline resistance genes encoding RPPs have been reported, including *tet*M, O, Q, S, T, W, 32, 36, 44, *B*(P), *otr*(A), and *tet* [Warburton et al., 2016]. Many of the RPP determinants are located on mobile genetic elements within *Salmonella*, including transposons or plasmids, which may have facilitated their spread throughout the eubacteria via lateral gene transfer events [Gargano et al., 2021]. Another example of modification of antimicrobial targets is the wide dissemination of the plasmid-encoded chloramphenicol-florfenicol resistance (cfr) methyltransferase, which specifically methylates the adenine at position 2503 in the 23S rRNA, thereby conferring resistance to a wide range of ribosome-targeted antimicrobials, including the phenicols, streptogramins, macrolides, and oxazolidinones (such as linezolid) [Kaminska et al., 2009].

For antimicrobials that target nucleic acid synthesis (for example, fluoroquinolones), resistance is primarily associated with chromosomal mutations in the bacterial genes encoding targeted enzymes, DNA gyrase (such as *gyrA* in Gram-negative bacteria, including *Salmonella*) or topoisomerase IV (such as *grlA* in Gram-positive bacteria or *parC* in Gram-negative bacteria).

Many of these mutations occur in the quinolone resistance determining region (QRDR) of the gyrase and topoisomerase genes and cause changes in the structure of the respective proteins that decreases or eliminates the ability of the antimicrobials to bind to the enzymes [Lee et al., 2021]. The emergence of fluoroquinolone-resistant *Salmonella* has resulted in treatment failure and high mortality rates [Lee et al., 2021].

Antimicrobials such as the sulfonamides and trimethoprim act as competitive inhibitors of essential steps in the folate biosynthetic pathway in bacteria. These drugs are structural analogs of the natural substrates (sulfonamides for p-amino-benzoic acid and trimethoprim for dihydrofolate) and bind to their respective enzymes through competitive inhibition by binding to the active site of the enzymes dihydropteroate synthase and dihydrofolate reductase, respectively. Resistance to these antimicrobials that inhibit metabolic pathways can be due to mutations in enzymes, such as dihydropteroate synthase or dihydrofolate reductase, that prevent binding of the competitive inhibitor [Reygaert et al., 2018]. Mutations in these enzymes at or near the active site result in structural changes in the enzyme that interfere with antimicrobial binding while still allowing the natural substrate to bind [Reygaert et al., 2018]. Additionally, overproduction of dihydrofolate reductase or dihydropteroate synthase can limit the competitive impact of sulfonamides and trimethoprim on the pathway function [Reygaert et al., 2018].

Antimicrobial inactivation by enzymes is another critical resistance mechanism. There are two main ways in which bacteria inactivate drugs, these include actual degradation of the drug or by the transfer of a chemical group (most commonly acetyl, phosphoryl, and adenyl groups) to the antimicrobials altering their function [Reygaert et al., 2018]. The β-lactamases are a very large group of drug hydrolyzing enzymes that inactivate β-lactam drugs (including penicillin, ampicillin, cephalosporins, carbapenems, and ceftazidime) by hydrolyzing a specific

site in the β-lactam ring structure, causing the ring to open, resulting in the inability of the antimicrobials to bind to their target penicillin-binding protein (PBP). To date, more than 1,300 distinct β-lactamases have been identified in clinical isolates, of which the most deleterious are the extended-spectrum β-lactamases (ESBLs) that hydrolyze most penicillins, cephalosporins, and the carbapenemases that can inactivate all β-lactam classes of drugs [Bush et al, 2013]. The production of β-lactamases, which can be encoded by genes located on the chromosome (such as bla_{SHV-12}, $bla_{CTX-M-9a}$, and bla_{MIR}) or on plasmids (for example, bla_{TEM} or $bla_{CTX-M-15}$), is the most common resistance mechanism used by Gram-negative bacteria against β-lactam drugs [Bush and Bradford, 2016; Reygaert et al., 2018]. Combined with decreased uptake or increased efflux of the drugs, resistance to the β-lactams in Gram-negative bacteria continues to be on the rise with the high-level resistance being a major clinical problem [Bush and Bradford, 2016].

Inactivation by enzymatic modification is also the most prevalent mechanism of resistance to aminoglycoside antimicrobials, which is currently considered to be one of the more formidable broad-spectrum antimicrobials used in the treatment of life-threatening infections in the clinical setting [Lalitha Aishwarya et al., 2020; Ramirez and Tolmasky, 2010]. Aminoglycoside-modifying enzymes (AMEs) are the most common cause of resistance to aminoglycosides [Ramirez and Tolmasky, 2010]. AMEs catalyze the covalent modification of aminoglycosides as they transport across the cytoplasmic membrane by modifying the amino or hydroxyl groups. There are three kinds of AMEs: 1) N-acetyltransferases (AAC), which acetylate the –NH2 (amino) group by N-acetylation; 2) O-nucleotidyltranferases (ANT), which adenylate the hydroxyl groups by O-nucleotidylation; and 3) O-phosphotranferases (APH), which phosphorylate the hydroxyl groups by O-phosphorylation [Ramirez and Tolmasky, 2010]. The modifications of the aminoglycoside reduces drug binding to the ribosome, which results in high

levels of resistance. The genes coding for AMEs are highly mobile with the ability to transfer as part of integrons, gene cassettes, transposons, integrative conjugative elements, or through conjugation as part of mobilizable or conjugative plasmids [Ramirez and Tolmasky, 2010]. To date, more than 85 AMEs have been reported in both Gram-positive and -negative bacteria [Lalitha Aishwarya et al., 2020]. The combination of large numbers, the ability of the genes coding for these enzymes to evolve, as well as the numerous mobile elements where they are located, result in a high adaptability AMEs to efficiently disseminate among bacteria, which has greatly reduced the efficacy of several aminoglycosides [Ramirez and Tolmasky, 2010]. Tetracyclines can also be inactivated by hydrolyzation via the TetX enzyme, whose gene is located on plasmid that catalyzes tetracycline degradation [Moore et al., 2005].

Antimicrobial inactivation by the transfer of a chemical group is another key resistance challenge, as a large number of transferases that have been identified contribute to resistance. Among them, acetylation is the most diversely used mechanism and has led to resistance against aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones [Reygaert et al., 2018]. The primary resistance mechanism for chloramphenicol is the enzymatic inactivation by acetylation of the antimicrobial drug molecule via different types of chloramphenicol acetyltransferases (CATs) [Schwarz et al., 2004]. There are two different types of CAT enzymes which are genetically unrelated and encoded by *catA* and *catB* groups, respectively [Roberts and Schwarz, 2017]. Both *catA* and *catB* genes are often associated with mobile elements such as plasmids, transposons, or gene cassettes and are able to be transferred between bacteria of different species and genera [Roberts and Schwarz, 2017]. In *Salmonella,* besides mutations in the QRDR and plasmid-mediated quinolone resistance (PMQR) determinants *qnr* genes; *aac(6')-*

Ib-cr encodes acetyltransferase which can acetylate the quinolone antimicrobials that reduces their activities and contribute to quinolone resistance [Robicsek et al., 2006].

The detection of AMR genes in bacteria has mainly relied on molecular methods. Due to their rapidity and sensitivity, nucleic acid-based detection methods have played an important role in the elucidation of resistance mechanisms [Fluit et al., 2001]. Based on the amplification of target genes, traditional PCR, multiplex PCR, qPCR (real-time PCR), RT-PCR (Reverse Transcriptase PCR), approaches have been developed and widely used in the laboratory to rapidly identify AMR genes [Galhano et al., 2021]. DNA microarray and hybridization approaches have also been used to simultaneously detect a large number of different AMR genes in a short time. Microarrays for resistance detection in both Gram-negative and Gram-positive bacteria have been developed [Galhano et al., 2021; Card et al., 2013; Perreten et al., 2005]. Microarray detection methods have been largely replaced by whole-genome sequencing (WGS) approaches that have become widely used and effective tools in detection of AMR genes [Galhano et al., 2021]. To detect AMR genes in WGS data, several AMR gene databases, such as ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation), RGI/CARD (Comprehensive Antibiotic Resistance Database), NCBI-AMRFinder, and PointFinder have been developed to identify AMR genes in the WGS contigs [Alcock et al., 2020; Feldgarden et al., 2021; Gupta et al., 2014; Zankari et al., 2017]. WGS has been proven to be an effective technique to detect AMR genes within bacteria such as *Salmonella* [Galhano et al., 2021].

1.3. Mobilome among *S. enterica* isolates:

The term "mobilome" refers to all mobile genetic elements (MGEs) and genes including plasmids, integrons, transposons, insertion sequences (ISs), integrative and conjugative elements

(ICEs), and resistance islands and/or genomic islands in bacteria which can translocate within and transfer between genomes [Cabezón et al., 2017; Frost et al., 2005]. MGEs play a key role in both the development and spread of AMR genes among the bacterial populations as well as in the evolution of bacterial genomes which rapidly respond to a selective pressure. These responses include alteration of antimicrobial exposure in different genera, species, and strains, including those of *Salmonella* isolates [Davies and Davies, 2010].

1.4. The mobility of the AMR genes through MGEs:

1.4.1. Plasmids

Understanding the transfer dynamics of AMR plasmids requires detailed monitoring of different pathogenic bacteria in both clinical and non-clinical environments. Plasmid-mediated transfer plays a critical role in the spread of AMR in *S. enterica* and related species [Han et al., 2012; San Millan, 2018; San Millan et al., 2015]. Several studies and review articles have described the importance of plasmid dynamics in Gram-negative bacteria, in particular *S. enterica*, and many have utilized DNA sequencing to help examine resistance transfer [Han et al., 2012; Lerminiaux and Cameron, 2019; McMillan et al., 2019; Zhao et al., 2003]. Baker et al. (2018) highlights the importance of whole genome sequencing (WGS) as a key technology for understanding the spatial dynamics of AMR evolution and for genomic epidemiology [Baker et al., 2018]. Tables 1 and 2 present multiple sequencing platforms and computational-based approaches that can be used for the detection of plasmid-based AMR genes.

Table 1. Approaches to detect plasmid-based antimicrobial resistance genes.

Approach	Sample types	Advantage	Limitation	References
Illumina-based WGS	Food/Clinical samples	Fast, applicable for studying the genetic diversity evolutionary history of plasmid type and epidemiological tracking.	Challenge for plasmid detection in short read length.	[Almeida et al., 2018]
Oxford Nanopore Technology (ONT)	Food/Clinical samples	Rapid tool Low instrument Cost and small footprint	Incomplete fragmentation of plasmid. High error rate. Performance often needs to be optimized with MiSeq reads.	[Lemon et al., 2017]
Pacific Bioscience single-molecule real-time sequencing (SMRT)	Food/Clinical samples	Generate long-read sequencing data that can lead to closed plasmid sequences.	The reads length of data relies on the individual polymerase.	[Chen et al., 2019]

Table 2. *In silico* analysis of resistance plasmid gene content and epidemiology.

Web Server	Advantages	Links	Ref
PLSDB	Used for short sequences to identify the specific AB genes in the plasmids.	https://ccb-microbe.cs.uni-saarland.de/plsdb/	[Galata et al., 2019]
ATLAS	Detect the plasmids carrying the antimicrobials resistance genes, and plasmid network.	http://www.patlas.site	[Jesus et al., 2019]
PlasmidSeeker	Able to identify the novel plasmids and detect plasmids from bacterial WGS data without read assembly.	https://github.com/bioinfo-ut/PlasmidSeeker	[Roosaare et al., 2018]
FeatureExtract	Able to identify the antimicrobial resistance genes and conjugal transfer system (IV).	https://cbsd-budk/servies/FeatureExtract	[Wernersson et al., 2005]

In studies of MDR plasmid transfer in different *Salmonella* strains*,* isolates have been sequenced and diverse AMR genes associated with chloramphenicol, tetracyclines, ampicillin, streptomycin, kanamycin, and β-lactam antimicrobials have been identified [Han et al., 2012]. When the rate of horizontal transfer outweighs the costs they impose on their bacterial hosts, plasmids are favored in the new hosts [Svara and Rankin, 2011]. Stalder et al. (2017) reported that common mechanisms of plasmid stabilization in bacterial pathogens could influence plasmid-host coevolutionary dynamics. Various concentrations of antimicrobials are encountered by bacteria in different environments and may affect the dynamics of the microbial population. For example, plasmid transfer between bacteria may lead to acquired resistance to different antimicrobials through the transfer of AMR determinants in response to selective pressure (Martinez, 2008). Svara and Rankin (2011) designed a model to examine the effect of antimicrobial treatment, specifically the dosage of antimicrobials and the interval between treatments, on the evolution of plasmid-borne resistance. Their results showed that different treatment regimens (different interval between antimicrobial treatments and the dosage of antimicrobials) can select for either plasmid-carried or chromosome-carried resistance. In the absence of competing non-resistant plasmids, high transmission favors plasmid-borne resistance. While in the presence of other plasmids, plasmid-borne resistance was favored over chromosomal resistance in the low frequency antimicrobial treatments, a high intensity of antimicrobial treatments is required for resistance plasmids to outcompete non-resistance plasmids [Svara and Rankin, 2011]. Therefore, changes in natural ecosystems, including the release of large amounts of antimicrobials, might alter the population dynamics of antimicrobial resistant microorganisms, which will affect the evolution and dissemination of AMR in nature

[Martinez et al., 2008]. Taken together, these findings emphasize the necessity of new therapeutics to reduce the spread of AMR.

According to their transmissibility by conjugation, there are two classes of transmissible plasmids: 1) conjugative plasmids, which contain a full set of conjugation genes; and 2) mobilizable plasmids, which contain only a minimal set of genes that allow them to be mobilized by conjugation when they coexist in the same donor cell with a conjugative plasmid [Garcillan-Barcia et al., 2011]. Conjugative plasmids possess a generally low copy number, while mobilizable plasmids tend to be a high copy number [Van Hoek et al., 2011; Watve et al., 2010]. Both conjugative and mobilizable plasmids can encode AMR genes and transfer them to a new host [Van Hoek et al., 2011]. Over the last decade, more than 1,000 unique plasmids have been identified in the family of *Enterobacteriaceae*, some of the most common ones include IncA/C, HI2, HI1, I1-γ, X, L/M, N, FIA, FIB, FIC, W, Y, P, T, K, B/O, FI1, U, R, ColE, and Q [Garcia-Fernandez and Carattoli, 2010]. Among these, some Inc types can be clustered into groups based on the genetic and pilus structure: 1) IncF group (consisting of IncF, D, J, and S); 2) IncI group (includes IncB, I, and K); 3) IncP group (containing IncM, P, U, and W); and Ti (tumor inducing) that may have implications on plasmid transfer dynamics [Van Hoek et al., 2011]. Specifically, many of the plasmids that have been identified in *Salmonella* strains include representatives of IncA/C, F, H, I, L/M, N, R and X groups [Foley et al., 2013; Fricke et al., 2009].

In the 1970s, IncA/C plasmids were initially described as large-molecular-weight (140 to 200 kb) and low copy number plasmids present in fish pathogens such as *A. hydrophila* and *Vibrio* spp. [Carraro et al., 2015; Ruggiero et al., 2018]. Subsequently, IncA/C plasmids were found to encode resistance genes to antimicrobial agents in food animals and agricultural settings

[Johnson et al., 2012]. To date, research on conjugative IncA/C plasmids has determined that these plasmids play an essential role in the spread of MDR among the species of *Enterobacteriaceae,* including *S. enterica* and *V. cholerae*, which is an important concern in the public health community [Carraro et al., 2015; Han et al., 2018]. The core genetic backbones of IncA/C plasmids are generally highly conserved, with greater than 98% nucleotide identity across the plasmid backbone. Most examples of IncA/C plasmids among non-typhoidal *S. enterica* isolates in the U.S. carry transfer-associated genes which accelerate conjugal transfer [Fricke et al., 2009; Han et al., 2018; Ruggiero et al., 2018; Welch et al., 2007; Wiesner et al., 2011]. Wiesner et al. (2011) described a MDR *S.* Typhimurium ST213 strain in Mexico which was associated with the carriage of IncA/C plasmids that carried a plasmid-borne bla_{CMY-2} gene encoding resistance to extended-spectrum cephalosporins. As predicted by Han et al. (2018), many IncA/C plasmids encode their own conjugal machinery and are able to be transferred within bacterial communities. However, others without the full cohort of transfer genes required other conjugative plasmids in the bacterial cell for transfer of the plasmids. Rankin et al. (2002) indicated that IncA/C plasmids were responsible for MDR phenotypes in *S.* Newport isolates and their resistance genes could be transferred both to other *Salmonella* as well as other susceptible organisms under antimicrobial selection pressure in the same environments. In *S. enterica* serovar Newport isolates, IncA/C plasmids emerged as the source of MDR phenotypes after 1980 [Johnson et al., 2012]. Moreover, these plasmids are considered as broad host range plasmids due to their ability to spread by conjugative transfer among the bacterial communities, meanwhile under *in vitro* conditions some IncA/C plasmids exhibit variation in their ability to transfer between *E. coli* and *S. enterica* [Johnson et al., 2012]. In addition to their presence in food animal environments, cases have also been documented of plasmids spreading in hospital

environments [Johnson et al., 2012]. In some of these studies it was demonstrated that IncA/C plasmids have mobilizable genes conferring resistance to a number of different antimicrobial agents including β-lactams, aminoglycosides, chloramphenicol, sulfonamide, trimethoprim, quinolones, and tetracyclines [Carraro et al., 2015]. These studies have been somewhat hampered by the presence of a large number of AMR elements carried on the plasmids. These AMR elements have been a challenge for studying their basic molecular biology and regulatory mechanisms because most of the molecular biology tools that are available rely on the antimicrobial selection for mutational studies [Carraro et al., 2015; Johnson et al., 2012].

1.4.2. Conjugative plasmids

One of the main mechanisms of the horizontal gene transfer is bacterial conjugation [Cabezón et al., 2017; Frost et al., 2005]. Bacterial conjugation is a highly specific process that occurs by transferring plasmid DNA from one bacterial cell (donor) to another bacterium (recipient) through a direct cell-to-cell contact [Partridge et al., 2018; Willetts and Wilkins, 1984]. Conjugative plasmids are considered to be self-transmissible and carry the genes, known as MOB (mobilization) or Dtr (DNA-transfer replication) genes, that are required for DNA transfer and mating-pair apparatus formation to initiate the transfer process at OriT [Jain and Srivastava, 2013]. The MOB functionality has also been used for the identification, classification and typing of plasmids and epidemiological tracking based on the conjugative and mobilization relaxase genes [Partridge et al., 2018]. The fundamental principle of the bacterial conjugation system involves the merging of the two archaic bacterial systems: rolling-circle replication (RCR) and the type IV secretion system (T4SS) [Llosa et al., 2002]. The T4SS forms a complex protein encoded by numerous genes called mating pore formation genes (MPF) [Cabezón et al.,

2017]. In the conjugation machinery, RCR has a similar function to a relaxosome, which initiates transfer through the T4SS [Llosa et al., 2002]. Howard et al. (1995) demonstrated that the two proteins (TraYp and TraLp) assemble the relaxosome site and bind at the origin of the transfer which causes a strand-specific nick found within conjugation machinery in the *E. coli* F plasmid, . There are two basic regions that contribute to conjugation machinery: Tral, also known as TrhX, which carries OriT, relaxosome genes and some MPF components; and Tra2, or TrhZ, which consists of genes encoding MPF proteins [Partridge et al., 2018]. It has been reported that the evolution of conjugation mobility systems, with their relaxases and type IV coupling protein (T4CPs) diverged from the prototypical T4SS [Smillie et al., 2010]. Studies in our lab demonstrated that some *Salmonella* strains with plasmids carrying a VirB/D4 T4SS survived better in epithelial cells and macrophages than those without the plasmid [Gokulan et al., 2013].

1.4.3. Mobilizable plasmids

Unlike conjugative plasmids which are self-transmissible, mobilizable plasmids lack the necessary genes for complete conjugation and are therefore not self-transmissible [Francia et al., 2004]. Mobilizable plasmids have an origin of transfer site (*oriT*), a region essential for replication, and a mobilization gene (*mob*). They have an ability to exploit conjugative plasmids for horizontal dissemination, but are non-mobile in cells that lack mobile elements carrying compatible mating-pore genes [Ramsay et al., 2016]. Like conjugative plasmids, mobilizable plasmids in *Salmonella* also can carry AMR genes.

The IncQ1 plasmids are examples of mobilizable plasmids. They are small in size (10 to 12 kb) and have broad host range [McMillan et al., 2020]. Generally, they are associated with AMR genes including *strAB*, *tetAR*, and *sul2*, and can be mobilized by large plasmids including the

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IncF, I1, N, P, W, or X plasmids [Francia et al., 2004; McMillan et al., 2020]. Baker et al. (2008) demonstrated that a small region in *Salmonella* pathogenicity island 7 (SPI-7) in *S.* Typhi was able to mobilize IncQ plasmid R300B [Baker et al., 2008]. Several examples of the the IncQ plasmid (MoB$_Q$) in different *Salmonella* strains are presented in Table 3. Other examples of mobilizable plasmids are the IncR plasmids (40 to 160 kb), which were first identified in 2009 from *Klebsiella* and *Salmonella* Montevideo [Garcia-Fernandez et al., 2009]. IncR plasmids have been reported to carry genes conferring resistance to antimicrobials belonging to numerous classes including: β-lactams, sulphonamides, quinolones, aminoglycosides, tetracyclines, chloramphenicol, and trimethoprim [Plumb. et al., 2019; Rozwandowicz et al., 2018].

Table 3. Examples of mobilizable IncQ plasmids identified in *S. enterica* strains.

Plasmid names	Antimicrobial resistance genes	Size (bp)	*S. enterica* serotypes	Ref
IncQ1	*sul2, strAB, tetAR*	10,867 bp	Reading	[Miller et al., 2020]
C0144011-and C01RNA1-like	*bla$_{TEM-1C}$*	10,384 bp	Reading	[Miller et al., 2020]
P28321a-1	*aph(3')-Ia, aph(6)-Id, bla$_{TEM-1}$, aph(3'')-Ib, sul2, tetB, aac(3)-IId*	219,745 bp	Typhimurium	[Zhao et al., 2020]
PSTU288-2	*sulII, strA, strB, tetA, cat*	11,067 bp	Typhimurium	[Hooton et al., 2014]

1.4.4. Integrons

Integrons are mobile genetic elements containing a site-recombination system that is able to integrate, express, and exchange specific DNA known as gene cassettes [Ravi et al., 2014; Silva et al., 2012]. Gene cassettes are defined as the smallest mobile elements with an AMR gene; they are located between two recombination sites (*attI* and *attC* 59-base elements) [Gillings, 2014; Rodriguez et al., 2006]. Generally, integrons contain three important major elements: (1) the

integrase, which is a gene encoding integration into the host genetic backbone, (2) the recombination site, which serves as the primary site to capture the resistance gene cassettes, and (3) the promoter. Each of these elements is required for transcription and drives expression of gene cassettes [Gillings, 2014; Rodriguez et al., 2006; Silva et al., 2012]. Integrons are divided into two different types: (1) mobile integrons (MIS), which carry a limited number of AMR gene cassettes (these include the class I integron) and are involved in the dissemination of AMR; and (2) chromosomal integrons (CIS), which have a different number of gene cassettes and are not involved in the dissemination of AMR [Domingues et al., 2012]. Moreover, integrons are found in a wide diversity of clinical bacterial strains and environmental isolates in both chromosomes and within MGE [Gillings, 2013]. The first discovered chromosomally -located superintegrons were identified in *V. cholerae* isolates [Ravi et al., 2014]. These superintegrons are located on the chromosome and typically carry more than 20 gene cassettes with unknown function [Ravi et al., 2014]. Integrons are immobile on their own and yet have been observed to transfer across numerous bacterial genomes [Gillings et al., 2014]. Integrons are divided into five classes based on the type of gene capture mechanism, gene cassettes, and the similarity of sequence which encodes integrases to facilitate insertion of the target DNA [Gillings et al, 2013, 2014; Ravi et al., 2014]. Each of the five different classes of integrons (class I-V) encodes a distinct integrase gene. The class I integrons play an important role in the spread of AMR genes and are the most-studied types from clinical samples [Gillings et al., 2013]. They are referred to as broad host range as they have been found both in commensal and pathogenic bacteria [Ravi et al., 2014] and are widely found across different *S. enterica* isolates [Rodriguez et al., 2006].

Recent comparative studies described how integrons contribute to MDR phenotypes [Deng et al., 2015; Gillings, 2014; Ravi et al., 2014]. The integron location within MGEs and the

chromosome has a unique advantage for generating genomic and phenotypic diversity [Gillings et al., 2014]. Three different classes of mobile integrons (class I, II, and III) can contribute to the MDR phenotypes [Mazel et al., 2006]. For example, class I and class II integrons are associated with the bacteria from animals and have an impact on the human gastrointestinal microbiota by transmission of AMR through the food supply. The horizontal transfer of class I integrons to commensal organisms via conjugation facilitates the development and spread of resistant bacteria [De Paula et al., 2018; Miller and Harbottle, 2018]. Class I integrons can also play a key role in the transmission of AMR in the aquatic environment and in fish pathogens, as well as in terrestrial pathogens such as *S. enterica*, *E. coli,* and other bacteria species [Miller and Harbottle, 2018]. Evershed et al. (2009) discovered IncA/C plasmids, which play an important role in the dissemination of AMR genes, have an unusual cassette in their class I integrons, with most carrying the same set of resistance genes including: *aacU, aphA, hph, sul2,* and *tetA*(D). Similar integrons have also been identified in *S. enterica* serovars Senftenberg and Ohio. IncA/C plasmids have a broad host range, having been recovered from numerous species, their conjugal transfer capacities are variable [Evershed et al., 2009; Wiesner et al., 2013].

Moreover, Villa and Carattoli (2005) reported that some plasmids in MDR *S.* Typhimurium strains carry *sprC, rck,* and *pefA* virulence genes, in addition to two class I integrons carrying AMR genes within the Tn22 and Tn1696 transposons. Thus, it was shown that the association between MDR and virulence determinants may contribute to virulence plasmid evolution [Villa and Carattoli et al., 2005]. Likewise, Rodriguez et al. (2006) reported that the class II integrons are present on self-transferable plasmids of *Salmonella*. It is well known, that the diversity of integrons plays a critical role in the spread of AMR genes and can drive evolution in *S. enterica*. Several integrons (both class I and class II) have been detected in clinical or environment isolates

of *Salmonella* [Yeh et al., 2018] and other *Enterobacteriaceae* from different geographical locations [Adelowo et al., 2018; Gomi et al., 2018; Leungtongkam et al., 2018; Phoon et al., 2018].

1.4.5. Transposons

Transposons,commonly referred to as "jumping gene systems", provide functionality for the transfer of a segment of bacterial DNA to and from chromosomal or plasmid DNA and often carry AMR genes [Bennett et al., 2008; Giedraitiene et al., 2011; Sultan et al., 2018]. Transposons are made up of a transposase and inverted repeat sequences on their ends. The inverted repeat sequences can accelerate target recognition and recombination; while the transposase catalyzes the movement of DNA segments to another part of the genome by a cut-and-paste mechanism, or by a replicative transposition mechanism, and functional genes (such as AMR genes) [Giedraitiene et al., 2011]. Furthermore, transposable elements (TEs) consist of a set of mobile elements that include small cryptic elements, IS elements, transposons, and transposing bacteriophages. The bacteriophage is a bacterial virus, which is also known as a transposing phage, that infects and replicates its own sequences within bacteria through either a lysogenic or lytic cycle [Balcazaret al., 2014]. In the lysogenic cycle, the phage is referred to as temperate or non-virulent, and occurs following the injection of bacteriophage DNA into the bacteria cell. During this phase the bacteriophage integrates its genome into the host genome via the phage-encoded integrases to form prophage where it replicates passively as part of the host genome. As the phage excises from the host chromosome, it may transport bacterial genes from one bacteria strain to another via a process called transduction [Bennett et al., 2008; Giedraitiene et al., 2011]. In the lytic cycle, also referred to as virulent infection, immediately after injecting

into the host cell, the bacteriophage genome synthesizes what are termed early proteins instead

of integrating its DNA into the host genome. These early proteins function to break down the

host DNA allowing the bacteriophage to control the host's cellular machinery to synthesize the

proteins required to build new phage particles. Eventually, the destruction of the infected

bacteria cell results in the release of the new phage progeny. Transduction mediated by

temperate bacteriophages can serve as a horizontal gene transfer mechanisms and has played an

important role in the dissemination of AMR genes among bacterial populations [Bennett et al.,

2008; Colavecchio et al., 2017; Giedraitiene et al., 2011]. Multiple bacteriophage and prophages

have been reported in *Salmonella* strains, such as Fels-1, Gifsy-2, P22, FelixO1[Colavecchio et

al., 2017; Moreno Switt et al., 2013]. Among them, *Salmonella* phage P22 has been widely used

in molecular biology for its ability to introduce foreign genes, including AMR genes into

recipient cells by transduction [Moreno Switt et al., 2013]. Since its emergence in the 1990s, the

MDR ACSSuT-type (resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and

tetracycline) *S.* Typhimurium phage type DT104, has become widely distributed worldwide.

Studies showed that the ACSSuT-type region of DT104 was derived from two separate

evolutionary events. One was the integration of a 43-kb *Salmonella* genomic island (SGI) 1 that

carries multiple AMR genes and the other is the integration of a P22-like phage into the

chromosome to form prophage PDT17 or ST104 [Chiu et al., 2006]. More information about the

contribution of bacteriophages to the spread of AMR genes among foodborne pathogens can be

found in a review article wrote by Colavecchio et al. (2017).

   The sizes of transposon sequences range from 3 to 40 kb depending on the variability of the

genes present in the transposons [Pagano et al., 2016]. Figure 3 shows a representative

transposon (Tn21) that is located within an IncA/C plasmid and carries a class 1 integron as well

as multiple antimicrobial and disinfectant resistance genes [Han et al., 2012]. There are two major types of TEs: (1) composite transposons (class I) and (2) complex transposons (class II). Class I transposons contain a variety of resistance genes and have little DNA homology in their central regions, which are flanked by IS elements [Pagano et al., 2016; Sultan et al., 2018]. In *S. enterica* serovars, such as Typhimurium and Choleraesuis, class I transposons often reside in conjugative plasmids, such as IncHI2, and are responsible for AMR gene dissemination [Sultan et al., 2018]. In contrast, class II transposons, constituting of three dissimilar, but interrelated families (Tn3, Tn21 and Tn2501), have more diverse genetic structures than class I transposons [Pagano et al., 2016; Sultan et al., 2018]. A wide diversity of class I transposons (for example, Tn1696, Tn10, Tn6088, Tn3, Tn9, and Tn1721-like transposon) carrying a variety of different AMR genes have been identified in *Salmonella* serovar [Cain and Hall, 2012; Cain et al., 2010; Chen et al., 2020; Frech and Schwarz, 1999; Orman et al., 2002]. The TEs that are transferred include: (i) the integrative and conjugative elements (ICEs), (ii) the integrative and mobilizable elements (IMEs), and (iii) decayed elements derived from ICEs or IMEs, such as cis-mobilizable elements (CIMEs) [Lao et al., 2020]. CIMEs are decayed elements originating from ICEs or IMEs that have lost their integration and transfer genes, but retained *attL* and *attR* sites [Guedon et al., 2017]. The CIMEs are known to be major vehicles for acquisition of a broad spectrum of AMR genes among bacteria and many other genes that can be advantageous for their hosts [Guedon et al., 2017]. The recombination between related or unrelated ICEs, IMEs, and CIMEs, likely integrated in tandem, plays a major role in the evolution and plasticity of ICEs and IMEs [Bellanger et al., 2014; Lao et al., 2020].
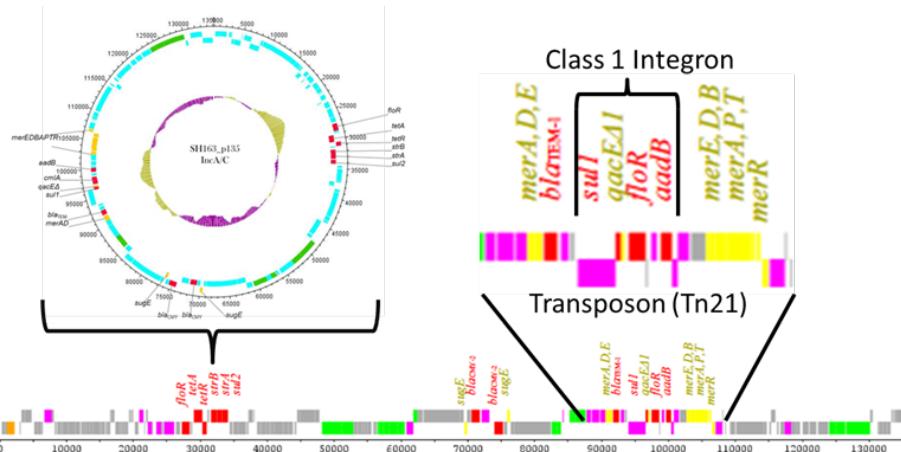
Figure 3. Results of the sequencing of a transmissible antimicrobial resistance plasmid from a *S. enterica* strain [Han et al., 2012]. The plasmid contains multiple antimicrobial resistance (denoted in red) and heavy metal/disinfectant (denoted in yellow) resistance genes, including those carried in a Tn21 transposon. Within the Tn21 transposon, there was a class 1 integron carrying multiple antimicrobial resistance genes. This nesting of MGEs within other MGEs provides significant opportunities for the generation and dissemination of diverse antimicrobial resistance elements. AMR regions and mobile genetic elements also often have a higher G+C content compared to the plasmid backbone as is highlighted in the inner ring of the circular plasmid diagram (gold vs. purple bars).

1.4.6. Integrative and conjugative elements (ICEs)

ICEs, also known as conjugative transposons, are genetic elements that are self-transmissible between chromosomes of different cells and are widely distributed among both Gram-negative and -positive bacteria [Johnson and Grossman, 2015; Seth-Smith et al., 2012; Van Hoek et al., 2011]. Contrary to conjugative plasmids which are extrachromosomal DNA elements within a cell that are physically separated from the host chromosome and replicate independently, ICEs are typically found integrated in the host bacterial chromosome [Johnson and Grossman, 2015]. They contain genes required for their integration and excision, through which they can excise

from the host chromosome to form a circular product prior to conjugal transfer [Johnson and Grossman, 2015]. Normally, the repression of transcription prevents the expression of the excision and conjugation genes, and the ICEs are maintained as inert elements in the host chromosome [Johnson and Grossman, 2015]. The de-repression/activation of expression of ICE genes under certain conditions lead to the induction of ICE genes, which in turn leads to their excision from the donor chromosome, formation of a circular intermediate, transfer by conjugation, and reintegrated into the recipient chromosome [Johnson and Grossman, 2015]. In addition to an integrase and excisionase genes, ICEs also encode a functional type IV secretion system, which facilitates the conjugative transfer [Johnson and Grossman, 2015]. While integrated in the chromosome, ICEs are passively propagated during chromosomal replication, segregation, and cell division [Johnson and Grossman, 2015]. Each ICE also contains a variety of cargo genes that confer different phenotypes to host cells, for example, virulence determinants, antimicrobial-resistant factors and/or genes coding for other beneficial traits, indicating they play important roles for bacterial evolution [Arai et al., 2019]. A well-known example is the *Salmonella* pathogenicity island (SPI)-7, which possesses features indicative of an ICE and carries genes implicated in virulence and has been found within some pathogenic strains of *S. enterica* [Seth-Smith et al., 2012]. Seth-Smith et al. (2012) identified and analyzed ICEs related to SPI-7 within the genus *Salmonella* and other *Enterobacteriaceae* and discovered two new ICEs with high levels of identity to SPI-7, which indicates that these elements may be more common than previously thought in the *Salmonella*. They also identified more distantly related ICEs, with distinct cargo regions in other strains of *Salmonella* and members of the *Enterobacteriaceae* [Seth-Smith et al., 2012].

Salmonella genomic island (SGI) 3 has been demonstrated to be an ICE and increases copper and arsenic tolerance in *Salmonella* strains [Arai et al., 2019]. ICEkp, an ICE in *Klebsiella pneumoniae*, mobilizes a *ybt* locus which encodes biosynthesis of the siderophore yersiniabactin and its receptor. Yersiniabactin and other siderophore systems are key bacterial virulence factors as they provide mechanisms for scavenging iron (an essential nutrient) from host transport proteins, thereby enhancing the ability of bacteria to survive and replicate within the host [Lam et al., 2018]. SXT/R391 family of ICEs, that is mainly associated with *Vibrio* spp, is one of the largest of the ICE families with more than 100 elements [Marrero and Waldor, 2007; Ryan et al., 2016]. These elements integrate into an *attB* site in the 5′ end of the chromosomal *prfC* gene by site-specific recombination with their *attP* site, catalyzed by the IntSXT tyrosine recombinase [Partridge et al., 2018]. ICEs of SXT/R391 family are able to mobilize adjacent sequences and have been recognized as major drivers of the dissemination of AMR genes among several species of *Enterobacteriaceae* and *Vibrionaceae* [Carraro et al., 2016; Partridge et al., 2018].

1.4.7. Integrative mobilizable elements (IMEs)/mobilizable transposons

Similar to ICEs, the integrative and mobilizable elements (IMEs), also known as mobilizable transposons, are mobile elements that lack the recombination module that allows for their autonomous integration and excision into host sequence [Guedon et al., 2017; Lao et al., 2020]. Unlike ICEs, which are self-transferable, IMEs cannot self-transfer. Instead they can be mobilized *in trans* by subverting the conjugation machinery of related or unrelated co-resident conjugative element to promote their own transfer [Guedon et al., 2017; Lao et al., 2020]. Beside AMR genes, IMEs can carry genes conferring various known or unknown functions that may

enhance the fitness of their hosts and contribute to their maintenance in bacterial populations [Doublet et al., 2005; Guedon et al., 2017]. An example is SGI1, which was classified within the group of IMEs since it is not self-transmissible, but mobilizable [De Curraize et al., 2021; Doublet et al., 2005]. SGI1 could be conjugally transferred from an *S. enterica* donor to *E. coli* recipient strains [Doublet et al., 2005]. SGI1 has been shown to exploit the conjugal apparatus of the IncA/C plasmids to facilitate SGI1 being conjugally mobilized [Douard et al., 2010; Harmer et al., 2016; Siebor et al., 2016]. SGI1 is extremely stable in the host chromosome; however, the presence of an IncA/C plasmid enables excision of SGI1 via the action of the IncC transcriptional activator complex AcaCD. This complex is required for expression of *tra* genes and hence for conjugative activity of IncC plasmids [Harmer et al., 2016; Huguet et al., 2016]. AcaCD binds to SGI1 upstream of *xis*, which encodes the recombination directionality factor Xis, resulting in the activation of *xis* expression and hence SGI1 excision which enables subsequent mobilization of SGI1 [Kiss et al., 2015]. The extra-chromosomal circle of SGI1 is integrated into the recipient chromosome in a site-specific manner [Doublet et al., 2005]. Paradoxically, although SGI1 needs a co-resident IncA/C plasmid for its excision from the host chromosome and transfer to a new host, it is incompatible with A/C plasmids [Harmer et al., 2016; Huguet et al., 2016; Huguet et al., 2020]. SGI1 promotes the loss of IncA/C plasmid, which is associated with SGI1 replication. The high-copy replication of SGI1 prevents the co-transfer of IncA/C plasmid into a recipient cell [Harmer et al., 2016; Huguet et al., 2016; Huguet et al., 2020]. The mobilization of SGI1 from *Salmonella* to the other bacterial pathogens probably contributes to the spread of AMR genes among them.

1.4.8. Insertion sequences (ISs)

In general, IS elements are small mobile elements (at least 0.5 kb) that perform as a complex type of transposable element and typically carry one or two transposase (Tnp) genes [Pagano et al., 2016; Partridge et al., 2018]. These elements are defined as the simplest autonomous mobile elements in bacterial genomes [He et al., 2015]. They are classified into different groups based on active site motifs in Tnp, designated by the key amino acids which are present in the active site including DDE (Asp and Glu), DEDO and H4H (a His split by a large collection of hydrophobic amino acids); and/or on whether transposition is a conservative, cut-and-paste mechanism, where the IS is simply excised from the donor and inserted into the recipient, or is replicative [Seth-Smith et al., 2012]. The transposition can be replicated by a copy-and-paste mechanism or copy-out-paste-in mechanism. In the copy-and-paste mechanism, the IS is replicated to join the donor and recipient in a co-integrate, which is subsequently resolved to provide the original donor plus the recipient with the IS; while in the copy-out-paste-in mechanism, the IS is replicated into a double-stranded circular intermediate that then integrates into the recipient [Partridge et al., 2018]. IS elements are widespread and can occur in very high numbers in bacterial genomes and have had an important impact on genome structure and function [Siguier et al., 2014]. IS-mediated gene inactivation can affect bacterial virulence, AMR and metabolism [Vandecraen et al., 2017]. For example, IS1 or IS10 insertion can up-regulate the AcrAB-TolC efflux pump in *Salmonella*, resulting in increased AMR [Ramirez-Santos et al., 1992; Siguier et al., 2014]. IS1, which is approximately 768 bp, can be detected in the majority of the family *Enterobacteriaceae* including *E. coli*, *Salmonella, Shigella*, and *Klebsiella* [Ramirez-Santos et al., 1992]. IS1 can be found in the chromosome and plasmids, respectively, and some strains carried IS1 in both the chromosome and plasmids [Ramirez-Santos et al.,

1992]. Ramirez-Santos et al. (1992) reported that the frequency of IS1 was higher in *Salmonella* than in *E. coli* and *Shigella*.

The relatively high prevalence of IS1 in plasmids of MDR clinical isolates suggests its role in the dissemination of AMR genes [Ramirez-Santos et al., 1992]. ISPa12, a member of IS4 family, can induce expression of ESBL PER-1 in a series of Gram-negative isolates, including *Salmonella*, resulting in resistance to penicillins, cefotaxime, ceftibuten, ceftazidime and aztreonam [Poirel et al., 2005]. IS200 is a *Salmonella*-specific IS that is found in almost all *Salmonella* serotypes examined, but is absent from most other enteric bacteria, thus it has been used as fingerprinting tool that is applied for strain discrimination [Beuzon et al., 2004]. IS elements have been reviewed thoroughly in previous publications [Siguier et al., 2014; Vandecraen et al., 2017].

1.4.9. Resistance islands/*Salmonella* genomic island (SGI)

Resistance islands are genomic islands that contain multiple resistance genes. They have been described mainly in the Proteobacteria, including *Salmonella*, *Shigella flexneri*, *V. cholerae*, *Staphylococcus aureus*, and *Acinetobacter baumannii* [Pagano et al., 2016]. Several AMR islands, also called *Salmonella* genomic islands (SGIs), have been identified in *Salmonella* strains [Miriagou et al., 2006; Silva et al., 2012]. The best known is SGI1, which was initially described in the epidemic MDR *Salmonella* Typhimurium phage-type DT104 [Boyd et al., 2001; Doublet et al., 2005]. Since then, several variants of SGI1 have been described in a wide variety of *Salmonella* serovars, including Typhimurium, Agona, Paratyphi B, Albany, Meleagridis and Newport, which indicates its horizontal transfer potentia [Levings et al., 2005]. SGI1 is 43 kb and contains 44 open reading frames (ORFs), many of which show no homology to known gene

sequences. As mentioned above, SGI1 is an integrative mobilizable element that contains a complex class 1 integron named In104, located within the AMR gene cluster which is 13 kb and is located at the 3' end of SGI1 [Doublet et al., 2005; Levings et al., 2005]. The SGI1 antimicrobial resistance gene cluster contains a complex class 1 integron that harbors five resistance genes (*aadA2, sul1, floR, tetA(G), blaPSE-1*) encoding ampicillin, chloramphenicol, florfenicol, streptomycin/spectinomycin, sulphonamides and tetracycline (ACSSuT) resistance, the so-called ACSSuT resistance type [De Curraize et al., 2021; Doublet et al., 2005; Levings et al., 2005].

1.5. Factors that impact resistance transfer

Environmental and genetic factors that regulate horizontal transfer of AMR genes in bacterial populations remain mostly unknown. MDR *Salmonella* often have multiple resistance-encoding plasmids. The exposure to some antimicrobial agents appears to influence plasmid transfer in enteric bacteria under certain circumstances [Liu et al., 2019; Lu et al., 2018; Moller et al., 2017; Schuurmans et al., 2014; Shun-Mei et al., 2018]. Several factors have been shown to contribute to the dissemination of resistance plasmids and other MGEs. Quorum-sensing (QS) and SOS responses have been known to promote horizontal gene transfer, which plays an essential role for the AMR development and dissemination among bacterial populations [Liu et al., 2019; Lu et al., 2017; Shun-Mei et al., 2018].

1.5.1. Quorum-sensing system

QS is an intercellular cell-cell communication mechanism that allows bacteria to control the expression of genes involved in a variety of cellular processes and plays a critical role in the

adaption and survival of bacteria in their environment [Kendall and Sperandio, 2007]. QS is mediated by secreted chemical signals called autoinducers (AIs), which are small diffusible molecules that are synthesized and released from bacterial cells in accordance with cell number and accumulate in the external environment [Taga et al., 2003]. When bacterial cell density reaches to a certain level that AIs concentration is over a minimal threshold, AIs bind to cognate receptors to alter and regulate bacterial gene expression accordingly in response to their population density [Kendall and Sperandio, 2007; Taga et al., 2003]. A large number of physiological processes in bacteria, including biofilm formation, virulence factor production, bioluminescence, sporulation, motility, and antibiotic production,are regulated by QS systems [Rutherford and Bassler, 2012; Wang et al., 2019a].

While most QS signals are species-specific, AI-2 has been observed throughout the bacterial kingdom and is considered as a universal QS signal [Wang et al., 2019a]. It is involved in interspecies cell-to-cell communication, as AI-2 produced by one species can influence gene expression in another. AI-2 is a metabolic byproduct of a *luxS* gene-encoded synthase, which is an enzyme that has been found in more than 55 bacterial species and is involved primarily in the conversion of ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor of AI-2 [Vendeville et al., 2005]. The LuxS/AI-2 QS system is biologically important as it is involved in numerous physiological processes such as altering bacterial growth characteristics, biofilm formation, conjugation, virulence regulation, AMR, and metabolism in *Salmonella* [Chen et al., 2008; Choi et al., 2007; Jesudhasan et al., 2010; Ju et al., 2018; Karavolos et al., 2008; Shi et al., 2018; Taga et al., 2001; Widmer et al., 2007]. It contributes to AMR through different mechanisms: MGE, efflux pumps, the VraSR two-component system, inhibition of the folate synthesis pathway, and biofilm formation. Detailed information on the

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relationships between the LuxS/AI-2 system and drug resistance has been reviewed by Wang et al. (2019a). In brief, the LuxS/AI-2 system has been shown to impact the transfer of resistance plasmids and the LuxS/AI-2-based QS enhances the expression of genes involved in conjugation-related activities, leading to increased conjugation frequency [Lu et al., 2004].

In the presence of AI-2, the conjugation frequency of the plasmid RP4 carrying the *tet(A)* gene in bacteria exposed to tetracycline increased significantly compared to a non-exposed control [Lu et al., 2004]. Increased AI-2 concentrations led to increased expression of plasmid-transfer associated gene *trbC*. Similar studies conducted by another group also showed that exposure to sub-MIC tetracyclines could facilitate the conjugative transfer of plasmid RP4 in *E. coli* and this process could be enhanced by AIs, but inhibited by quorum sensing inhibitors (QSIs) [Zhang et al., 2018]. Therefore, the LuxS/AI-2 could be a potential target for preventing the spread of bacterial resistance.

1.5.2. SOS response

The SOS response is a global stress response to DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis is induced [Baharoglu et al., 2010; Beaber et al., 2004; Qin et al., 2015; Zgur-Bertok, 2013]. It is an inducible DNA repair pathway controlled by two key regulators, LexA, a repressor, and RecA, an inducer [Podlesek and Žgur Bertok, 2020] (Figure 4). In bacteria, upon DNA damage, SOS is induced when an increase in intracellular single-stranded DNA (ssDNA) is generated when DNA polymerase III stalls at a lesion while helicase continues unwinding DNA [Podlesek and Žgur Bertok, 2020]. Stimulated by ssDNA, RecA is activated by binding to ssDNA [Maslowska et al., 2019]. The formation of a ssDNA/RecA nucleofilament stimulates auto-proteolysis of the LexA repressor, leading to de-

repression of genes comprising the SOS regulon [Baharoglu et al., 2010; Podlesek and Žgur Bertok, 2020]. In addition to several endogenous triggers (for example UV irradiation, oxidants, and chemical mutagens) that cause the accumulation of ssDNA, thus inducing the SOS response, ssDNA is also produced by several mechanisms of exogenous DNA uptake involved in lateral gene transfer, including conjugation, transformation, and occasionally transduction [Baharoglu et al., 2010]. The SOS response can also be induced by numerous antimicrobials, presumably because these antimicrobials cause the production of ssDNA. At least two classes of antimicrobials, fluoroquinolones (such as ciprofloxacin) and dihydrofolate reductase inhibitors (such as trimethoprim), can activate the SOS response [Beaber et al., 2004; Qin et al., 2015]. Besides being initially recognized as a regulator of DNA damage repair, it has been noted that the SOS response plays a much broader role in bacteria. As an error-prone repair system, the SOS response contributes significantly to DNA changes observed in a wide range of species by promoting an elevated mutation rate which generates genetic diversity and adaptation, including the development of resistance to antimicrobials [Podlesek and Žgur Bertok, 2020]. The SOS response also plays a key role in AMR by stimulating gene transfer, inducing mutation and genomic rearrangements, and the formation of biofilms which are highly recalcitrant to antimicrobials [Podlesek and Žgur Bertok, 2020].
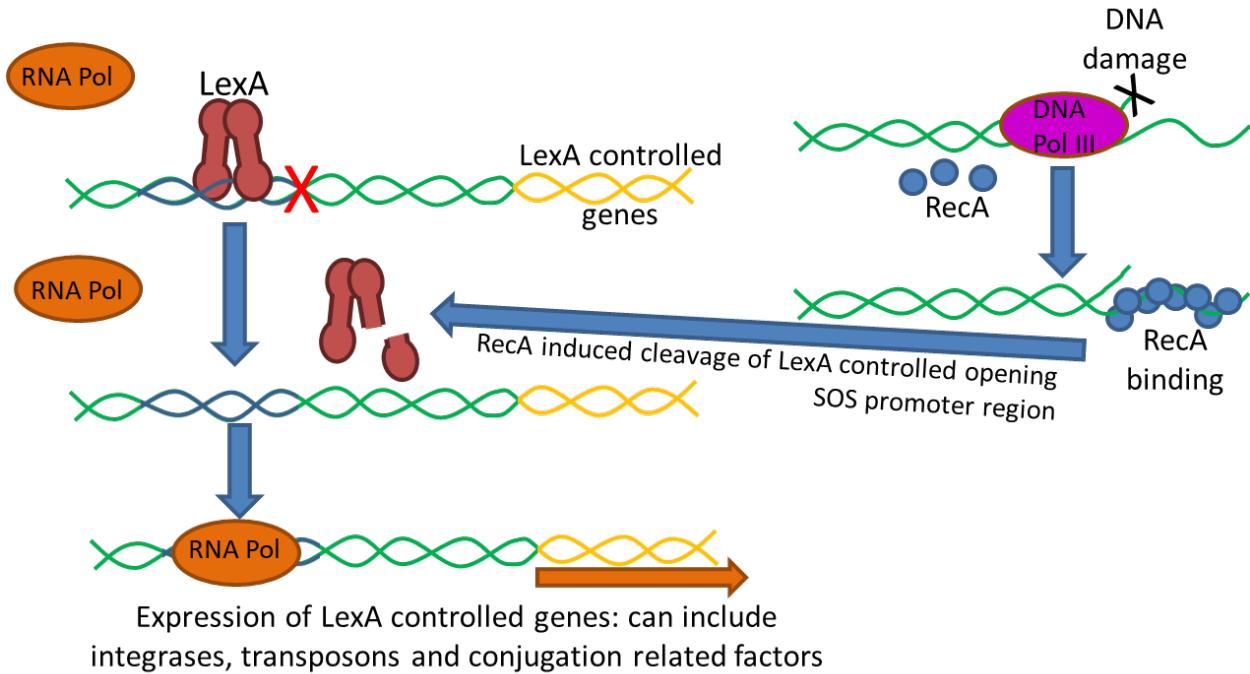
Figure 4. The SOS response is an inducible DNA repair pathway controlled by two key regulators, the LexA repressor of gene expression and the RecA inducer. Under normal conditions, LexA is bound to the promoter of SOS-associated genes preventing binding of RNA polymerase (RNA Pol) and expression of the regulated genes [Maslowska et al., 2019]. Following DNA damage, such as following certain antimicrobial exposures, the DNA polymerase III (DNA Pol III) enzyme senses the damage and the increase in single-stranded DNA (ssDNA) causes RecA to become activated and bind to the ssDNA. The ssDNA/RecA complex leads to proteolysis of the LexA repressor allowing RNA polymerase to bind facilitating expression of genes under LexA control. LexA has been shown to impact the expression of integrases and transposases, as such the activation of the SOS response has been shown to promote horizontal dissemination of resistance genes. Additionally, conjugation can also trigger the SOS response as ssDNA is generated and transferred during the conjugation process which can up-regulate integrase and transposase expression and triggering recombination of plasmids in the recipient cells.

The SOS response has been shown to promote horizontal dissemination of resistance genes in the presence of antimicrobial agents [Beaber et al., 2004; Hastings et al., 2004]. Guerin et al.

(2009) observed that the antimicrobial compounds found in manure can activate the SOS response in bacteria, which then increases the activity of integrases and transposases, and thereby transposition rates of gene cassettes and other translocative elements. They concluded that LexA controlled expression of most integron integrases and consequently regulated cassette recombination. Baharoglu et al. (2010) found that conjugative transfer of plasmids R388, R6Kdrd, and RP4 in *E. coli* and *V. cholerae* induces the SOS response and up-regulates integrase expression, triggering cassette recombination in recipient cells. Additional studies have shown that the SOS response can lead to an increase in cell membrane permeability and down-regulation of the *korA* repressor of various transfer associated genes in the RP4 plasmid [Wang et al., 2019b].

The SOS induction leads to genetic diversification of these mobile elements and their transfer to surrounding bacteria, resulting in the dissemination of AMR genes [Baharoglu et al., 2010]. It was also observed that ICEs could utilize the SOS response to mobilize themselves from the bacterial chromosome and infect other cells [Hastings et al., 2004]. The transfer of SXT, an approximately 100kb ICE derived from *V. cholerae* that encodes a variety of antimicrobial resistance genes, is markedly enhanced when the SOS response is induced by two DNA-damaging antimicrobials (mitomycin C and ciprofloxacin), resulting in the transfer of MDR genes [Hastings et al., 2004].

Other studies have outlined the connections between horizontal gene transfer, bacterial stress response (SOS), and recombination of gene cassettes in integrons, which provides new insights into the development of the AMR within a population [Baharoglu et al., 2010; Guerin et al., 2009]. Since several commonly used antimicrobials can induce the SOS response, which in turn can enhance the conjugative transfer of the plasmids, the use of certain antimicrobial agents,

either clinically or in agricultural settings, might potentiate the horizontal dissemination of AMR genes to a broad range of bacterial species and hasten genetic change and the evolution to resistance in pathogenic populations.

1.6. Conclusions:

AMR acquisition can occure via either vertical or horizontal transfer. In vertical transmission, the accumulated genetic errors in existing genes (either in the chromosome or plasmid) is passed on from parent cells to progeny cells, leading to the observed resistance. While in the horizotal transfer, also called as acquired resistance, the AMR genes are exchanged within and among different bacteria species [Founou et al., 2016]. The resistant genes harbored on MGEs can be acquired by the recipient strains. Both vertical transmission and horizontal transfer of AMR genes play important roles in AMR acquisition. The MGE-mediated horizontal transfer of foreign genes among different bacteria plays an important role in bacterial evolution and contributes signaficatnly in both the AMR and virulence gene acquisition [Gyles and Boerlin et al., 2014]. This review article examined MGEs that can be found in *Salmonella* as well as discussed their roles in the development and dissemination of antimicrobial resistance. Key examples of MGEs that are important in antimicrobial resistance dissemination include SG1, which has been associated with the stable acquisition of multiple resistance genes at a site in the chromosome, yet requires an IncC resistance plasmid to facilitate its transfer. Plasmids are likely the most important vehicle of resistance transfer between different *Salmonella* strains and/or from related species. Plasmids can harbor other MGEs including transposons and integrons that can lead to the transfer of genes between the chromosome and other co-resident plasmids, thus providing more genetic diversity among the bacterial populations. The dynamic resistance of

Salmonella isolates to antimicrobial agents is considered a serious problem and developing

methods and strategies to reduce and control the spread of resistance genes and their evolution

are vital. Another trend observed in *S. enterica* is IS element activation which plays a critical role

in efflux mechanisms. A recent study suggested that a combination of conjugation inhibition and

plasmid elimination would be considered an effective method to reduce the conjugation-assisted

persistence of antimicrobial resistance [Lopatkin et al., 2017; San Millan et al., 2018]. Since the

induction of SOS response promotes horizontal dissemination of AMR genes, inhibiting the

bacterial SOS response would be a suitable target to prevent the acquisition of AMR genes, and

could be used in combination with antibiotics for the treatment of infections [Baharoglu et al.,

2010]. The different types MGEs are summarized in Table 4, which describes the antimicrobial

resistance mobilome of zoonotic pathogens, particularly in *S. enterica*, that is critical to

understand the epidemiology, dynamics and evolution of antimicrobial resistance.

Table 4. Summary of terms and definitions of mobile genetics elements (MGEs) in *Salmonella enterica.*

Mobile Genetic Elements	Definition
Plasmids	Plasmids are genetic elements that are typically circular DNA in a cell that can replicate independently of the chromosomes. They have variable host-ranges and can be conjugative and replicate in diverse genera.
Integrons	Integrons are genetic elements with a site-specific recombination system that can integrate, express and exchange specific DNA elements (gene cassettes), including resistance genes.
Transposable elements/ Transposons (Tn)	Transposable elements/ Transposons (Tn) are known as "jumping genes" that can move themselves (and associated resistance genes) almost randomly to new locations in the same or different DNA molecules within a single cell.
Resistance islands	Resistance islands are genomic islands (GIs) containing MDR genes.
Bacteriophages	Bacteriophages are bacterial viruses that infect and replicate only in bacterial cells.
Insertion sequences	Insertion sequences are small mobile elements (generally 700~2,500 bp) and only code for proteins implicated in the transposition activity. They can move themselves almost randomly to new locations in the same or different DNA molecules within a single cell.

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CHAPTER TWO

RESEARCH ARTICLE

In silico Analyses of Diversity, and Dissemination of Antimicrobial Resistance Genes and Mobile Genetics Elements for Plasmids of Enteric Pathogens

Suad Algarni[1,2], Jing Han[1], Dereje D. Gudeta[1], Bijay K. Khajanchi[1], Steven C. Ricke[3], Young Min Kwon[2], Douglas Rhoads[2], and Steven L. Foley[1,2]

[1]Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson AR, 72079

[2]Cell and Molecular Biology program, University of Arkansas, Fayetteville AR, 72701

[3]Meat Science & Animal Biologics Discovery Program, Dept. of Animal and Dairy Sciences, University of Wisconsin, Madison WI, 53706

Corresponding Author: Steven L. Foley, National Center of Toxicological Research, Food and Drug Administration, 3900 NCTR Rd, Jefferson, AR 7209. Phone: (870) 543-7547. Email: steven.foley@fda.hhs.gov

Running Title: Enteric plasmid-associated resistance mobilome

Abstract:

The antimicrobial resistance (AMR) mobilome plays a key role in the dissemination of resistance genes encoded by mobile genetics elements (MGEs) including plasmids, transposons (Tns), and insertion sequences (ISs). These MGEs contribute to the dissemination of multidrug resistance (MDR) in enteric bacterial pathogens which have been considered as a global public health risk. To further understand the diversity and distribution of AMR genes and MGEs across different plasmid types, we utilized multiple sequence-based computational approaches to evaluate AMR-associated plasmid genetics. A collection of 1309 complete plasmid sequences from Gammaproteobacterial species, including 100 plasmids from each of the following 14 incompatibility (Inc) types: A/C, BO, FIA, FIB, FIC, FIIA, HI1, HI2, I1, K, M, N, P except W, where only 9 sequences were available, was extracted from National Center for Biotechnology Information (NCBI) GenBank database using BLAST tools. The extracted FASTA files were analyzed using the AMRFinderPlus web-based tools to detect antimicrobial, disinfectant, biocide, and heavy metal resistance genes and ISFinder to identify IS/Tn MGEs within the plasmid sequences. *In silico* prediction based on plasmid replicon types showed that the resistance genes were diverse among plasmids, yet multiple genes were widely distributed across the plasmids from enteric bacterial species. These findings provide insights into the diversity of resistance genes and that MGEs mediate potential transmission of these genes across multiple plasmid replicon types. This notion was supported by the observation that many IS/Tn MGEs and resistance genes known to be associated with them were common across multiple different plasmid types. Our results provide critical insights about how the diverse population of resistance genes that are carried by the different plasmid types that can allow for the dissemination of AMR across enteric bacteria. The results also highlight the value of computational-based approaches

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and *in silico* analyses for the assessment of AMR and MGEs, which are important elements of molecular epidemiology and public health outcomes.

2.1. Introduction:

Technological and bioinformatics advancements in *in silico* lab-based tools now allow

for the replacement of several of the traditional microbiology laboratory methods, potentially

increasing analytical throughput and reducing costs. The value of this replacement was

particularly evident during the coronavirus-19 (COVID-19) pandemic, when in-person staffing

numbers were reduced to maximize physical distancing and remote work became more common.

Whole genome sequencing (WGS) is a key approach that provides comprehensive data

assessment on the genetics of bacterial pathogens and can potentially replace laboratory-

intensive methods. Indeed, several tools and approaches have already been developed to identify

the serotype of the isolates, resistance genotypes, plasmid replicon sequence and putative

virulence gene content using WGS data [Carattoli et al., 2014; Mao et al., 2015; Zhang et al.,

2015]. The online bioinformatics tools PlasmidFinder and ResFinder developed by the Center for

Genomic Epidemiology, and AMRFinderPlus available through the National Center for

Biotechnology Information (NCBI), analyze DNA sequences to identify the resistance genes and

factors that can contribute to the transmission of resistance [Zankari et al., 2012; Tyson et al.,

2015; Feldgarden et al., 2019]. These are important services for antimicrobial resistance

diagnostics, epidemiological surveillance, and outbreak investigations.

The mobilome encompasses mobile genetic elements (MGEs), including transposons

(Tns), insertion sequences (ISs), gene cassettes, integrons, resistance islands, and plasmids, that

can contribute to the spread of genes in a microbial population [Algarni et al., 2022]. ISFinder is

a bioinformatics tool used for identifying and classifying IS elements and Tn elements which

usually carry accessory genes encoding different resistance and/or virulence functions [Siguier et

al., 2006; Algarni et al., 2022]. One of the major challenges of utilizing WGS data to predict

bacterial functions has been the computational horsepower needed to analyze large scale datasets and the bioinformatics expertise to execute some of analyses programs to achieve the analyses [Tyson et al., 2015]. To help overcome some of these limitations, investigators have worked to develop more user-friendly interfaces and to provide resources that allow for analyses to be done remotely utilizing core computing capacity. This study takes advantage of some these *in silico* tools to identify the plasmid replicon sequences, predict AMR content, and MGEs in enteric bacterial species, including *Salmonella enterica, Escherichia coli, Klebsiella pneumoniae* and *Shigella* spp. The rapid implementation of WGS for epidemiological investigations and the development of improved plasmid sequencing approaches has made more data available to further understand AMR epidemiology and identify the mechanisms that lead to development of AMR phenotypes and improve the detection of resistant strains [Zankari et al., 2012].

These molecular epidemiology efforts aid in understanding the transmission of antimicrobial resistant organisms between humans and other animal species, which can escalate AMR spread across species (e.g. among household pets and owners) or through the food supply [Blondeau et al., 2017]. Multidrug resistance has been increasing in prevalence as shown in the many studies examining the relationship between enteric bacterial species and the prevalence of antimicrobial, disinfectant, biocide, and heavy metal resistance encoded on plasmids [Deng et al., 2018; Johnson et al., 2007; Mc Carlie et al., 2020]. Disinfectants, biocides and metal ions are extremely important for the control of infection and/or microbial contamination in the environment. The investigation of heavy metal resistance (HMR) genes in different environments, specifically in *S. enterica* and *E. coli*, suggests that they are often mediated by plasmid. Multiple studies have shown that disinfectant/biocide resistance (DBR), HMR and

AMR genes can be co-located on MGEs [Deng et al., 2018; Johnson et al., 2007; Han et al., 2012].

Plasmids are well known as key components of bacterial strains that display high levels of antimicrobial resistance and provide a source for the dissemination of antimicrobial resistance genes [Mathers et al., 2015]. Most emerging multidrug-resistant strains rely on a variety of plasmids that can be classified into various incompatibility (Inc) groups, with some of the key ones for Enterobacteriaceae include IncA/C, B/O, FIA, FIB, FIC, FIIA, HI1, HI2, I1, L/M, N, P and W which are associated with clinically important antibiotics [Carattoli et al., 2009; Rozwandowicz et al., 2018]. These groups that fall into larger number of different Inc groups of plasmids have been detected in the Enterobacteriaceae (McMillan et al., 2020). In addition to Inc grouping, some other classification schemes for plasmids that rely on different sequences have been developed, such as those associated with mobilization, to define different plasmid groups associated with conjugation experiments [Petersen et al., 2011; Rozwandowicz et al., 2018]. Although the extensive plasmid Inc typing scheme has already been used to investigate the dissemination of antimicrobial resistant pathogens, further efforts are still required to facilitate epidemiological tracking for identification and classification purposes as there can be significant diversity within some Inc groups as well as co-integration of multiple replicon types in a single plasmid [Partridge et al., 2018; Hsu et al., 2019].

The distribution and genetic identification of resistance genes among individual plasmid types across different bacterial strains have been documented [Venturini et al., 2013; Carattoli et al., 2015; Seiffert et al., 2017; Majewski et al., 2021; Chang et al., 2020; Moran and Hall, 2019; Reid et al., 2015]. In the current study, we applied several bioinformatic tools including BLAST, AMRFinderPlus, and ISFinder for detecting genes in assembled plasmid sequence data and

assessed the combination of resistance genes and MGEs, and individual plasmid types from enteric bacterial species to better elucidate AMR epidemiology as an effort to respond to this important public health threat. Understanding the AMR mobilome is important for the development of strategies that can limit the transfer of resistant pathogens to humans via contaminated foods [Algarni et al., 2022; Johansson et al. 2021]. A schematic of the flow of the experiments is provided in Figure 1.
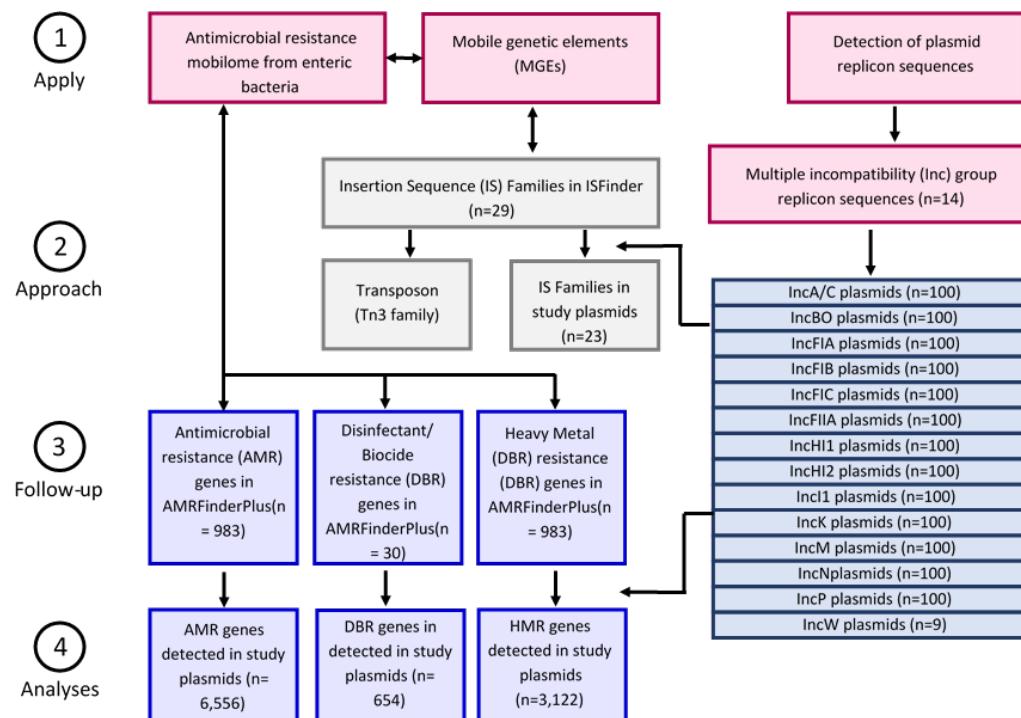
Figure 1. Workflow of the study to examine the resistance gene and MGE content of multiple plasmids representing those plasmid types most commonly associated with carrying antimicrobial resistance genes in enteric bacterial pathogens.

2.2. Methods and Materials:

2.2.1. Selection of enteric plasmids for sequence analyses and data acquisition

The GenBank database was screened for 14 different plasmid replicon type sequences: A/C, B/O, FIA, FIB, FIC, FIIA, HI1, HI2, I1, L/M, N, P and W [Carattoli et al., 2009; Rozwandowicz

et al., 2018]. To select the sequences for further analyses, representative plasmid replicon sequences described by Carattoli et al. (2005) were used for BLAST searching (Supplemental Table S1). Individual replicon sequences were queried by selecting "Microbe" genomes and "Complete plasmids" in the Microbial Nucleotide BLAST database using default settings (i.e., optimize for "highly similar sequences "megablast"). These queries were conducted in January 2022. The DNA sequences for the initial set of 100 plasmids identified for each replicon type were downloaded as a FASTA file (with the exception of IncW, where there were only 9 sequences available using the search parameters for the study). This set of extracted sequences provided 1309 plasmids to assess the presence of resistance genes and MGEs across the plasmid replicons. The identity and accession numbers of these plasmids are provided in Supplemental Table S2. In addition, to evaluate the use of 100 plasmids per group, a validation set of 563 IncA/C plasmid sequences was extracted from GenBank. This set included all of the plasmid IncA/C sequences detected at the time of data extraction using the search criteria noted above.

2.2.2. Identification of resistance genes using the AMRFinderPlus database

Analyses were performed by analyzing the FASTA files for the individual replicon types described above using the latest AMRFinderPlus database V3.2.1 (available at https://www.ncbi.nih.gov/pathogens/antimicrobial-resistance/AMRFinder). The AMRFinderPlus database has sequences for 983 unique AMR genes (including 6,060 total variants), 30 DBR genes (including 55 total variants) and 82 HMR genes (including 182 total variants). The analyses were run using the AMRFinderPlus plugin within the GalaxyTrakr (https://account.galaxytrakr.org) operating environment to identify AMR genes, disinfectant, biocide and metal heavy resistance genes located within the submitted sequences. The input file

for each replicon (Inc) type contained the sequences for 100 plasmids (with the exception of IncW, n=9). A report outlining the identity and numbers of the AMR genes belonging to different antimicrobial compounds classes, as well as DBR and HMR genes was downloaded from GalaxyTrakr and parsed out based on their functions (AMR, disinfectant/biocide, and heavy metal) to allow for comparison between the different plasmid types.

2.2.3. Identification of IS and Tn sequences using the ISFinder database

The combined FASTA files described above for each Inc type were used to identify the IS elements, including Tns, using the ISFinder program (https://isfinder.biotoul.fr/) (accessed on 02/17/2022). The FASTA files were uploaded into the ISFinder BLAST (blastn) interface using the default parameters. The database included sequenced for 5,735 different IS elements that were divided amongst 29 different IS families. The output provided information on the sequences producing significant alignments based on BLAST e-value, IS family, group, score (bits) and e-value.

2.2.4. Statistical analyses

The majority of statistics for this project are descriptive in nature. Since for all but IncW, the denominator for the numbers of plasmids is 100, the percentages are equal to the number of positive values. For comparison of the distribution of AMR, DBR and HMR and IS/Tn MGEs traits across the different plasmid replicon types, student t-test was used with statistical significance observed at $p < 0.05$.

2.3. Results and Discussion

To explore the diversity between different plasmid types and the presence/absence AMR genes within the plasmid database, we performed AMRFinderPlus analyses to extract AMR genes and extrapolate the resistance to the antibiotic compounds that are related to each gene. We selected 14 plasmid replicon or Inc types that are associated with antimicrobial resistance in enteric bacteria [Carattoli et al., 2009; Rozwandowicz et al., 2018]. From each Inc type, with the exception of IncW, we used a convenience sample of 100 whole plasmid sequences (1,309 in total) for analyses. These plasmids originated from members of the Gammaproteobacteria, with the great majority being from members of the Enterobacteriaceae family, a small number of representatives from the *Morganellaceae* and *Vibrionaceae* families (Supplemental Table S2).

2.3.1. AMR gene analysis

With AMRFinderPlus, 195 unique resistance genes were detected in at least one of the 1309 sequences screened, with the distribution of genes varied across each of the plasmid types (Table 1). Among the different plasmid types, IncHI2 (n=1033), N (n=932), A/C (n=820), P (n=733), HI1 (n=732), and FIA (n=574) exhibited the highest abundance of AMR genes. When the diversity of the resistance genes (number of unique genes/total number of genes detected) the IncFIA plasmids had lowest diversity of genes (0.06; 34 unique genes/574 genes detected). Indeed, several IncFIA resistance genes were detected in at least 48 percent of the plasmids, including *aadA5, aph(3")-Ib, aph(6)-Id, bla*CTX-M-27*, dfrA17, mph(A), sul1, sul2,* and *tet(A)* (range is n=48-55) (Table 1). In contrast, the IncW plasmids showed the highest calculated diversity of resistance genes (0.68), which was driven by the observation that there was a cumulative total of 34 genes detected which represented 23 distinct genes. It is important to note that for IncW, only nine plasmids were screened, which likely contributed to the high perceived level of diversity as

there may have been a bias in the plasmids that were sequenced due to the carriage of resistance genes. Among those with 100 sequences analyzed, the IncFIIA had the highest calculated diversity (0.34) of resistance genes, which was driven by the low numbers of total genes detected (N=82).

An interesting observation among some of the genes was that there were greater than 100 *sul1* genes in the IncA/C (n=121) and the IncHI2 (n=127) plasmids and 100 *tet(A)* genes detected in the IncP plasmids (Table 1). The sizes of these plasmid types are generally large, with some of the IncHI2 and IncP plasmids over 400KB in size (e.g. GenBank accession numbers NZ_CP043927.1 and NZ_MN256757.1, respectively) and IncA/C plasmids routinely over 100KB in size with multiple resistance operons (13). When the numbers of unique plasmids carrying the resistance genes were examined in these cases, there were 80 IncA/C and 78 IncHI2 plasmids with a *sul1* gene and 99 IncP plasmids with *tet(A)*.

Table 1. Numbers of antimicrobial resistance genes detected in 100[A] representative sequences for each plasmid replicon type identified using AMRFinderPlus

AMR GENES	PRODUCTS	Resistance Genes Compounds	IncA/C	IncBO	IncFIA	IncFIB	IncFIIA	IncFIC	IncHI1	IncHI2	IncI1	IncK	IncM	IncN	IncP	IncW[A]
aac(3)-Ia	aminoglycoside N-acetyltransferase AAC(3)-Ia	Gentamicin			2					1			6			
aac(3)-Ib	aminoglycoside N-acetyltransferase AAC(3)-Ib	Gentamicin								2						
aac(3)-IId	aminoglycoside N-acetyltransferase AAC(3)-IId	Gentamicin	12		17	6		6	23	7	4		1	6	7	
aac(3)-IIe	aminoglycoside N-acetyltransferase AAC(3)-IId	Gentamicin	9	1		7	1	3	1	4		1	4	2	9	
aac(3)-IIg	aminoglycoside N-acetyltransferase AAC(3)-IIg	Gentamicin								20				2		
aac(3)-IVa	aminoglycoside N-acetyltransferase AAC(3)-IVa	Apramycin/Gentamicin/ Tobramycin				1	2	4	2		2	2	1	17	8	
aac(3)-VIa	aminoglycoside N-acetyltransferase AAC(3)-VIa	Gentamicin		2						2	7				7	
aac(6')-Ia	aminoglycoside 6'-N-acetyltransferase AAC(6')-Iae	Aminoglycoside											1			1
aac(6')-Ib	AAC(6')-Ib family aminoglycoside 6'-N-acetyltransferase	Aminoglycoside	14					2		2			6			4
aac(6')-Ib3	aminoglycoside N-acetyltransferase AAC(6')-Ib3	Amikacin/ Kanamycin/ Tobramycin	36			3				13			8	1		
aac(6')-Ib4	aminoglycoside N-acetyltransferase AAC(6')-Ib4	Gentamicin								15			5	1	5	
aac(6')-Ib-cr5	fluoroquinolone-acetylating aminoglycoside 6'-N-acetyltransferase AAC(6')-Ib-cr5	Amikacin/ Kanamycin/ Tobramycin /Quinolone	11	2		11		1		14		2	10	13		
aac(6')-II	aminoglycoside N-acetyltransferase AAC(6')-IIc	Aminoglycoside	7							28			1	2		
aacA34	aminoglycoside 6'-N-acetyltransferase AacA34	Beta-lactam	1													

Gene	Description	Antibiotic															
aadA1	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA1	Gentamicin	23	9	2	3	2	5	20	27	20	9	16	26	60	
aadA11	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA11	Streptomycin											1			
aadA12	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA12	Streptomycin												1		
aadA13	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA13	Streptomycin								2						1
aadA15	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA15	Streptomycin	1													
aadA16	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA16	Streptomycin	1							1			1	1		
aadA2	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA2	Streptomycin	29	2	1	13	2	6	16	34	13	1	3	29	26	1
aadA22	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA22	Streptomycin		1			1	1	17		5	1		6	1	
aadA25	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA25	Streptomycin							1							
aadA5	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA5	Streptomycin			48	13		4	10	2			1	9	3	
aadA7	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA7	Streptomycin								4						
aadA8	ANT(3")-Ia family aminoglycoside nucleotidyltransferase AadA8	Streptomycin								1						
ant(2")-Ia	aminoglycoside nucleotidyltransferase ANT(2")-Ia	Bleomycin	14							11			18			
aph(3')-Ia	aminoglycoside O-phosphotransferase APH(3')-Ia	Streptomycin	12	8	3	2	2	10	23	17	3	3	7	24	29	
aph(3")-Ib	aminoglycoside O-phosphotransferase APH(3")-Ib	Streptomycin	14	22	53	20	8	16	49	47	7	17	40	41	45	2
aph(3')-IIa	aminoglycoside O-phosphotransferase APH(3')-IIa	Kanamycin							1	5		1		12		
aph(3')-VI	APH(3')-VI family aminoglycoside O-phosphotransferase	Sulfonamide	4													
aph(3')-VIa	aminoglycoside O-phosphotransferase APH(3')-VIa	Sulfonamide	4									1				

Gene	Description	Drug														
aph(3')-VIb	aminoglycoside O-phosphotransferase APH(3')-VIb	Amikacin/ Kanamycin											14			
aph(3')-XV	aminoglycoside O-phosphotransferase APH(3')-XV	Amikacin/ Kanamycin						1								
aph(4)-Ia	aminoglycoside O-phosphotransferase APH(4)-Ia	Hygromycin			1	2	4	2		2		1	16	8		
aph(6)-Ic	aminoglycoside O-phosphotransferase APH(6)-Ic	Streptomycin						1								
aph(6)-Id	aminoglycoside O-phosphotransferase APH(6)-Id	Streptomycin	20	22	54	20	8	1	49	47	7	17	11	41	43	2
aph(7)-Ia	aminoglycoside O-phosphotransferase APH(7)-Ia	Streptomycin			1											
aphA16	APH(3') family aminoglycoside O-phosphotransferase AphA16	Amikacin/ Kanamycin										5				
armA	ArmA family 16S rRNA (guanine(1405)-N(7))-methyltransferase	Gentamicin	20	1				1	1			1		4	1	
arr	NAD(+)--rifampin ADP-ribosyltransferase	Rifamycin								20		1	3			
arr-2	NAD(+)--rifampin ADP-ribosyltransferase Arr-2	Rifamycin	8				1		2	9	1			4		
arr-3	NAD(+)--rifampin ADP-ribosyltransferase Arr-3	Rifamycin	5					1					4	13		
*bla*ACC-1	cephalosporin-hydrolyzing class C beta-lactamase ACC-1	Cephalosporin								15						
*bla*ACC-4	cephalosporin-hydrolyzing class C beta-lactamase ACC-4	Cephalosporin		1								1				
*bla*AFM-1	subclass B1 metallo-beta-lactamase AFM-1	Carbapenem														1
*bla*CARB-2	PSE family carbenicillin-hydrolyzing class A beta-lactamase CARB-2	Beta-lactam										1				
*bla*CMY-2	class C beta-lactamase CMY-2	Cephalosporin	6	13					1		20	14			5	
*bla*CMY-4	class C beta-lactamase CMY-4	Cephalosporin	12	2								2				
*bla*CMY-6	class C beta-lactamase CMY-6	Cephalosporin	34			1										
*bla*CTX-M	class A extended-spectrum beta-lactamase CTX-M family	Cephalosporin				1				4		2				

Gene	Description	Class														
*bla*CTX-M-1	class A extended-spectrum beta-lactamase CTX-M-1	Cephalosporin		2					12	1	1			11	4	
*bla*CTX-M-101	class A extended-spectrum beta-lactamase CTX-M-101	Cephalosporin								1						
*bla*CTX-M-134	class A extended-spectrum beta-lactamase CTX-M-134	Cephalosporin				1										
*bla*CTX-M-14	class A extended-spectrum beta-lactamase CTX-M-14	Cephalosporin	2	2	2	3	1	3	1	2	10	6	20	7		
*bla*CTX-M-15	class A extended-spectrum beta-lactamase CTX-M-15	Cephalosporin	11	5	11	12			2	5	3	4	3	2		
*bla*CTX-M-2	class A extended-spectrum beta-lactamase CTX-M-2	Cephalosporin							1		1				2	
*bla*CTX-M-27	class A extended-spectrum beta-lactamase CTX-M-27	Cephalosporin			48										1	
*bla*CTX-M-3	class A extended-spectrum beta-lactamase CTX-M-3	Cephalosporin		1								1	1	1		
*bla*CTX-M-32	class A extended-spectrum beta-lactamase CTX-M-32	Cephalosporin		1								1		1		
*bla*CTX-M-55	class A extended-spectrum beta-lactamase CTX-M-55	Cephalosporin	2			1		5	3		8			18		
*bla*CTX-M-63	class A extended-spectrum beta-lactamase CTX-M-63	Cephalosporin												1		
*bla*CTX-M-65	class A extended-spectrum beta-lactamase CTX-M-65	Cephalosporin						3		1				8	7	
*bla*CTX-M-8	class A extended-spectrum beta-lactamase CTX-M-8	Cephalosporin											1	1		
*bla*CTX-M-9	class A extended-spectrum beta-lactamase CTX-M-9	Cephalosporin								6						
*bla*DHA	DHA family class C beta-lactamase	Cephalosporin	9							1						
*bla*DHA-1	class C beta-lactamase DHA-1	Cephalosporin								9			2	2		
*bla*DHA-27	class C beta-lactamase DHA-27	Cephalosporin								1						
*bla*FOX-7	cephalosporin-hydrolyzing class C beta-lactamase FOX-7	Cephalosporin											1			
*bla*GES	GES family class A beta-lactamase	Cephalosporin														1

Gene	Description	Drug class											
*bla*GES-24	carbapenem-hydrolyzing class A beta-lactamase GES-24	Cephalosporin	7										
*bla*IMP-1	subclass B1 metallo-beta-lactamase IMP-1	Carbapenem						5					1
*bla*IMP-14	subclass B1 metallo-beta-lactamase IMP-14	Carbapenem	1										
*bla*IMP-4	subclass B1 metallo-beta-lactamase IMP-4	Carbapenem						2			4	5	1
*bla*IMP-68	subclass B1 metallo-beta-lactamase IMP-68	Carbapenem						1					
*bla*KPC	carbapenem-hydrolyzing class A beta-lactamase KPC family	Carbapenem		2									
*bla*KPC-2	carbapenem-hydrolyzing class A beta-lactamase KPC-2	Carbapenem	1	6				1			8	1	
*bla*KPC-4	carbapenem-hydrolyzing class A beta-lactamase KPC-4	Carbapenem						1			1		
*bla*LAP-2	class A beta-lactamase LAP-2	Beta-lactam					1	1					
*bla*NDM-1	subclass B1 metallo-beta-lactamase NDM-1	Carbapenem	54		3		1	4				1	1
*bla*NDM-15	subclass B1 metallo-beta-lactamase NDM-15	Carbapenem											
*bla*NDM-5	subclass B1 metallo-beta-lactamase NDM-5	Carbapenem		1	9		3	7		2		1	3
*bla*NDM-7	subclass B1 metallo-beta-lactamase NDM-7	Carbapenem	1										
*bla*NDM-9	subclass B1 metallo-beta-lactamase NDM-9	Carbapenem										1	
*bla*OXA-1	oxacillin-hydrolyzing class D beta-lactamase OXA-1	Beta-lactam	10	2	2	11	1		16		2	10	12
*bla*OXA-10	oxacillin-hydrolyzing class D beta-lactamase OXA-10	Beta-lactam	11				1		2		1	4	
*bla*OXA-101	OXA-10 family class D beta-lactamase OXA-101	Beta-lactam											1
*bla*OXA-129	OXA-5 family class D beta-lactamase OXA-129	Beta-lactam						1					
*bla*OXA-181	OXA-48 family class D beta-lactamase OXA-181	Beta-lactam					1						

Gene	Description	Category														
*bla*OXA-2	oxacillin-hydrolyzing class D beta-lactamase OXA-2	Beta-lactam		2										2	6	
*bla*OXA-4	oxacillin-hydrolyzing class D beta-lactamase OXA-4	Beta-lactam	3													
*bla*OXA-48	carbapenem-hydrolyzing class D beta-lactamase OXA-48	Beta-lactam													20	
*bla*OXA-9	oxacillin-hydrolyzing class D beta-lactamase OXA-9	Beta-lactam	6						2						4	
*bla*SCO	SCO family class A beta-lactamase	Beta-lactam													1	
*bla*SCO-1	class A beta-lactamase SCO-1	Beta-lactam		1										1		
*bla*SFO-1	class A extended-spectrum beta-lactamase SFO-1	Beta-lactam							3							1
*bla*SHV-11	class A extended-spectrum beta-lactamase SHV-11	Beta-lactam	1													
*bla*SHV-12	class A extended-spectrum beta-lactamase SHV-12	Beta-lactam	1						30						4	1
*bla*SHV-18	class A extended-spectrum beta-lactamase SHV-18	Beta-lactam													2	
*bla*SHV-2	class A extended-spectrum beta-lactamase SHV-2a	Beta-lactam	1												1	
*bla*SHV-30	class A extended-spectrum beta-lactamase SHV-30	Beta-lactam													13	
*bla*SHV-5	class A extended-spectrum beta-lactamase SHV-5	Beta-lactam													3	
*bla*SHV-7	class A extended-spectrum beta-lactamase SHV-7	Beta-lactam	2												5	
*bla*TEM	TEM family class A beta-lactamase	Beta-lactam			1	2		2	4	2				3	18	2
*bla*TEM-1	class A broad-spectrum beta-lactamase TEM-1	Beta-lactam	20	26	31	20	15	16	60	37	8	20	23	44	56	1
*bla*TEM-135	class A broad-spectrum beta-lactamase TEM-135	Beta-lactam													3	
*bla*TEM-190	class A beta-lactamase TEM-190	Beta-lactam						1								
*bla*TEM-2	class A broad-spectrum beta-lactamase TEM-2	Beta-lactam	1													1
*bla*TEM-3	class A broad-spectrum beta-lactamase TEM-3	Beta-lactam													1	

Gene	Description	Class																	
*bla*TEM-20	class A extended-spectrum beta-lactamase TEM-20	Beta-lactam			7													
*bla*TEM-32	inhibitor-resistant class A broad-spectrum beta-lactamase TEM-32	Beta-lactam		1									1					
*bla*VEB-5	class A extended-spectrum beta-lactamase VEB-5	Beta-lactam	1															
*bla*VIM-1	subclass B1 metallo-beta-lactamase VIM-1	Beta-lactam	8						11									
*bla*VIM-4	subclass B1 metallo-beta-lactamase VIM-4	Carbapenem							5					2				
ble	bleomycin binding protein Ble-MBL	Bleomycin	52		1	9		4	7	3	3				14	3		2
bleO	bleomycin binding protein	Bleomycin			1			5	4	1				1	23	3		
catA1	type A-1 chloramphenicol O-acetyltransferase	Chloramphenicol	2	3	2			2	41	15		2	3		14			
catA2	type A-2 chloramphenicol O-acetyltransferase CatII	Chloramphenicol	4				4			30			1		4			
catB3	type B-3 chloramphenicol O-acetyltransferase CatB3	Chloramphenicol	9	2	1	11		1		17		2	10		13	5		1
cfr	23S rRNA (adenine(2503)-C(8))-methyltransferase Cfr	Macrolide													3			
cmlA1	chloramphenicol efflux MFS transporter CmlA1	Chloramphenicol					3	1		8	2	10		1	20	11		
cmlA5	chloramphenicol efflux MFS transporter CmlA5	Chloramphenicol	12					1		2	2	1			4			
dfrA1	trimethoprim-resistant dihydrofolate reductase DfrA1	Trimethoprim	8	4		2		2	2	4	2	4	1	1		27		
dfrA10	trimethoprim-resistant dihydrofolate reductase DfrA10	Trimethoprim	2															
dfrA12	trimethoprim-resistant dihydrofolate reductase DfrA12	Trimethoprim	19	1	1	12	2	6	16	4	5	1	1		19	15		
dfrA14	trimethoprim-resistant dihydrofolate reductase DfrA14	Trimethoprim	9	2		6	1	10	3	12	1	2	4		16	10		1
dfrA15	trimethoprim-resistant dihydrofolate reductase DfrA15	Trimethoprim							1		1				1	5		
dfrA16	trimethoprim-resistant dihydrofolate reductase DfrA16	Trimethoprim	1							7					1			
dfrA17	trimethoprim-resistant dihydrofolate reductase DfrA17	Trimethoprim			48	16		7	10	1					9	3		

Gene	Product	Antibiotic														
dfrA19	trimethoprim-resistant dihydrofolate reductase DfrA19	Trimethoprim						26		1						
dfrA21	trimethoprim-resistant dihydrofolate reductase DfrA21	Trimethoprim						1								
dfrA22	trimethoprim-resistant dihydrofolate reductase DfrA22	Trimethoprim								1						
dfrA23	trimethoprim-resistant dihydrofolate reductase DfrA23	Trimethoprim						2								
dfrA25	trimethoprim-resistant dihydrofolate reductase DfrA25	Trimethoprim			4											
dfrA27	trimethoprim-resistant dihydrofolate reductase DfrA27	Trimethoprim	1					1		1	1					
dfrA35	trimethoprim-resistant dihydrofolate reductase DfrA35	Trimethoprim									4					
dfrA3b	trimethoprim-resistant dihydrofolate reductase DfrA3b	Trimethoprim									3					
dfrA5	trimethoprim-resistant dihydrofolate reductase DfrA5	Trimethoprim			6						4				2	
dfrA7	trimethoprim-resistant dihydrofolate reductase DfrA7	Trimethoprim					3		20						1	
dfrA8	trimethoprim-resistant dihydrofolate reductase DfrA8	Trimethoprim						1							8	
dfrB2	trimethoprim-resistant dihydrofolate reductase DfrB2	Trimethoprim														1
ere(A)	EreA family erythromycin esterase	Trimethoprim						21			2					
erm(42)	23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(42)	Trimethoprim	1													
erm(B)	23S rRNA (adenine(2058)-N(6))-methyltransferase Erm(B)	Trimethoprim		2	3	3			2			6	2			
floR	chloramphenicol/florfenicol efflux MFS transporter FloR	Chloramphenicol	3	1		7	1	9	31	4	6	1	2	36	12	1
fosA	FosA3/FosA4 family fosfomycin resistance glutathione transferase	Fosfomycin					1									
fosA3	fosfomycin resistance glutathione transferase FosA3	Fosfomycin				2		4	2	3	1			23	5	
fosA4	fosfomycin resistance glutathione transferase FosA4	Fosfomycin							2					3		

Gene	Description	Antibiotic														
fosL	fosfomycin resistance glutathione transferase FosL	Fosfomycin							1							
lnu(F)	lincosamide nucleotidyltransferase Lnu(F)	Lincosamide					1	1	1		1			7		
lnu(G)	lincosamide nucleotidyltransferase Lnu(G)	Lincosamide							16					1		
mcr-1.1	phosphoethanolamine--lipid A transferase MCR-1.1	Colistin						1	8					17	10	
mcr-2.3	phosphoethanolamine--lipid A transferase MCR-2.3	Colistin												1		
mcr-3.1	phosphoethanolamine--lipid A transferase MCR-3.1	Colistin							1			1				
mcr-3.2	phosphoethanolamine--lipid A transferase MCR-3.2	Colistin												1		
mcr-3.40	phosphoethanolamine--lipid A transferase MCR-3.40	Colistin												1		
mcr-5.1	phosphoethanolamine--lipid A transferase MCR-5.1	Colistin					1		3					1	1	
mcr-9.1	phosphoethanolamine--lipid A transferase MCR-9.1	Colistin							64			1				
mef(B)	macrolide efflux MFS transporter Mef(B)	Macrolide							5		1			5	1	
mph(A)	Mph(A) family macrolide 2'-phosphotransferase	Macrolide	16	3	55	21		7	12	10	3	2	3	34	16	
mph(B)	Mph(B) family macrolide 2'-phosphotransferase	Macrolide													6	
mph(E)	Mph(E) family macrolide 2'-phosphotransferase	Macrolide	19	1				1	12		1			1		1
msr(E)	ABC-F type ribosomal protection protein Msr(E)	Macrolide	19	1				1	12		1					1
oqxA	multidrug efflux RND transporter periplasmic adaptor subunit OqxA	Quinolone					1		4	1	1			18	2	
oqxA2	multidrug efflux RND transporter periplasmic adaptor subunit OqxA2	Quinolone												7		
oqxB	multidrug efflux RND transporter permease subunit OqxB	Quinolone					1		4	1	1			20	2	
oqxB2	multidrug efflux RND transporter permease subunit OqxB2	Quinolone												5		

Gene	Description	Drug class													
qepA1	fluoroquinolone efflux MFS transporter QepA1	Quinolone			1									5		
qnrA1	quinolone resistance pentapeptide repeat protein QnrA1	Sulfonamide	14						23				1		
qnrA9	quinolone resistance pentapeptide repeat protein QnrA9	Quinolone							1						
qnrB19	quinolone resistance pentapeptide repeat protein QnrB19	Quinolone											4		
qnrB1	quinolone resistance pentapeptide repeat protein QnrB1	Quinolone	3						8						
qnrB2	quinolone resistance pentapeptide repeat protein QnrB2	Quinolone							5						
qnrB4	quinolone resistance pentapeptide repeat protein QnrB4	Quinolone							10			2	2		
qnrB6	quinolone resistance pentapeptide repeat protein QnrB6	Quinolone	1						2				1		
qnrB9	quinolone resistance pentapeptide repeat protein QnrB9	Quinolone	4												
qnrE1	quinolone resistance pentapeptide repeat protein QnrE1	Quinolone											3		
qnrS	QnrS family quinolone resistance pentapeptide repeat protein	Quinolone												11	1
qnrS1	quinolone resistance pentapeptide repeat protein QnrS1	Quinolone	1	3		2	1	4	26	5	1	3	5	16	2
qnrS2	quinolone resistance pentapeptide repeat protein QnrS2	Quinolone							1				3		
qnrS11	quinolone resistance pentapeptide repeat protein QnrS11	Quinolone													1
rmtB	RmtB family 16S rRNA (guanine(1405)-N(7))-methyltransferase	Aminoglycoside											1		
rmtB1	16S rRNA (guanine(1405)-N(7))-methyltransferase RmtB1	Aminoglycoside			2						2		8		1
rmtC	RmtC family 16S rRNA (guanine(1405)-N(7))-methyltransferase	Aminoglycoside	34				1		1						
rmtD1	16S rRNA (guanine(1405)-N(7))-methyltransferase RmtD1	Aminoglycoside											2		

Gene	Description	Drug class														
sat2	streptothricin N-acetyltransferase Sat2	Streptothricin		4								4			1	
sul1	sulfonamide-resistant dihydropteroate synthase Sul1	Sulfonamide	121[C]	9	54	26	4	8	40	147[C]	9	8	34	34	74	6
sul2	sulfonamide-resistant dihydropteroate synthase Sul2	Sulfonamide	7	29	53	19	6	17	49	18	5	21	4	44	32	
sul3	sulfonamide-resistant dihydropteroate synthase Sul3	Sulfonamide				4	1	5	14		10			22	12	
tet(A)	tetracycline efflux MFS transporter Tet(A)	Tetracycline	5	13	54	44	8	20	13	18	9	8	7	55	100	1
tet(B)	tetracycline efflux MFS transporter Tet(B)	Tetracycline	1	9	2	5		4	58	18	3	7	2		1	
tet(C)	tetracycline efflux MFS transporter Tet(C)	Tetracycline		1				1		2						1
tet(D)	tetracycline efflux MFS transporter Tet(D)	Tetracycline	2							25					3	
tet(M)	tetracycline resistance ribosomal protection protein Tet(M)	Tetracycline					1	1		8		1			9	2
tet(X)	tetracycline-inactivating monooxygenase Tet(X)	Tetracycline								15					1	

[A]For IncW there were only nine plasmids that met the inclusion criteria and were analyzed

[B]The colors are based on a progression of the numbers of specific genes detected in a plasmid type moving from a darker green for the lowest number (1) detected to lighter green to yellow to orange and red for the highest number of genes.

[C]Please note that some plasmids have more than one copy of an individual gene, which can lead to having numbers greater than 100.

2.3.2. DBR and HMR gene analyses

Furthermore, we used the AMRFinderPlus tool to characterize the DBR and HMR genes across the plasmid replicons to count the number of each gene present in the dataset. We identified 5 unique biocide resistance genes, with the highest cumulative numbers being for IncHI2 (n=146), A/C (n=115), P (n=90), FIA (n=58), M (n=58) and HI1 (n=54) (Table 2). By far the most common biocide resistance gene was *qacE*Δ1, which accounted for 464/654 (70.9%) of the DBR genes. For the HMR, 40 unique genes were detected among the strains tested (Table 3). One of the key features of the HMR genes is that they often occur as part of multigene operons, including arsenite efflux (*ars* genes), mercury resistance (*mer* genes), nickel resistance (*ncr*), copper resistance (*pco* genes), copper/silver resistance (*sil* genes), and tellurium resistance (*ter* genes) (Table 3). The highest numbers of HMR genes were found in the IncHI2 (n=1,166), P (n=639) and HI1 (n=404) plasmids. As was observed with the AMR genes, the IncP and HI2 and I1 plasmids have greater than or very near counts of 100 for some of the HMR genes. For example, with the IncP plasmids, three of the genes in the mercury resistance operon (*merP, R* and *T*) have between 103-110 gene copies, while for IncHI2 plasmids there were 97 *merR* and *terD* and 99 *terW* and *terZ* genes detected (Table 3).

Table 2. Disinfectant and biocide resistance genes detected in 100[A] representative sequences for each plasmid replicon type identified using AMRFinderPlus.

BIOCIDE GENES	PRODUCTS	IncA/C	IncBO	IncFIA	IncFIB	IncFIIA	IncFIC	IncHI1	IncHI2	IncI1	IncK	IncM	IncN	IncP	IncW[A]
qacE	Quaternary ammonium compound efflux SMR transporter QacE	29		4	2			1	47	1		4	13	5	2
qacEΔ1	Quaternary ammonium compound efflux SMR transporter QacE delta 1	82	9	54	26	4	10	39	96	9	8	30	23	68	6
qacG2	Quaternary ammonium compound efflux SMR transporter QacG2								1				1	5	1
qacL	Quaternary ammonium compound efflux SMR transporter QacL	4			3	1	4	14		11		2	21	12	
smr	Multidrug efflux SMR transporter Smr								2						

[A]For IncW there were only nine plasmids that met the inclusion criteria and were analyzed
[B]The colors are based on a progression of the numbers of specific genes detected in a plasmid type moving from a darker green for the lowest number (1) detected to lighter green to yellow to orange and red for the highest number of genes.
[C]Please note that some plasmids have more than one copy of an individual gene.

Table 3. Heavy metal resistance genes detected in 100[A] representative sequences for each plasmid replicon type identified using AMRFinderPlus

METAL RESISTANCE GENES	PRODUCTS	IncA/C	IncBO	IncFIA	IncFIB	IncFIIA	IncFIC	IncHI1	IncHI2	IncI1	IncK	IncM	IncN	IncP	IncW[A]
arsA	arsenite efflux transporter ATPase subunit ArsA	6						1				2	1		
arsB	arsenite efflux transporter membrane subunit ArsB	5						1				2	1		
arsC	glutaredoxin-dependent arsenate reductase	5						1	81	1		3	1		
arsD	arsenite efflux transporter metallochaperone ArsD	7						9				2	1		
arsR	arsenite efflux transporter ATPase subunit ArsR	7						9				2	1		

Gene	Description														
klaB	tellurium resistance system protein klaB													2	
klaC	tellurium resistance system protein klaC													2	
merA	mercury(II) reductase	13						1	60			25		14	1
merB	organomercurial lyase MerB	11							20			2	1	14	
merC	organomercurial transporter MerC	14	16	3	4	1	4	50	17	9	11	1	11	89	
merD	mercury resistance co-regulator MerD	13						7	75	1		23	1	15	1
merE	broad-spectrum mercury transporter MerE	13						7	59			21		12	1
merG	phenylmercury resistance protein MerG								3						
merP	mercury resistance system periplasmic binding protein MerP	25	16	3	4	1	4	56	24	8	11	2	8	103[c]	1
merR	mercury resistance transcriptional regulator MerR	57	16	2	6	1	5	60	97	9	11	29	13	110	1
merT	mercuric transport protein MerT	29	16	3	4	1	4	60	80	9	11	27	12	106	1
ncrA	Metal Resistance								1						
ncrB	nickel-sensing transcriptional repressor NcrB								1						
ncrC	Ni(II)/Co(II) efflux transporter permease subunit NcrC								1						
pcoA	multicopper oxidase PcoA	1		1				7	9	1			9	8	
pcoB	copper-binding protein PcoB	1		1				7	8	1			4	8	
pcoC	copper resistance system metallochaperone PcoC	1		1				7	9	1			5	8	
pcoD	copper resistance inner membrane protein PcoD	1		1				2	8	1			1	8	
pcoE	copper resistance system metallochaperone PcoE	1						5	8				5	6	
pcoR	copper response regulator transcription factor PcoR	1		1				2	8	1			1	8	
pcoS	copper resistance membrane spanning protein PcoS	1		1				8	82	1		1	5	8	
silA	Cu(+)/Ag(+) efflux RND transporter permease subunit SilA	1		1	2			9	26	1			12	8	
silB	Cu(+)/Ag(+) efflux RND transporter periplasmic adaptor subunit SilB	1		1	2			9	26	1			12	8	
silC	Cu(+)/Ag(+) efflux RND transporter outer membrane channel SilC	1		1	2			9	26	1			12	8	
silE	silver-binding protein SilE	1		1	2			9	29	1			12	8	

Gene	Description													
silF	Cu(+)/Ag(+) efflux RND transporter periplasmic metallochaperone SilF	1		1	2			9	26	1			12	8
silP	Ag(+)-translocating P-type ATPase SilP	1		1	2			9	28	1			11	8
silR	copper/silver response regulator transcription factor SilR	1		1	2			9	30	1			12	8
silS	copper/silver sensor histidine kinase SilS	1		1	2			9	29	1			12	8
terB	tellurium resistance membrane protein TerB	1		1				8		1			2	
terC	tellurium resistance membrane protein TerC	1		1				8		1			2	
terD	tellurium resistance membrane protein TerD	1		1		2	1	8	97	2		1	34	18
terE	tellurium resistance membrane protein TerE	1		1				8		1			2	
terW	tellurium resistance protein TerW					2	1		99			1	32	18
terZ	tellurium resistance-associated protein TerZ					2	1		99	1		1	32	18

[A]For IncW there were only nine plasmids that met the inclusion criteria and were analyzed

[B]The colors are based on a progression of the numbers of specific genes detected in a plasmid type moving from a darker green for the lowest number (1) detected to lighter green to yellow to orange and red for the highest number of genes.

[C]Please note that some plasmids have more than one copy of an individual gene.

2.3.3. Comparison of resistance genes

We next compared the pattern of the data of three types of resistance genes including AMR, DBR and HMR to determine the more global diversity of resistance genes across the 14 plasmid types associated with enteric bacteria (Table 4, Figure 2). When the percentage the three resistance gene types across of each plasmid replicons were evaluated in relation to the total numbers of acquired resistance genes in the AMRFinderPlus database, the results were variable across the different plasmid types, with the overall average percentage of AMR genes detected being 5.5%; and 9.0% for DBR and 22.5% for HMR: 14.4 (Table 4).
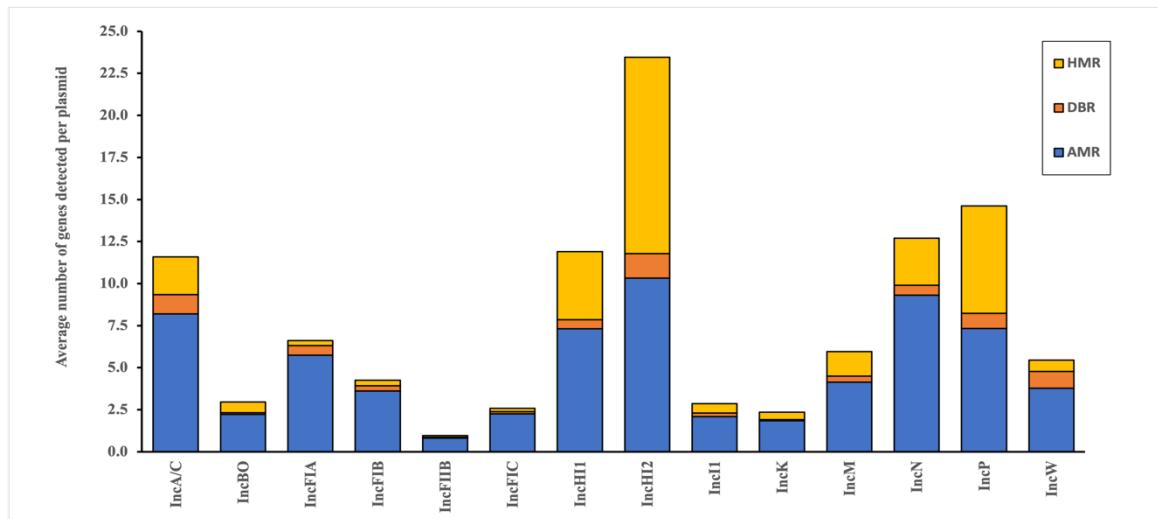
Figure 2. Average number of AMR, DBR and HMR genes detected per plasmid in each of the plasmid replicon types.

Likewise, according to the overall body of dataset it showed that there were six plasmid types with relatively high prevalence of resistance genes (>15% average percentage) compared to other individual plasmid types, which these included IncN (20.5%), HI2 (20.0%), A/C (18.7%), HI1 (18.2%), P (17.9%), and I1 (15.0%) (Table 4, highlighted in red text). These observed elevated average resistance gene levels were largely driven by the HMR genes, as those

with the highest overall percentages had the highest metal gene percentages; however, many of

these types also had higher than average AMR gene percentages as well. In contrast, the IncBO

and K plasmids had the lowest numbers of unique resistance genes detected compared to the

other plasmid types, however none of the differences in resistance gene content between plasmid

types reached statistical significance.

Table 4. Relative percentage of the different gene types compared to the number of corresponding genes in AMRFinderPlus (AMR: n=983; Biocide: N=30; Metal: N=82) and ISFinder (N=29).

Replicon types	Percent of genes present among plasmid replicons				Average Resistance Genes	P value
	AMR	Biocide	Metal	IS/Tn Families		
A/C	7.2	10.0	39.0	58.6	18.7	0.30
BO	4.2	3.3	4.9	62.1	4.1	0.09
FIA	3.5	6.7	26.8	65.5	12.3	0.50
FIB	4.6	10.0	14.6	62.1	9.7	0.34
FIIB	2.8	6.7	8.5	58.6	6.0	0.16
FIC	4.7	6.7	8.5	65.5	6.6	0.17
HI1	6.7	10.0	37.8	65.5	18.2	0.31
HI2	8.9	13.3	37.8	58.6	20.0	0.25
I1	4.5	10.0	30.5	62.1	15.0	0.40
K	4.2	3.3	4.9	62.1	4.1	0.09
M	7.7	10.0	20.7	58.6	12.8	0.47
N	9.2	13.3	39.0	62.1	20.5	0.24
P	6.1	13.3	34.1	55.2	17.9	0.30
W	2.3	10.0	7.3	55.2	6.6	0.18
Average	5.5	9.0	22.5	60.8	12.3	

Average resistance gene percentages highlighted in red font had >15% of genes detected and discussed further in the body of the manuscript.

The association of the common resistance genes across the different incompatibility

groups may suggest mobility of AMR, DBR, and HMR within bacterial populations through

plasmid transfer [Tamminen et al. 2012]. These observations further indicate importance of

plasmid transfer for the dissemination of resistance across the Enterobacteriaceae [Che et al.,

2021; Rodrigues et al., 2020; Li et al., 2021]. Moreover, previous studies also showed the prevalence of AMR, DBR and HMR in *S. enterica* isolates was mainly plasmid mediated [Deng et al., 2018; Mourao et al., 2015]. Looking specifically at the individual plasmid types with the highest prevalence of resistance genes (IncN, HI2, I1, HI1, P and A/C), there are some key features. For example, the IncA/C plasmids have been isolated from multiple *S. enterica* and *E. coli* associated with infections of animals and human patients and often found to simultaneously contain multiple AMR, DBR and HMR genes [Octavia et al., 2020; Zhao et al., 2020; Harmer and Hall, 2014; Mattioni Marchetti et al., 2020]. The IncI1-complex of plasmids also have a global diversity in Enterobacteriaceae, and many representatives carry multiple resistance genes, including those for clinically important agents, and they have been reported as a major driver of antimicrobial resistance in *Salmonella, E. coli* and *Klebsiella pneumoniae* [Zhang et al., 2019; Oladeinde et al., 2021; Sekizuka et al., 2017; Abraham et al., 2018; Foley et al., 2021]. The IncHI plasmids tend to be very large plasmids, often greater than 200 kB in size and carry multiple AMR, HMR and DBR operons, as well as virulence factors that are important for enteric fever [Phan and Wain, 2008, Han et al., 2012]. The IncN and IncP plasmids have relatively broad host ranges and are found to disseminate of AMR, which has been demonstrated in *K. pneumoniae, E. coli* and *S. enterica* isolated from healthy humans and clinically ill patients (Lu et al., 2017; Wailan et al., 2015). These diverse groups of medically important plasmids have shown the utility of sequence data to characterize plasmids in Gram-negative bacteria and the use of sequencing has been proposed as a method to assess the functions of plasmids to improve health outcomes (Harmer and Hall, 2014).

2.3.4. IS element analyses

To assess the presence of IS and Tn elements in the sequenced plasmids, ISFinder was utilized. This program has been used for the analyses the mobilome of *S. enterica*, *E. coli*, *K. pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Shigella* spp. which are included in the World Health Organization (WHO)'s list of the important pathogens [World Health Organization, 2020; Zhang et al., 2022]. In this study, ISFinder predicted over 250 MGEs that were either ISs or Tns. Among these, there were 24 (from a total of 29) different families of IS/Tn elements identified in at least one plasmid Table 5. The prevalence of ISs was highly diverse and variable within the data set, with 11 of 29 IS families identified in all 14 plasmid types. Among these, Tn3 was among the most abundant of the sub-families identified across all the individual plasmid replicons (Table 5). The genetic variability of the IS elements contributes to the dynamic diversity observed in the corresponding host genetics [Mahillon and Chandler et al., 1998; Vandecraen et al., 2017]. Further study is required to evaluate the combination of resistance genes type and IS/Tn in the individual plasmid replicon types.

To gain a fuller understanding of the distribution of the MGEs across the plasmid types, a comparison was performed between resistance genes elements and MGEs (IS/Tn) in the plasmid data set. As noted above, six replicons including IncA/C, I1, HI1, HI2, N, and P possessed the highest number of different resistance genes and MGEs as determined by calculating the percentage of each in individual plasmid replicons (each had an average of at least 15% presence among the different resistance groups; Table 4). When the numbers of different families of IS/Tn elements were detected, these plasmid types did not have a significantly increased number of IS/Tn families (Table 5). The overall range of IS/Tn families was 17 to 21 per plasmid type

(average 18.9; SD 1.1); among the six replicons, the range was 17 to 20 (average 18.7; SD 1.1), thus the increase in numbers of genes was not due to an increase in IS/Tn element.

Table 5. IS families detected (brown box) in the representative plasmid types using ISFinder

IS Family	PRODUCT	IncA/C	IncBO	IncFIA	IncFIB	IncFIIA	IncFIC	IncHI1	IncHI2	IncI1	IncK	IncM	IncN	IncP	IncW
IS110	IS110 family transposase ISMav6	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IS21	IS21 family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IS256	Mutator IS256 family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IS3	IS3 element transposase of IS3 family	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IS4	IS4 family transposases	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IS5	IS5 family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IS6	IS6 family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IS66	IS66 family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	■
ISL3	ISL3 family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	■
ISNCY	ISNCY family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Tn3	Type II transposons of Tn3 family	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IS630	IS630 family transposase	■	■	■	■	■	■	■	■	■	■	■	■	■	
IS30	IS30 family transposase IS1062	■	■	■		■	■	■	■	■	■	■	■	■	■
ISKra4	ISKra4 family transposases	■	■	■	■	■	■	■	■	■	■	■	■		■
IS481	IS481 family transposase		■	■	■	■	■	■	■	■	■	■	■	■	
IS1182	IS5/IS1182 family transposase	■		■	■	■	■	■	■		■	■	■	■	■
IS1	IS1 family transposase	■	■					■	■		■		■	■	■
IS200/IS605	IS200/IS605 family transposase		■					■	■	■		■	■	■	■
ISAs1	ISAs1 family transposase	■	■					■	■		■		■	■	■
IS91	IS91 family transposase		■			■	■								■
IS1380	IS1380 family transposase ISEc9			■		■		■				■			
IS1634	IS1634 family transposase IS1549							■				■	■	■	
ISAzo13	ISAzo13-like element ISCfu1 family transposase														■
ISH6	ISH6 family transposase														■
IS1595	IS1595 family transposase														
IS701	IS701 family transposase														
ISH3	ISH3 family transposase														
IS607	IS607 family transposase														
IS982	IS982 family transposase														

Several of the HMR elements are part of the larger set of operons (*mer, ars, pco, sil,* and *ter*) with multiple genes per operon, which led in part to IncA/C, I1, HI1, HI2, N, and P plasmids having the highest percentage of genes (Tables 3 and 4). These HMR operons are typically part of a IS element or Tn on a plasmid, thus the increase in genes does not lead to a proportional

increase in IS/Tn elements (i.e., unlike AMR, where a single gene leads to resistance, for HMR multiple genes in an operon are required to encode the resistance) [Han et al., 2012].

Because these elements are often located on plasmids, it is reasonable to believe that plasmids are required components for understanding of dissemination of resistance genes in isolates from food producing animals and humans [Wang et al. 2014]. It has been reported the MDR regions carried on different plasmids can be present novel mobile elements (MGEs) in clinical bacterial strains [Cheng et al., 2019]. For example, MGEs located on transmissible plasmids, particularly IncI and IncF groups, have been demonstrated previously to facilitate resistance transmission [Venturini et al., 2013]. The Tn3-type Tns play critical role in the evolution of both MDR plasmids and chromosomal islands in the Enterobacteriaceae [Venturini et al., 2013]. Several conserved and variable resistance gene type (RGs)-MGEs combinations have been observed across unrelated enteric pathogens and were located on different individual plasmid replicons based on the results of on the current study.   In our data, there was significant overlap in the presence of the genes *sul1* and *qacEΔ1*, which are common parts of 3'-conserved region of class 1 integrons [Gillings et al., 2008]. These integrons are also associated with a variety of IS elements that allow for the accumulation of antimicrobial resistance gene cassettes with in the integrons [Gillings et al., 2008; Gillings et al., 2009]. Moreover, a composite transposon-like structure carrying multiple resistance genes has been identified on various plasmid Inc groups and in genomic islands identified in *Salmonella* and *E. coli* isolates from diverse food, animal human and environment sources [Reid et al., 2015].

One of the distinctive findings of this study, which is highlighted in Table 4, is that, among the different families of IS elements, these elements are widely distributed across the different plasmid types. Indeed, 18 of the 24 (75%) different IS families were found in at least

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75% (n>10/14) of the plasmid types, with 11 (45.8%) IS families being detected in all of the different plasmid types. This widespread dissemination of these MGEs likely provides opportunities for the wide distribution of resistance genes associated with the elements to spread between the different plasmid types when they are co-resident within a host bacterium. The distribution of the resistance genes is further evident in the heat map that was produced to illustrate the similarities and differences of AMR, BRG and HMR genes detected across the different plasmid replicons (Figure 3). The heat map was used to cluster the groups based on the resistance gene types. According to the analysis, IncB/O and IncK are most closely related as are IncFIA and IncFIB plasmids based on the cumulative composition of resistance genes (Figure 3). These particular plasmid types have previously been shown to have similar transfer functions as well [Shirakawa et al., 2020; Zhao et al., 2020].
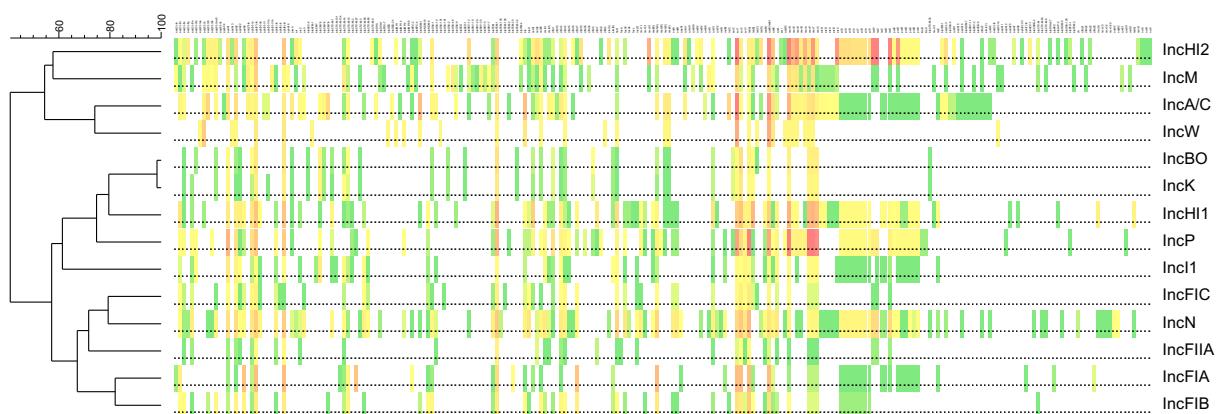
Figure 3. Illustration of clustering heat map association between different replicon type based on a combination of AMR, BDR and HMR data from Tables 1, 2 and 3, respectively. Clustering analysis was performed using Pearson correlation of numerical values using BioNumerics software to generate the dendrogram using the unweighted pair group means with averages (UPGMA) algorithm. The colors of the boxes correspond to those in the tables.

The use of databases and *in silico* tools allow for the prediction of bacterial function. Resistance gene prediction databases for plasmid sequences have given insights into human health care challenges in a timelier fashion [Darphorn et al., 2021]. Previously, it was complex and time-consuming to characterize large population of *S. enterica* and other enteric pathogens by their plasmid replicon typing in a laboratory setting. However, in the present *in silico* study, identified resistance gene types and MGEs at the same time became possible and facilitated the characterization over that 1,300 plasmid sequences from enteric pathogens that contained representative plasmid replicon types and different gene types. Indeed, the epidemiological observation of the presence of diverse mechanisms suggests extremely broad dynamic potential for transfer across the range of plasmids [Che et al., 2021]. Our data analysis results provide extremely helpful way to analyze the plasmid replicons sequences as part of molecular epidemiology techniques.

A major limitation of our research is the need of extensive systemic validation of the data processing. For example, we used 100 representative plasmid sequences for each plasmid type and a question may be how representative these are to the entire plasmid population. This will likely get easier with the increasing availability of high-quality closed plasmids that are becoming available. In the interim, we conducted an analysis to determine the potential impact of selection of the 100 plasmids vs. the total available at the time of selection. To do this analysis, we compared the 100 IncA/C plasmid sequences that were returned through GenBank with the total number that were available at the time (validation set, n=563). With the validation set, 214 AMR genes were identified compared to 71 for the test set (Supplemental Table S3, Validation tab). Of the 143 genes that were not detected in the test set, but present in the validation set, 51 (36%) were present in only one plasmid and 104 (73%) were present in 5 or

less plasmids, which would correspond to less than 1% of the 563 total plasmids in the validation set. These lower abundance genes are at a threshold below the 1% which is the level for a single positive in the test set of 100. There were 12 (8%) instances, where a gene was detected in at least 2% of the validation set, but not in the detected in the test set, with a range of 2.1 to 6.8%. This largest percentage was for *aac(6')-Ib4*, for which some of the related variants of *aac(6')-Ib* were more common in the test set. There were some other variabilities in the genes that were detected among the test and validation sets, with the gene bla_{NDM-1} detected in 54% of the test set isolates compared to 20% of the validation set. There were six additional genes that were detected in at least 20% of the test that were present at least a 10% lower level in the validation set, these included *ble, rmtC, blaCMY-6, aac(6')-Ib3, sul1,* and *armA* Conversely, *sul2* was detected in 7% of the test set plasmids and 66% of the validation set isolates. There were also six additional genes that were detected in at least 20% of the validation set, but at least a 10% lower level in the test set, these included bla_{TEM-1}, bla_{CMY-2}, *aph(6)-Id, tet(A), floR*, and *aph(3'')-Ib*. Altogether, of the 214 AMR genes, 189 (88%) had less than 5% difference in detection rates between test and validation sets and 158 (74%) were within 2% variance of one another. Thus overall, the findings from the test set of 100 plasmid sequences was, in general, representative of the larger validation set of plasmids; as even in cases where specific genes were much more commonly detected in the validation set, they were still detected in the test set, albeit at a lower percentage.

Another caveat with the study is that there may be a bias to resistance-related plasmids, as these have a potential be more important to public health due to their clinical relevance and may be targeted for WGS compared to those not associated with AMR. Future prospects of our research include the expansion of the current results to look at more expansive sets of data for

each of the plasmid types. Additionally, it may be important to have greater detailed assessment of all accessory genes on plasmids including *mob* and *tra* genes that impact the dissemination of MGEs among enteric pathogens. These detailed analyses can also include a detailed linkage analyses of resistance genes to specific IS/Tn elements that will give more detail to the distribution of specific genetic elements through bacterial populations.

2.4. Conclusions

In conclusion, the study demonstrated that AMR, BRG and HMR genes were widely distributed among the Enterobacteriaceae regardless of the source origin. Based on the *in silico* analyses, RG-MGE combinations likely demonstrate the dynamic interactions that contribute to the plasticity of multidrug-resistant pathogens and to the mobility of genes among plasmids. Overall, these findings advance the level on the current understanding of the interplay of resistance genes, IS elements, plasmid replicons and the scope of the significant combinations between plasmid genetic content interaction and other MGE components, which effects the dissemination of antimicrobial resistance genes.

Acknowledgements

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Supplemental Table S1. Replicon typing reference sequences used in the BLAST program to detect plasmids for inclusion in the study (Carattoli et al. 2005)

IncA/C
ACGACAAACCTGAATTGCCTCCTTGATTTCGTTCAGGTTGTAACCGTGACCCATCTTC
GAGAGCTCTTTCTGGAGTTCGTACAGAGTGAACATGACTCCAGCCTTGCCCTCGATG
ATATGGCCTTTCCCGTTCACTGCGAGCTTGCGTAGAGCATCCTCAAGGATTTCCTCTC
GCTGTCCCGCGTAGATCAGCACGGTTCTTCCGTCATCCTTCTCGATGATGGCTGGCTT
CACCTTCACCGTGAAATGCTGGCCTCTGATGGTGCATTGTCGTGTCACTACAGCGTT
GGATAAATCTTCATGCTCACGCTTTTGGTCCCAAATGTACTTCGGCAGCGCATCGTA
GATCTCGATGGTGTTGGAGTAGTCGTCTTTCTTGGCCGGAGATTCAATGATCTCAAA
CAGGCTCATTTGCGGTGAGTCTTTGATTACGTCGAGTTTCTCCAGGTCTTTGTCTTTG
GTTCTC

IncB/O
TCTGCGTTCCGCCAAGTTCGAGGAAAAATAGTGGGGGTTTTCCTTTATGGTCTTCTG
AAACTTTTTTCAGCCTTCCGGCACCGGCAAACTGCCGTCGTTTCTGCCATACGTCCTG
AATGCCTTATGGCGCATTCTGCCGTTTTCTGGCTTTCCGGACCGC

IncFIA
CCATGCTGGTTCTAGAGAAGGTGTTGTGACAAATTGCCCTTTCAGTGTGACAAATCA
CCCTCAAATGACAGTCCTGTCTGTGACAAATTGCCCTTAACCCTGTGACAAATTGCC
CTCAGAAGAAGCTGTTTTTTCACAAAGTTATCCCTGCTTATTGACTCTTTTTTATTTA
GTGTGACAATCTAAAAACTTGTCACACTTCACATGGATCTGTCATGGCGGAAACAGC
GGTTATCAATCACAAGAAACGTAAAAATAGCCCGCGAATCGTCCAGTCAAACGACC
TCACTGAGGCGGCATATAGTCTCTCCCGGGATCAAAAACGTATGCTGTATCTGTTCG
TTGACCAGATCAGAAAATCTGATGGCACCCTACAGGAACATGACGGTATCTGCGAG
ATCCATGTTGCTAAATATGCTGAAATATTCGGATTGACCTCTGCGGAAGCCAGTAAG
GATATAC

IncFIB
GGAGTTCTGACACACGATTTTCTGTTTATTCTTTTACTGTCCACAGGCTGGAGGCTTT
CTGGAAAACGAAAATTCAGACATCAAAAAACTGTTCGGCGAGGTGGATAAGTCGTC
CGGTGAGCTGGTGACACTGACACCAAACAATAACAACACCGTACAACCTGTGGCGC
TGATGCGTCTGGGCGTTTTTGTACCGACCCTTAAATCACTGAAGAACAGTAAAAAAA
ATACACTGTCACGTACTGATGCCACGGAAGAGCTGACACGTCTTTCCCTGGCCCGTG
CTGAGGGATTCGATGAGGTTGAGATCACCGGCCCCCGCCTGGATATGGATAACGATT
TCAAGACCTGGGTGGGGATCATTCATTCCTTTGCCCGCCATAACGTGATTGGTGACA
AAGTTGAACTGCCTTTTGTTGAGTTTGCAAAACTGTGTGGTATACCTTCAAGCCAGT
CATCCCGCATGCTGCGTGAGCGCATCAGCCCTTCCCTGAAGCGCATTGCCGGTACCG
TGATCTCGTTTTCCCGTACCGATGAGAAGCACACCCGGGAATACATCACCCATCTTG
GACAATCAGCCTACTACGATACTGAGCGGGATATTTGTTAAGTTCAGGCTTGATCCCCGCT
GGTTGAACTGTACCAGTTTG

IncFIC
TTCTCCTCGTCGCCAAACTAGATGAAGATTATCGGGGGTTTTTGCTTTTCTGGCTCCTGTAAATCCACATC
AGAACCAGTTCCCTGCCACCTTATGGCGTGGCCAGCCACAAAATTCCTTAAACGATCAGCAATCTATC

ACTCACGCCTGAGATAAGCAAGAATGTGAATATTTACAAAGTCGCTCTGCGTTTCAGCTCTGATTCAAT
CAGTTTTTCAAGCATCTGCGCCTGGGTAATACCTTCCTCATCTGCCAGTTCAC

IncFIIA
CTGTCGTAAGCTGATGGCGAAAGCCGAAGGGTTCACGTCCCGTTTTGATTTTTCCGT
CCATGTGGCGTTCGTTCGTTCGCTGGGAAAGCGTCACCGGATGCCGCCTTTGCTGCG
CCGTCGTGCCATCGATGCGCTGCTTCAGGGGTTGTGCTTCCATTATGATCCACTGGCC
AACCGTGTACAGAGATCCATCACCAATCTGGCTATAGAGTGCGGTCTGGCCACTGAG
TCAAAAAGTGGTAATCTGTCCATCACCCGCGCCACACGGGCGCTGAAGTTTGTGGCA
GAG

IncHI1
GGAGCGATGGATTACTTCAGTACGCTTAGGCGCATTAATATAACGGGATTTGTCGTT
CCCGATATGCTGCTCATCACTCATACATTACCCCCTTGCTTCACTGAAAAACTCTTAA
TCTCATTAATAAATTGCTGGTAGACCGCCATCACGGATTCTTCGGCAACATCAAGCT
GTTTGGGAGAGCAGAGCTCTTCTGATTTTTTGATATCCAATACGGTTCTACCCCTAGA
AGCTGCGGCTTTAAACGCTTCTGAGTTTTTGATATTTGCAGACATAAAGAGTTCCTG
CACTGTCCTTACCAGTTTATCCAGGACAATCTTTTCATAGGGACTCTTATCGTCGACA
CTCACTGCCAACACTTTGAACCAGCTTAGATTTTCGCCCTTGCTCGGCGATTGTTTGA
ATCGCTCACTGATCGTCAACATGAAATCAGTTGTTGAGGCATAATCATACTCACGAG
GTGAAACGGCA

IncHI2
TTTCTCCTGAGTCACCTGTTAACACCCGGTTTCTACGCTTTACTTCATTGGCCAGTAC
ATCCAACGGAAAATATCGTAACGGGGTGACAATAGCAGTGAGTTGACCCACTCACT
ACCATCGACATTCAAATCCCGGCTTCTCTGGTAGCTATAGTGACAATGGTAATGATG
TGTACCTTACTGGCATTGTCATTGTCCTCCGAGTGTGCGTTCAGCTTTGGGAGGACAC
CGGAACTTAATGCGGCGAGTCACGGCCATTGTGAATCTCGATTGGATTGGCGAATAC
GGCTTCCGGTTGGGCCAGATGTCAATGGTTATGCTTCATCCAGCTCATTACTGTAGTC
ATTGCCGTTCAGCTGAGCTGATGCTCCTCTCATTCTATGTGGCGACAACGGTAGTGA
GCCCAGAAAGTGACTACCGTTGTCATGGCCGTCCAGTAGAGCGGGTCAACCTCTTAA
ATCACAGGGTGACAACGGTATTGAGCCCAGAAAGTCACTACCGATGTCATGGACGT
CTGGTTAAGCGGATCAACCTCTCAATTACAGGGTGCAATGGTAGTGAGGCCAGCAA
GGCACTACCGTTGCATAGTCGTCCCGGTGNGCAGATCAACCTCTTAACTTACAGGAT
GACAACGGTAGTGAGCC

IncI1
CGAAAGCCGGACGGCAGAATGCGCCATAAGGCATTCAGGAGAGATGGCATGTACGG
GCAGTAAGTCAGAAGACTGAAGATGTTCCGGAAGCCATAAAAGGAAAACCCCCACT
ATCTTTCTTACGAACTTGGCGGAACGA

IncK
GCGGTCCGGAAAGCCAGAAAACGGCAGAATGCGCCATAAGGCATTCAGGATGTATG
GCAGAAACGACGGCAGTTTGCTGGGGCCGGAAGGCTGAAAAAGTTTCAGAAGGCC
ATAAAGGAAAACCCCCACTATCTTTCCTCGAACTTTGGCGGGCTCGTGAAAGA

IncL/M

CTGCAGGGGGCGATTCTTTAGGGGACTGGCTTTCAAGCCAGGAGATGAACTCCGGCG
AAAGACCTTCTACTGACTCAATGTCAGAAAGCTGTAGGTATGGATTCTTTTGATTCA
CCAGCCCCTGCACATATTGGCCGGGCTGTATTGCAATAGGGTACGCATTTGCTATAA
TCCTTTCTGCCAGTGGGCAGTTTGCACCCCCTGATTCTATTCCGAGCCGGCCAAAGTT
CTGAAATAGGATCAGGGGGGTTTTACTTTTTGTGGCTCCTGCCACTCCTAAGCGGAAC
ATCTCCGGGCCGCTCTTCCCGTGCTAAATTCTGTAATAACCCCGTAAGGTTATGTAA
ACGCCGCTTAACATATCATGCGTTTCTAAGCGGGGCAATACCTGCGATCCCGCAGTT
ACAGAGTAACCTCACTCTTCAGAATCGTCAAATTTTAAGCCGTCGTAATAACTTTTC
ACAAGAAATTCAATAACCTCCGCCTGAGTGAGCTTTTTTACTTTTGTGATCTCTATCA
ACATTCTTTTTGCATCTTTGGGCAGATAAAGATTTAGCTGGTCATGAGTATCTCTGAT
GCGATTCCTGTAATCCTTCTGCTTATCCAGGTTACTCTTATGCACCTGCCGCTCAAAT
ACTCTGCTNANAAGCCTTCGACTACAACGACTNTTACCNCCTGNGTATCNCCAAAAA
ACCCGCCATANTCGGCGGGTAAACTCAAAAAATTCTGTTTATGAA

IncN

GTCTAACGAGCTTACCGAAGCTGCTTACTACCTCTCGCTAAAAGCAAAGCGCGTTCT
CTGGTTATGTCTTATGCAGACGTATTTCACAGCTTCAGTAAGCGAAGATGATGATGA
GATGGCTGTACTCGGTGACTCTACTTTCAAAGTAAAGGTGGCTGACTATCAGCAAAT
TTTTCAGGTAAGCCGTAACCAGGCTATCAAGGATGTTAAAGAAGGCGTGTTTGAGTT
AAGCCGTTCTGCGGTAATCTTTTACCCGAAAGAAGGGAGTTTTGACTGCGTCGCGCG
CCCCTGGCTAACAGAGGCTGGCAGCCGATCAGCTCGTGGTATCTGGGAAATCGAATT
TAACCATAAACTCCTGCGGTACATTTACGGCCTGACGAACCAGTTCACCACCTACTC
GCTCCGCGATTGTGGCAGTCTTCGAAATCCACGGACGATCCGCCTTTATGAAAGTCT
TGCTCAATTCAAATCTTCAGGCTTATGGGTTACTACTCATGCTTGGTTAAATGACCGT
TTCCTTTTGCCGGAATCCCAACAGAAGAACTTGGCAGAGTTGAAAC

IncP

CTATGGCCCTGCAAACGCGCCAGAAACGCCGTCGAAGCCGTGTGCGAGACACCGCG
GCCGCCGGCGTTGTGGATACCTCGCGGAAAACTTGGCCCTCACTGACAGATGAGGG
GCGGACGTTGGCACTTGAGGGGCCGACTCACCCGGCGCGGCGTTGACAGATGAGGG
GCAGGCTCGATTTCGGCCGGCGACGTGGAGCTGGCCAGCCTCGCAAATCGGCGAAA
ACGCCTGATTTTACGCGAGTTTCCCACAGATGATGTGGACAAGCCTGGGGATAAGTG
CCCTGCGGTATTGACACTTGAGGGGCGCGACTACTGACAGATGAGGGGCGCGATCC
TTGACACTTGAGGGGCAGAGTGCTGACAGATGAGGGGCGCACCTATTGACATTTGA
GGGGCTGTCCACAGGCAGAAAATCCAGCATTTGCAAGGGTTTCCGCCCGTTTTTCGG
CCACCGCTAACCTGTCTTNTAACCTGNCTTTAAACCAATATTTATAAACCTTGTTTTT
AACCAGGGCTGCGCCCTGGCGCGTGA

IncW

GGTGCGCGGCATAGAACCGTAGGGCAGGCCGATGCTCGGCTTGCCCATGATCGACA
AGGTGACGATGCCATTGGTGCGCTCAAAGTAGCTGGTCTTGGGGTCGGTGTGGGGC
ATGGTCGCTTGCACAAGGCAACGGGCCCGTAGCCGACTAAGCCAGCTTCGCGGGCA
TCCTCCATTTCGAGCGCGAGGCTCGTCTTGATGATCTCGTTGATACGATGGCCGGGG
GCTTTGTTGTTCTTAGG

Supplemental Table S2. Plasmids included in the study

Supplemental Table S3. Output data from AMRFinderPlus analyses

RESEARCH ARTICLE

Evaluation of transfer-associated genes from antimicrobial resistance plasmids in enteric bacteria

Suad Algarni[1,2], Steven L. Foley[1], Hailin Tang[3], Shaohua Zhao[4], Dereje D. Gudeta[1], Bijay K. Khajanchi[1], Steven C. Ricke[5], Young Min Kwon[2], Douglas D. Rhoads[2], and Jing Han[1*]

[1]Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson AR, 72079

[2]Cellular and Molecular Biology Graduate Program, University of Arkansas, Fayetteville AR, 72704

[3]Division of Bioinformatics and Biostatistics, National Center for Toxicological Research, Food and Drug Administration, Jefferson AR, 72079

[4]Center for Veterinary Medicine, Food and Drug Administration, Laurel MD, 20708

[5]Meat Science & Animal Biologics Discovery Program, Dept. of Animal and Dairy Sciences, University of Wisconsin, Madison WI, 53706

*Corresponding Author: Jing Han, National Center of Toxicological Research, 3900 NCTR Rd, Jefferson, AR 7209. Phone: (870) 543-7528. Email: jing.han1@fda.hhs.gov

Running Title: Plasmid-transfer gene database

Abstract:

Type IV secretion systems (T4SSs) are integral components of the conjugation process in enteric

bacteria. These secretion systems are encoded within the transfer (*tra*) regions of plasmids,

including those that harbor antimicrobial resistance genes. The conjugal transfer of resistance

plasmids can lead to the dissemination of antimicrobial resistance (AMR) among bacterial

populations. To facilitate the analyses of the conjugation-associated genes, transfer related genes

associated with key groups of AMR plasmids where identified, extracted from GenBank and

used to generate a Plasmid Transfer Gene Database that served as the foundation for

computational tools for the comparison of the conjugal transfer genes. Two comparison tools, the

Plasmid Transfer Factor Assessment and Plasmid Transfer Factor Comparison tools, were

developed to evaluate the transfer genes located on plasmids and to facilitate the comparison of

plasmids from multiple sequence files. To assess the database and associated tools, plasmid and

whole genome sequencing (WGS) data were extracted from GenBank along with prior WGS

data from our lab and assessed using the analysis tools. According to the datasets, the plasmid

transfer genes from IncBO and IncK where highly similar/identical to one another and in some

cases closely related to IncI1 plasmids. Additionally, when genes/proteins of the same name

(e.g., *traI*/TraI) or predicted function (VirD4 ATPase homologs) were compared across the

different plasmid types, there was minimal sequence similarity among the different plasmid

replicon types, with only a few exceptions. Overall, the plasmid transfer database and associated

tools proved to be very useful approaches for evaluating the different plasmid types and their

T4SSs. Based on the study findings, there are some potentially necessary modifications to be

added to the database including combining the IncBO and K gene sets and ensuring that all T4SS

genes are present. These modifications will improve database and increase the utility for

understanding how conjugative plasmids contribute to the dissemination of antimicrobial resistance genes.

3.1. Introduction:

With the increasing utilization of DNA sequencing for the characterization of bacterial pathogens, such as *Salmonella* and other enteric species, improved analysis tools are needed to more fully capture the value of the data which can contribute to improved public health. Whole genome sequencing (WGS) is being widely used by academic, public health and regulatory laboratories to better understand microbial genetics and physiology. *Salmonella enterica*-related infections affect millions of humans and animals in both developed and under-developed countries, which costs the global economy billions of dollars; therefore, tools for epidemiological studies which can improve prevention and treatment approaches are important [Zafar et al., 2019]. *Salmonella* strains can be divided into more than 2,600 serotypes based on their surface antigen profiles. However, most human infections in the United States (U.S.) are associated with a smaller number of serotypes including Typhimurium, Enteritidis, Newport, Javiana and Heidelberg [Algarni et al., 2022].

Among *Salmonella* and related pathogens, antimicrobial resistance (AMR) is a significant concern due to potential treatment failures. Often, AMR genes are encoded on transmissible plasmids; as such, research on plasmids has mainly been focused on a limited number of types of conjugative plasmids associated with the multidrug resistance (MDR) in bacterial pathogens [Algarni et al., 2022]. Conjugative plasmids are known to be self-transmissible and encode genes associated with mobilization (MOB) and/or DNA transfer and replication (Dtr). The Dtr factors are important for the formation of the mating-pair apparatus and initiating DNA transfer that originates from the relaxases at origin of transfer (OriT). The structural machinery associated with conjugal transfer in Gram-negative organisms is a type IV secretion system (T4SS) that is encoded by multiple genes carried on the plasmid. T4SSs are

known to be central to the spread of several genetic determinants among bacterial strains. T4SSs contain 11 to 13 core proteins that form structural and energetic elements that facilitate the transmission of DNA or proteins directly from the cytoplasm of one bacterium to that of the recipient cell, which is in conjugation with another bacterium [Lewley et al., 2003]. Plasmid mobility can be essential for microorganisms under a number of external stresses and understanding the distribution and epidemiology of plasmids is very important [Tamminen et al., 2012].

As WGS has been widely adopted as basic laboratory technique in numerous public health laboratories, DNA sequences have become widely available for a wide range of bacterial isolates. These expansive sequencing efforts, coupled together with the development of bioinformatics tools, allows for an improved ability to discern bacterial strain transmission dynamics and resistance plasmid spread across strains, which enables investigators to speed up their understanding of gene mobility among plasmids [Orlck et al., 2017; Tamminen et al., 2012]. In addition to their role in plasmid mobilization, MOB genes can be used for multiple functions including the identification, classification and typing of plasmids based on the mobilization relaxase genes [Algarni et al., 2022; Chen et al., 2022]. Besides MOB typing, additional plasmid classification schemes, such as replicon and plasmid taxonomic unit (PTU) typing schemes are useful for providing insights into the epidemiology of plasmid-mediated antimicrobial resistance [Carattoli et al., 2014; Orlek et al., 2017; Redondo-Salvo et al., 2021].

While these typing approaches provide data on the plasmid classification, an area where increased value can be obtained is by better understanding the diversity and functionality of the genes that make up the functional T4SSs which facilitate the transfer of plasmids from a donor to recipient bacterium.   To enable this analysis of the transfer-associated genes, the current study

was undertaken to identify the transfer-associated genes that are part of specific conjugal T4SSs in key groups of AMR plasmids. A plasmid transfer gene database was developed along with computational tools that can be utilized for transfer gene identification in the Enterobacteriaceae and allow for a comparison of the different plasmid gene profiles among different isolates. This database was used to characterize strains and identify potential utility for predicting the factors that contribute to the spread of virulence and AMR-associated plasmids. Several key attributes are important to utilize the database for the characterization of plasmid genes. These attributes include that the sequences in the database are able to distinguish among the different plasmid replicon types, that the transfer genes detected correspond to the predicted replicon types, and that the data can be utilized to better understand the genetics of the plasmids present in the strains. To these ends, several analyses were undertaken to assess the functionality and utility of the database including a comparison of the relatedness of genes with similar names or predicted functions (e.g., VirD4-like ATPases of T4SSs) utilizing multiple alignment of nucleic acid sequences or/and protein primary sequences [Chang et al., 2011; Orlek et al., 2017]. In addition to the database, tools were developed to identify the specific plasmid transfer genes present in individual strains and to facilitate comparison of the genes between multiple strains carrying plasmids.

3.2 Methods and Materials:

3.2.1 Generation of non-redundant, comprehensive list of plasmid-transfer associated genes from enteric bacteria:

Based on the literature of the different plasmid Inc groups that are most commonly associated with carrying and transmitting AMR genes in *Salmonella enterica* and related members of the

Enterobacteriaceae [Carattoli et al. 2009; Rozwandowicz et al. 2018], the following

incompatibility groups were selected for inclusion in the initial database: IncA/C, B/O, FIA, FIB,

HI1, HI2, I1, I2, K, M, N, P, and W. To generate a non-redundant, comprehensive list of plasmid

transfer factors, a multistep process was utilized. In the first step, the GenBank nucleotide

database (https://www.ncbi.nlm.nih.gov/nuccore/) was searched using the terms

"Enterobacteriaceae" and the specific plasmid type (e.g., "IncA/C") [Madeira et al. 2022]. From

the list of plasmids returned, those sequences that were in the range of size of the predicted

plasmids (~50-400 kb) and listed as "circular" were selected for further analyses.   Plasmid DNA

sequences were downloaded as GenBank (.gb) formatted files and the files were processed for

further analysis using the Feature Extract 1.2 server

(https://services.healthtech.dtu.dk/service.PhP?FeatureExtract.1.2) to parse out the sequences of

the different coding genes in the selected plasmid types [Wernersson et al. 2005]. The parsed

data was transferred to Excel software (Microsoft, Redmond, WA, USA) for sorting and further

assessment. Based on the identities of the gene products, the transfer-associated genes for

representative plasmids were identified and compared to other plasmids of the same Inc type to

determine a plasmid-type specific, non-redundant list of putative transfer-related genes.

Representative plasmids were identified for each of the different Inc types carrying the cohort of

sequences were used to select the reference sequences for the Plasmid Transfer Factor Database

(Table 1).

3.3.2 Development of Comprehensive Plasmid Transfer Factor Database:

The gene-level nucleotide and amino acid sequence data and other related information (e.g.,

locus tag, product, DB_Xref and any notes) for each transfer factor was extracted from GenBank

using a customized Python and Biopython program, the extracted data was normalized to a delimited form and imported to into the PostgreSQL (https://www.postgresql.org) relational database that serves as the backend data for the sequence analysis tools.

3.2.3 Analysis Tools Development

Two different analysis tools were developed in this study. To predict which putative plasmid transfer genes are present in multiple sequenced strains the Plasmid Transfer Factor Comparison tool was developed using simple matching algorithms developed using a BLAST-based strategy [Altschul et al., 1997]. The input files were multiple FASTA-formatted sequence files that were compared to the gene sequences in the Plasmid Transfer Factor Database (Supplemental Table S1). The output of the comparison was a table that provides a binary matrix of the presence or absence of the transfer genes in the sequences. For a gene to be called "present in the sequence", it must meet the Expect (e)-value cutoff of 10^{-3}, which is the threshold used in NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To assess the function of the Plasmid Transfer Factor Comparison tool, a convenient set of 159 previously sequenced *Salmonella enterica* strains, that had undergone plasmid replicon typing (Supplemental Table S2), were analyzed to assess congruence of the results of plasmid types identified and to determine whether there were putative false positive calls for specific genes (i.e. factors for one plasmid type being identified as present, even though the plasmid was not detected in the strain with PlasmidFinder). In addition, the sets of IncA/C and IncBO plasmids (100 each) that were analyzed in Chapter 2 were tested to compare the diversity of plasmids within a particular group of plasmids.

A second tool termed the Plasmid Transfer Factor Assessment provides more detailed information on the plasmid transfer genes identified within a submitted FASTA file. In this tool, a single FASTA file is uploaded, and the resulting output is a detailed table containing the names of the plasmid transfer genes, the % identity to the reference sequence, number of mismatched base pairs, location of the gene in both the query and reference sequences, e-value, and bit score of the gene. The e-value cutoff for inclusion in the gene table was 10^{-3}. To evaluate and demonstrate the function of the tool, we utilized the WGS data from *S. enterica* isolate 142, which is a multidrug resistant strain that carries IncA/C, HI2 and I1 plasmids [Han et al., 2018].

3.2.4 Phylogenetic analyses of the transfer genes:

The nucleotide and amino acid sequences for each of the identified transfer factors (Supplemental Table S1) were imported into Microsoft Word and reformatted into the FASTA format, with each gene as an individual entry, and saved as .txt files (one for nucleotide and one for amino acid sequences). The nucleotide file with FASTA formatted sequences was uploaded into Clustal Omega Multiple Sequence Alignment program (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the alignment run on the sequences using the default parameters [Madeira, et al., 2022]. From the results, distance coordinates for a phylogenetic tree were downloaded and saved for further analyses. To display the tree, the data were imported into the interactive Tree of Life (iTOL; https://itol.embl.de/upload.cgi) [Letunic and Bork, 2021]. A rectangular tree displaying the branch lengths was generated from the file. Based on the initial analyses, two tree subsets with 10 and 12 members, who had relatively high degrees of similarities (short overall branch lengths), were selected for re-analyses to determine the impact of the large number of diverse genes on the calculated phylogenetic relationships. A

third subset containing *traI* genes, the most commonly genes across the plasmid types, was chosen for analyses to determine the similarities of plasmid genes with the same name. A fourth subset included genes that were predicted to encode VirD4-like ATPases to determine if the predicted common gene function had tighter phylogenetic relatedness than other subsets [Gunton et al. 2005; Raleigh and Low, 2013; Christie et al., 2014; Guglielmini et al. 2014; Foley et al. 2021].

3.3 Results

3.3.1 Development of a bioinformatics tools for construction and selection of Plasmid Transfer Factor Database website:

An overview of the plasmid transfer factor database workflow is shown in Figure 1. We identified representative plasmids from the Enterobacteriaceae across the 13 different plasmid types (Table 1). The initial assessment of transfer associated genes identified many genes for each of the plasmid Inc groups. The results of the genes that were found in the different plasmid types are shown in Table 2. To develop the Plasmid Transfer Factor Database, plasmid sequences from the different host species of enteric bacteria were utilized to build the set of reference genes that make up the backend of the Plasmid Transfer Factor Database (Supplemental Table 1). This reference set contains 359 genes across the 13 different plasmid types assessed. Among the gene names identified, there were significant overlaps in the names of the genes across the different plasmid types. For example, genes identified as *traI* were noted in 12 of the 13 different plasmid types (Table 2). Some plasmid types, such as IncW and to some extent IncH plasmids, appear to have plasmid-type specific naming as each are named with "*trw*" or "*trh*", respectively, rather than the more generic "*tra*" (Table 2).
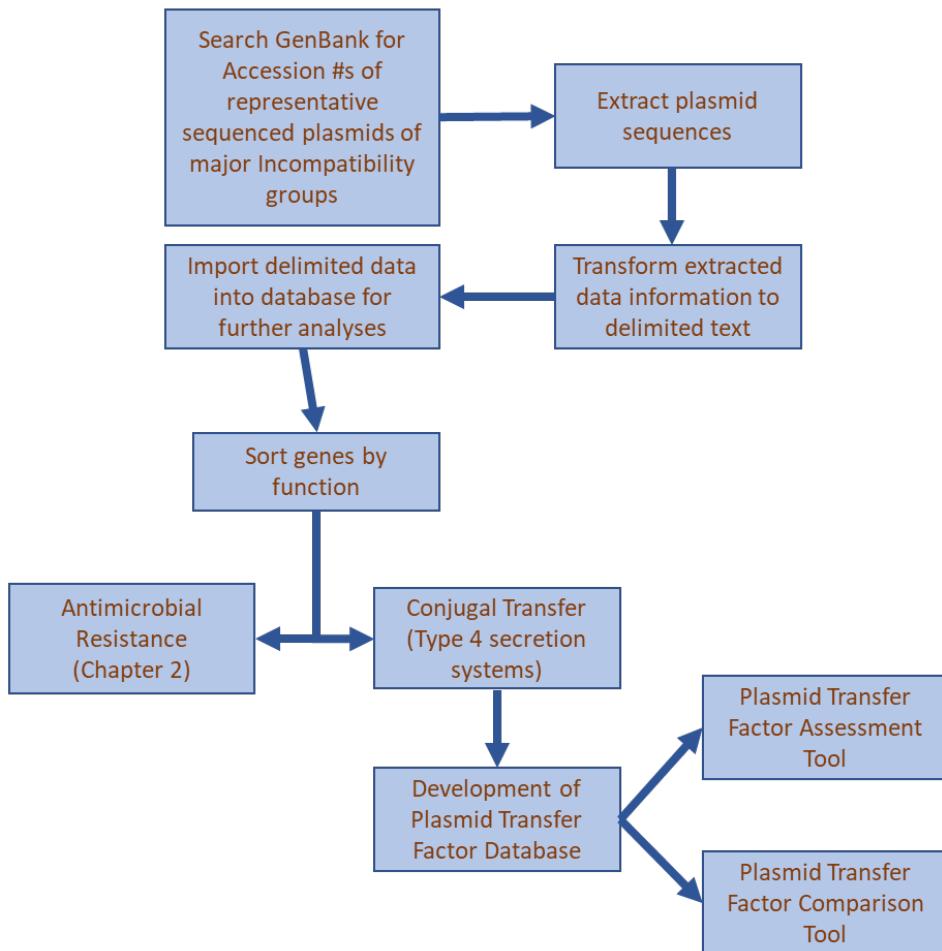
Figure 1. The workflow for the creation of the Plasmid Transfer Factor Database.

In order, to utilize the data that was curated in the initial part of the study, tools were developed to analyze WGS data. The first of these tools was termed the Plasmid Transfer Factor Assessment tool. An overview of the analyses processes is shown in Figure 2. The second tool that was developed by using Plasmid Transfer Factor Comparison tool, which is highlighted in Figure 3 and allows for the comparison of multiple different FASTA files simultaneously to give a presence/absence matrix for the comparison of the comprehensive transfer gene profiles among

multiple enteric bacteria. As the evaluation of Plasmid Transfer Gene Database is an ongoing effort, we continue to update a new transfer-gene information for these organisms and extending the workflow to evaluate *Salmonella* isolates and other enteric bacteria.

Table 1. Source isolates used in the generation of the Plasmid Transfer Factor Database

Plasmid Type	Accession #	Host Species	Plasmid Name
IncA/C	NC_012692	*Escherichia coli*	pAR060302
IncBO	MK088173	*Salmonella enterica*	R805a
IncFIA	NZ_CP014498	*Escherichia coli*	pZH193
IncFIB	J01724	*Escherichia coli*	plasmid F
IncHI1	KP899804	*Salmonella enterica*	pF8475
IncHI2	MH287084	*Escherichia coli*	pSDE-SvHI2
IncI1	AP005147	*Salmonella enterica*	R64
IncI2	LN623683	*Salmonella enterica*	STH21_InHI2
IncK	NC_014477	*Escherichia coli*	pCT
IncM	KM406490	*Salmonella enterica*	pSEM
IncN	JX065631	*Escherichia coli*	pKT58A
IncP	KX377410	*Klebsiella pneumoniae*	pMCR_1511
IncW	BR000038	*Escherichia coli*	R388

3.3.2 Assessment of the Plasmid Transfer Factor Assessment tool:

To assess the function of the initial part of current study, we analyzed the WGS data using the Plasmid Transfer Factor Assessment tool. *S. enterica* isolate 142, which was determined to be multidrug resistant and carry multiple plasmids including IncA/C, HI2 and I1 plasmids was used for the initial assessment [Han et al., 2018]. As is shown in Table 3, the transfer genes for the IncA/C, HI2 and I1 plasmids were detected in the strain, along with their percent identity to the reference sequences (Table S1), alignment length, mismatches and gaps, and alignment information. All the Inc A/C, HI2 and I1 transfer genes had at least a 98.9% identity to the reference. Conversely, highlighted in red text in the table are five genes (*pilV* from IncBO and IncK, and KP899804_186327, *trhB* and *trhN* from IncHI1) that were detected,

even though those plasmid types were not identified in isolate 142. When comparing the percent

identity and alignment information, it appears that these are "false positive" calls, since their

identity values were relatively low (<82.2%) and their alignments overlapped with corresponding

genes in either the IncI1 (*pilV*) or IncHI2 (KP899804_186327, *trhB* and *trhN*) detected in

plasmids identified by replicon typing (these are highlighted in the green text in Table S1).

3.3.3 Assessment of the Plasmid Transfer Factor Comparison tool:

The Plasmid Transfer Factor Comparison tool is highlighted in the Figure 3 and

facilitates the comparison of multiple different FASTA files simultaneously to provide a

presence/absence matrix for the comparison of the transfer gene profiles among multiple strains.

To assess this tool, three sets of data including the cohort of IncA/C and IncBO plasmids

described in Chapter 2, as well as WGS data from 159 *Salmonella* strains that were previously

sequenced [Aljahdali et al., 2020; Han et al., 2018; Kaldhone et al., 2018; Khajanchi et al, 2017,

2019] were analyzed.

The results of the analyses of the IncA/C plasmid sequences are shown in Figure 4. In

99/100 cases, at least one of IncA/C transfer genes was detected. And among these 99 cases, 85

(85.9%) had all the IncA/C plasmid transfer genes included in the database. In the next most

common genotype (N=7, 7.1%), the plasmids were missing *traC, traN, traU, traW* and *trhF*, and

the third most common genotype (N=3, 3.0%) was lacking *traD, traN, traU, traW* and *trhF*

genes (Figure 4).

Table 2. Transfer genes detected in the representative plasmid types

Transfer Genes	IncA/C	IncBO	IncFIA	IncFIB	IncHI1	IncHI2	IncI1	IncI2	IncK	IncM	IncN	IncP	IncW
eex											■		
htdA					■	■							
htdF					■	■							
htdK					■	■							
htdO					■	■							
htdT					■	■							
htdV					■								
kikA											■		
korA											■		
korB											■		
mobA										■			
mobB										■			
mobC										■			
orf4							■						
orf9							■						
orf16							■						
orf17							■						
pilI		■					■		■				
pilJ							■						
pilK		■					■		■				
pilL		■					■	■					
pilM		■					■	■					
pilN		■					■	■					
pilO		■					■	■					
pilP		■					■	■					
pilQ		■					■	■					
pilR		■					■	■					
pilS		■					■	■					
pilT		■					■	■					
pilU		■					■	■					
pilV		■					■	■					
traA	■		■	■					■		■		
traB		■	■	■	■		■		■		■	■	
traC		■	■	■	■		■		■		■	■	
traD		■		■	■		■				■	■	
traE		■	■	■			■	■	■		■		
traF		■	■		■		■		■		■	■	
traG		■	■	■	■		■	■			■	■	
traH		■	■	■	■		■	■	■		■	■	
traI		■	■	■	■		■	■	■		■	■	
traJ		■		■	■				■		■		
traK		■	■	■	■		■		■		■	■	
traL		■	■	■			■		■		■	■	
traM		■	■	■			■						
traN	■	■		■			■		■				
traO		■	■	■								■	
traP		■	■	■								■	
traQ		■	■	■			■		■			■	
traR		■	■	■			■		■			■	
traS		■		■				■					
traT		■	■	■			■		■				

Transfer Genes	IncA/C	IncBO	IncFIA	IncFIB	IncHI1	IncHI2	IncI1	IncI2	IncK	IncM	IncN	IncP	IncW
traU	■	■	■	■			■		■	■			
traV	■	■	■	■			■		■				
traW	■	■	■	■			■		■				
traX		■	■	■			■		■			■	
traY		■		■			■		■				
trbA		■	■	■			■		■			■	
trbB		■	■	■					■			■	
trbC		■	■	■					■			■	
trbD		■	■	■								■	
trbE		■		■								■	
trbF		■		■								■	
trbG		■		■								■	
trbH		■										■	
trbI		■		■								■	
trbJ		■						■				■	
trbL								■				■	
trbM												■	
trbN					■							■	
trhA					■	■							
trhB					■								
trhC					■								
trhE					■	■							
trhF	■				■	■							
trhG					■								
trhH					■								
trhI					■								
trhK					■								
trhL					■								
trhN					■								
trhO					■								
trhP					■								
trhR					■								
trhU					■								
trhV					■								
trhW					■	■							
trhY					■	■							
trhZ					■	■							
trwA													■
trwB													■
trwC													■
trwD													■
trwE													■
trwF													■
trwG													■
trwH													■
trwI													■
trwJ													■
trwK													■
trwL													■
trwM													■
trwN													■

Plasmid Transfer Factor Assessment

Interested in assessing the plasmid transfer factor for a sequence? Fill in the form below or upload a FASTA file.

Enter query sequence

Or, upload file
Choose File | DRR022715_contigs_renamed.fasta

Reset Submit

Plasmid Transfer Factor Assessment Results

| query acc.ver	subject acc.ver	location	% identity	alignment length	mismatches	gap opens	q. start	q. end	s. start	s. end	evalue	bit score
NODE_264_length_915_cov_1.937817	IncFIB_traK	plasmid	100.000	72	0	0	1	72	658	729	1.40e-32	134
NODE_313_length_536_cov_3.236802	IncI1_pilV	plasmid	100.000	336	0	0	201	536	1425	1090	1.41e-179	621
NODE_292_length_673_cov_2.712454	IncFIB_traY	plasmid	100.000	396	0	0	278	673	1	396	0.0	732
NODE_221_length_1734_cov_3.240821	IncFIB_traD	plasmid	100.000	198	0	0	1	198	1957	2154	2.42e-102	366
NODE_241_length_1294_cov_2.640960	IncI1_pilR	plasmid	100.000	1073	0	0	1	1073	1073	1	0.0	1982
NODE_173_length_3081_cov_3.590724	IncI1_pilU	plasmid	100.000	657	0	0	1230	1886	1	657	0.0	1214
NODE_173_length_3081_cov_3.590724	IncI1_pilT	plasmid	100.000	561	0	0	685	1245	1	561	0.0	1037
NODE_236_length_1479_cov_3.022929	IncI1_pilK	plasmid	100.000	68	0	0	1412	1479	1	68	3.81e-30	126
NODE_236_length_1479_cov_3.022929	IncI1_pilI	plasmid	100.000	255	0	0	613	867	1	255	4.24e-134	472
NODE_173_length_3081_cov_3.590724	IncI1_pilR	plasmid	100.000	43	0	0	1	43	1056	1098	6.30e-16	80.5
NODE_135_length_5410_cov_3.363619	IncFIB_traW	plasmid	100.000	633	0	0	1778	2410	633	1	0.0	1170
NODE_182_length_2923_cov_3.935265	IncI1_traC	plasmid	100.000	684	0	0	98	781	684	1	0.0	1264
NODE_182_length_2923_cov_3.935265	IncI1_traA	plasmid	100.000	174	0	0	2124	2297	174	1	8.99e-89	322
NODE_182_length_2923_cov_3.935265	IncI1_traD	plasmid	100.000	84	0	0	1	84	84	1	9.65e-39	156

Figure 2. Plasmid transfer gene assessment database developed using the different transfer genes noted in Table 2 and Supplemental Table S1. For the Plasmid Transfer Factor Assessment, a single sequence (FASTA) file is selected (**A**) and the sequence uploaded into the system (**B**) and a BLAST-based analyses is conducted. When a gene is identified the resulting information related to identity to reference, locations, etc. is provided for all the genes present (**C**).
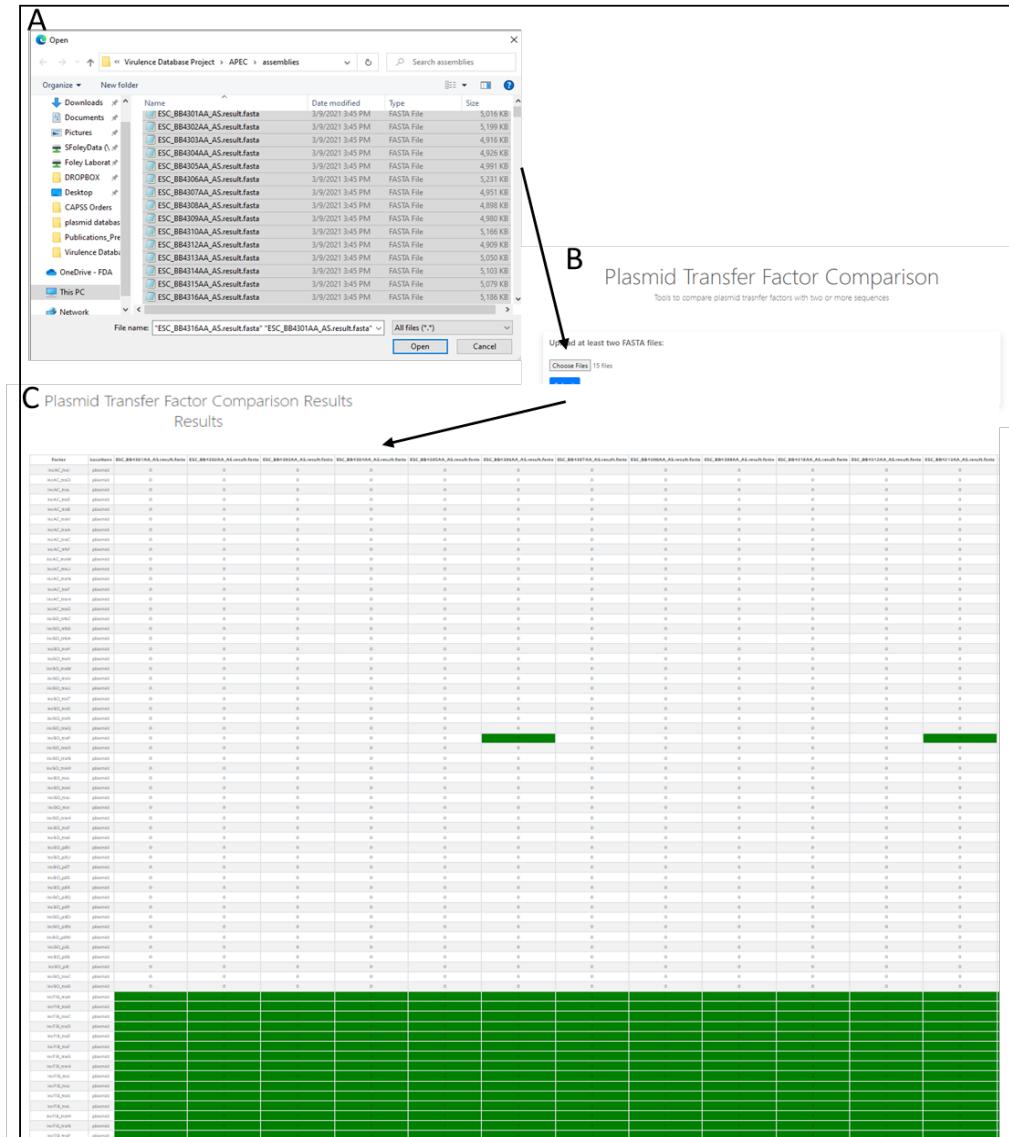
Figure 3. Plasmid transfer gene assessment database developed using the different transfer genes noted in Table 2 and Supplemental Table S1. For the Plasmid Transfer Factor Comparison, multiple sequence (FASTA) files are selected (A) and uploaded into the system (B) and a BLAST-based analyses is conducted. When genes are detected a resulting presence/absence matrix is generated to facilitate comparison among strains (C). These analyses can be extracted for further phylogenetic analyses.

We subsequently analyzed the IncBO plasmids, with at least one IncBO gene being detected in 99/100 plasmids analyzed. There was a diversity of different genotypes, with the most common (N=38, 38.4%) being the presence of all of the IncBO transfer genes (Figure 5).

Another common set of genotypes was the absence of multiple genes in the *pilK-U* transfer gene

region. A key observation was the presence of a mirror image of the gene profiles for the IncK

genes as well as IncBO genes, which is indicative of having identical or at least extremely high

similarity in the transfer gene systems. In addition, sequences mapping to the *pilV* genes for

IncI1 and IncI2 were also detected in some cases, which based on the data from Table 3, is likely

due to a false positive detection due to some similarity of the sequences across the different

plasmid types. To examine this overlap further, an alignment completed with the IncBO, K, and

I1 *pilV* genes using Clustal Omega was undertaken and the result shows that there was extensive

overlap of the gene sequences, with the IncBO and K sequences being identical, this observation

is highlighted with the red boxes in Figure 6.
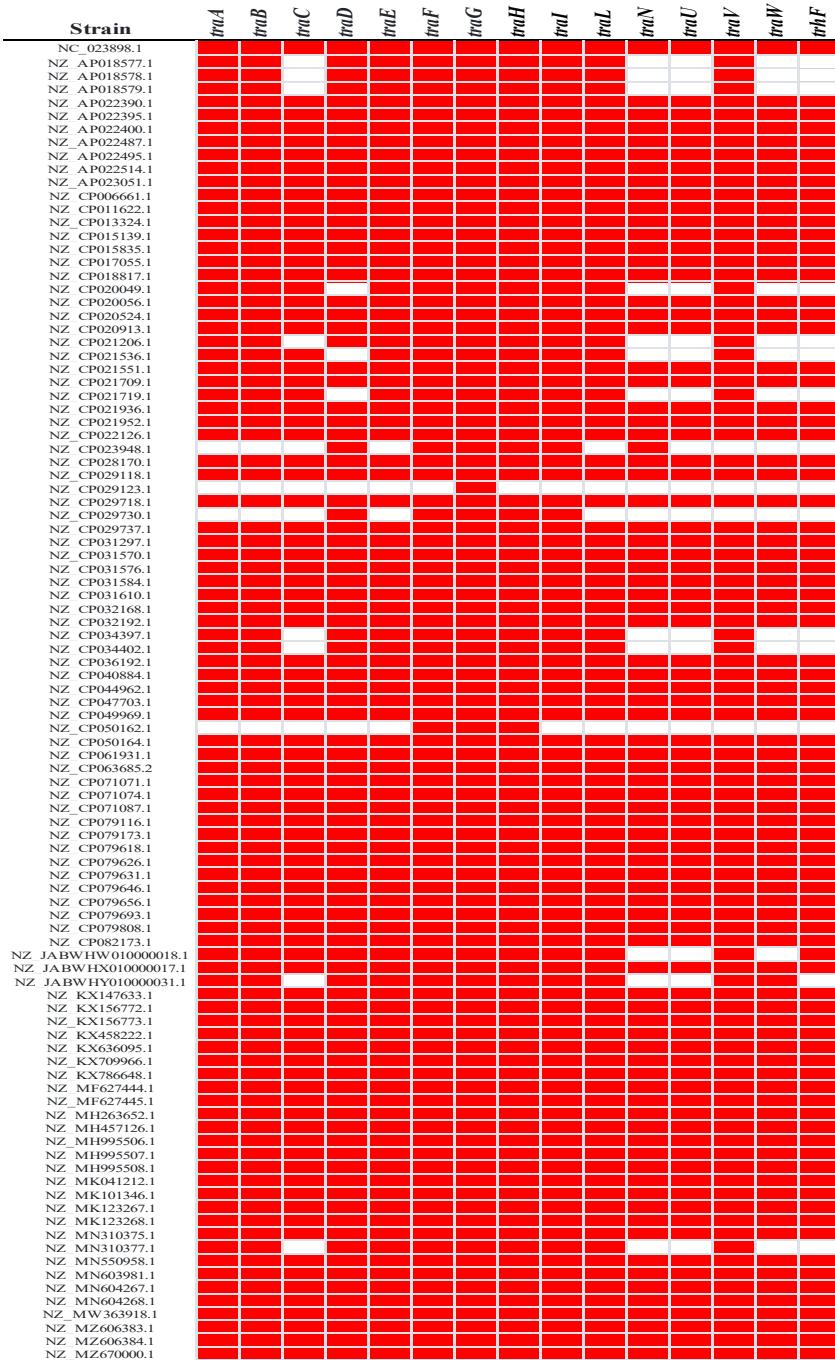
Figure 4. The Plasmid Transfer Factor Comparison tool for the IncA/C plasmids transfer gene assessment database developed using the different Type IV system (T4SS) transfer genes.

Table 3: Analyses of *Salmonella enterica* isolate 142 (Han et al., 2018) using the Plasmid Transfer Factor Assessment tools

query acc.ver	subject acc.ver	% identity	alignment length	Mis-matches	gap opens	q. start	q. end	s. start	s. end	E value	bit score
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traA	100.0	393	0	0	27053	27445	1	393	0	726
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traB	100.0	1317	0	0	25158	26474	1	1317	0	2433
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traC	100.0	2448	0	0	38469	40916	1	2448	0	4521
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traD	100.0	1866	0	0	17959	19824	1	1866	0	3446
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traE	100.0	627	0	0	23634	24260	1	627	0	1158
NODE_39_length_43773_cov_54.8262_ID_77	IncAC_traF	100.0	1029	0	0	13426	14454	1	1029	0	1901
NODE_39_length_43773_cov_54.8262_ID_77	IncAC_traG	100.0	3615	0	0	15902	19516	1	3615	0	6676
NODE_39_length_43773_cov_54.8262_ID_77	IncAC_traH	100.0	1434	0	0	14456	15889	1	1434	0	2649
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traI	100.0	2973	0	0	14990	17962	1	2973	0	5491
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traL	100.0	282	0	0	23356	23637	1	282	1.34 E-147	521
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traN	100.0	2277	0	0	44779	47055	1	2277	0	4205
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traU	100.0	1008	0	0	43668	44675	1	1008	0	1862
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traV	100.0	579	0	0	26471	27049	1	579	0	1070
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_traW	100.0	1266	0	0	41738	43003	1	1266	0	2338
NODE_35_length_47182_cov_55.9495_ID_69	IncAC_trhF	100.0	531	0	0	41245	41775	1	531	0	981
NODE_18_length_107400_cov_89.6844_ID_35	IncBO_pilV	77.1	144	33	0	106696	106839	841	984	1.70 E-15	84.2
NODE_42_length_30602_cov_44.011_ID_83	IncHI1_KP899804_186327	82.2	409	71	2	2232	2639	79	486	4.33 E-96	350
NODE_22_length_90538_cov_42.1913_ID_43	IncHI1_trhB	75.0	1379	324	18	6650	8016	2	1371	1.14 E-175	616
NODE_22_length_90538_cov_42.1913_ID_43	IncHI1_trhN	76.9	3200	698	29	34145	37330	2	3175	0	1784
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_htdA	100.0	453	0	0	24783	25235	1	453	0	837
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_htdF	100.0	426	0	0	25225	25650	1	426	0	787
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_htdK	99.8	555	1	0	25659	26213	1	555	0	1020

NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_htdO	99.8	462	1	0	6210	6671	1	462	0	848
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_htdT	100.0	981	0	0	8397	9377	1	981	0	1812
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_htdV	99.8	483	1	0	8024	8506	1	483	0	887
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_MH287084_3 0570	100.0	135	0	0	32927	33061	1	135	1.34 E-65	250
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_orf16	99.5	837	4	0	20836	21672	837	1	0	1524
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_orf17	100.0	522	0	0	20322	20843	522	1	0	965
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_orf4	99.9	873	1	0	37361	38233	873	1	0	1607
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_orf9	100.0	4305	0	0	26333	30637	1	4305	0	7950
NODE_42_length_30602_cov_44.011_ID_83	IncHI2_traH	99.8	486	1	0	2154	2639	1	486	0	893
NODE_42_length_30602_cov_44.011_ID_83	IncHI2_traI	99.9	3147	2	0	3383	6529	1	3147	0	5801
NODE_42_length_30602_cov_44.011_ID_83	IncHI2_traJ	100.0	663	0	0	9709	10371	1	663	0	1225
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhA	100.0	354	0	0	3416	3769	1	354	0	654
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhB	99.9	1356	1	0	6661	8016	1	1356	0	2499
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhC	99.9	2682	2	0	10346	13027	1	2682	0	4942
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhE	100.0	789	0	0	4149	4937	1	789	0	1458
NODE_30_length_61375_cov_42.0677_ID_59	IncHI2_trhF	99.4	1047	6	0	530	1576	1	1047	0	1901
NODE_30_length_61375_cov_42.0677_ID_59	IncHI2_trhG	100.0	3954	0	0	2990	6943	1	3954	0	7302
NODE_30_length_61375_cov_42.0677_ID_59	IncHI2_trhH	99.4	1416	9	0	1566	2981	1	1416	0	2566
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhI	99.9	1785	1	0	38413	40197	1	1785	0	3291
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhK	99.9	1272	1	0	4937	6208	1	1272	0	2344
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhL	99.7	318	1	0	3820	4137	1	318	1.16 E-165	582
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhN	100.0	3189	1	0	34144	37332	1	3189	0	5884
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhO	99.7	948	3	0	21669	22616	948	1	0	1735
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhP	100.0	513	0	0	30915	31427	1	513	0	948
NODE_42_length_30602_cov_44.011_ID_83	IncHI2_trhR	100.0	801	0	0	595	1395	801	1	0	1480
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhU	99.9	1062	1	0	33064	34125	1	1062	0	1956

NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhV	100.0	951	0	0	9387	10337	1	951	0	1757
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhW	100.0	1512	0	0	31414	32925	1	1512	0	2793
NODE_42_length_30602_cov_44.011_ID_83	IncHI2_trhY	99.6	513	2	0	81	593	513	1	0	937
NODE_22_length_90538_cov_42.1913_ID_43	IncHI2_trhZ	100.0	390	0	0	19936	20325	390	1	0	721
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilI	100.0	255	0	0	94890	95144	1	255	3.11 E-132	472
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilJ	99.3	453	3	0	95187	95639	1	453	0	821
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilK	99.8	591	1	0	95689	96279	1	591	0	1086
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilL	99.9	1068	1	0	96313	97380	1	1068	0	1967
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilM	100.0	438	0	0	97380	97817	1	438	0	809
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilN	100.0	1683	0	0	97831	99513	1	1683	0	3109
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilO	99.9	1296	1	0	99506	100801	1	1296	0	2388
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilP	100.0	453	0	0	100788	101240	1	453	0	837
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilQ	100.0	1554	0	0	101251	102804	1	1554	0	2870
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilR	100.0	1098	0	0	102817	103914	1	1098	0	2028
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilS	99.8	615	1	0	103932	104546	1	615	0	1131
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilT	100.0	561	0	0	104556	105116	1	561	0	1037
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilU	100.0	657	0	0	105101	105757	1	657	0	1214
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_pilV	100.0	1425	0	0	105757	107181	1	1425	0	2632
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traA	100.0	174	0	0	91843	92016	1	174	3.31 E-87	322
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traB	100.0	534	0	0	92572	93105	1	534	0	987
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traC	100.0	684	0	0	93359	94042	1	684	0	1264
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traD	100.0	567	0	0	94056	94622	1	567	0	1048
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traE	100.0	825	0	0	2464	3288	1	825	0	1524
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traF	100.0	1203	0	0	3373	4575	1	1203	0	2222

NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traG	100.0	585	0	0	4635	5219	1	585	0	1081
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traH	100.0	459	0	0	5614	6072	1	459	0	848
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traI	100.0	819	0	0	6069	6887	1	819	0	1513
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traJ	100.0	1149	0	0	6884	8032	1	1149	0	2122
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traK	99.7	291	1	0	8029	8319	1	291	1.41E-150	532
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traL	100.0	348	0	0	12760	13107	1	348	0	643
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traM	99.6	693	3	0	13104	13796	1	693	0	1264
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traN	99.8	984	2	0	13807	14790	1	984	0	1807
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traO	99.8	1290	2	0	14793	16082	1	1290	0	2372
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traP	100.0	705	0	0	16082	16786	1	705	0	1303
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traQ	99.4	528	3	0	16786	17313	1	528	0	959
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traR	99.5	405	2	0	17364	17768	1	405	0	737
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traS	100.0	189	0	0	17832	18020	1	189	1.52E-95	350
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traT	100.0	801	0	0	18004	18804	1	801	0	1480
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traU	99.8	3045	6	0	18894	21938	1	3045	0	5590
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traV	99.2	615	5	0	21938	22552	1	615	0	1109
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traW	98.9	1203	13	0	22519	23721	1	1203	0	2150
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traX	99.5	585	3	0	23750	24334	1	585	0	1064
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_traY	95.7	1466	60	3	24362	25826	1	1464	0	2355
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_trbA	99.0	1209	12	0	29597	30805	1	1209	0	2167
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_trbB	99.3	1071	7	0	30824	31894	1	1071	0	1940
NODE_18_length_107400_cov_89.6844_ID_35	IncI1_trbC	99.8	2292	5	0	31887	34178	1	2292	0	4205
NODE_18_length_107400_cov_89.6844_ID_35	IncK_pilV	77.1	144	33	0	106696	106839	841	984	1.70E-15	84.2

*Note genes highlighted in green text are predicted false positive gene detections for corresponding genes that are shown in the red text based on identity % and sequence location.
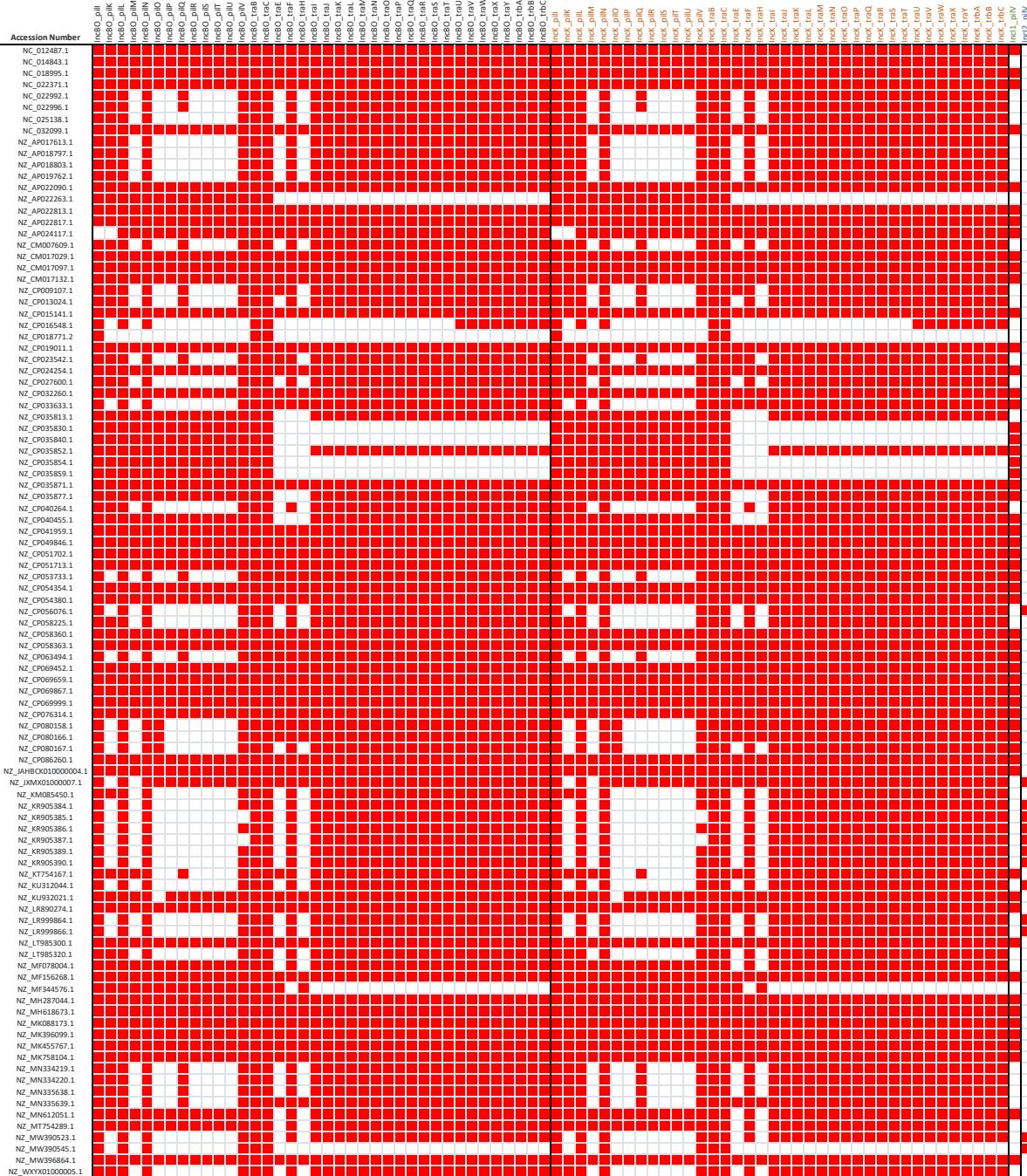
Figure 5. The Plasmid Transfer Factor Comparison tool for the IncBO plasmids transfer gene assessment database developed using the different transfer genes. Of note is that there is duplication of the genes being positive for the IncK plasmid genes (orange gene names) and *pilV* of IncI1 and I2 (far right columns).
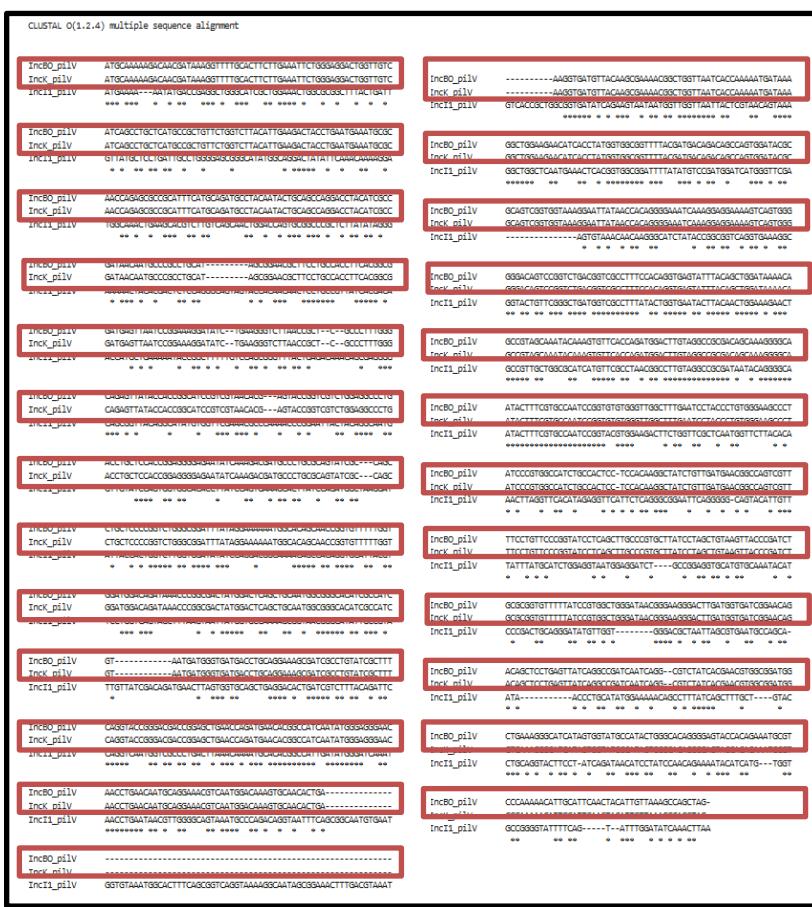
Figure 6. Clustal Omega alignment for the sequences of the *pilV* genes of the IncBO, K and I1 plasmids.

The third set of analyses explored a diverse group of WGS data from *S. enterica* isolates previously characterized and representing multiple serotypes and plasmid replicon types [Aljahdali et al., 2020; Han et al., 2018; Kaldhone et al., 2018; Khajanchi et al, 2017, 2019]. In most cases, the transfer genes identified correlated well with the plasmid replicon typing with some noted exceptions (Figure 7 and Table S2). In several instances plasmids were predicted to have both IncFIA and IncFIB plasmid replicons but did not have the corresponding IncFIA transfer genes.

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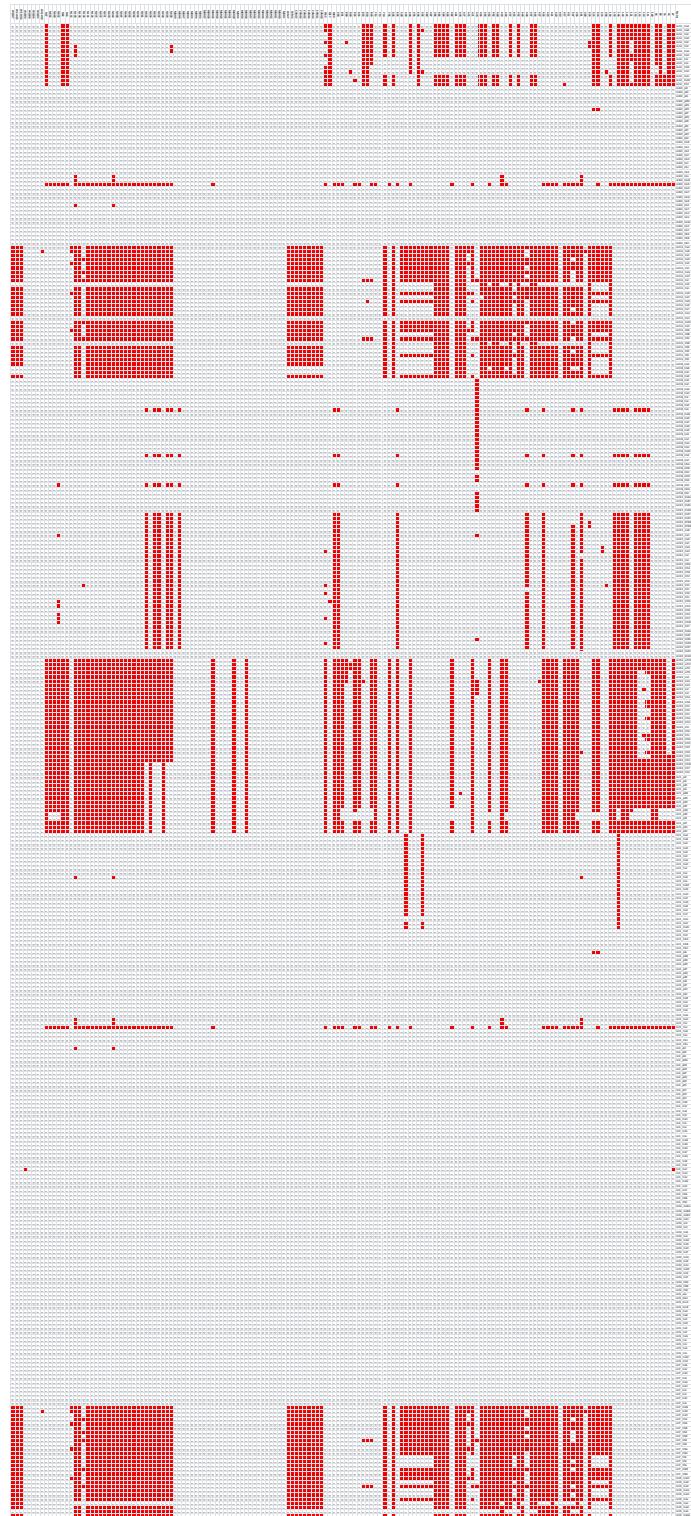
Figure 7. The Plasmid Transfer Factor Comparison tool for 159 previously sequenced strains to assess the overall function of the database to predict the presence of transfer genes. The detailed information on the results presented are shown in Supplemental Table S2.

3.4.4 Phylogenetic analyses of the transfer genes:

Multiple sequence alignment of nucleotide sequences of the 359 genes transfer system across the different plasmid type replicons among strains were analyzed using Clustal Omega and the results demonstrating a large amount of diversity among the sequenced analyzed is shown in Figure S1. The plasmids and transfer genes used in the alignment is listed in Figure S2.

We identified two clusters (of at least 10 genes) that were more closely related across the different types of plasmids based on the distance correction (Clustal neighbor-joining tree distance of <0.26) from panel A in Figure 8 (which is a highly condensed version of Figure S1). The genes from the two clusters (Figures 8B and C) were re-analyzed to determine the impact of sample size on the calculated relatedness of the genes (Figures 9A and B) and in some cases the nearest neighbors changed, indicating the importance of sample size and diversity when looking at large, diverse genetic data.

Another key observation of the initial global analyses was that genes with the same name are often quite divergent/unrelated on the trees. Building on the example noted above for *traI*, which was identified in 12 of the 13 plasmid types (TraI is present in all types, except IncW, as shown in Table 2). Figure 10 shows a subset analysis of the *traI* genes from each of the plasmid types and what was evident in most cases are that the genes and amino acid (data not shown) sequences are not very closely related, with the exceptions of IncBO, K and to a lesser degree I1, and IncFIA and FIB plasmids. These overall observations of sequence diversity likely indicate that even though the genes have the same name, they likely encode divergent functions within the T4SSs.
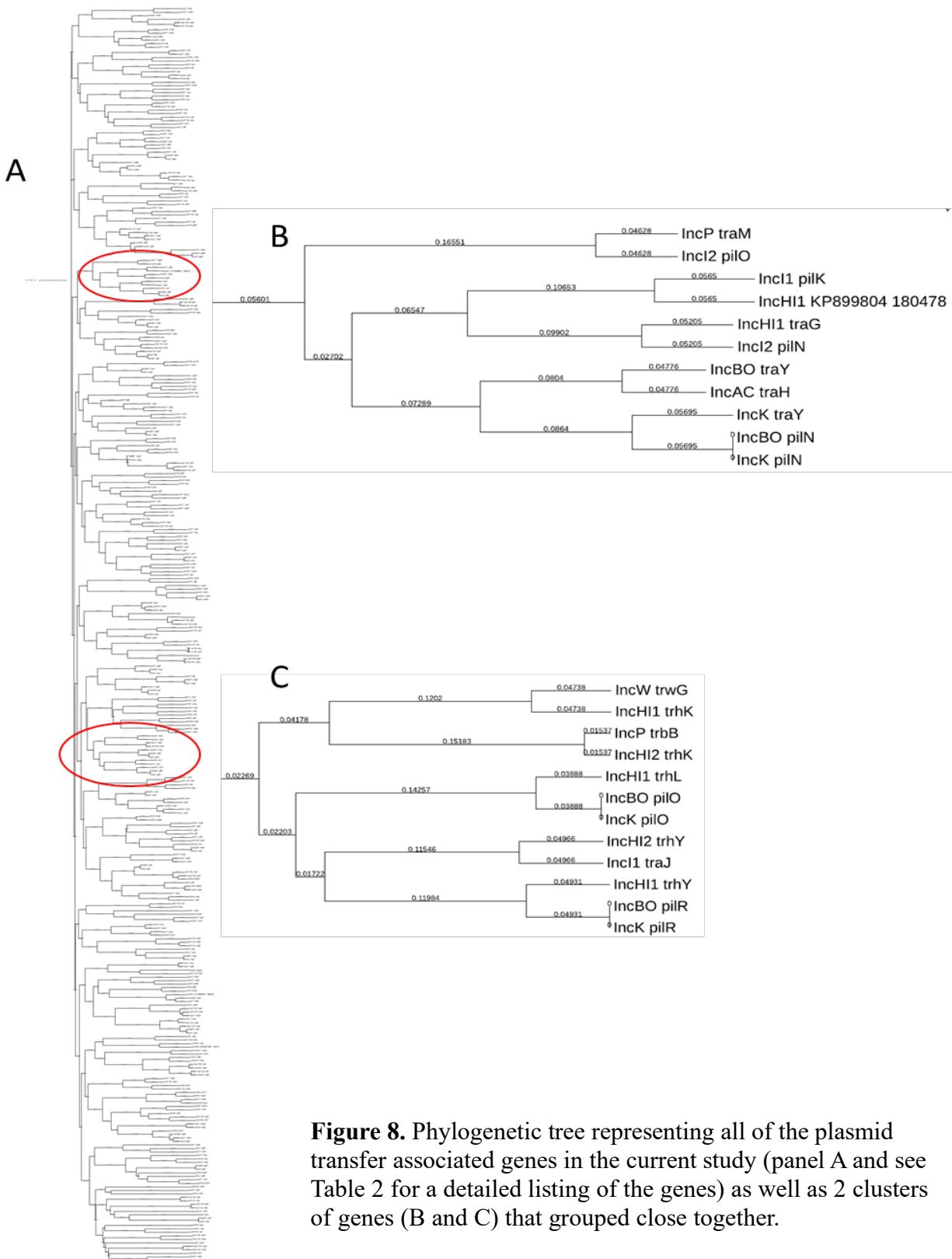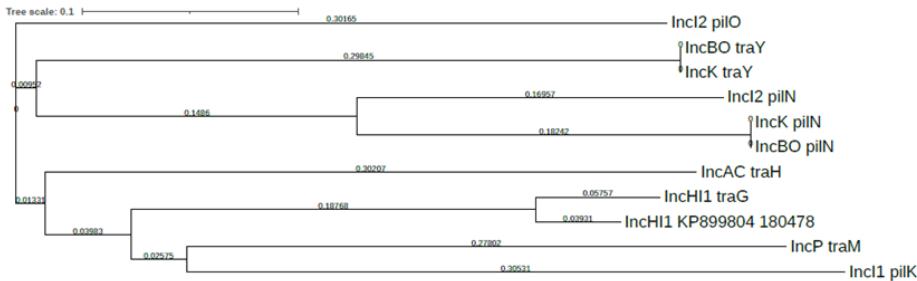
Figure 8. Phylogenetic tree representing all of the plasmid transfer associated genes in the current study (panel A and see Table 2 for a detailed listing of the genes) as well as 2 clusters of genes (B and C) that grouped close together.

Cluster A

Cluster B
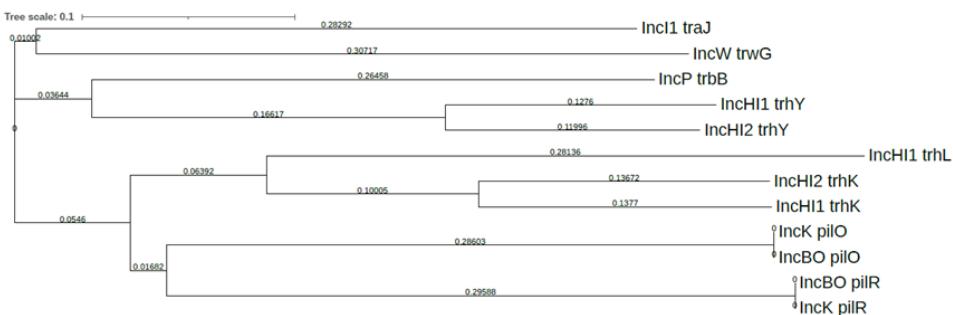
Figure 9. Re-analysis of the subgroups from Figures 8B and 8C that were closely clustered in the global analyses. Cluster A represents the members in subgroup B in Figure 8B after re-analysis. Cluster B represents the member in subgroup C in Figure 8C after re-analysis.
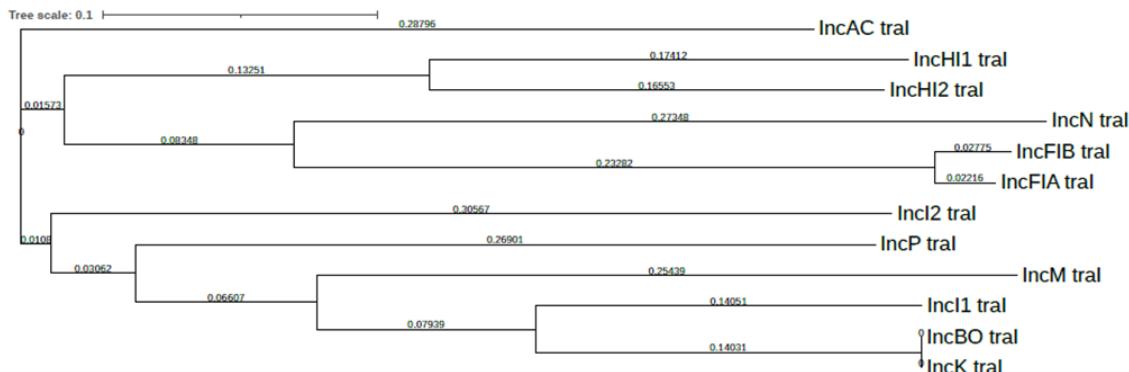
Figure 10. Phylogenetic tree representing the sequences of genes named *traI* in the various plasmid types. Genes with the name *traI* were the most common among the different plasmid types examined.

Since those genes with the same name are not always closely related, we looked at a group of sequences where the proteins are predicted to have a common function across different plasmid types. This analysis, which is shown in Figure 11, examined genes that are predicted to encode the VirD4-like ATPases in the T4SS of Gram-negative organisms [Gunton et al. 2005; Raleigh and Low, 2013; Christie et al., 2014; Guglielmini et al. 2014; Foley et al. 2021]. There was diversity among the sequences of the amino acids of the proteins with a common function, with the exceptions of the amino acid sequences of TrbC from IncBO, K and I1 and to a lesser extent TrbC from IncM plasmids. Similarly, the TraD amino acid sequences of the IncFIA and FIB showed similarity to one another and to some extent to TraJ and TrwB from IncN and W plasmids, respectively. The VirD4 reference sequence (NCBI accession number WP_032488284.1) did not display close amino acid sequence to the other predicted VirD4-like ATPases. The closest protein to the VirD4-like ATPases in reference sequence was the TraG protein from IncP plasmids (Figure 11).
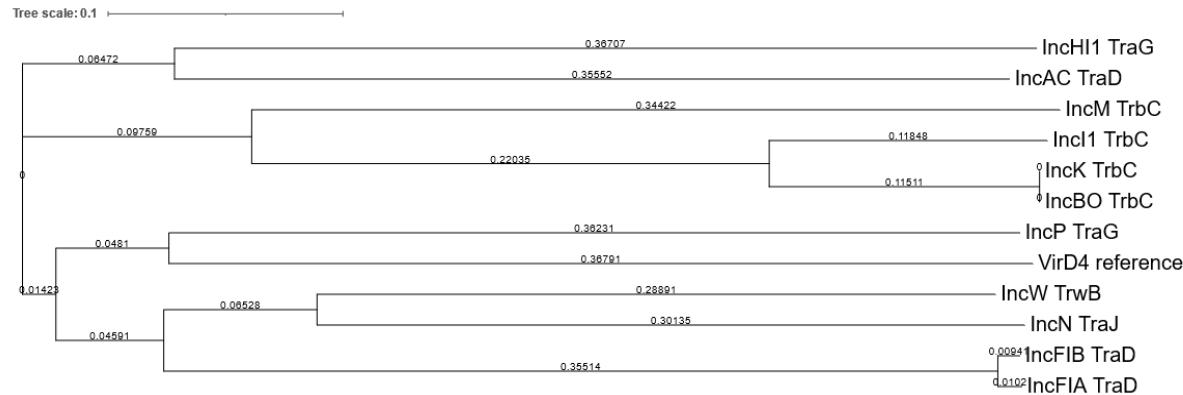

Figure 11. Phylogenetic tree representing the sequences of ATPases associated with the conjugal type IV secretion systems.

3.5 Discussion

The results of this study will allow researchers to further utilize the data generated from WGS studies and gain a more complete understanding of genetics and diversity of plasmid transfer systems. The overall project was conducted to develop and evaluate a plasmid transfer gene database and associated tools known as the Plasmid Transfer Factor Assessment and Plasmid Transfer Factor Comparison tools. The initial database was created by examining whole plasmid sequences that were deposited in GenBank for some of the key plasmid replicon types that are associated with antimicrobial resistance in *Salmonella* and other enteric bacteria (see Chapter 2). The plasmid data were extracted, the genes associated with the conjugal transfer of the different types of plasmids were identified, and the DNA and amino acid sequences were downloaded and used to create the Plasmid Transfer Factor Database (Figure 1).

The initial set of transfer associated genes included 359 sequences from 13 different plasmid types (Table 1, Table S1). Figures 2 and 3 show the basic interfaces and outputs for the Plasmid Transfer Factor Assessment and Plasmid Transfer Factor Comparison tools, respectively. The output of the Plasmid Transfer Factor Assessment tool can be exported into an Excel spreadsheet format for easy sorting and data analyses. The ability to show the % identity to reference and the start/stop locations for the genes is quite valuable to understand the order of the genes within the transfer operon, whether the genes are conserved or not, and whether there are potential false positive results for genes (such as those highlighted in red text in Table 3).

The Plasmid Transfer Factor Comparison tool allows for the upload and comparison of multiple sequences simultaneously. The result is a binary output representing presence or absence of genes in the database. An initial evaluation was the screening of the 100 IncA/C plasmid sequences described in Chapter 2 to determine specificity of the database for transfer

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genes within the single plasmid type. IncA/C plasmids are key elements for the simultaneous dissemination of multiple antimicrobial resistance genes [Hegyi et al., 2017; Hoffmann et al., 2017]. Ninety-nine of the plasmids had at least one IncA/C associated transfer gene, although not all genes were present in each of the plasmids (Figure 4). This diversity of IncA/C transfer genes has been described previously and may play a role in the ability of IncA/C plasmids to transfer among strains [Han et al., 2018; Unpublished data, Chapter 4]. The assessment of the Comparison tool was broadened to evaluate 159 *Salmonella enterica* strains that had undergone WGS and analyzed using the PlasmidFinder tool [Aljahdali et al., 2020; Han et al., 2018; Kaldhone et al., 2018; Khajanchi et al., 2017, 2019]. In general, when a plasmid type was identified by PlasmidFinder, at least some of the corresponding transfer associated genes were detected (Figure 7 and Supplementary Table S2). A couple of exceptions were with the IncF plasmids, where in several instance both IncFIA and FIB plasmid replicons were detected, but only contained the IncFIB transfer genes. In several *Salmonella*-associated plasmids there appears to be a co-integration of the IncFIA replicon sequence within the backbone of the IncFIB plasmids that explains these observations [Hsu et al., 2019; Liu et al., 2021].

To evaluate whether the different genes were appropriate for incorporation in the Plasmid Transfer Factor Database, a series of different analyses were conducted. Initially a global phylogenetic analysis of all the transfer associated genes selected for the database was conducted in order to see if there was significant overlap in the genes from the different plasmid types. As was shown in Figure 8A and Supplemental Figure 1, there was very little overlap in the genes from the different plasmid types with the noted exception of those from the IncBO and IncK plasmid groups. Within the global analyses of the transfer genes there were a few clusters of genes that displayed a higher degree of similarity (shorter branch lengths), so we wanted to

see the impact of the sample size on the phylogenetic analyses, which could impact the interpretation of the data from the larger tree. Figure 9 shows the results of individual re-analysis of the genes from two clusters (Figures 8B and 8C). In instances were closely related/identical sequences were observed in each analysis, such as those from IncBO and K, the genes continued to cluster together, but others had different nearest neighbors in the trees.

The significant overlap of the IncBO and K plasmid types was quite evident from Figure 5, where we screened nearly 100 identified IncBO plasmids. In each of these cases the genes identified in the IncBO plasmids were mirrored by those of the same name in the IncK plasmids. Additionally, with the *pilV* gene, there was also some cross positivity with the IncI1 and to a lesser extend IncI2 plasmids. This cross reactivity was also observed when the WGS data from isolate 142 were assessed and found to carry the genes associated with an IncI1 T4SS, as well as some similarity to the *pilV* genes from IncBO and K plasmids (Table 3). To further delineate the similarity of the IncBO, K and I1 plasmids further, we looked at the *pilV* gene sequences in each of these types and carried out a sequence alignment (Figure 6) and concluded that the genes that were identical for the IncBO and K plasmids and the I1 plasmids had significant overlap. The *pilV* gene encodes for an adhesin that is present at the tip of the conjugal pilus (T4SS) which interacts with the lipopolysaccharide (LPS) on the recipient cell during conjugation in liquid environments [Ishiwa and Komano, 2000, 2003; Foley et al., 2021]. This overlap between the transfer genes has been reported elsewhere in papers exploring IncI-related plasmids. Zhang et al. (2019) undertook comparative genomics analyses of multiple IncI, BO, K and Z plasmids and found high degrees of similarities (> 95%) across the transfer genes of IncBO and K plasmids [Zhange et al., 2019]. Likewise, PlasmidFinder now groups IncBO, K and Z as a single replicon type, IncB/O/K/Z, in their analyses [Shirakawa et al., 2020; Darphorn et al., 2021]. Overall, the

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results of the comparison of the IncBO and IncK plasmid in this study and the data from others indicate that these plasmid types could likely be merged into a single plasmid type in the Plasmid Transfer Gene Database (IncB/O/K). The benefits of this change would be to eliminate confusion on the plasmids present; and by removing 38 genes from the database, it creates a more streamlined set of data to assess and is consistent with the current plasmid typing tools, such as PlasmidFinder.

Another feature of the global phylogenetic analysis was the identification that genes with identical names are not always closely related in the phylogenetic trees. For example, the *tral* gene was the most common gene present across plasmid replicon types, found in all plasmid types except the IncW plasmid. When the phylogenetic analyses were conducted on just the *tral* genes from the different plasmid types, there was significant diversity in the gene sequences (Figure 10). This is likely due to the naming issues with the genes in different plasmid types being named in an *ad hoc* fashion as the secretion systems were characterized initially. For example, in the IncF plasmids, TraI serves as a relaxase and there is a domain responsible for conjugative repression that resides in the central region of TraI, which plays a role in the regulation of conjugation [Haft et al., 2006]. Moreover, TraI in the IncF-like plasmids was observed to serve as translocation signals mediating type 4 secretion [Zatyka et al. 1998; Waksman et al., 2019]. In contrast, in the IncI-like plasmids, TraI is part of a multiprotein structural complex in the outer membrane that helps to stabilize the conjugal pilus [Foley et al., 2021]. As these two examples show, the naming of the plasmid transfer genes is quite confusing and should probably be reassessed to help clarify the nomenclature. One possible way to rectify these issues could be to use naming systems such as shown with the IncW plasmids, where the genes are named with the precursor "*trw*", which indicates that there are transfer genes (*tr*) from

IncW (*w*) plasmids. It appears that there is some movement in this direction as some of the

IncHI1 and IncHI2 transfer genes are also annotated as *"trh_"*. A possible challenge with this

approach is the long-standing existing nomenclature that is embedded in the literature that would

need to be cross-referenced with a revised nomenclature.

As the *traI* example showed, the naming of plasmid factors is quite variable. When we

looked at proteins that are predicted to have the same function, they are often named quite

differently. For example, when we look at the predicted homologs of VirD4 ATPases or type

four coupling protein (T4CP) from prototypical T4SS of the bacterium *Agrobacterium*

tumefaciens [Ztyka et al., 1998; Raleigh et al 2013; Christie et al., 2014; Foley et al., 2021], there

are several different gene/protein names (Figure 11). In the process of identifying the T4CP, it

was noted that *trwB* from IncW was not included among the 359 genes in the database, as it was

not in the R388 reference strain used in the database development (Table 1). As is shown in

Figure 11, the level of amino acid similarity between the reference VirD4 and the other predicted

ATPases was quite divergent. Some of the predicted ATPases displayed similarity including

TrbC belonging to IncBO, IncK, IncI1 and IncM and TraD from IncFIA and FIB, which also

displayed similarity to TrwB and TraJ belong to IncW and IncN, respectively. However, these

protein sequences that are clustered together are divergent from the other predicted ATPase

genes from the other plasmid types. In general, T4CPs of Gram-negative conjugation machines

stimulate the ATPase activity of the coupling protein, which is required to initiate the

relaxosome complex of the T4SS [Hegyi et al, 2017]. The T4CPs influences the nucleotide

binding properties that facilitate loading of plasmid DNA into the T4SS for transfer [Alvarez-

Martinez et al., 2009; Raleigh et al., 2013].

3.5. Concluding remarks and future directions:

Multidrug resistance in *Salmonella* and other enteric pathogens remains a worldwide challenge, in large part due to the dissemination of AMR by plasmids. A key challenge to mitigate AMR is trying to predict the potential for plasmid transfer and AMR spread. The Plasmid Transfer Factor Database and tools evaluated in the current project provide very extensive bioinformatics tools that can enable the identification and characterization of the transfer associated genes. Key findings from this study have identified potential needs for refinement of the Plasmid Transfer Factor Database, such as consolidation of overlapping replicon associated T4SSs, potential genes that should be added to the database, and issues with confusing nomenclature. The refined database will allow for the detection of differences in the transfer gene content that can potentially have an impact of the abilities of different plasmids to transfer, allowing for risk assessment of the different plasmids from a public health standpoint. Additionally, the tools allow for a direct phylogenetic comparison of replicon types and transfer genes among bacterial strains, which can help understand the distribution of plasmids across bacterial populations. The database and tools are currently only available within the FDA; however, efforts are ongoing to make them available to the public. Currently, scientists can access the database through FDA/NCTR to upload data to the Plasmid Transfer Factor Assessment and Plasmid Transfer Factor Comparison tools at NCTR/FDA server.

Acknowledgements:

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Supplemental Table S1: see attached Chapter 3 Supplemental Tables spreadsheet tab Table S1

Supplemental Table S2: see attached Chapter 3 Supplemental Tables spreadsheet tab Table S2

CHAPTER FOUR

RESEARCH ARTICLE

Impact of Chloramphenicol and Tetracycline Exposures on the Transfer Efficiency of Multidrug Resistance Plasmids in *Salmonella enterica*

Suad Algarni[1,2], Jing Han[2], Joanna Deck[2], Ashlyn Carlton[2], Nancy Bao[2], Steven C. Ricke and Steven L. Foley[1,2]

[1]Cellular and Molecular Biology Graduate Program, University of Arkansas, Fayetteville, Arkansas, 72704

[2]Division of Microbiology, FDA National Center for Toxicological Research, Jefferson, Arkansas, 72079

[3]Meat Science & Animal Biologics Discovery Program, Dept. of Animal and Dairy Sciences, University of Wisconsin, Madison WI, 53706

*Corresponding Authors: Dr. Steven L. Foley, Mailing Address: National Center of Toxicological Research, Food and Drug Administration, 3900 NCTR Rd, Jefferson, AR 7209. Phone: (870) 543-7390

Running Title: Chloramphenicol and tetracycline exposure and AMR transfer

Abstract:

Background: *Salmonella enterica* is a zoonotic pathogen that causes bacterial foodborne disease in the U.S. Dissemination of multidrug resistance (MDR) phenotypes are considered a public health concern. Antimicrobial agents impart selective pressure that contributes to the emergence MDR bacteria; however, there is minimal knowledge about the impact of the antimicrobial exposure on plasmid transfer.

Methods: Six MDR *S. enterica* isolates were examined to assess their ability to transfer resistance plasmids following antimicrobial exposure with different concentrations of tetracycline or chloramphenicol (0 and 0.063 to 64 µg/ml). Whole genome sequence (WGS) analyses were also used predict plasmid replicon types, antimicrobial resistance profiles and plasmid transfer genes present in the strains.

Results: The results indicated the dose-dependent influence following tetracycline and chloramphenicol exposure on plasmid transfer efficiency in some strains where there was a pattern of increasing dose-dependent conjugation efficiency compared to the non-exposed control. WGS analyses showed diverse plasmid replicon types, resistance gene profiles, and conjugal transfer genes among the *Salmonella* strains. The distribution of plasmids transfers among transconjugants varied in several isolates.

Conclusions: Tetracycline and chloramphenicol exposure had a significant impact on the transfer of antimicrobial resistance among some *Salmonella* isolates. There is distribution of impacts of tetracycline and chloramphenicol exposure during conjugation for each of the *Salmonella* isolates. The data also show the importance of using WGS as an effective tool to characterize plasmids and antimicrobial resistance in *Salmonella* particularly as costs drop and techniques improved.

4.1. Introduction:

According to the Centers for Disease Control and Prevention (CDC), *Salmonella* infections annually result in approximately 1.2 million illnesses, 19,000 hospitalizations, and 350 deaths [Scallan et al. 2011; Brunelle et al. 2017]. *Salmonella enterica* can adapt to external and internal environments by interacting with other bacteria in the intestinal microbiota and the environment [Kaldhone et al. 2008]. The composition of the *Salmonella* genome can change in response to antimicrobial exposure through the process of horizontal gene transfer (HGT) [Hoffmann et al. 2017].

Bacterial conjugation is one of the primary mechanisms of HGT and occurs when plasmids transfer DNA from one bacterial cell to another through a direct link between the donor and recipient bacterial cells [Cabezón et al. 2017; Frost et al. 2005]. The conjugation system contains several unique features including the conjugal transfer protein system (i.e., type 4 secretion system, T4SS) and rolling-circle replication (RCR) that can transmit the plasmids, as well as the related integrated conjugative elements (ICEs), to other bacterial cells [Llosa et al. 2002; Wawrzyniak et al. 2017; Algarni et al. 2022].

The ICEs and conjugative transposons (CTns) are self-mobile genetic elements which can manage their own transfer among bacterial genomes utilizing the conjugation machinery [Seth-Smith et al. 2012; Hoek et al. 2011; Mebrhatu et al. 2014]. Many plasmids carry genes that expedite their own conjugal transfer among bacteria communities; while other plasmids are missing some, or all of the conjugal transfer genes, but can transfer with assistance from another conjugative plasmid in the bacterium [Han et al. 2018; also see Chapter 3].

S. enterica can develop multidrug resistance (MDR), which is a major concern when an individual develops a more severe *Salmonella* infection. The fluoroquinolones and third-

generation cephalosporins are most often used as treatment for severe case of human

salmonellosis; however, exposure to these and other antibiotics can potentially lead to the

development of antimicrobial resistance (AMR), which can be life threatening due to limited

treatment options [Hoffmann et al. 2017]. Several AMR genes are encoded on the mobile

genetic elements including plasmids, transposons and integrons; which can be transferred

between human pathogens [Hoffmann et al. 2017].

Plasmids can replicate independently of the bacterial chromosome and contain AMR and

virulence genes that augment bacterial function [Kaldhone et al. 2008]. Several *Salmonella*

strains have been identified that encode the MDR-AmpC resistance phenotype to multiple

clinically relevant antimicrobial drugs, including the cephalosporins, ampicillin,

chloramphenicol, streptomycin, sulfonamide, and tetracycline [Hoffmann et al. 2017].

Additionally, the MDR genotypes can be detected on different plasmids representing multiple

plasmid incompatibility (Inc) groups including IncA/C, IncI1 and IncHI2 [Han et al. 2018].

Previous studies have indicated that these plasmid types can be transferred between multiple

Salmonella isolates [Han et al. 2018].

Over the last half century, predominant laboratory-confirmed serotypes associated with

human illnesses include Typhimurium, Enteritidis, Newport, Heidelberg, Infantis and Javiana;

these serotypes are associated with over 47% of the human infections caused by *Salmonella* [Han

et al. 2018; CDC 2013]. In the U.S., *S. enterica* serotype Typhimurium strains contribute to at

least 7% of the *Salmonella* deaths and 84,000 illnesses [Han et al. 2012]. Therefore, due to the

potential severity of illness, antimicrobial treatment may be required in some cases. Indeed, data

from the National Antimicrobial Resistance Monitoring System (NARMS) showed that the most

observed resistance phenotypes of *S.* Typhimurium isolated from human patients included

resistance to tetracycline (33%), ampicillin (31%), streptomycin (31%), and chloramphenicol (24%) [Brunelle et al. 2015]. In addition to human illnesses, *S.* Typhimurium is one of the most prevalent serotypes detected in multiple food animal species; which could facilitate an increase in the transmission of AMR genes through the food supply for this specific serotype [Brunelle et al., 2015; Brunelle et al., 2017; Angulo et al., 2005].

Furthermore, antimicrobial resistance data from NARMS for *S.* Heidelberg isolates show that the most observed resistance phenotypes are to streptomycin (32%), tetracycline (30%) and kanamycin (24%) [Kaldhone et al., 2008]. *S.* Heidelberg strains have been commonly isolated from human patients, retail meats and the food supply [Han et al., 2011]. Data from NARMS showed an increase in the percentage of *S.* Heidelberg strains from both human and poultry (chicken and turkey) that were resistant to cephalosporins from 1997 to 2008 [Han et al., 2012].

Several isolates from serovars Heidelberg (2%) and Typhimurium (15%) were also shown to carry IncFIB (ColV-like) plasmids found in MDR serovar Kentucky (73%) isolated from poultry [Johnson et al., 2010]. The development of AMR limits treatment options, this is especially true for pediatric patients where the fluoroquinolones are limited due to toxicity concerns. It has been estimated that approximately 40% of patients with laboratory confirmed *Salmonella* infections cases are treated with antimicrobial medication in the U.S. [Angulo et al., 2005]. To combat resistance challenges, World Health Organization (WHO, 2011) and other public health entities have encouraged the research for the development of new alternative AMR options [Brunelle et al., 2017]. These efforts can be enhanced by limiting the dissemination of antimicrobial resistance among enteric pathogens through understanding how factors such as antimicrobial selective pressure impact horizontal gene transfer helps develop new strategies to reduce the emergence of resistant bacteria and minimize resistance transfer [Han et al., 2018].

Two of the key drugs where resistance has been observed on the transmissible plasmids is chloramphenicol and tetracycline [Han et al., 2012]. Chloramphenicol was initially described as chloromycetin and isolated from *Streptomyces venezuelae* in 1947. Similarly, tetracycline was initially described in 1948 and originally isolated from *Streptomyces aureofaciens.* Both drugs are broad-spectrum antibiotics and potent inhibitors of protein synthesis and have been commonly used in both human and veterinary medicine targeting bacterial pathogens of different species and genes, including *Salmonella* [Sultan et al., 2018; Van Hoek et al., 2011; Holman et al., 2018; Schwarz et al., 2004]. Chloramphenicol and tetracycline resistance was common susceptibility among the *Salmonella* isolates, with 13.1% of *S.* Typhimurium and 8.8% of *S.* Heidelberg isolated display cross resistance, whereas overall tetracycline resistance for *S.* Typhimurium and *S.* Heidelberg were 17.8% and 20.6%, respectively. Overall chloramphenicol resistance was detected among the non-typhoidal *Salmonella* from human patients (4.6%), and from animal sources including chickens (14.3%), turkeys (1.5%), cattle (11.1%) and swine (2.2%); while for tetracycline resistance among the non-typhoidal *Salmonella* from human patients was 11.8%, and in animal sources including chickens (54.6%), turkeys (44.7%), cattle (19.2%) and swine (39.4%) (CDC 2021; FDA 2021). Accordingly, the main focus of the current study is to assess the efficiency of plasmid transfer following exposure to different concentrations of chloramphenicol and tetracycline in multiple *S. enterica* isolates as part of selective pressure experiments; this helps to assess the mechanisms and proliferation on the dissemination antimicrobial resistance plasmids in vitro.

4.2. Methods and Materials

4.2.1. Bacterial strains:

Six *S. enterica* isolates from serovars Typhimurium and Heidelberg were phenotypically identified by standard microbiological methods (Table 1) [Han et al. 2018; Aljahdli et al. 2020]. For phenotypic testing, the antimicrobial drug susceptibility testing (AST) was performed for each isolate using disc diffusion or broth microdilution following standard Clinical and Laboratory Standards Institute (CLSI) guidelines, as previously reported [Foley et al., 2006; Kaldhone et al., 2008; Lynne et al., 2009b]. These strains were chosen for the experiments because they were resistant to at least eight antimicrobial agents among the 15 different antimicrobials tested in the NARMS program (Centers for Disease Control and Prevention, 2015). The six MDR isolates belong to serovars Typhimurium (n=4) and Heidelberg (n=2) and originate from different sources and locations within the U.S. from 1992 to 2002 [Han et al., 2018] (Table1). Isolates were frozen at -80°C in brain heart infusion broth (Remel, Lenexa, KS, USA) containing 20% glycerol for long-term storage. For experiments, the isolates were streaked on tryptic soy agar (TSA) supplemented with 5% sheep's blood (Blood Agar, Remel) and then incubated at 37°C for 18 to 24 h. Isolates were screened by PCR to confirm the presence of the IncAC, IncFIB, IncFIA, IncI1, IncHI1, and/or IncHI2 replicons that were previously reported [Han et al., 2018; Carattoli et al., 2005; Johnson et al., 2007]. This step was important to ensure that a plasmid was not lost during long-term storage or re-culturing. The sodium azide resistant *Escherichia coli* J53 was utilized as recipient strain and the six *Salmonella* strains were selected as donors for both conjugation and selective pressure experiments [Jacoby and Han, 1996; Han et al., 2018; Aljahdali et al., 2020].

Table 1. Information on the *Salmonella enterica* isolates included in the antimicrobial exposure studies that were analyzed using whole genome sequencing

Isolate	Serotype	States	Source	Year	Accession Number
SE142	Heidelberg	Indiana	Swine	2002	NPFC00000000
SE143	Heidelberg	Minnesota	Swine	2002	NPEL00000000
SE163	Typhimurium*	Ohio	Turkey-diagnostic	2002	LSZ 00000000
SE452	Typhimurium	Unknown	Turkey-ground	1999	LYRS0000000
SE462	Typhimurium	Unknown	Turkey-ground	1999	VTSU00000000
SE710	Typhimurium*	North Dakota	Turkey-diagnostic	1992	LXGZ00000000

*Isolates were initially serotype as Heidelberg using traditional methods and later identified as Typhimurium following whole genome sequence analyses.

4.2.2. Selective pressure and conjugation assays:

Two types of selective plates were made, either with Luria-Bertani (LB) agar or MacConkey agar containing sodium azide (350 µg/ml) and tetracycline (32 µg/ml). These two selective plates were used for both the selective pressure and the transconjugants screening experiments. To initiate conjugation analysis, six *Salmonella* isolates were plated on blood agar plates and served as the donor strains and the sodium azide-resistant *E. coli* J53 strain was used as the recipient. For each conjugation experiment, a donor and recipient were mixed at the ratio of 1:1 to form the mating mixture. The final antimicrobial concentrations of chloramphenicol and tetracycline used to provide selection pressure ranged from 0.0625 to 64 µg/ml and were generated by carrying to serial 2-fold dilutions starting at 128 µg/ml in LB broth and 100 µl of each dilution was combined with 100 µl of mating mixture in a centrifuge tube and the tubes were incubated at 37°C for two hours (the final chloramphenicol concentrations ranged from 0.0625 to 64 µg/ml). Each experiment included non-antimicrobial exposed samples (100 µl of LB broth and 100 µl of

mating mixture) that served as control/baseline samples. After incubation, the tubes were centrifuged at 10,000 rpm for 5 min and the supernatants were discarded to remove the antibiotics from bacterial cells, then the pellets were suspended in 200 µl of sterile LB broth. This step was repeated twice to ensure all antimicrobials were removed prior to suspend the cells in 200 µl of BHI broth and incubated at 37 °C for 4 h to facilitate cell recovery. Following incubation, the bacterial cell mixture was serially diluted (1:10, 1:100, 1:1000, and 1:10000) in sterile LB broth for each isolate, and the diluted mixtures were plated in triplicate onto LB-agar selection plates described above containing the sodium azide and tetracycline. The culture plates were incubated at 37°C for up to 48 h and the numbers of bacterial colonies were counted and recorded. Each selective pressure experiment for six *Salmonella isolates* was done in triplicate for a total on nine data points for each isolate.

After the selective pressure experiment was completed, five colonies were randomly selected and plated onto MacConkey agar selection plates to confirm the transconjugants were *E. coli* with resistance plasmids transferred. The plates were incubated overnight and observed. A pink colony morphology indicated the presence of *E. coli* strains that ferment lactose. Transconjugants were picked randomly from each plate and subjected to PCR-based replicon typing using primers to identify the plasmids that transferred in the conjugation studies [Han et al., 2018; Carattoli et al. 2005].
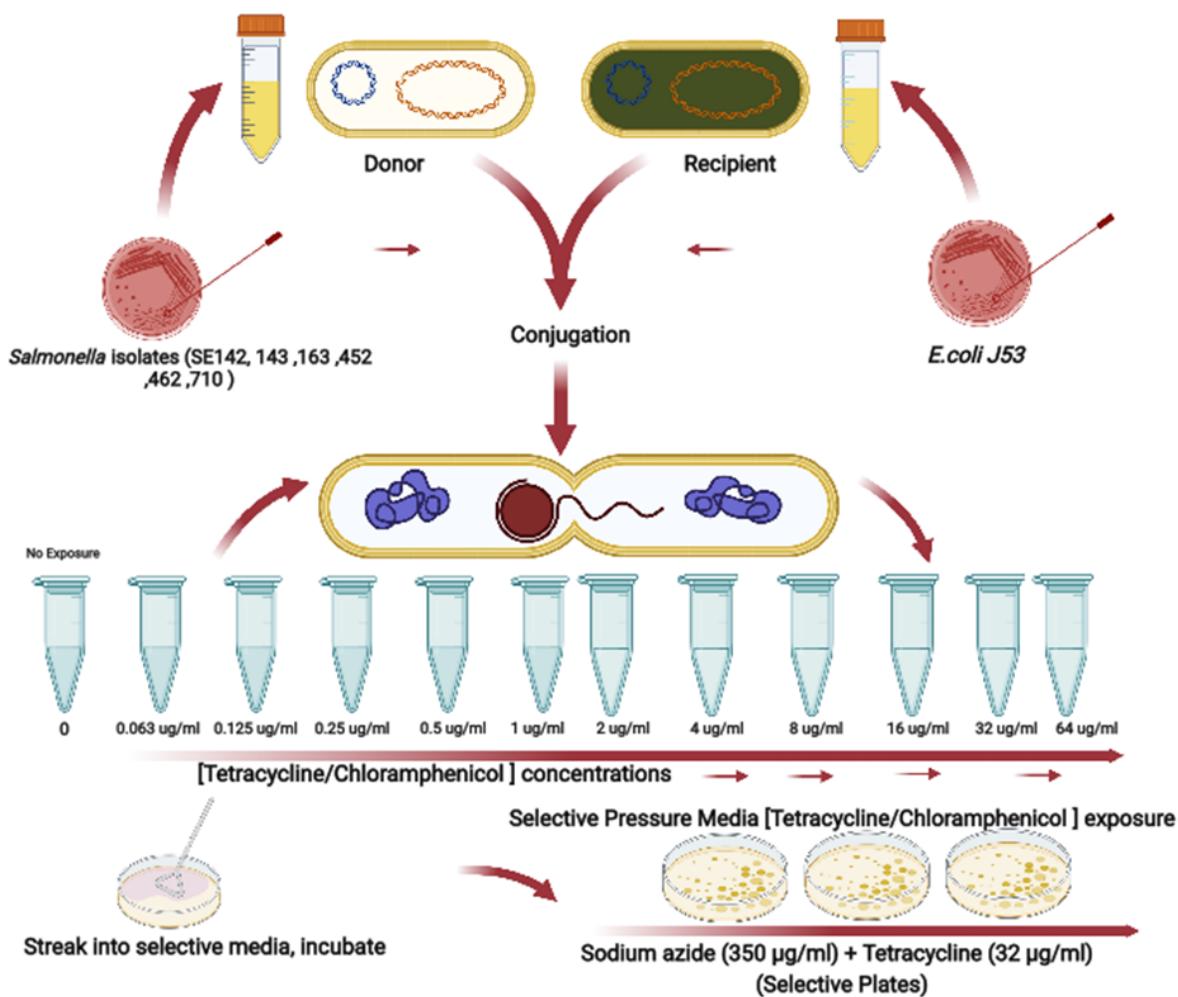
Figure 1. Schematic of conjugation process of six donor *S.* Heidelberg and *S.* Typhimurium serotypes and recipient *E. coli* J53 indicated the steps of the selective pressure experiments. Figure was created using BioRender (biorender.com).

4.2.3. Bioinformatic analyses from *Salmonella* isolates:

The donor *Salmonella* isolates were sequenced as part of previous studies [Kaldhone et al., 2017; Aljahdali, et al., 2020a, 2020b]; however, further characterization of the genome sequences was undertaken in this study. FASTA files of sequences assembles from each strain were analyzed

using Plasmid Finder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) to extract the plasmids

replicon (Inc) group types [Carattoli et al., 2014], the ResFinder web server

(https://cge.cbs.dtu.dk/services/ResFinder/) to determine the antimicrobial resistance gene

present [Zankari et al., 2012], and the Plasmid Transfer Gene database as described in Chapter 3,

which generated the Basic Local Alignment Search Tools (BLAST)-based comparison to the

plasmid transfer genes.

4.2.4. Statistical analysis:

All data were imported into Excel (Microsoft, Redmond, WA, USA), the mean and standard

deviations were calculated for each experiment and T-tests were used to determine statistical

different between six *Salmonella* isolates across a different concentration of antimicrobial agents

to non-exposed cell ratio with a p-value ≤ 0.05 considered as a significant different among

Salmonella isolates.

4.3. Results:

Antimicrobial agent modulation of *Salmonella enterica* was assessed to determine whether

antimicrobial selective pressure impacted resistance plasmid transfer efficiency in a dose-

dependent fashion. Six *S. enterica* isolates, representing different serotypes, sources and

geographic locations were examined in the present study (Table 1) [Khajanchi et al., 2016, 2017;

Kaldhone et al., 2017]. From these isolates, selective pressure was initially conducted to assess

the impact of two antimicrobial exposures, particularly tetracycline and chloramphenicol, on

plasmid transfer dynamics in MDR *S. enterica* (Figure 2 and 3). The plasmid transfer phenotype

of all MDR *S.* Typhimurium and *S.* Heidelberg isolates was observed following exposure to

tetracycline (0.063-64 µg/ml). Two resistant isolates (163 and 462) had significantly increased

numbers of transconjugants following exposure to tetracycline (0.0625-0.5µg/ml) at low

concentrations. Interestingly, the higher concentrations of tetracycline (2, 4, 8, and 16 µg/ml)

reached statistical significance for MDR *S.* Typhimurium isolate 462 compared to the non-

antimicrobial control (Figure 2). Notably tetracycline displayed a pattern of increasing

conjugation efficiency as the antimicrobial concentration increased to a concentration where the

numbers began to decrease, potentially due to elevated toxicity at the antimicrobial

concentrations.
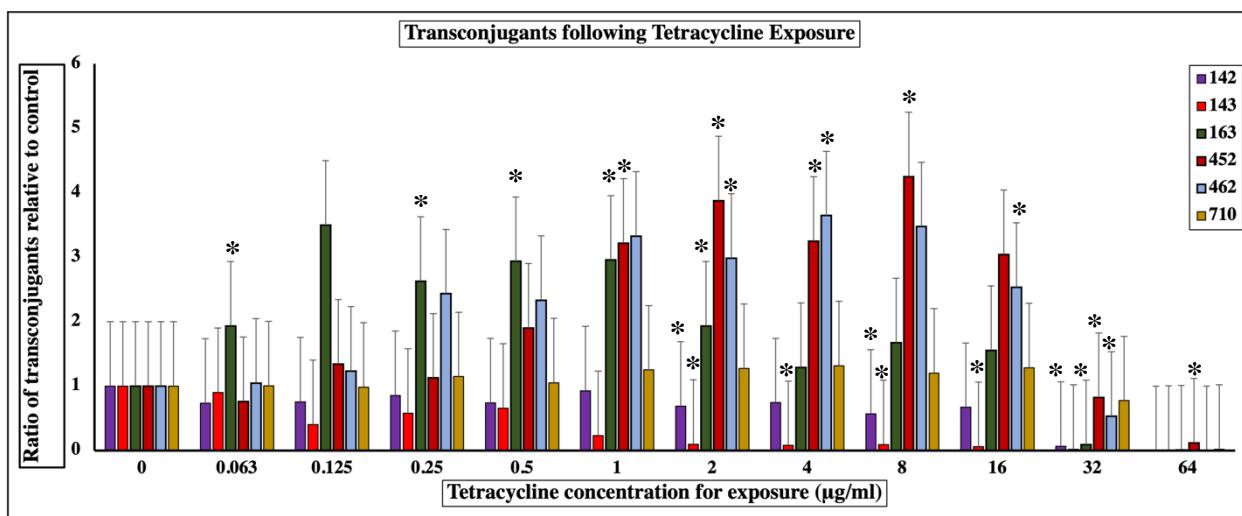
Figure 2. Results of the tetracycline selective pressure for the six *Salmonella* isolates donor and *E. coli* J53 recipient. The bars indicate the ratio of transconjugants to non-exposed cells. Each set of experiments were done in triplicate and repeated a total of three times (9 total replicates). The error bars show the standard deviation across the tetracycline concentrations for each isolate. * Indicates statistically significant difference of the transconjugants between the exposed isolate and the non-exposed control ($P < 0.05$).
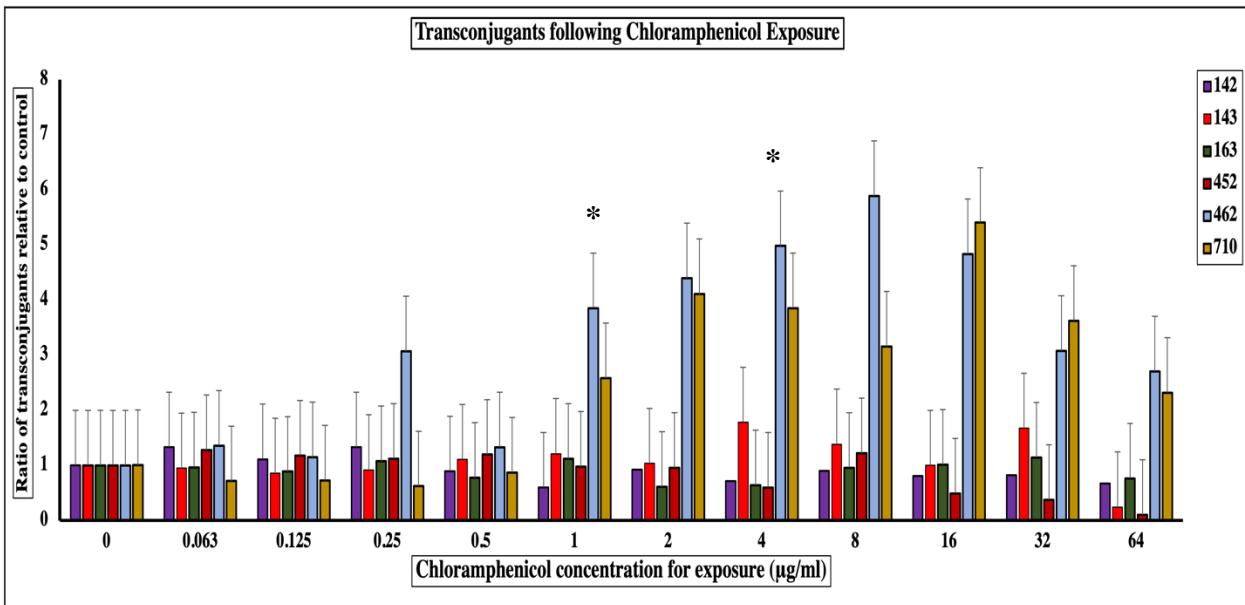
Figure 3. Results of the chloramphenicol selective pressure for the six *Salmonella* isolates donor and *E. coli* J53 recipient. The bars indicate the ratio of transconjugants to non-exposed cells. Each set of experiments were done in triplicate and repeated a total of three times (9 total replicates). The error bars show the standard deviation across the chloramphenicol concentrations for each isolate. * Indicates statistically significant difference of the transconjugants between the exposed isolate and the non-exposed control ($P < 0.05$).

The MDR *Salmonella* isolate conjugation efficiencies were also determined following the same exposure process to chloramphenicol (0.063-64 µg/ml). An increased number of transconjugants did not reach statistical significance for isolates 142, 143, 163, 452, and 710 following exposures to chloramphenicol (0.063-64 µg/ml), respectively. Conversely, there was statistical significance with an increased conjugation efficiency for isolate 462 at 1 and 4 µg/ml concentrations of chloramphenicol compared to the non-exposed bacterial control (Figure 3). The results of the selective pressure assays generated detectable responses from multiple isolates and range of antimicrobial concentrations for tetracycline and chloramphenicol. In many cases those isolates resistant to tetracyclines, and chloramphenicol demonstrated the pattern of increased conjugation efficiency as the antimicrobial concentration increased to specific point

where the efficiency gradually declined (see Figure 2 and 3). Conversely, for some strains, the plasmid transfer did not appear to be impacted by exposure as the efficiencies and isolates were close to the baseline efficiencies across different exposure concentrations before dropping further at higher exposure concentrations. These studies demonstrated that for some strains, the exposure concentrations led to a dose-dependent impact on plasmid transfer efficiency.

To determine the plasmid and AMR characteristics of the isolates, WGS analyses were performed by using PlasmidFinder analyses which confirmed that all isolates contained IncA/C plasmids along with several other plasmid replicon types including IncI1 (n=3; 142, 143, and 163), IncHI2 (n=2; 142 and 143), IncI2 (n=1; 143), IncFIB (n=4; 163, 452, 462, and 710), IncX4 (n=2; 163 and 452), IncFIA (n=3; 452, 462, and 710), IncFII(pCoo) (n=4; 163, 452, 462, and 710), and IncColpVc (n=1; 710) (Table 2).

Table 2. List of *Salmonella enterica* isolates plasmid replicon (Inc) types predicated by in silico PlasmidFinder server.

Donor	Plasmids (Kb)	Replicon (Inc) Types by PlasmidFinder
SE142	>100, 10, 4	A/C, I1, HI2
SE143	>165x2, 92, 34	A/C, I1, I2, HI2
SE163	135, 120, 34, 6, 4	A/C, FIB, X4, I1, FII(pCoo), FIA
SE452	124,4,1	A/C, FIB, FII(pCoo), FIA
SE462	>150, 6, 4, 2	A/C, FIB, FII(pCoo), FIA
SE710	>120, 34, 8, 3, 2	A/C, FIA, FIB, X4, ColpVC, FII(pCoo)

The distribution of plasmids transferred to the recipient *E. coli* J53 were evaluated following tetracycline selection using PCR-based replicon typing and the patterns of the transmission were a highly variable. All donor isolates were IncA/C positive, while only transconjugants for 143 and 163 were IncA/C positive, compared to the transconjugants for the

other isolates (142, 710, 452, and 462) which lacked IncA/C plasmids [Han et al., 2018]. In

contrast, for isolate 142 only the IncI plasmid transferred, while isolates 452, 462, and 710 had

the IncFIB/FIA plasmid transferred (Table 3).

Table 3. List of *Salmonella enterica* isolates, conjugation efficiency and the plasmids that were transferred during *in vitro* conjugation experiments (wet lab).

Isolate	Donor Replicon	Efficiency	Transconjugants Replicons
SE142	IncA/C, IncI1, IncHI2	2.03E-07	IncI1 (3/4)
SE143	IncA/C, IncI1, IncHI1, IncHI2	5.13E-03	IncA/C (4/4), Inc I1 (4/4)
SE163	IncA/C, IncFIB	4.60E-07	IncA/C (4/4), IncFIB (4/4)
SE452	IncA/C,IncFIA,IncFIB	1.22E-04	IncFIA (3/4), IncFIB (3/4), NT (3/4)
SE462	IncA/C, IncFIA, IncFIB	1.22E-03	IncFIA (3/4), IncFIB (3/4)
SE710	IncA/C, IncFIA, IncFIB	6.81E-07	IncFIA (2/4), IncFIB (2/4)

* non-typeable (NT) results indicated the plasmid transfer but an identified replicon was not amplified from the corresponding plasmids transferred to the transconjugants.

WGS data were analyzed using ResFinder software to detect antimicrobial resistance

genes and predict the resistance phenotypes for each donor isolate [Zankari et al., 2012]. A

complete list of each isolate and its predicted resistance phenotype are shown in Table 4. Among

the *Salmonella* isolates resistance was detected for amoxicillin (AMC)(n=6, 100%), ampicillin

(AMP) (n=6, 100%), ceftiofur (TIO) (n=6, 100%), streptomycin (STR) (n=6, 100%), gentamicin

(GEN) (n=6, 100%), cefoxitin (FOX) (n=6, 100%), chloramphenicol (CHL) (n= 6, 100%),

ceftriaxone (CRO) (n=6 ,100%), tetracycline (TET) (n=6,100%), sulfisoxazole (FIS) (n=6,

100%), kanamycin (KAN) (n=6, 100%), and trimethoprim/sulfamethoxazole (SXT) (n=1,

16.7%). The resistance genes detected among the isolates and their % identity included *aph(3')-

lla* (n=2, 33.3%), *strA* (n= 2, 33.3%), *strB* (n= 2, 33.3%), *bla*CMY-2 (n= 6, 100%), *aph(6)-lc* (n= 2,

33.3%), *aadA1* (n= 3, 50%), *aac(3')-Via* (n= 3, 50%), *floR* (n= 6, 100%), *sul1* (n= 4, 66.7%),

sul2 (n= 4, 66.7%), *tet(A)* (n= 6, 100%), *tet(B)* (n= 6, 100%), *dfrA1* (n=1, 167%), *aph(3'')-lb* (n=

4, 66.7%), *ant(2'')-la* (n= 3, 50%), *aph(6)-ld* (n= 3, 50%), *bla*TEM-1*B* (n= 4, 66.7%), *cmlA1* (n= 4,

66.7%) and *aph(3')-la* (n=5, 83.3%), with their distribution shown in Table 4. These results

demonstrate that the donor cells can transmit multiple resistance phenotypes through

conjugation.

Table 4. List of *Salmonella enterica* isolates, resistances genes and extracted phenotypes by ResFinder server.

Donor	Resistance Genes by ResFinder File	Extracted Phenotype
SE142	*aph(3')-lla, strA , strB, blaCMY-2, aph(6)-lc, aadA1, aac(3')-Via , floR, sul1, sul2, tet(A), tet(B)*	STR, AMP, FIS, AMC, GEN, CHL, TET, CRO
SE143	*aph(6)-lc, aph(3')-lla, aph(3')-la, strA, strB aac(3')-Via, blaCMY-2, floR, sul1, sul2,tet(A), tet(B), dfrA1*	STR, AMP, AMC, FIS, KAN, CRO, GEN, CHL, TET, SXT
SE163	*aph(3")-lb, ant(2")-la, aph(3')-la, aac(3)-Via, aph(6)-ld, aadA1, blaTEM-1B, blaCMY-2, cmlA1, floR, sul1, sul2, tet(A), tet(B)*	KAN, STR, AMC, AMP, CRO, GEN, CHL, FIS, TET
SE452	*strA, aph(3')-la, strB, ant (2")-la, blaTEM-1B, blaCMY-2, cmlA1, floR, sul2 ,tet(A), tet(B)*	KAN, STR, AMC, AMP, CRO, FOX, TIO, GEN, CHL, FIS, TET
SE462	*strA, aph(3')-la, ant (2")-la, strB , blaTEM-1B, blaCMY-2,* cmlA1, floR, sul2, tet(A), tet(B)	KAN, STR, AMC, AMP, CRO, FOX, TIO, GEN, CHL, FIS, TET
SE710	*strA, strB , ant (2")-la, aph(3')-la, aac(3')-Via, aadA1, blaTEM-1B, blaCMY-2,* cmlA1, floR, *sul1, sul2, ,* tet(A), tet(B).	KAN, STR, AMC, AMP, CRO, FOX, TIO, GEN, FIS

STR, streptomycin; AMP, ampicillin; GEN, gentamicin; AMC, amoxicillin; KAN, kanamycin; FOX, cefoxitin; TIO, ceftiofur; CRO, ceftriaxone; FIS, sulfisoxazole; SXT, trimethoprim/sulfamethoxazole; CHL, chloramphenicol; TET, tetracycline.

BLAST analyses of the conjugal transfer gene content across plasmid types in all isolates

also showed variability. For the IncA/C transfer operon, 11 out of the 15 conjugal transfer genes

(*traA, traB, traC, traD, traE, traF, traG, traH, traI, traL,* and *traV*) were present in all six

isolates, however, only isolates 142 and 143 had the remaining IncA/C tra gene region

containing a conjugative transfer genes *traN, traU, traW* and *trhF*; while other isolates (163, 452,

462, and 710) lack of these genes. The transfer genes associated with IncFIA/IncFIB plasmids

are present on four isolates 163, 452, 462, and 710, and other isolates were absent of these (Table

5). Two isolates (142 and 143) contained 32 of conjugative transfer genes associated with IncHI2 plasmids, which were absent in the other isolates. The IncI1 plasmid-associated conjugal transfer genes were present in three isolates (142 143, and 163) as is shown in Table 5.

Table 5. Conjugal transfer genes identified from the WGS data from *Salmonella enterica* isolates identified through in silico analysis.

Transfer Genes (Inc type_gene)	Isolates					
	SE142	SE143	SE163	SE452	SE462	SE710
IncAC_traA	X	X	X	X	X	X
IncAC_traB	X	X	X	X	X	X
IncAC_traC	X	X	X	X	X	X
IncAC_traD	X	X	X	X	X	X
IncAC_traE	X	X	X	X	X	X
IncAC_traF	X	X	X	X	X	X
IncAC_traG	X	X	X	X	X	X
IncAC_traH	X	X	X	X	X	X
IncAC_traI	X	X	X	X	X	X
IncAC_traL	X	X	X	X	X	X
IncAC_traN	X	X				
IncAC_traU	X	X				
IncAC_traV	X	X	X	X	X	X
IncAC_traW	X	X				
IncAC_trhF	X	X				
IncFIA_traA				X	X	X
IncFIA_traB			X	X	X	X
IncFIA_traC			X	X	X	X
IncFIA_traD			X	X	X	X
IncFIA_traE			X	X	X	X
IncFIA_traF				X	X	X
IncFIA_traG			X	X	X	X
IncFIA_traH				X	X	X
IncFIA_traI			X	X	X	X
IncFIA_traK			X	X	X	X
IncFIA_traL			X	X	X	X
IncFIA_traN			X	X	X	X
IncFIA_traP			X	X	X	X
IncFIA_traQ			X	X	X	X
IncFIA_traR			X	X	X	X
IncFIA_traT			X	X	X	X
IncFIA_traU			X	X	X	X
IncFIA_traV			X	X	X	X
IncFIA_traW			X	X	X	X
IncFIA_traX			X	X	X	X
IncFIA_trbA				X	X	X
IncFIA_trbB			X	X	X	X
IncFIA_trbC			X	X	X	X
IncFIA_trbD			X	X	X	X

IncFIA_trbE			X	X	X	X
IncFIA_trbI			X	X	X	X
IncFIA_trbJ			X	X	X	X
IncFIB_traA			X	X	X	X
IncFIB_traB			X	X	X	X
IncFIB_traC			X	X	X	X
IncFIB_traD			X	X	X	X
IncFIB_traE			X	X	X	X
IncFIB_traF			X	X	X	X
IncFIB_traG			X	X	X	X
IncFIB_traH			X	X	X	X
IncFIB_traI			X	X	X	X
IncFIB_traJ			X	X	X	X
IncFIB_traK			X	X	X	X
IncFIB_traL			X	X	X	X
IncFIB_traM			X	X	X	X
IncFIB_traN			X	X	X	X
IncFIB_traP			X	X	X	X
IncFIB_traQ				X	X	X
IncFIB_traR			X	X	X	X
IncFIB_traT			X	X	X	X
IncFIB_traU			X	X	X	X
IncFIB_traV			X	X	X	X
IncFIB_traW				X	X	X
IncFIB_traX			X	X	X	X
IncFIB_traY			X	X	X	X
IncFIB_trbA			X	X	X	X
IncFIB_trbB			X	X	X	X
IncFIB_trbC			X	X	X	X
IncFIB_trbD			X	X	X	X
IncFIB_trbE				X	X	X
IncFIB_trbF			X	X	X	X
IncFIB_trbG			X	X	X	X
IncFIB_trbI			X	X	X	X
IncHI2_htdA	X	X				
IncHI2_htdF	X	X				
IncHI2_htdK	X	X				
IncHI2_htdO	X	X				
IncHI2_htdT	X	X				
IncHI2_htdV	X	X				
IncHI2_orf16	X	X				
IncHI2_orf17	X	X				
IncHI2_orf4	X	X				
IncHI2_orf9	X	X				
IncHI2_traH	X	X				
IncHI2_traI	X	X				
IncHI2_traJ	X	X				
IncHI2_trhA	X	X				
IncHI2_trhB	X	X				
IncHI2_trhC	X	X				
IncHI2_trhE	X	X				
IncHI2_trhF	X	X				
IncHI2_trhG	X	X				
IncHI2_trhH	X	X				

IncHI2_trhI	X	X				
IncHI2_trhK	X	X				
IncHI2_trhL	X	X				
IncHI2_trhN	X	X				
IncHI2_trhO	X	X				
IncHI2_trhP	X	X				
IncHI2_trhR	X	X				
IncHI2_trhU	X	X				
IncHI2_trhV	X	X				
IncHI2_trhW	X	X				
IncHI2_trhY	X	X				
IncHI2_trhZ	X	X				
IncI1_pilI	X	X	X			
IncI1_pilJ	X	X	X			
IncI1_pilK	X	X	X			
IncI1_pilL	X	X	X			
IncI1_pilM	X	X	X			
IncI1_pilN	X	X	X			
IncI1_pilO	X	X	X			
IncI1_pilP	X	X	X			
IncI1_pilQ	X	X	X			
IncI1_pilR	X	X	X			
IncI1_pilS	X	X	X			
IncI1_pilT	X	X	X			
IncI1_pilU	X	X	X			
IncI1_pilV	X	X	X			
IncI1_traA	X	X	X			
IncI1_traB	X	X	X			
IncI1_traC	X	X	X			
IncI1_traD	X	X	X			
IncI1_traE	X	X	X			
IncI1_traF	X	X	X			
IncI1_traG	X	X	X			
IncI1_traH	X	X	X			
IncI1_traI	X	X	X			
IncI1_traJ	X	X	X			
IncI1_traK	X	X	X			
IncI1_traL	X	X	X			
IncI1_traM	X	X	X			
IncI1_traN	X	X	X			
IncI1_traO	X	X	X			
IncI1_traP	X	X	X			
IncI1_traQ	X	X	X			
IncI1_traR	X	X	X			
IncI1_traS	X	X	X			
IncI1_traT	X	X	X			
IncI1_traU	X	X	X			
IncI1_traV	X	X	X			
IncI1_traW	X	X	X			
IncI1_traX	X	X	X			
IncI1_traY	X	X	X			
IncI1_trbA	X	X	X			
IncI1_trbB	X	X	X			
IncI1_trbC	X	X	X			

4.4. Discussion:

MDR strains of *Salmonella* are a major public health concern leading to difficult-to-treat infections globally. Historically, *S.* Typhimurium and *S.* Heidelberg serovars are found widespread in foods, food animals and among the most common serovars associated with human infection, with *S.* Heidelberg having relatively high percentage of more severe invasive infections [Kaldhone et al., 2008; Brunella et al., 2015; Han et al., 2011; CDC 2013].

Antimicrobial agents can provide selective pressure that can lead to the emergence of drug-resistant *Salmonella*. The resistance mobilome plays a key role in the spread of AMR genes and how the bacterial genomes respond to selective pressure are shaped by antimicrobial exposure in particular *S. enterica* isolates [Algarni et al. 2022]. Studies comparing the relative ability for the different antimicrobial agents to facilitate the spread of plasmids that confer multidrug resistance among *Salmonella* are limited and hence a key focus of the efforts of the current study. Just as importantly, in studies of MDR plasmid transfer among *Salmonella* isolates where strains have been sequenced and identified, diverse plasmids and AMR genes associated with resistance to chloramphenicol, tetracycline, ampicillin, streptomycin, kanamycin and β-lactam antimicrobials have been detected [Algarni et al., 2022].

Tetracyclines are broad-spectrum antimicrobial agents that are used for treating and prophylaxis against human infections, as well as for the prevention and control of bacterial infections in veterinary medicine [Gargano et al., 2021]. Therefore, the proportion of *Salmonella* isolates presenting with phenotypic resistance to tetracycline is relatively high (FDA, 2021). Due to the excessive use of tetracycline, resistance to it is often found in MDR *Salmonella* isolates either from human or livestock [Khoshbaleht et al., 2018; Patchanee et al., 2008; Etter et al., 2019]. In the present study, we characterized the six MDR *S.* Typhimurium and Heidelberg

strains for their ability to transfer resistance plasmids following exposure to two antimicrobials, chloramphenicol and tetracycline. An earlier study demonstrated that the majority of MDR *S.* Typhimurium isolates are resistant to tetracycline [Brunelle et al. 2013]. The possibility that these resistance genes can possibly be transferred to human intestinal microbiota with the consumption of contaminated foods is still a concern [Hernadez et al. 2002]. This study showed the effect of tetracycline exposure related to the plasmid transmission under four parameters: tetracycline resistance phenotype, tetracycline concentration, conjugal transfer genes and plasmid transfer. Multiple tetracycline concentrations were used to evaluate if the selective pressure induction was linked to the dose-dependent response and/or if the tetracycline exposure would be effect transmission in *Salmonella* isolates. We chose several concentrations of tetracycline (0, 0.63, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 µg/ml; Figure 2) to study the relative impact on plasmid transfer phenotype among different MDR *Salmonella* isolates after exposure of the bacterial cells. Tetracycline's exposure impact on plasmid transfer can be dependant on differences in bacterial strains and dose range of antimicrobials concentrations [Holman et al., 2018; Brunelle et al., 2013]. The resistance transfer dynamics was significantly increased in response to exposures for three isolates (163, 452 and 462) following exposure to various tetracycline concentrations. These alterations were observed at concentrations of 2-16 µg/ml for strain 462, at 0.063 and 0.25-2 µg/ml for strain 163 and at 1 to 8 µg/ml for 452. In the cases of isolates 142 and 143, there was a significant decrease in the number of transcongates beginning in as low 2 µg/ml of tetracycline. This phenomenon was likely due to a low rate of plasmid transfer and toxicity to the recient cell, since both donors were tetracycline resistant. Only one isolate (710) did not have a significantly variable response across all concentrations following exposure to tetracycline. Our data for isolates that were exposed to tetracycline were

variable across the strains. However, there have been limitied studies that evaluated the effect of tetracycline on these isolates, thus it was important to evaluate the selective pressure under tetracycline exposure to assess the impact on plasmid transfer. The differences in response among *Salmonella* isolates could be due to genetic content, including the presence of resistance genes and conjugal transfer genes associated with the plasmid transfer in a specific response. Importantly, resistance to tetracycline has been reported in multiple *Salmonella* isolates which were linked to causing more severe disease including bloodstream infections, where these factors contributed to increased morbidity among MDR *Salmonella* patients [Brunelle et al., 2013; Mukherjee et al., 2019]. The response to tetracycline exposure demonstrated a selective benefit in some strains due to increase in antimicrobial resistance phenotypes following exposures. Our data identified that tetracycline exposure can impact phenotypes which promoted the formation of transconjugants.

Tetracycline resistance genes were observed in *S.* Typhimurium and *S.* Heidelberg isolates, with both carrying the same *tet(A)* and *tet(B)* genes. Both *tet(A)* and *tet(B)* have been observed in multiple *Salmonella* serotypes as reviewed previously [Han et al., 2012]. A previous study by Delgarb-Suarz et al. (2021), examined *Salmonella* isolates and characterized the mechanisms of tetracycline efflux pumps. Efflux pumps are employed by bacteria to regulate their internal environment through remove toxic materials including antimicrobial agents [Aljahdali et al., 2020].

Chloramphenicol was also tested in the current project to evaluate if there are responses dependent on bacterial strains or/and particular dose concentrations that impact plasmid transfer. In this study, we used a selective pressure phenotypic assays to evaluate the plasmid transfer in response to chloramphenicol exposure to the same set of six MDR *Salmonella S.* Typhimurium

and S. Heidelberg isolates. Chloramphenicol is a broad-spectrum antimicrobial agent commonly applied in both veterinary medicine and hospital medications. It can be used as inhibitor to MDR *Salmonella* strains and lethal to sensitive isolates [Cherubin et al., 1977; Holman et al., 2018]. According to a previous study, most of MDR S. Typhimurium and *S.* Heidelberg isolates are resistant to chloramphenicol [Hernandez et al., 2002]. Our objective was to clarify and confirm the impact of selective pressure on MDR *Salmonella* following chloramphenicol exposure. We used the same conditions for tetracycline exposure by employing several concentrations of chloramphenicol (0 and 0.063 to 64μg/ml; Figure 3) to study the relationship of four conditions in vitro: chloramphenicol resistant phenotype, chloramphenicol concentration, conjugal transfer genes and plasmid transfer. Isolates 142, 143, 452, 163, and 710 were not significantly different from the control following chloramphenicol exposure. Only isolate 462 displayed significantly increased conjugation efficiency following exposure to chloramphenicol, although isolate 710 demonstrated a non-significant increase in the numbers of transconjugants with increasing concentrations. The data provides valuable results to clarify the selective pressure dynamics under chloramphenicol exposure. Until now, no previous studies examined the effect of chloramphenicol on these isolates using selective pressure assay. All isolates encoded the chloramphenicol resistance genes *floR* and *cmlA1* that were found to encode efflux pumps in previous studies [Delgaob-Suarez et al., 2021].

The exposure to both antimicrobial agents, tetracycline and chloramphenicol, associated with a selective pressure indicated the interrelatedness of factors including resistance genes, antimicrobials concentration, plasmid transfer and conjugal transfer genes in many cases. The current results raised multiple questions on the mechanism associated with the selective pressure findings. For instance, is the observed differentiation generated by a stress response that leads to

increased plasmid transfer or more targeted response such as resistant gene utilizing specific quorum sensing-like autoinducers (AI-2) that trigger plasmid transfer and impact resistance gene conjugation frequency? For example, the presence of AI-2 associated with conjugation of the plasmid RP4 carrying the *tet(A)* gene in the bacteria exposed to tetracycline exposure (Algarni et al., 2022). Indeed, many of the isolates contained IncA/C plasmids, which have been associated with co-localization with *Salmonella* genetic island 1 (SGI1) and can impact the mobility of the plasmids [Han et al., 2018]. Likewise, SGI1-encoded antimicrobial resistance genes can confer resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide and tetracycline (ACSSuT) [Algarni et al., 2022]. Further research is required to merge WGS and transcriptomic analyses to assess and identify what genes and processes that are affected by tetracycline and chloramphenicol exposures following antimicrobials agent exposure in *Salmonella* strains.

In our earlier work, we observed that most transconjugants harbored plasmids of multiple replicon types including IncA/C, FIB, FIA, and I1. These plasmid types can carry antimicrobial resistance genes that are transferred during conjugation experiments as discussed in the previous study (Table 3). However, some plasmids were not transferable to *E. coli* J53 due to multiple factors, potentially including plasmid size, mutations/deletions in *pil/tra/trh* regulatory genes, and different efficiency of conjugation methods (broth versus solid mating, antimicrobial selective pressure exposure concentrations, incubation temperature and donor/recipient ratio). Although all isolates carried IncA/C plasmid in the donor replicons, only transconjugants of two isolates (143 and 163) contain IncA/C replicons occurred, indicating that they had the ability to transfer IncA/C plasmids (Table 3). Many of the strains where the IncA/C plasmids were not transferred, the plasmids lacked some or all of the IncA/C transfer related genes, which may have impacted the ability of the plasmid to transfer.

One of the key benefits of using WGS analysis is that it allows for the identification of multiple plasmids that may not have been screened for by PCR, which is focused on specific target genes [Louden et al., 2012]. The PlasmidFinder database was used for the WGS to identify the plasmid replicon types of each isolate [Carattoli et al., 2014, Han et al., 2018]. In *S.* Heidelberg isolates 142 and 143 collected from swine, both isolates carried IncA/C, I1, and HI2 plasmids and isolate 143 had an additional IncI2 replicon. The other isolates (163, 452, 462, and 710) were *S.* Typhimurium strains sharing the replicon including IncA/C, FIA, FIB, and FII (pCoo) [Gokulan et al., 2013], and isolate 710 also containing the colpV replicon (Table 3). One key limitation of the short-read sequencing approach for characterization of the plasmid that is assembling full plasmid sequences is still problematic and assigning the specific replicon types to specific plasmids can be a challenge [Li et al., 2012]. In particular cases, especially with the IncF-type plasmids, they may carry several plasmid replicons sequences that seem to show the detection of multiple plasmids in a strain, even though they are the same plasmid [Hsu et al., 2019]. Thus, potentially present in the case of *S.* Typhimurium isolates where the IncF plasmid carries the FII replicon together with FIA and FIB, as discussed previously [Rozwandowicz et al., 2018]. With increasing availability of a long-read sequencing, additional studies may be warranted to characterize the plasmid genetic content in the strains.

WGS was also used for the identification of antimicrobial resistance genes and their predicted product phenotypes utilizing the ResFinder program (Table 4). Among the experimental *Salmonella* isolates, the following genes detected in all strains bla_{CMY-2}, *floR, aph(3')-lb* (also known as *strA*), *aph(6)-ld* (also known as *strB*), *sul2, tet(A),* and *tet(B)*. Other resistance genes including bla_{TEM-1B}, *cmlA1, sul1, ant(2'')-la*, and *aph(3')-la* were detected in four of the six isolates. These resistance genes encode resistance to multiple antibiotics

including, β-lactam, chloramphenicol, streptomycin, sulfonamides and tetracycline were observed [Frye et al., 2013]. Among the resistance genes that were encoded in all isolates, the *tet(A)* and *tet(B)* belong to tetracycline resistance MFS efflux pump family which is carried in mobile genetic elements such as SGI1, integrons and plasmids [Han et al., 2012], and the genes resistant to chloramphenicol (*cmlA1* and *floR*) which are associated with efflux mechanisms are similar to those identified in strains as previously reported [Delgado-Suárez et al., 2021; Han et al., 2012]. It was noted that a relatively high percent of *Salmonella* isolates display resistance to tetracycline for *Salmonella* serovars from human, animal origins and other sources worldwide and presented phenotypic resistance to tetracycline greater than previously suggested [Gargano et al., 2021]. Streptomycin resistance is also commonly found in *S.* Heidelberg isolates from human patients, animal and food sources [Han et al., 2011; Rodrigues et al., 2020]. Our data indicated that that the resistance in isolates 142, 143, 452, 462, and 710 was likely encoded by *strA/strB* which defined as streptomycin phosphotransferase protein product (Table 4). Resistance genes *sul1, sul2* and *bla*$_{CMY-2}$ may confer resistance against sulfonamides and ceftiofur, respectively. These resistance genes are frequently detected in the *Salmonella* resistome from animal and food sources [Bythwood et al., 2019; Rodrigues et al., 2020]. Marrero-Ortiz et al. (2012) also reported high-levels MDR phenotypes occurring among *Salmonella* isolates from dairy cattle and the presence of multiple plasmids which carry many resistance genes associated with resistance to β-lactams, streptomycin, tetracycline, chloramphenicol, and the sulfonamides. Similarly, Kaldhone et al., (2008) and Welch et al., (2007) demonstrated the plasmids can transfer the same types of MDR genes from donor strains to a susceptible recipient.

Building on the efforts described in Chapter 3 of this dissertation, the genetic foundation of plasmids containing conjugal transfer genes among several plasmid types including IncA/C, FIA, FIB, HI2 and I1 were analyzed by both *in silico* analysis and laboratory-based experiments. BLAST analyses of genomes (as detailed in Chapter 3) show the plasmid transfer gene content of the *S.* Heidelberg and Typhimurium isolates by detecting the *tra/trb/pil* genes and their corresponding products by the presence/absence profiles (Table 5), indicating the types IV secretion-like conjugative plasmid transfer systems for each isolate. Interestingly, the IncA/C plasmid harbored a conserved set of *tra* genes that were present in all isolates which may indicate the IncA/C plasmid conserved backbone. Particularly, strain 143 containing all of the IncA/C transfer genes demonstrated the highest conjugation efficiency studies carried out previously, which may indicate the importance of the region for transfer of resistance [Han et al., 2011, 2018]. More notably, *traV* gene is considered as unique single conserved gene, which involved conjugative transfer (*tra* genes) and replication (*repA*) among comparative genomic studies of IncA/C plasmid isolated from MDR *S. enterica* (Table 5) [Carraro et al., 2014]. Most of the transfer genes have a known predicted function/product described as cytoplasmic transfer protein and pilus extension. There have been comprehensive reviews (see Chapter 3) examining the conjugative transfer and describing the function/products for each gene [Virolle et al., 2020; Foley et al., 2021].

4.5. Conclusions:

In the present study, tetracycline and chloramphenicol exposure on MDR *Salmonella* isolates appeared to influence plasmid transfer among multiple *Salmonella* strains in vitro. These findings highlighted the impact of plasmid transfer, which plays a vital role in the dissemination

of antimicrobial resistance in enteric bacteria. This dissemination is problematic since it can potentially lead to the transfer of resistance genes within the GI tract microbiota due to the diversity of strains carrying multiple resistance plasmids [Algarni et al., 2022]. Our data demonstrated both tetracycline and chloramphenicol exposure can induce plasmid transfer in MDR *Salmonella* serotypes, and the specific response relied upon antimicrobial concentration, transconjugants ratio and isolates, which may indicate a phenotypic response *in vitro*.

The role of genetic content of plasmids was extensively explored for the transfer of other plasmids within these isolates in response to multiple antimicrobial agents. According to the current data, the increased plasmid transfer increased the dissemination of resistance gene types following the antimicrobials exposure. The results of WGS analyses demonstrated that generically diverse group of plasmids were detected across the MDR *Salmonella* which may help to explain why many of the isolates with the greatest ability (i.e. highest transconjugants ratio and conjugal transfer genes) carried multiple large plasmids. The combination of using the wet and *in silico* approaches in the study provided a unique set of tools to evaluate the diversity of plasmid transfer from MDR donor isolate to recipient by carrying multiple resistance plasmids.

The results indicated the need for ongoing efforts to develop methods to better understand the roles of plasmids and selection factors that can potentially drive the dissemination of plasmids among MDR *Salmonella enterica* isolates, particularly related to the dynamics of plasmid transfer. Generally, multidrug resistant *Salmonella* have multiple resistance-encoding plasmids; these could lead to some challenges, such as attempting to elucidate the mechanism(s) impacting plasmids transfer. Our data will allow us to identify the specific plasmid-encoded resistance genes and the dynamic impact of bacterial pathophysiology on human health as well

as allowing the veterinarians and physician to decide which antimicrobial treatment is best to treat for bacterial infections.

In future studies, additional efforts will focus on investigating the role of antimicrobials exposure on MDR *Salmonella* plasmid transfer using transcriptomic approaches to provide insights into both the relation of genetics pathways and the effect of the antimicrobial exposure on the MDR enteric strains.

Acknowledgements

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GENERAL CONCLUDING REMARKS AND FUTURE DIRECTIONS

The improved understanding of the dynamics of the antimicrobial resistance (AMR) mobilome and the quantitation of the transfer efficiency of multidrug resistance plasmids for diverse groups of plasmids in *Salmonella enterica* strains are key results of the work described in this dissertation. Comparisons of the plasmid-associated resistance genes of *Salmonella* strains and among the plasmid sequences emphasize the plasticity of plasmid evolution. The finding that different antimicrobial exposures impact the efficiency of AMR plasmid transfer is key, as it shows it is important to pay more attention to resistance spread in the health care systems and consider the spread as a factor during hospitalization and treatment due to the potential development of resistance to multiple antimicrobials agent. The plasmid mobilome of *Salmonella* and related enteric bacteria is made up of a diverse group of mobile genetic elements (MGEs) which have a significant impact on the spread of AMR genes.

The further development methods and strategies to understand AMR plasmid evolution to limit and control the spread resistance genes is still required. The initial steps in this project used publicly available tools to assess the antimicrobial resistance mobilome of *S. enterica* and other members of the Enterobacteriaceae, which provided very useful knowledge of connections of resistance elements and specific plasmid types. These findings served as a major contribution to our understanding of the epidemiology, dynamic and evolution of antimicrobial resistance.

The diversity of antimicrobial resistance genes is spread among plasmid replicons and between *S. enterica* and related species. *In silico* tools have the potential to assess the several different groups of genes including AMR, biocide, disinfectant and heavy metal resistance genes,

and associated MGEs. The study found that plasmid replicons IncA/C, HI1, HI2, I1, N and P had the highest numbers of resistance genes among bacterial plasmid analyzed and these levels were likely influenced by the dynamic interaction between resistance genes and MGEs. A major intersection between the plasmid genetic content and other parts of MGEs can provide a significant avenue for the dissemination of AMR genes among plasmids within a strain or between strains. Understanding the diversity of MGEs may help to understand phylogenetic relationships to assess the diversity of each plasmid sequence among representative of *Salmonella* strains and other enteric pathogens and gain insights into resistance plasmid evolution. Plasmids are key MGEs that often encode for their own transfer through the carriage of genes that encode the conjugal type 4 secretion systems (T4SSs). Differences in the T4SS genes can potentially lead to differences in the ability of plasmids to spread among bacteria, the Plasmid Transfer Factor Database and associated tools provided a resource to assess the T4SS genes.

A key driver of AMR dissemination is conjugation, which has a vital role in the spread of resistance in enteric bacteria. To understand factors that influence plasmid transfer, in vitro exposure of resistant donors and a recipient to antimicrobials including tetracycline and chloramphenicol at different concentrations was undertaken and found to influence plasmid transfer efficiency in some MDR *Salmonella* strains. In some cases, the plasmids that transferred the AMR genes were different and likely influenced by the T4SS genes carried on the different plasmids. These studies demonstrated the need for future directions to investigate the role of antimicrobial exposure on MDR *Salmonella* isolates plasmids transfer using transcriptomic

approaches. These studies will give insights into both the correlation of genetic pathways as well as the effect of antimicrobial exposure on the MDR strains.

Multidrug resistance in *Salmonella* and other enteric pathogens remains a worldwide challenge, in large part due to the plasmid spread of AMR. According to the dissertation efforts, tools were developed and assessed to utilize WGS data to identify transfer-associated genes from different plasmids replicon types that may potentially influence the evolution of AMR. These tools allow for the comparison of gene-transfer region pattern in corresponding plasmids from *S. enterica* isolates and other enterica bacteria to assess the transfer potential among bacteria strains. The tools, knowledges and approaches described in the dissertation are available for researchers, scientists who are interested in the field. Overall, the research presented in this dissertation expanded our understanding of the distribution of resistance genes and provide a useful method for a comprehensive molecular studies of plasmid transfer dynamics. The studies increased our knowledge of AMR plasmid transfer dynamics and the provided the scientific community tools that can be used to better understand AMR gene transfer among *S. enterica* isolates and related species, which can potentially impact the transfer of resistance genes within the GI microbiome. This improved knowledge can have a positive impact to further plasmid biology research and improve our understanding of plasmid evolution and epidemiology.