A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Prevalence, Transmission, and Molecular Mechanisms of Antimicrobial Resistance of *Escherichia coli* from Food-producing Animals in Korea

국내산업동물유래 *Escherichia coli* 항생제 내성의 분포, 전달성 및 분자유전학적 특성

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Abstract

Prevalence, Transmission, and Molecular Mechanisms of Antimicrobial Resistance of *Escherichia coli* from Food-producing Animals in Korea

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Antimicrobial resistance is one of the top health challenges facing the 21st century. Although antimicrobial resistance is an inevitable consequence of the evolutionary adaptation of microbes, there is a clear connection between consumption of antimicrobial agents and the subsequent development of antimicrobial resistant organisms both in human and veterinary medicine. Generally, food-producing animals are regarded as an important reservoir and play an immense role in transmission of antimicrobial resistance between animals and

humans. A rapid rise was observed both in the prevalence and newly emergence of antimicrobial resistance. This is particularly common in pathogenic and commensal *Escherichia coli* harboring antimicrobial resistance determinants for various classes of antimicrobials currently in use. Hence, the results presented in this study offer a contribution to understand the evolving mechanisms of antimicrobial resistance and the pattern of different classes of antimicrobials resistance in *E. coli* isolates obtained from food-producing animals in South Korea.

First, a total of 247 *E. coli* isolates collected from four cattle farms were investigated for aminoglycoside-modifying enzyme (AME) genes, their plasmid replicons and transferability. The isolates showed high prevalence of resistance to various antimicrobials. Streptomycin resistance was the most detected in the isolates 139 (56.3%) and the isolates were mainly categorized under the non-pathogenic phylogroups (B1 and A). The principal aminoglycoside resistance determinate observed were *aph3"-1a* and *aph3"-1b*. Some strains showed phenotypic resistance without carrying AME gene. Conjugation experiments showed the majority of the isolates (63.2%) were capable of transferring AME genes and *aph3"-1a* was the dominant transferred gene via the IncFIB replicon-type. Pulsed-field gel electrophoresis (PFGE) analysis showing 14 distinct cluster types indicated higher degree of genetic diversity in the isolates. This suggests the role of commensal *E. coli* isolates in carrying and dissemination of transferable AME genes and posing a potential public health risk.

The second study was performed with the objective of characterizing class 1

integrons, their associated diverse gene cassettes and related antimicrobial resistance mechanisms were characterized in the isolates. Little is known about the relationship between class 1 integron carriage with respect to phylogroups and patterns of tetracycline resistance. Of the 92 isolates included in this study, all the isolates at least carried one of the tetracycline resistant determinants. *tet(A)* and *tet(B)* were identified as the predominant *tet* genes. The prevalence of *tet* gene carrying isolates were significantly higher in isolates harboring class 1 integrons gene (*intI1)*. Compared to other phylogroups, a significant lower presence of *intI1* gene was observed in B2 phylogroup. Out of 92 isolates, 38 (41.3%) isolates carried the *intI1* gene and among them 27 isolates had *sul1* and *qacE*Δ*1* at the 3′-CS. Sulfonamide resistance gene (*sul1)* was also detected in 39.1% of isolates as a 3′-CS component of class 1 integrons. Seven gene cassettes were identified, either alone or in combination with another gene cassette. These genes, in the cassettes, encode versatile resistance spectrum that confer resistance to different classes of antimicrobials. Class 1 integrons have spread into a commensal *E. coli* strains and associated with carriage of tetracycline resistant determinants. While on the contrary, isolates in the pathogenic phylogroups carried *intI1* gene to a lesser extent.

Lastly, a total of 636 commensal *E. coli* isolates recovered from fecal samples obtained from clinically healthy animals were used to investigate the prevalence, genetic mechanism, and transferability of plasmid-mediated colistin resistance. Non-susceptibility to colistin was detected in 9 (1.4%) isolates, and among those isolates the *mcr-3* and *mcr-1* genes were detected in 2 and 3 isolates, respectively.

All isolates harboring *mcr-1* and *mcr-3* genes were multidrug resistant (MDR) and harboring various antimicrobial resistant genes that were confirmed to be transferred to a recipient *E. coli* J53 AZ^R strain via conjugation assay. *E. coli* isolate, 17S-208, harboring *mcr-3* gene was selected for further plasmid analysis using whole genome sequencing (WGS). Plasmid p17S-208 (260,339bp) containes 241 predicted ORFs and encodes genes resistant to various antimicrobial agents. The *in silico* plasmid finder tool demonstrated that p17S-208 exhibited a typical IncHI2-type backbone. Plasmid MLST showed that p17S-208 belonged to sequence type ST3. Comparison of the p17S-208 plasmid's with deduced amino acid sequence of 28 other bacterial species depicted that p17S-208 had 35% identity with *mcr-2* isolated from a swine in Belgium and *mcr-1* isolated from a patient in South Korea, whereas 100% identity with *mcr-3* isolated from swine in China. Care when applying colistin for therapeutic purposes has to be encouraged and regular WGS-based surveillance is a requisite to early detection of *mcr* genes, and to describe novel *mcr* genes.

In conclusion the issue of antimicrobial resistant bacteria is extremely complex and widespread. Besides, there is a high possibility for those resistant bacteria can spread from one living thing to another. Moreover, a regular surveillance using appropriate diagnosis technique to limit the further spread and emergence of resistance bacteria originated from food-producing animals is warranted. Therefore, future research direction should focus on characterization of different resistance mechanisms, development of effective novel drugs and control strategy through

coordinated interdisciplinary approach.

Keywords: *Escherichia coli*, AME genes, transferability, class 1 integrons, tetracycline resistance, phylogenetic group, *mcr* genes, IncHI2-ST3 type plasmid.

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List of abbreviations

AGAs	Aminoglycoside antibiotics
AIEC	Adherent invasive *E. coli*
AMC	Amoxicillin-clavulanic acid
AMEs	Aminoglycoside modifying enzymes
AMP	Ampicillin
APQA	Animal and plant quarantine agency of Korea
ATCC	American type culture collection
BRIG	Blast ring image generator
C	Chloramphenicol
CDC	Centers for Disease Control and Prevention
CF	Cephalothin
CFU	Colony forming unit
CIP	Ciprofloxacin
CL	Colistin
CLSI	Clinical Laboratory Standards Institute
CRE	Carbapenem-resistant *Enterobacteriaceae*
CSs	Conserved segment
DAEC	Diffusely adherent *E. coli*

DOX	Doxycycline
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility testing
GEN	Gentamicin
KAN	Kanamycin
KFDA	Korean Food and Drug Administration
LEV	Levofloxacin
LPS	Lipopolysaccharide
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MIN	Minocycline
MLST	Multilocus sequence typing
NA	Nalidixic acid
NCBI	US National Center for Biotechnology Information
NEO	Neomycin
OIE	Oficina Internacional de Epizootias

OM	Outer membrane
OMP	Outer membrane protein
ORF	Open reading frame
OXY	Oxytetracycline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
QRDR	Quinolone resistance-determining regions
RPPs	Ribosomal protection proteins
S/STR	Streptomycin
ST	Sequence type
STEC	Shiga toxin-producing *E. coli*
SXT	Sulfamethoxazole/Trimethoprim
TE/TET	Tetracycline
TOB	Tobramycin
TSA	Tryptic soy agar
TSB	Tryptic soy broth
USFDA	United States Food and Drug Administration
WGS	Whole genome sequencing

General introduction

Antimicrobial resistance has emerged in the past few years as a major problem and threatening the return of untreatable infections on a massive scale in human and veterinary medicine. Antimicrobial-resistant bacterial infections now account for many emerging infectious diseases worldwide (Alonso *et al.*, 2017). There is a lack of knowledge on the importance of commensal microorganisms in the carriage and transfer of resistance determinants, thereby spreading resistance both to humans and animals. Moreover, the impact of antimicrobial usage in veterinary medicine and its impact in increasing prevalence and emergence of resistance to various classes of antimicrobials agents also need to be elucidated. *E. coli* strains can be both commensal and pathogenic with diverse pathotypes have increasingly been recognized as a major public health concern. Due to its high genomic flexibility there is a tendency for *E. coli* isolates to disseminate resistance via horizontal gene transfer and clonal spread (Bajaj *et al.*, 2016). Multidrug resistance in *E. coli* has created a complex problem in the treatment of infections by limiting the available treatment options. The global prevalence of multidrug resistant (MDR) *E. coli* has continued to increase through time (Sidjabat *et al.*, 2014).

Bacteria have been furnished with various resistance mechanisms to cope with aminoglycosides, the most common being chemically modifying aminoglycosides by aminoglycoside-modifying enzymes (AMEs) (Wachino and Arakawa, 2012).

Most of these AME genes are encoded on plasmids, transposons and integrons, which give them high mobility and readily facilitate the spreading of resistance (Becker and Cooper, 2013). Moreover, the genes coding for those enzymes have the capability to evolve continually; enabling them to exploit new antimicrobials as substrate and also efficient dissemination among bacteria giving rise to a multidrug resistance (Ramirez and Tolmasky, 2010). Knowledge of the molecular characteristics associated with a higher degree of resistance to aminoglycosides in commensal *E. coli* strains would be of profound significance to clinical practice, infection control measures and treatment options in veterinary and human medicine.

Multidrug-resistance was determined in many cases to be associated with transmissible plasmids, and the importance of integrons in the acquisition of resistance genes constitute the major vector of multidrug-resistance in Gram-negative and to a lesser extent in Gram-positive bacteria (Cambray *et al.,* 2010). A considerable number of resistance determinants in *E. coli* inhabit on integron classes that are capable of capturing and expressing genes comprised in cassette-like structures (Ponce-Rivas *et al.,* 2012). There are various observations on the interrelationship of integron carriage and different *E. coli* phylogroups for environmental, human and animal isolates. Tetracycline has been used in human and veterinary medicine and as a growth promoter in animal husbandry. Although there are many mechanisms of tetracycline resistance in *Enterobacteriaceae* efflux pump activity via *tet* genes remains the predominant mechanism (Koo and Woo, 2011; Karami *et al.,* 2006).

Emergence of resistance via plasmid mediated carriage of antimicrobial resistance genes has severely limits the treatment options for the infections. For instance, colistin resistance mediated by plasmid encoded *mcr* genes in *Enterobacteriaceae* has attracted a global attention (Liu *et al.,* 2016). Since, colistin is the last resort drug for treating serious infection caused by carbapenem-resistant *Enterobacteriaceae,* colistin-resistance became a great concern to public health (Yao *et al.,* 2016). Colistin-resistant *E. coli* isolates from different sources, including humans, animals and foods are continued to be reported from different countries and they harbored this important resistance mechanism (Malhotra-Kumar *et al.,* 2016B; Zurfuh *et al.,* 2016; Carattoli *et al.,* 2017; Belaynehe *et al.,* 2018B). Colistin use in animals' husbandry is mainly for treatment of infections caused by *Enterobacteriaceae* and also to treat digestive disorder related cases in pig production (Kempf *et al.,* 2016). This uncontrolled practice of colistin usage might contribute to the occurrence of colistin resistance. More importantly, studies support the need to be aware of the possible dangers of spread of plasmid mediated colistin resistant *Enterobacteriaceae* to humans via the food chain which necessitates strict regulation of the use of colistin in veterinary medicine for treatment and as a feed additive.

The present study was aimed to describe the situation of antimicrobial resistance in *E. coli* isolates of food-animals origin in Korea, with particular focus on prevalence of resistance and molecular based investigation of resistance determinants for various antimicrobial classes, and their transferability. The thesis

was organized in to three different chapters. In chapter 1, pattern of aminoglycoside resistance in *E. coli* isolates originated from cattle farms was assessed. Subsequently, the distribution and transferability of AMEs from the isolates were investigated. The second chapter stated on the importance of integrons and their associated diverse gene cassettes in mediating antimicrobial resistance in commensal *E. coli* isolated from cattle. This chapter also looks into the relationship between class 1 integron carriage with respect to phylogroups and patterns of tetracycline resistance. The third chapter focused on surveillance and genetic characterization of plasmid-mediated colistin resistance genes in *E. coli* strain collected from food-producing animals in Korea.

Literature Review

I. ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI*

Escherichia coli is a notable commensal bacterium of the gastrointestinal tract of vertebrates, including humans and animals, and it is related in extra-intestinal and intestinal pathologies (Skurnik *et al.*, 2015). *E. coli* is a Gram-negative, rod-shaped, oxidase-negative, non-sporulating and facultative anaerobe bacterium from the family *Enterobacteriaceae* inhabiting the gut microbiota. The gut microbiota comprising of not less than 500 bacterial species ranges from 10^{10}-10^{11} bacterial cells per gram of intestinal content. Generally intestinal anaerobic bacteria dominate the intestinal microbiota than *E. coli* by 100/1 to 10,000/1; however, *E. coli* dominates aerobic organisms in the gastrointestinal tract (Tenaillon *et al.*, 2010).

E. coli, due to its pathogenic capacity, can cause significant diarrheal and extra intestinal infections. Pathogenic variants of *E. coli* (pathovars or pathotypes) have drawn huge attention globally and in-depth studies have been undertaken in humans, animals, food, and the environment. Diarrhea causing enteric *E. coli*, otherwise known as diarrheagenic *E. coli*, categorized in to six major pathotypes, namely, enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC),

enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC) (e.g., enterohemorrhagic *E. coli* [EHEC]), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC), as well as a new pathotype, adherent invasive *E. coli* (AIEC) (Croxen *et al.*, 2013).

E. coli can be both a commensal and a pathogen and can be easily isolated and grown in the laboratory, it has been used as a model organism in population genetics studies for so many decades, and researchers have benefited from each technical or conceptual advance. Generally it is accepted that commensal microbiota, and particularly the intestinal microbiota, has been demonstrated to have a significant role in the emergence of drug-resistance (Andremont, 2003). Owing to the extensive application of antibiotics in both human and veterinary medicine, a large density of bacteria with a high gene pool exposed to high environmental antibiotics, as a consequence, selection of antibiotic resistance ensued in the commensal microbiota (Tenaillon *et al.*, 2010).

There are various available methods for microbiological isolation and typing of *E. coli*. Culturing on agar media is the oldest method of isolation and, among many, MacConkey agar is commonly employed to differentiate between lactose-fermenting and non-lactose-fermenting strains, as lactose-positive *E. coli* colonies will appear red or pink color (Quinn *et al.*, 2011). *E. coli* serotyping bases the Kauffman classification scheme using the O (somatic) and H (flagellar) surface antigen (Nataro and Kaper, 1998). While substantial information can be obtained

for pathotypes such as STEC O157:H7, there are circumstances where serotyping has limited use due to cross-reactivity between antigens or some isolates being untypeable. Molecular identification can be performed by PCR amplifying genes coding for O (*wzx* and *wzy*) and H (*fliC*) antigens (Croxen *et al.*, 2013).

Although it is time consuming and needs skilled professionals, pulsed-field gel electrophoresis (PFGE) is regarded as the gold standard for typing *E. coli* isolates during epidemiological investigations to discern between outbreak isolates (Swaminathan *et al.,* 2001). Recently, typing pathogenic *E. coli* strains and establishing their relatedness has been done using multilocus sequence typing (MLST). This is based on amplifying and sequencing a small number of housekeeping genes and assigning a unique allele, followed by assigning a sequence type number using the allelic profile of the housekeeping genes (Lacher *et al.,* 2007; Okeke *et al.,* 2010; Á lvarez-Suárez *et al.,* 2016). At present four publically curated MLST schemes are available managed by different institutes, namely, Pasteur institute *E. coli* MLST database (http://www.pasteur.fr/mlst), the EcMLST (http://www.shigatox.net), MLST website of the Warwick database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), and at the center of genetic epidemiology (https://cge.cbs.dtu.dk/services/MLST-1.4/).

E. coli strains recovered from various animal populations that were differentially exposed to human contact have been investigated for antimicrobial resistance and integron carriage. Integrons are molecular machineries that are of significant role in the spread and expression of antimicrobial resistance genes (Mazel, 2006).

Multidrug resistant (MDR) *E. coli* mostly belong to the extra-intestinal pathogenic *E. coli*. Multidrug resistance in *E. coli* has created a complex problem in the treatment of infections by limiting the available treatment options. The global prevalence of MDR *E. coli* has continued to increase through time (Sidjabat *et al.*, 2014).

Worldwide a lot of studies have been conducted on *E. coli* and their resistance to different classes of antimicrobials. Emergence of resistance to most first-line antimicrobial agents growingly complicates treatment of infections caused by *E. coli* (Rasheed *et al.*, 2014). Over the last few years the number of studies focusing on the epidemiology of antimicrobial resistant *Enterobacteriaceae* has increased, with particular focus to carbapenemase production in *Enterobacteriaceae*, extended spectrum b-lactamase (ESBL), plasmid mediated *AmpC* β-lactamase (p*AmpC*) and plasmid mediated colistin resistance (*mcr* genes). Most of these reports have been performed in *E. coli*, generally regarded as an important indicator of antimicrobial resistance because of its presence in a wide range of hosts and its medical significance (Alonso *et al.*, 2017). In general the burden caused by antimicrobial resistance can be summarized into 3 different mechanisms in both human and veterinary medicine; firstly longer duration of illness and high mortality rate due to infections caused by antimicrobial resistance bacteria, secondly, unable to do procedures that rely on effective antibiotics to prevent infection and at thirdly, treatment costs of resistant infections are rising

(Laxminarayan *et al.*, 2013). The prevalence and incidence of multidrug resistant strains of *E. coli* in animals and humans can be minimized by applying strict hygienic practices in the farm and abattoirs. Preventing contamination of animal's product with manure, sewage line, animal intestinal content, soil and unclean water is crucial to avoid clinical infections by resistance strains (Figure 1).

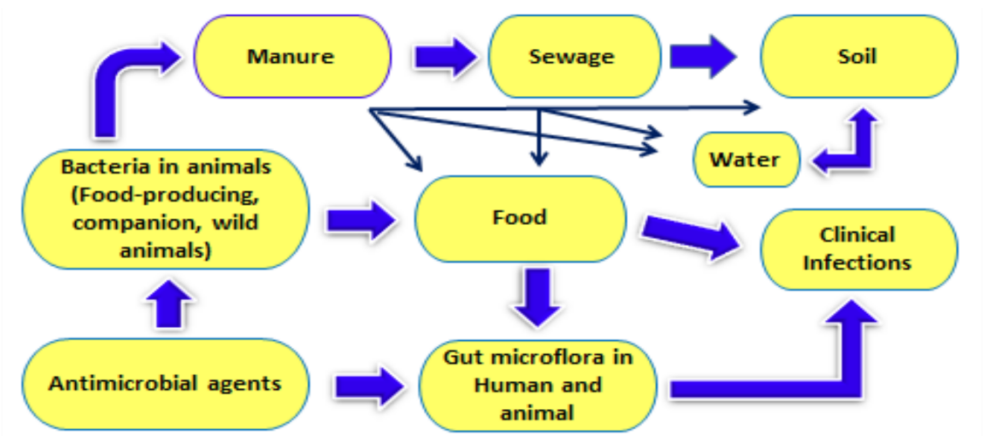


Figure 1. Antibiotic resistance flow chart in bacteria and the environment (Modified from Tham, 2012)

II. PRINCIPAL FORMS OF ANTIBIOTIC RESISTANCE

2.1. Acquired antibiotic resistance

There are three main mechanisms for acquired antibiotic resistance: (1) genetic adaptation, such as, through drug target change; (2) resistance genes transfer between susceptible and resistant microorganisms; and(3) phenotypic adaption of

existing cellular machinery, for example, efflux pumps (Van der Horst *et al.*, 2011). In most cases, several of these mechanisms combine to give a high level of resistance against a particular antibiotic.

2.2. Adaptive antibiotic resistance

Takes place as a consequence of an environmental trigger (e.g., sub-inhibitory levels of antibiotics or changes in the concentration of nutrient) that causes temporary changes in protein and/or gene expression levels leading to the tolerance of antibiotics. Bacteria growing on surfaces as biofilms maintain an adaptive resistance (often referred to as tolerance) to antibiotics (Garneau-Tsodikova and Labby, 2016). Bacteria can alter their transcription swiftly in response to changes around their surroundings with the objective of enhancing their chances of survival. In return these alterations confer on the bacterium a great deal to defy the challenges from antimicrobial drugs (Fernández *et al.*, 2011). On the other hand, adaptive resistance to cationic antimicrobial peptides, such as aminoglycosides and polymyxins, is known to occur in response to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} cations which led to the upregulation of bacterial lipopolysaccharide (LPS) operon modification and subsequent reduction in the drug uptake (Fernández *et al.*, 2010).

2.3. The intrinsic resistance

Intrinsic resistance of a bacterial species to a particular antibiotic is the capability to resist the action of that antibiotic as a consequence of inherent functional or structural characteristics. One of the most instance of intrinsic resistance in an individual species results from the lack of a susceptible target to a particular antimicrobial agent (Blair *et al.,* 2015). Detail investigations led to the identification of many genes that are accountable for intrinsic resistance to different classes antimicrobial, such as aminoglycosides, fluoroquinolones and β-lactams. This was accomplished by employing high-throughput screens of high-density genome mutant libraries that were created by random transposon mutagenesis or targeted insertion in bacteria, including *E. coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*.

The inability of many antimicrobial agents to cross the outer membrane (OM) of some Gram-negative bacteria is due to intrinsic resistance to these compounds: for instance, vancomycin, a class of glycopeptide antibiotics, acts by binding to target d-Ala-d-Ala peptides to inhibit the peptidoglycan crosslinking but it is only effective in Gram-positive bacteria; as in Gram-negative organisms it cannot cross the OM and access these peptides in the periplasm. While acquired and intrinsic resistance, which are stable and elements are passed on vertically during bacterial reproduction to subsequent generations, adaptive resistance has an unstable

property and generally reverses back after removal of the environmental triggering factor (Rizi *et al.,* 2018).

III. A MULTITUDE MECHANISMS OF ACQUIRED ANTIBIOTIC RESISTANCE

3.1. Mutations

Point mutations: Point mutations, resulting in phenotypic resistance, often change the binding targets of antimicrobials ensuing in lowered affinity to bind. For instance, point mutations of the DNA gyrase encoding gene can decrease the efficacy of quinolones by altering their binding efficiency. Multiple point mutations of DNA topoisomerase genes, such as *gyrA*, *gyrB*, and *parC*, found in the quinolone resistance-determining region (QRDR), can lead to higher levels of resistance thereby leading to higher minimum inhibitory concentrations (MICs) and therefore reduced susceptibility to quinolone antimicrobials (Harbottle *et al.,* 2006). Although the principal mechanism of resistance for β-lactam antibiotics is the production of β-lactamase enzymes, accumulation of point mutations in certain porin genes restrict antibiotic permeability inside the bacterial cell by energy-dependent efflux systems has also been associated with resistance (Mc Dermott *et al.,* 2003).

Deletions: Gene deletion is rare and extreme mechanism of resistance to antimicrobial agents. Deletion of a base in the structure of a gene alters all the codons following the point of nucleotide suppression. As a result of nucleotide elimination, a protein having shorter or longer amino acid sequence could be translated. Therefore, deletion transforms a triplet with meaning into a nonsense triplet (terminator codon) then the sequence of amino acids will be much shorter than normal. In contrary, if the nonsense codon transformed in to amino acid coding triplet, then the resulting amino acid will have a much longer sequence (Bennett, 2008).

Previous study demonstrated that altered expression of oligopeptide-binding protein due to the deletion of the gene resulted in the aminoglycoside resistance in clinical and laboratory *E. coli* strains (Acosta *et al.,* 2000). Similarly, reduced tigecycline susceptibility has been demonstrated in *E. coli* mutants having a deleted LPS encoding gene (Linkevicius *et al.,* 2013), and tigecycline-related methyltransferase gene, *trm*, deletion was found to decrease susceptibility to tigecycline, doxycycline and minocycline in *Acinetobacter baumannii* (Chen *et al.,* 2014).

Insertions: Insertions are mutations in which extra base pairs are inserted into a new place in the DNA. The number of base pairs inserted can range from one to thousands. Deletion followed by insertion affects those codons located between the insertion point and the deletion point. The codons coming after the insertion point

will have normal structure. The study by Siu *et al.* (2003) demonstrated that the hyper-production of *AmpC* β-lactamase in *E. coli* clinical isolates induced by the creation of an optimal distance for gene expression by the insertion of either one nucleotide or two nucleotides, which resulted in the over expression of *AmpC* RNA compared to the level of transcription in the wild-type strain.

3.2. Horizontal gene transfer

Genes, including specific antibiotic resistant genes, can be transferred from one bacterium to another. This process, described as horizontal gene transfer, can contribute to acquired resistance against antimicrobial agents in a rapid manner (Gogarten and Townsend, 2005). Horizontal transferring of genes occasionally passes beyond phylogenetic borders; if certain conditions are met, gene exchange can happen within unrelated bacteria. In practice, genes carried on a bacterium of animal origin can be exchanged to environmental organisms which cause the development of a reservoir of resistant genes. Similarly, bacteria of animal's origin can transfer their genes to bacteria of human origin and vice versa, for example when pathogens or commensals are taken up by animals or humans through ingesting contaminated water or food, they interact with indigenous microflora and possible exchange of resistance genes can take place (Huddleston, 2014).

Where horizontal gene transfer takes place between unrelated organisms, the genes most probably to be engaged tend to belong to the simplest sets of functional networks. Therefore, informational genes of the central cellular machinery such as DNA replication, transcription or translation have a tendency to spread slowly, even if they confer antibiotic resistance, in comparison to single-function-resistance determinants, for instance in the case of aminoglycoside-modifying enzymes or β-lactamases. However, the nature of the transfer mechanism can also be depending on the bacteria and genes that are most often involved (Thomas and Nielsen, 2005). Generally there are three main mechanisms by which bacteria transfer genes horizontally, namely, bacterial conjugation, transduction, and natural transformation.

Natural transformation: It is the process based on the intrinsic capacity of bacteria cells whereby it takes up free DNA from the environment and incorporates it into their own genomes by using homologue recombination (Figure 2). This process not only allows the uptake of plasmid DNA but also chromosomal DNA. Accordingly, it is generally regarded as the major mechanism in the evolution of microbes (Huddleston, 2014).

Over the last two decades naturally transformable bacterial species has almost doubled. A total of 82 known different species confirmed to be naturally transformable. All transformable bacteria, except *Helicobacter pylori*, are believed to share common mechanisms of DNA uptake and processing that depend on

conserved proteins, which are mainly encoded by a set of genes that are simultaneously expressed during the start of competence (Johnston *et al.,* 2014).

Bacterial transduction: Is a process in which DNA is accidentally transferred to a new host via bacteriophages (Figure 2). Bacteriophages defined as viruses that infect bacteria and integrate itself into the host genome to become prophages until environmental conditions trigger their lytic growth (Huddleston, 2014). Compared to the other mechanisms there is limited information about this mechanism. Since many antibiotic resistance genes are plasmid-encoded, bacteriophage related antimicrobial resistance genes have attracted less attention and less effort to study them in comparison to plasmid mediated resistance. Despite this fact, a lot of available studies showed that bacteriophages can mobilize antimicrobial resistance genes and confer resistance, and some authors propose that mobilization can happen via generalized transduction (Banks *et al.,* 2004; Colomer-Lluch *et al.,* 2011). Some studies also suggest phages are reservoirs of resistance genes in the environment (Colomer-Lluch *et al.,* 2011) and from food animals (Brabban *et al.,* 2005).

Bacterial conjugation: It is the transfer of DNA fragments; it could be large chromosomes or very small, from one bacterium to the other by direct contact (Figure 2). Stepwise process of conjugation includes; cell-to-cell contact, formation of mating pair and at last mobile genetic elements (e.g. transposons, plasmids, and mobile genetic elements) transfers through a conjugative pilus (Bennett, 2008). It is

the principal and the most studied mechanism of horizontal transfer of plasmids. The conjugation of plasmids coding for antimicrobial resistance machinery has resulted in a global distribution of resistance genes, in particular in *Enterobacteriaceae*. Resistance encoded on these plasmids can be to extended spectrum β-lactamases (CTX, VEB), β-lactamases (CMY, DHA, GES, LAP, NDM, SHV, TEM), metallo β-lactamases (IMP), aminoglycoside resistance (*AAC*, *Arm*, *RmtB*), carbapenemases (KPC, VIM), tetracycline resistance (*Tet*), sulfonamide resistance (*Sul*), quinolone resistance (*Qep*, *Qnr*) or colistin resistance (*mcr*). Moreover, they encode for oxazolidinones, streptogramin A, pleuromutilins, lincosamides, and phenicols resistance gene, namely, Cfr rRNA methyltransferase (Marchaim *et al.*, 2011; Khajuria *et al.*, 2014; Schlüter *et al.*, 2014; Huddleston, 2014).

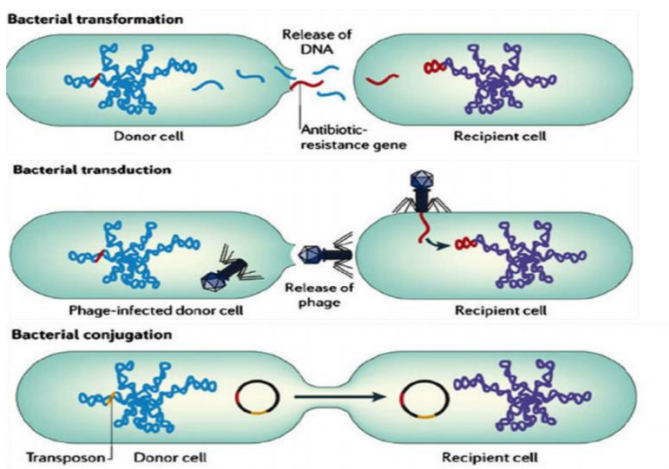


Figure 2. Horizontal gene transfer between bacteria by transformation, transduction and conjugation (Adapted from Furuya and Lowy, 2006).

3.3. Integrons: As agent of emergence and spread of antibiotic resistance

Reported for the first time in 1989 (Stokes and Hall, 1989), integron was considered as a novel resistance determinant and exhibited mobility for its inner excision and integration among gene cassettes. Integrons are genetic elements that allow bacteria to evolve rapidly through the acquisition, efficient capture and expression of exogenous open reading frames (ORFs) ensuring their correct expression. They were first described by virtue of their significance in spreading and emergence of antibiotic-resistance genes among bacterial pathogens. The ability of integrons to spread antimicrobial resistance genes within bacteria was evidenced by discovery of classes of integrases in multiple bacterial genera of both commensal and pathogenic organisms associated with diverse animal production environment (Goldstein *et al.,* 2001). Now integrons universally present in the commensal bacteria of food-producing animals, then there is a possibility for this bacterium made its way into the human commensal flora via food. Once integrons made its way into the human commensal or pathogenic flora, it then exposed to various selection pressures, eventually leading to the acquisition of many more different resistance genes (Ghaly *et al.,* 2017).

Structure of integrons: There are three essential core aspects shared by all integrons, whose consortium action is to capture and afterwards express exogenous genes as part of gene cassettes (Gillings, 2014). The first feature of the integron

functional platform is the *intI* gene, which encodes a member of the tyrosine recombinase family. This enzyme is responsible for catalytic activity of specific excision and recombination of dedicated and discrete genetic elements, known as gene cassettes (Cambray *et al.*, 2010). There is a minor genetic diversity exhibited by *IntI* among each integron class suggesting their recent emergence from a much larger and diverse pool of integrons (Diaz-Mejia *et al.*, 2008; Cury *et al.*, 2016), perhaps carried on chromosome (Hall, 2012). The second core feature, primary recombination site *attI*, which situated immediately adjacent to incoming gene cassettes. Transcription of the captured genes is facilitated by a third core feature, an integron-associated promoter (Pc). Successive integration at the *attI* site ensures integrons to acquire new genes as part of gene cassettes. Gene cassettes are minimal functional elements aimed to be mobilized by the catalytic activity of the integrase enzyme. This cassette array constitutes a single open reading frame (ORF) bounded by a cassette-associated recombination site referred to as *attC*, which is specifically recognized by *IntI* (Figure 3). The integron *IntI* mediates the integration of circular gene cassettes by site-specific recombination between *attI* and *attC* (Cambray *et al.*, 2010; Gillings, 2014; Cury *et al.*, 2016). The integron machinery has genomic innovation advantages for the bacteria since the newly integrated gene is instantly ready to be subjected to natural selection. As a result the newly generated variants will immediately express genes that might confer advantageous phenotypes.

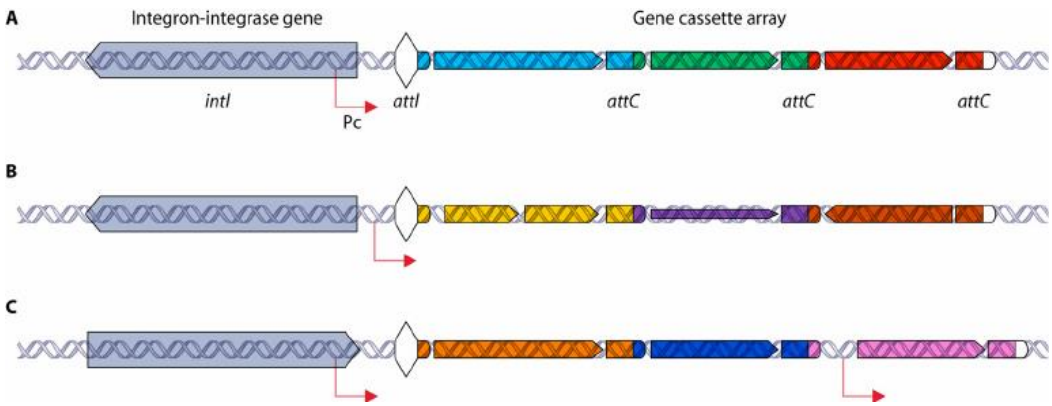


Figure 3. General structure of inegrons (Adapted from Gillings, 2014).

Integron classes as antimicrobial resistance machinery: At present, five classes of mobile integrons, numbered 1 to 5, are recognized to have a role in the spreading of antibiotic-resistance genes (Gillings, 2014). Compared to other classes of integrons, class 1 integrons are the most studied and ubiquitous in clinical pathogens, on this account constitute the major experimental model of integron. In addition it was also the most clinically important integron found in 22–59% of Gram-negative bacteria; such as *Escherichia* (Belaynehe *et al.,* 2018A; Dessie *et al.,* 2013), *Klebsiella* (Rao *et al.,* 2006), *Pseudomonas* (Poirel *et al.,* 2005), *Enterococcus* (Yu *et al.,* 2017), *Salmonella* (Guerra *et al.,* 2001), as well as in some Gram-positive isolates, *Streptococcus* (Shi *et al.,* 2006) and *Staphylococcus* (Xu *et al.,* 2007A; Xu *et al.,* 2008). The majority of the known antibiotic resistance gene cassettes belong to class 1 integrons and widely spreading in clinical isolates (Mazel, 2006). There is a link between class 1 integrons with functional and

nonfunctional transposons derived from Tn*402* and it further embedded in larger transposons, such as Tn*21* (Partridge *et al.*, 2018).

Similar in organization, class 2 integrons are exclusively associated with Tn*7* transposon derivatives and display a dozen different cassette arrays (Ramírez *et al.*, 2010; Li *et al.*, 2018). *IntI2* of class 2 integrons only possesses less than 50% homologous to *IntI1* integrase in amino acid sequences. Moreover, it normally contains a nonsense mutation in codon 179, replacement of the internal termination codon with a codon for glutamic, which yields a nonfunctional protein (Cambray *et al.*, 2010). The gene cassettes in class 2 integron were verified to be relatively low. The most frequently detected gene cassettes in class 2 integron were *aadA1*, *sat1* and *dfrA1* which contribute in resistance to the streptomycin/spectinomycin, streptothricin, and trimethoprim (Partridge *et al.*, 2018). Class 3 integrons were identified for the first time from carbapenem-resistant *Serratia marcescens* strain of hospitalized patient in Japan (Arakawa *et al.*, 1995). They are less prevalent and at times environmental isolates harboring class 3 integrons were detected with no link to any antimicrobial resistance gene was detected (Xu *et al.*, 2007B). They also did not carry much diverse gene cassettes, possibly because of less active class 3 integron integrase in contrast to other classes (Collis *et al.*, 2002). Although rare in occurrence class 4 and 5 integrons were discovered embedded in a subset of the integrative and conjugative element SXT reported in *Vibrio cholerae* (Hochhut *et*

al., 2001) and in transposon harbored on the pRSV1 plasmid of *Alivibrio salmonicida,* respectively (Sørum *et al.,* 1992).

IV. EVIDENCE FOR TRANSMISSION OF ANTIMICROBIAL RESISTANCE BETWEEN ANIMALS AND HUMANS

Antimicrobials are applied in veterinary medicine for therapeutic purpose as well as in the form of feed additives. Antimicrobials are majorly used in the cattle, swine and poultry production, but also applied in aquaculture. Over the last 60 years this relatively recent practice has caused organizational transformation of agriculture. It is equally vital to understand how this practice has considerably changed the relationship between animals and humans with regard to spreading of infectious disease via food-chain and other means (Silbergeld *et al.,* 2008). Although the relationship of antimicrobial resistant bacteria in human to antibiotic use in food animals continues to be a point of debate, there are direct and indirect pieces of evidence linking animal use to antibiotic resistance confronting humans. Among these are a number of studies which strongly support the concern that use of antibiotics in food animals impacts the health of humans via the food chain.

There is a persistent fecal shedding of *E. coli* into the immediate environment of the animals that contaminate floor, litter, and the pens of housed animals and the

soil for outdoor animals. *E. coli* is capable of persisting in the environment for longer period and be spread via the slurry and manure to contaminate vegetation, and ground and surface water. The transmission of *E. coli* to other animals can be achieved via contaminated feed, drinking water, animal attendants, possibly farm to farm by shared utensils and vehicles such as transport trucks. Infection in animal can take either oral route or via inhalation of contaminated dust in the case of chicken farms. A possible transmission of drug-resistant organism from animal to humans can encounter via direct contact, or ingestion of food or water or consumption of meat following contamination of carcasses at the slaughterhouse (Figure 4). The situation becomes more complicated if the organism carries multidrug-resistant genes for different classes of antimicrobial agents (Holmberg *et al.*, 1984A).

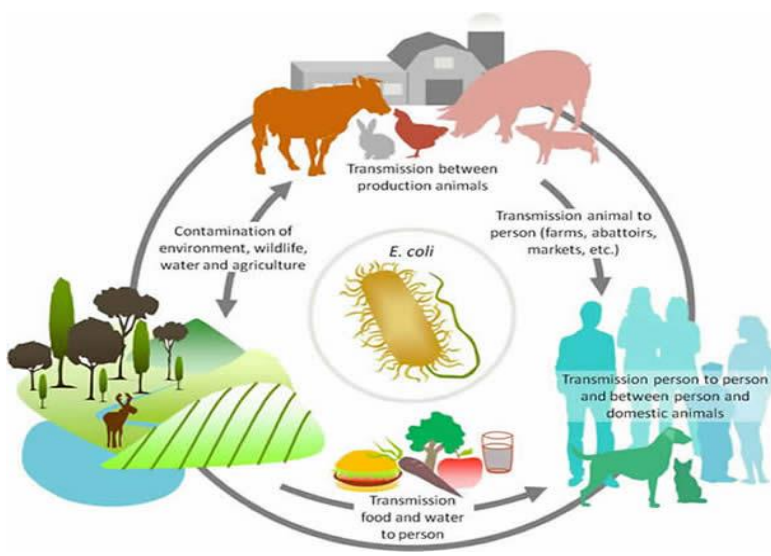


Figure 4. Contamination and transmission of *E. coli* between the environment, animals and humans (Adapted from http://www.ecl-lab.com/en/ecoli/index.asp).

Levy *et al.* (1976) reported for the first time the direct spread of bacteria from animals to human showing the same plasmid mediated tetracycline-resistant *E. coli* strains from the gut microflora of an animal attendant as in the chickens on feed mixed with tetracycline. Since then a lot of studies demonstrated an increased in the rate of spread of resistant bacteria from animals to the environment, and, more significantly, in the food production chain, are key determinants for the spread to humans (Table 1). Animal attendants, abattoir workers, veterinarians, and other personnel who are in close contact with farm workers are directly at risk of being colonized or contracting infection with resistant bacteria via close contact with infected or colonized animals (Marshall and Levy, 2011).

Commensal *E. coli* recovered from several human and animal populations that have variable contacts to one another showed an increased antimicrobial resistance score when there was nearby contact between them. Hence, strains isolated from humans living in a close contact with animals had a significant higher resistance score than in those living without a close contact, similarly, strain of animals living in close proximity to humans showed a significantly higher score in comparison to those animals living with an occasional contact to humans (Skurnik *et al.*, 2015). When the event of antimicrobial resistance are examined sequentially, reservoirs of resistance among animals could bring emergence of resistance across the food chain, as a sequel, increased resistance in food products consumed by humans can possibly occur. This notion is substantiated by the occurrence of the extended-

spectrum β-lactamase (ESBL) CTX-M-2 gene in *S. enterica*. Chronologically, this resistant determinant first described in poultry and then in poultry meat and, eventually, from human isolates (Bertrand *et al.*, 2006).

The creation of reservoir of bacteria resistant to antimicrobial growth promoters in food-producing animals and subsequent impact on the medically important last resort antibiotics, such as vancomycin and colistin is evident. For this reason many countries banned the use of antimicrobial growth promoters that belonged to antimicrobial classes also used in human medicine (Wegener, 2003; Silbergeld *et al.*, 2008; Woolhouse *et al.*, 2015). Even application of low-dose antibiotics for a prolonged course among food-producing animals creates a potential selective pressure for the propagation of resistant strains (Marshall and Levy, 2011). Although a significant reduction in occurrence of resistant bacteria in food-producing animals, owing to the imposed ban on the use of antimicrobials for growth promotor, the resistant strains are still not completely disappeared in the food-producing animals, farm environment and even in the foodstuff at low levels (Wegener, 2003).

Table 1. Evidence for transmission of antimicrobials resistance between animals and humans

Type of transfer	Bacterial species	Recipient host	Host animal	Antibiotic resistance	Evidence	Reference
Human colonization via direct or indirect animal contact	*E. coli*	Animal caretakers, farm family	U.S. chickens	Tetracycline	Following introduction of tetracycline on a farm, resistant *E. coli* strains with transferable plasmids were found in caretakers' gut floras, with subsequent spread to the farm family	Levy *et al.*, 1976
	S. aureus, *Streptococcus* spp., *E. coli* and other *Enterobacteriaceae*	Swine farmers	French swine	Erythromycin, penicillins, nalidixic acid, chloramphenicol, tetracycline, streptomycin, cotrimoxazole	Phenotypic antibiotic resistance was significantly higher in the commensal floras (nasal, pharyngeal, and fecal) of swine farmers than in those of non-farmers	Aubry-Damon *et al.*, 2004
	E. coli	Poultry workers	U.S. chickens	Gentamicin	Increase in phenotypic gentamicin resistance in workers through direct contact with chickens receiving gentamicin	Price *et al.*, 2007
	E. coli	Farm workers	Chinese swine and chickens	Apramycin (not used in human medicine)	Detection of *aac*(*3*)-*IV* apramycin resistance gene in humans, with 99.3% homology to that in animal strains	Zhang *et al.*, 2009
	E. coli	Companion animal owners	Companion animal	Quinolone	Detection of plasmid mediated quinolone resistance genes	Chung *et al.*, 2017
Human infection via direct or indirect animal contact	*Salmonella Newport*	*Salmonella*-infected patients with diarrhea	Beef cattle (ground beef) receiving chlortetracycline AGP	Ampicillin, carbenicillin, tetracycline	Direct genetic tracking of resistance plasmid from hamburger meat to infected patients	Holmberg *et al.*, 1984B

	E. coli	Swine farmers, family members, community members, UTI patients	German swine (ill)	Streptothricin	Identification of transferable resistance plasmids found only in human gut and UTI bacteria when nourseothricin was used as swine AGP	Hummel *et al.*, 1986
	E. coli, *Salmonella enterica* (serovar Typhimurium)	Hospital inpatients	Belgian cattle (ill)	Apramycin, gentamicin	Plasmid-based transfer of *aac(3)-IV* gene bearing resistance to a drug used only in animals (apramycin)	Chaslus-Dancla *et al.*, 1991
	Enterococcus faecium	Hospital patients with diarrhea	Danish swine and chickens	Vancomycin	Clonal spread of *E. faecium* and horizontal transmission of the *vanA* gene cluster (Tn*1546*) found between animals and humans	Hammerum *et al.*, 2000
	E. coli	Bacteremic hospital patients	Spanish chickens (slaughtered)	Ciprofloxacin	Multiple molecular and epidemiological typing modalities demonstrated avian source of resistant *E. col*	Johnson *et al.*, 2007A

V. FOOD ANIMALS AND ANTIMICROBIAL RESISTANCE

5.1. ESBL-producing *E. coli* in husbandry animals

Broad-spectrum β-Lactam antibiotics resistance in *E. coli*, through the production of extended-spectrum β-lactamase (ESBL), has been regarded as the most significant MDR in *E. coli*. However, *E. coli* isolates producing ESBL are not only resistant to multiple antimicrobials, but also they harbor virulence determinants that trigger extra-intestinal infections (Pitout, 2012). Since there emergence in the 1980s ESBL-producing *E. coli* has continued to be dominant until the mid-2000s. It became a major treat by further limiting the available treatment options for infections, since there is strong connection between ESBL-producing *E. coli* and the resistance to fluoroquinolones, sulfonamides and aminoglycosides (Sidjabat and Paterson, 2015). There is a high variability in the prevalence of ESBL-producing *E. coli*. Apart from differences in the methodology used, other factors, such as, farming practices, type of antimicrobial used in the area, geographical predominance of specific clones, type of animal breed under investigation (local/exotic) or age have proved to influence the carriage percentages of ESBL among animals (Reist *et al.*, 2013; Seni *et al.*, 2016).

The three key enzyme types which are involved in plasmid-mediated β-lactamases of *E. coli* are SHV, CTX-M and TEM (Bush and Jacoby, 2010). TEM is the first

recognized β-lactamase in Gram-negative bacteria when it was discovered in the 1960s. SHV confers a wide range of resistance to β-lactams, including the third-generation cephalosporins and aztreonam. However, both SHV and TEM are susceptible to β-lactamase inhibitors (sulbactam, clavulanate and tazobactam) (Sidjabat and Paterson, 2015). The CTX-M family of ESBL enzymes is comprised of over 165 gene variants with the predominance of CTX-M-15 in many regions of the world (Cantón *et al.,* 2012). This CTX-M type was also the dominant ESBL enzyme determined among livestock in many African countries (Alonso *et al.,* 2017). However, it is important to note that geographical variation can be a factor in the occurrence of different ESBL variants. CTX-M-9 variant dominantly reported in Spain as opposed to CTX-M-1 in the Northern European countries (Cantòn and Coque, 2006), whereas, in Switzerland CTX-M-1 and CTX-M-15 had showed more dominance in animal and human isolates, respectively (Lartigue *et al.,* 2007; Geser *et al.,* 2012). Whereas, in other study by Shin *et al.* (2017) and his colleagues, CTX-M-15 and CMY-2 were the predominant ESBL genotypes detected in *E. coli* isolates originated from Pig and Chicken farms from Korea. In general, most data from Korea depicted that, *E. coli* from food-producing animals and farm personnel mainly carry the CTX-M type of ESBL and CTX-M-14 was the most predominant type (Tamang *et al.,* 2013A; Tamang *et al.,* 2013B).

Further worrying development in the dissemination of ESBLs are a growing number of isolates carrying *bla* genes has been increasingly reported in food-

producing animals and was proven to be associated with the human epidemic clone sequence type (ST131). This is the case for ST131-CTX-M-15-producing *E. coli* isolates identified from chickens and healthy swine (Ahmed *et al.*, 2013; Seni *et al.*, 2016). Not only limited to husbandry animals, but *E. coli* from companion animals also found to harbor this important ST along with *bla* gene, ST-131-CTX-M-27 (Harada *et al.*, 2012). Similar to the clonal spread of ESBL, horizontal transfer of *bla* genes by plasmids and/or other transferable genetic elements are also vital in the epidemiology of ESBL genes (Alonso *et al.*, 2017). For instance, *E. coli* isolates carrying the $bla_{\text{CTX-M-15}}$ in IncF-type plasmids was reported in animals and humans (Seni *et al.*, 2016; Ojo *et al.*, 2016). Similarly, animal origin *Enterobacteriaceae* isolates carrying IncI1 plasmids were widely linked in the spread of ESBL genes (Girlich *et al.*, 2007; Ben Sallem *et al.*, 2014). In general, a high genetic diversity among *Enterobacteriaceae* isolates of food-producing animals and their high ESBL production is a big worry and an indication of established reservoir in farm animals (Geser *et al.*, 2012).

5.2. Carbapenemase-producing *E. coli* in husbandry animals

Carbapenems have served as an important antimicrobial class for the treatment of *Enterobacteriaceae* resistance to broad-spectrum antimicrobials, such as the extended-spectrum cephalosporins, penicillins, and the monobactam aztreonam

(Morrill *et al.*, 2015). Due to its greatest potency and broadest spectrum of action against Gram-positive and Gram-negative bacteria it was considered as drugs of last resort on the shelf (McKenna, 2013).

Unfortunately, the recent emergence of MDR pathogens critically poses a threat to this class of lifesaving drug (Papp-Wallace *et al.*, 2011). Carbapenem-resistant *Enterobacteriaceae* (CRE) are a family of bacteria that are difficult to treat because they have high levels of resistance to antibiotics due to this it is regarded as an important emerging threat to public health (CDC, 2015). CRE is generally mediated by two principal mechanisms: (i) production of carbapenemases enzymes (e.g. KPC-type, VIM-type, NDM-type, and OXA-type) capable of hydrolyzing almost all β-lactams (ii) via modifications to the cell OM, specifically by reducing cell membrane permeability from porin modification, and/or production of efflux pumps (Nordmann *et al.*, 2012; Diene and Rolain, 2014).

According to the world organization for animal health (OIE, 2015), carbapenems are not approved for use in livestock production anywhere in the world; as a result, use in food-animals is assumed to be rare. Due to this fact little is known about the prevalence of carbapenem-resistant bacteria (CRB), and more specifically CRE, in livestock populations and their associated environments. Animal origin carbapenem resistant bacteria surveillance is still in preliminary stages. However, it is highly credible, taking into account the high prevalence of co-resistance to other antimicrobial classes among most clinically relevant carbapenems, if brought into

animal husbandry, CRE would further spread to humans through food, animal contact, or the environment. Hence, it is crucial to look into the animal husbandry systems to early detect and to characterize bacteria isolated from animals that displays resistance to carbapenems (Webb *et al.,* 2016).

There are few instances were CRB have been reported by different investigators, chronologically, *bla*$_{\text{OXA-23}}$ was detected in *Acinetobacter* isolated from horses in Belgium (Smet *et al.,* 2012), and dairy cattle in France (Poirel *et al.,* 2012), *bla*$_{\text{NDM-1}}$ was detected in *Acinetobacter* isolates from a chicken and pig farm in China (Wang *et al.,* 2012; Zhang *et al.,* 2013), *bla*$_{\text{VIM-1}}$ was detected in *Salmonella* isolates recovered from a broiler farm and fattening pig farms in Germany (Fischer *et al.,* 2013), *bla*$_{\text{NDM-9}}$ was detected in *E. coli* strain recovered from a chicken meat sample in china (Yao *et al.,* 2016), *E. coli* and *A. baumannii isolates* harboring *bla*$_{\text{CMY-2}}$ and a novel *bla*$_{\text{OXA}}$ gene (*bla*$_{\text{OXA-497}}$), respectively, reported in fecal samples from dairy cattle in USA (Webb *et al.,* 2016), *bla*$_{\text{IMP-27}}$ was detected in *E. coli* and *Proteus mirabilis* from environmental samples collected from a litter of pigs in USA (Mollenkopf *et al.,* 2017), and *bla*$_{\text{KPC-2}}$ gene reported in *K. pneumonia* isolated from fecal sample in beef cattle from USA (Vikram and Schmidt, 2018).

5.3. Plasmid-mediated colistin resistance in husbandry animals

Polymyxins, including colistin, are used as antibiotics of last resort to deal with serious infections caused by CRE (Yao *et al.,* 2016). Since it was first described in China in 2015 (Liu *et al.,* 2016), the plasmid-mediated *mcr-1* gene has become a serious problem worldwide gaining a huge attention. Following this report a different variant of the plasmid-mediated colistin resistance *mcr-2* gene was identified in *E. coli* strain recovered from swine and cattle in Belgium (Xavier *et al.,* 2016), and in June 2017, *mcr-3* was detected in the 261 kb plasmid (IncHI2) of *E. coli* isolated from swine (Yin *et al.,* 2017). This third variant showed nucleotide sequence similarities of 45% and 47% with previously detected *mcr-1* and *mcr-2* genes, respectively (Yin *et al.,* 2017). Novel *mcr-4* gene in *E. coli* strains from pigs in Europe also reported by Carattoli *et al.* (2017).

Despite the use of colistin in veterinary medicine for either prophylactic or metaphylactic practices, in many countries, there is low prevalence of colistin resistant bacteria isolated from animals (Figure 5) (Wasyl *et al.,* 2013; Kempf *et al.,* 2013; Quesada *et al.,* 2014). Colistin mainly prescribed in animals' husbandry for treatment of infections caused by *Enterobacteriaceae.* Mainly in pig production system colistin used to treat digestive disorder related cases (Kempf *et al.,* 2016). For instance, annual colistin usage in food-producing animals has increased gradually in Korea over recent years (Lim *et al.,* 2016). Likewise, in China

widespread use of colistin, especially, in poultry and swine farms have been practiced for long (Huang *et al.,* 2017). This practice might contribute to the occurrence of colistin resistance. More importantly, studies support the need to be aware of the possible dangers of transferring *mcr-1* producing *Enterobacteriaceae* to humans via the food chain which necessitates strict regulation of the use of colistin in veterinary medicine for treatment and as a feed additive.

Organisms harboring *mcr-1* have been recovered from various sources–such as livestock (Malhotra-Kumar *et al.,* 2016A), the environment (Zurfuh *et al.,* 2016), food (Hasman *et al.,* 2015; Huizinga *et al.,* 2016) and from human clinical isolates (Cannatelli *et al.,* 2016; Prim *et al.,* 2016). Generally, the prevalence and the number of reported *mcr* genes have been more in animal and food isolates than in human strains. Quite recently *mcr-3* harboring *E. coli* isolates were reported from swine farms in Korea (Belaynehe *et al.,* 2018B). Interestingly, those reported isolates were proven to display multidrug- resistance for 3 different antimicrobials phenotypically and harboring various antimicrobial resistant genes.

A more sensitive and quick detection methods among animals of colonization with bacteria harboring *mcr* genes is vital to early trace and confine the spread of these genes. Therefore, on this account, few methods are developed, such as, a single real-time polymerase chain reaction to detect *E. coli* carrying the colistin-resistance *mcr-1* and *mcr-2* genes (Chalmers *et al.,* 2018) and a multiplex PCR that quickly detects all *mcr* genes reported in *Enterobacteriaceae* to date (Rebelo *et al.,*

2018). Currently, the epidemiology of transferable colistin resistance *mcr* genes in animal husbandry is changing quickly; hence updated information on molecular epidemiology and prevalence of *mcr*-positive isolates is mandatory to control and prevent further spread of plasmid-mediated colistin resistance genes (Alba *et al.*, 2018).

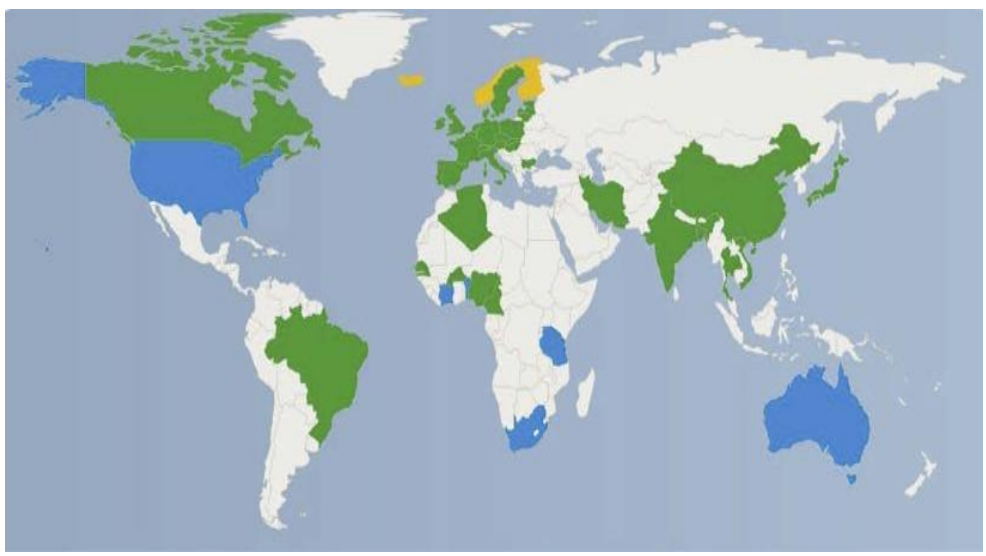


Figure 5. Worldwide colistin usage in animal husbandry practice. The green, blue and white color represents colistin, polymyxins and no available information on colistin/polymyxin usage, respectively (Adapted from Kempf *et al.*, 2016).

VI. NATURE AND CHARACTERISTICS OF IMPORTANT ANTIMICROBIALS BOTH IN VETERINARY AND HUMAN MEDICINE

6.1. Aminoglycoside antibiotics

Aminoglycoside antibiotics (AGAs) are among the first antibiotics discovered and most clinically important classes of antibiotics. Nowadays, with the striking rise in the rate of infections caused by multidrug-resistant bacteria, a shift towards AGAs has returned as one of the few treatment options left, in particular for Gram-negative pathogens (Becker and Cooper, 2013). In recent years, the potential of AGAs as a potential treatment option for parasitic infections, genetic diseases and for fungal infections have been reexamined in clinical settings (Garneau-Tsodikova and Labby, 2016; Fosso *et al.*, 2014). Presently, however, AGAs have a therapeutic application for treating infections caused by Gram-negative bacteria, such as *E. coli*, *P. aeruginosa*, *Enterobacteriaceae* spp, *K. pneumoniae*, and *A. baumannii*. Despite its clinical significance, the application of aminoglycosides was not left unchallenged and different resistance mechanisms were originated through a variety of acquired and intrinsic mechanisms (Figure 6).

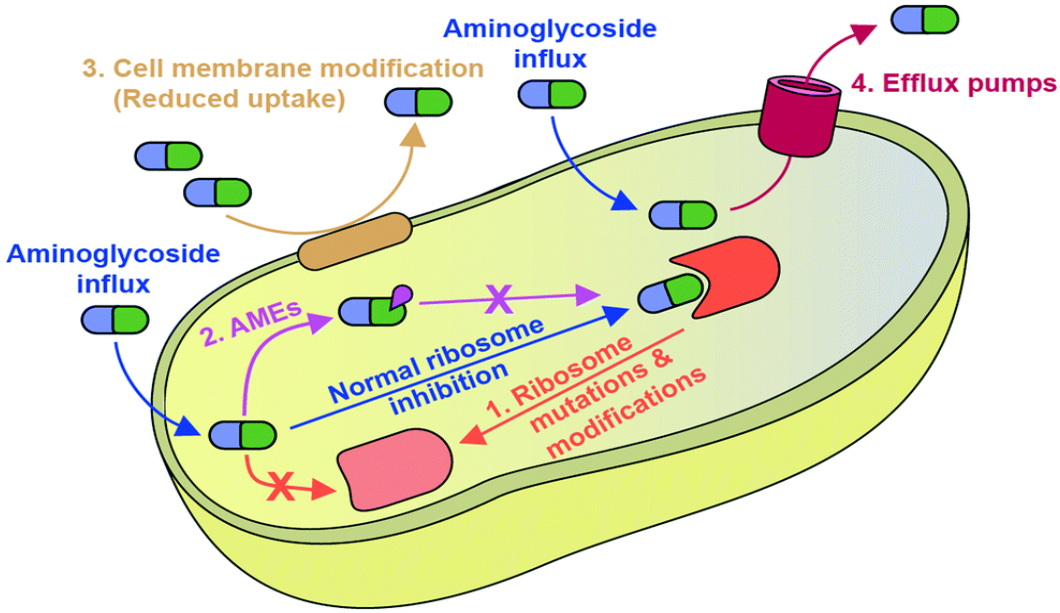


Figure 6. Schematic overview of the overall mechanisms of resistance to AGAs (Adapted from Garneau-Tsodikova and Labby, 2016)

6.1.1. Antibiotics in the class of aminoglycoside

The first AGAs were isolated from the soil-dwelling bacterial strains of *Micromonospora* and *Streptomyces*. Notable active former AGAs consists of streptomycin (1944), neomycin (1949), kanamycin (1957), paromomycin (1959), spectinomycin (1961), gentamicin (1963), tobramycin (1967), and sisomycin (1970). The more widespread clinical application of these antibiotics, resistance became more obvious. Due to this fact attempts have been made to make better the pharmacological profile of AGAs and achieved in the discovery and subsequent introduction of second generation of AGAs in the market, namely, dibekacin

(1971), arbekacin (1973), isepamicin (1975), and amikacin (1976) (Zhanel *et al.*, 2012). Plazomicin, originally known as ACHN-490, is a next-generation, semisynthetic aminoglycoside antibiotic, derived from sisomicin and incorporates structural modifications that enables this molecule stable even in the presence of most enzymes that inactivate the antibiotic. Currently, this antibiotic is under development for the treatment of infections caused by multidrug-resistant *Enterobacteriaceae* (Cox *et al.,* 2018; Castanheira *et al.,* 2018).

6.1.2. Chemical nature

Chemically aminoglycosides are characterized by a central aminocyclitol ring replaced with amino and hydroxyl groups (Figure 7). The amino and hydroxyl groups, which can also comprised of further substituents, are the central binding elements that act on the RNA of the 30S subunit of the ribosome where they intervene with translation of protein. The structure of AGA defines its susceptibility to various enzymes produced by bacteria and contributes in the development of non-susceptibility. Aminoglycosides are basic molecules, polar, poorly soluble in organic solvents and extremely soluble in water. Moreover, they are poorly absorbed from the gastrointestinal tract and do not promptly cross the blood–brain barrier, since they could not easily cross cellular membranes and due to the fact that at physiological pH they are positively ionized. For systemic use, intramuscular or intravenous route of administration is important, and intrathecal

administration is needed to attain therapeutic concentrations in the central nervous system (Shakil *et al.*, 2008; Zhanel *et al.*, 2012). Aminoglycosides are cationic in nature which makes them readily attach with variety of negatively charged biomolecules such as RNA, DNA and membrane lipids. This affinity is accountable for the ribosome targeting activity of aminoglycosides and could add to their fast bactericidal action.

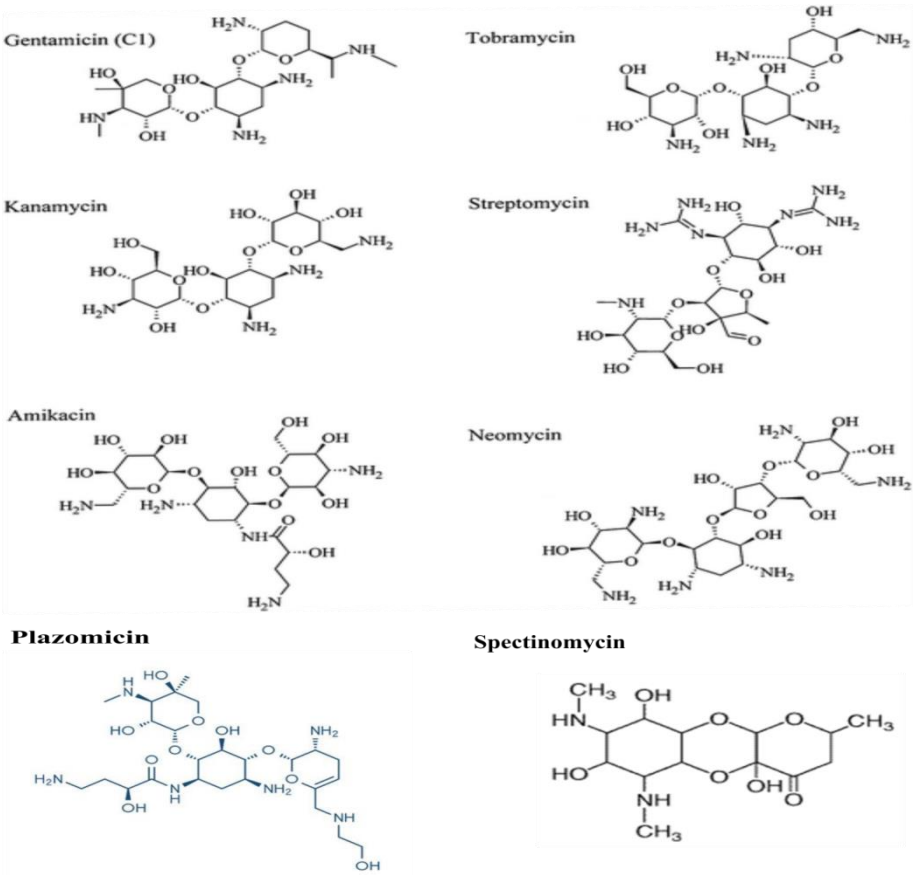


Figure 7. Chemical structures of the main aminoglycoside class antibiotics (AGAs) (Adapted from Becker and Cooper, 2013; Zhanel *et al.*, 2012).

6.1.3. Mechanism of action

In susceptible bacteria, class aminoglycosides display bactericidal activity, with the exception of spectinomycin and kasugamycin, whose principal mechanism of action is through inhibition of normal protein synthesis by binding to the A-site of the bacterial ribosome. The 16S RNA is the place where the A site located which is the decoding center of the ribosome (The 30S subunit of the ribosome is composed of about 21 proteins together with 16S RNA). Understanding the mechanisms by which aminoglycosides inhibit ribosome function will aid in creating new clinically useful drugs that selectively target the bacterial ribosome (Borovinskaya *et al.,* 2007). Aminoglycosides get across the bacterial cell through a three-step process; firstly energy-independent process and next by two energy-dependent steps (Ramirez and Tolmasky, 2010).

6.1.4. Mechanisms of aminoglycoside resistance

AGAs Inactivation by a family of enzymes named AMEs: The most widespread and clinically important resistance mechanism to aminoglycosides is by aminoglycoside-modifying enzymes (AMEs) that are commonly disseminated among Gram-positive and Gram-negative bacteria. Many of these AMEs are highly mobile; their genes are encoded on mobile genetic structures and transferred on plasmids, transposons, integrons, and other transposable gene elements, often co-

harboring β-lactamases and other resistance genes. (Ramirez and Tolmasky, 2010; Castanheira *et al.,* 2018). These enzymes catalyze covalent bonding of a specific transfer group to an amino or hydroxyl group on the aminoglycoside molecule, as assigned in the name (Figure 8) (Zhanel *et al.,* 2012). When the enzyme's transferase molecule binds with the aminoglycoside substrate resulted in the limited binding affinity of the drug for their target in the ribosome thereby affecting the bactericidal action of the drug.

Aminoglycoside O-phosphotransferases (APHs): Phosphorylation of hydroxyl groups, by the catalytic activity of APHs and using the phosphate group of ATP as a cofactor, makes the molecule of aminoglycoside antibiotics negatively charged; as a consequence their binding potential to the A-site in the ribosome diminishes resulting in non-susceptibility (Becker and Cooper, 2013). APHs includes the following classes and subclasses of enzymes; APH(2″)-I to –IV, APH(3″) I, APH(3′)-I to –VII, APH(4)-I, APH(6)-I, APH(7″)-I, and APH(9)-I.

Aminoglycoside N-acetyltransferases (AACs): they belong to the GCN5-related N-acetyltransferase (GNAT) proteins superfamily. GNAT enzymes catalyze the acetylation of amino groups (–NH2) in aminoglycosides using acetylCoA as a cofactor for the activity of the enzyme. AACs are grouped into four basic classes and a number of subclasses, namely, AAC(1), which has no subclasses, AAC(3)-I to X, AAC(2′)-I, and AAC(6′)-I and –II.

Aminoglycoside O-nucleotidyltransferases (ANTs): ANTs catalyze inactivation of aminoglycosides by transferring an AMP group from the donor substrate ATP to hydroxyl group in the AG molecule. There are five different classes of ANTs that catalyze the adenylylation of AGAs; namely, ANT (2″), ANT (3″), ANT (4′), ANT (6), and ANT (9). All the ANTs do not have subclasses with the exception of ANT (4′), which has two subclasses. In contrast with the other AME family, ANT has few classes but with crucial clinical significance, since ANT (2″) bestow a broad spectrum of resistance for amikacin, gentamicin and tobramycin.

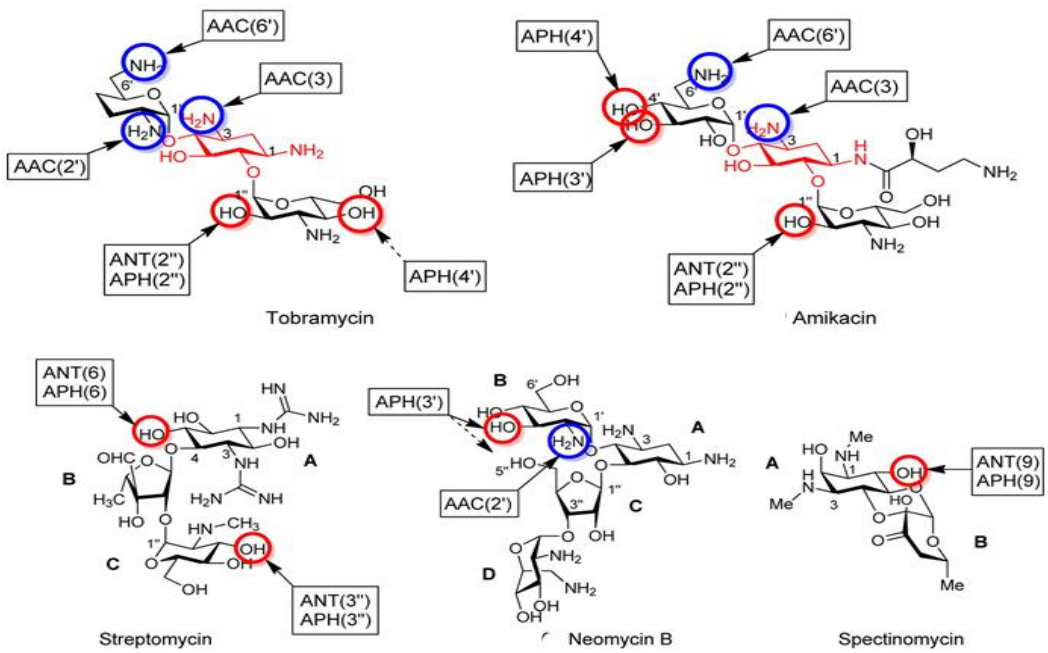


Figure 8. Sites of enzymatic modifications on tobramycin, amikacin, streptomycin, neomycin and specinomycin by various ANTs, APHs, and AACs (Adapted from Becker and Cooper, 2013).

The various types of AEM genes varies in different reports, for instance, *E.coli* strains isolated from hospitalized patients in Poland (Ojdana *et al.,* 2018) demonstrated *aac(6')-Ib* and *aph(3")-Ib* as the most common AEMs, whereas among Chinese clinical isolates of *E. coli*, *aac(3)-II* and *aac(6')-Ib* genes were reported as the dominant AME genes (Xiao and Hu, 2012.). In another study by Miró *et al.* (2013) and Fernández-Martínez *et al.* (2015) from Spain reported the predominant AME genes detected were *aph(3")-Ib*, and *aac(6')-Ib* and *ant(2")-Ia*, respectively. In Korea *aph3'-1a* and *aph3'-1b* were reported to be the most prevalent AME genes (Belaynehe *et al.,* 2017 and Shin *et al.,* 2014) and Fihman *et al.* (2008) described *aac(6')-Ib* gene as the common AEM gene among *E.coli* isolated from France.

AGAs resistance by mutations of the target ribosome: Changes in the antibiotic target site mostly occurred from chromosomal spontaneous mutation of a bacterial gene and selection due to the presence of the drug. RNA polymerase mutations in the 16S rRNA gene confer resistance to the aminoglycosides. The most frequent substitutions, from A to G, at positions 1400, 1401, and 1483 have been found in some kanamycin-resistant clinical isolates (Lambert, 2005). Streptomycin interacts with the ribosomal protein S12, and mutations in this protein can alter binding and ensued resistance. Whereas, mutation in the ribosomal 30S subunit binding site located in the single-stranded region of the 16S rRNA loop confers high-level of resistance to gentamicin and the neomycin (Becker and Cooper, 2013).

Ribosomal modification by a family of methyltransferase enzymes (16S rRNA methylases)*:* Natural aminoglycosides producers of the genera *Actinomycetes*, such as *Streptomyces* spp. and *Micromonospora* spp., are naturally resistance to aminoglycoside antibiotics, since they intrinsically harbor 16S rRNA methylase (also referred as RMTases/16S-RMTase) enzymes (Wachino and Arakawa, 2012). The AGAs binding site of the specific nucleotide residues, either the N-7 position of nucleotide G1405 or the N-1 position of nucleotide A1408, in 16S rRNA can be enzymatically modified by various 16S rRNA methyltransferases (Doi and Arakawa, 2007; Moric *et al.,* 2010). There are ten acquired N7-G1405 16S-RMTase genes (*ArmA*, *RmtA*, *RmtB*, *RmtC*, *RmtD*, *RmtD2*, *RmtE*, *RmtF*, *RmtG*, and *RmtH*) and only one acquired N1-A1408 16S-RMTase gene (*NpmA*) confer resistance exclusively to aminoglycosides (Figure 9). The first 16S-RMTase was discovered in japan in plasmid harboring *RmtA* in 1997 from AGAs resistant *P. aeruginosa* strain (Yokoyama *et al.,* 2003). Later in the early 2000s significant progress in the field of genetic analytical techniques, leads to discovery of another family of 16S rRNA methylase gene, *ArmA*, on *K. pneumoniae* isolated from a hospitalized patient in France (Galimand *et al.,* 2003). Quite recently, O'Hara *et al.* (2013) identified RmtH from a clinical strain of *K. pneumoniae* in a wounded male USA solder returned from Iraq war.

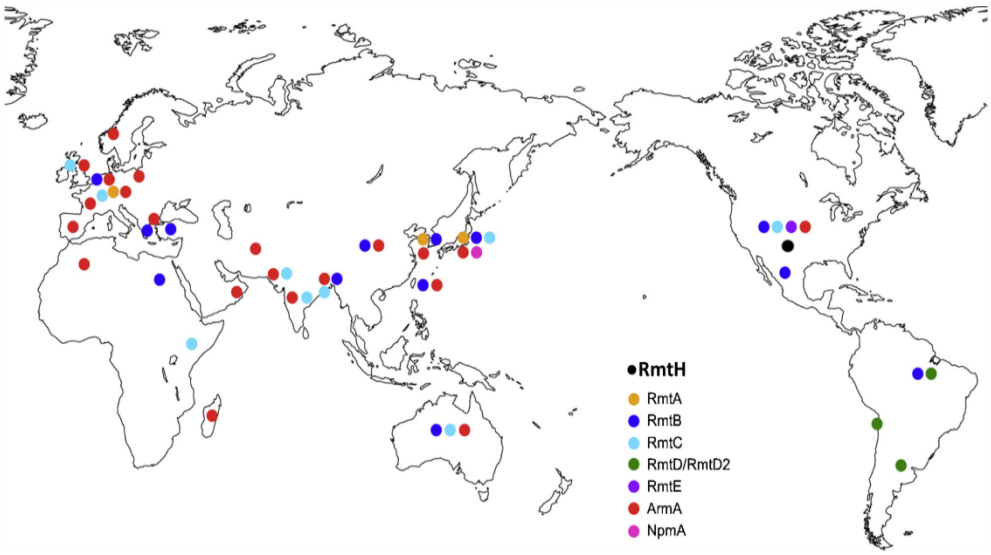


Figure 9. Worldwide distribution of aminoglycoside resistance by 16S-RMTases. (Modified from Wachino and Arakawa, 2012)

Modification of outer membrane's permeability: Gram-negative bacteria's OM carries out an important role of furnishing an extra layer of protection to the bacteria at the same time not compromising the transfer of necessary molecules (Delcour, 2009). The sensitiveness of many bacteria to different classes of drugs depends on the protein and lipid composition of the OM molecules; this in turn paves the way for antibiotic resistance by modification of these molecules. Hydrophilic antibiotics, such as aminoglycosides, get accesses of entry to bacteria via water filled channels made by outer membrane proteins (OMP). Hence, the permeability and, accordingly, the antibiotic resistance or susceptibility of the bacteria depends upon the type and number of porins possessed (Fernández and

Hancock, 2012). The existence of AGAs resistant strains in a large number of bacterial species can be by modification of their OM permeability via down regulation of porins or alteration of their outermost lipopolysaccharides (Garneau-Tsodikova and Labby, 2016). Cationic molecules like AGAs are attracted by the OM, since OM comprises of sugar functionalized LPSs which bear a net negative charge (Nikaido and Pagès, 2012; Li *et al.*, 2015). A modification of LPS by incorporating cationic molecule, 4-amino-4-deoxy-L-arabinose sugar, increases the overall positive charge of OM and changes the net negative charge of the LPS layer, thereby reducing affinity for AGAs (Kwon and Lu, 2006; Fernández *et al.*, 2010). Overall, modification of OM's permeability resulted in no expression, reduced expression, or structural mutations of porins that, consequently, brings in narrowing of the channel size to exclude relatively large AGAs.

Active efflux Pumps: All bacteria have drug efflux pumps or multidrug transporters inserted into the cell-membrane to get rid of toxic substances from the inside (Putman *et al.*, 2000; Buffet-Bataillon *et al.*, 2016). In Gram-negative bacteria the most prominent and clinically relevant efflux systems belong to a member of resistance-nodulation-division (RND) family of efflux pumps. In *E. coli* and *P. aeruginosa* AcrAD-TolC and Mex-OPr, respectively, are the main aminoglycoside efflux pumps typically composed of an OMP channel, a periplasmic protein and a cytoplasmic membrane pump (Soto, 2013). These efflux pumps decrease the available antibiotics concentration inside the cell and are then

called MDR pumps (Putman *et al.*, 2000; Poole, 2007). For instance the principal tobramycin resistance mechanism is via acquisition of AMEs, whereas for amikacin resistance mainly occurs due to over-expression of efflux pumps (Kos *et al.*, 2015).

6.2. Tetracycline antibiotics

Tetracyclines are part of a family of broad spectrum antimicrobials. It is the most widely used antibiotic in livestock farming, including aquaculture due to its low cost, efficacy, ease of administration, and less pronounced side effects (Koo and Woo, 2011). Chlortetracycline, the first drug in the tetracycline family, was discovered from Streptomyces aureofaciens in 1948 by Dr. Benjamin Duggar working at Lederle Laboratories (American Cyanamid) and the same year received approval for clinical use (Duggar, 1948). Tetracycline antibiotics represent a large and diverse group of compounds well recognized for their broad spectrum action against a broad range of Gram-negative and -positive bacteria, obligate intracellular bacteria, spirochetes and protozoan parasites.

After the first tetracyclines discovered more than 60 years ago, a step by step optimization of the core scaffold has developed different generation of tetracyclines for clinical application and this developments are aimed at preventing many of the resistance mechanisms (Figure 10).

6.2.1. Antibiotics in the class of Tetracycline

1st generation tetracyclines: The first members of the tetracycline family discovered in the late 1940s were chlortetracycline and oxytetracycline, and they were obtained from biosynthesis of *Streptomyces aureofaciens* and *S. rimosus*, respectively. Then later tetracycline was identified as naturally occurring molecules. Chlortetracycline, oxytetracycline, and tetracycline have been widely used as feed additives for animal growth promoters for decades (Koo and Woo, 2011). Widespread and imprudent application of those antibiotics both in human and veterinary medicine has resulted in selection for resistant bacteria and a high prevalence of tetracycline resistance (Chopra and Roberts, 2001; Roberts, 2005).

2nd generation tetracyclines: Was developed by Pfizer and Lederle through a semisynthetic approach, after a series of chemical modifications of ring C. This generation has a better antimicrobial potency, decreased toxicity and improved pharmacokinetic properties than the former generation. Second-generation semisynthetic tetracyclines developed and received market approval includes, doxycycline in late 1960s and minocycline in the early 1970s. Particularly doxycycline is used in companion animals like cats and dogs. Doxycycline is one of the most commonly used tetracyclines to date and minocycline was the last tetracycline to be introduced into the market in the 20th century (Nguyen *et al.*, 2014).

3rd generation tetracyclines: Earlier in 1990s, a group of researchers from Lederle Laboratories developed third-generation tetracycline antibiotics through expansion of known antibiotic classes by synthetic improvement aiming to overcome problems of resistance to earlier tetracyclines (Barden *et al.*, 1994). Tigecycline, derivative from minocycline, was the first of a novel class of semisynthetic glycylcyclines with expanded-spectrum properties (Pournaras *et al.*, 2016). Therefore, due to its expanded antimicrobial spectrum it is used for the treatment of different organisms, including Gram-positive and-negative, anaerobic, and atypical bacteria. Tigecycline has received a huge attention and has been considered as the last resort for treating infections caused by pandrug-resistant bacteria (Sun *et al.*, 2013; Pournaras *et al.*, 2016). However, resistance to tigecycline has been detected in *A. baumannii* and *Enterobacteriaceae*, especially in MDR strains (Sun *et al.*, 2013).

4th generation tetracyclines: Eravacycline is a synthetic fluorocycline antibacterial agent, currently under phase III clinical trial, having structural analogy to tigecycline with two modifications to the D-ring of its tetracycline core. Eravacycline proven to show broad-spectrum activity against Gram-negative, Gram-positive and anaerobic bacteria except in *P. aeruginosa* (Zhanel *et al.*, 2016; Markley and Wencewicz, 2018). Eravacycline, like tigecycline, remains active even in the presence of the most common tetracycline resistance mechanisms such as ribosomal protection proteins and efflux pumps (Bassetti and Righi, 2014).

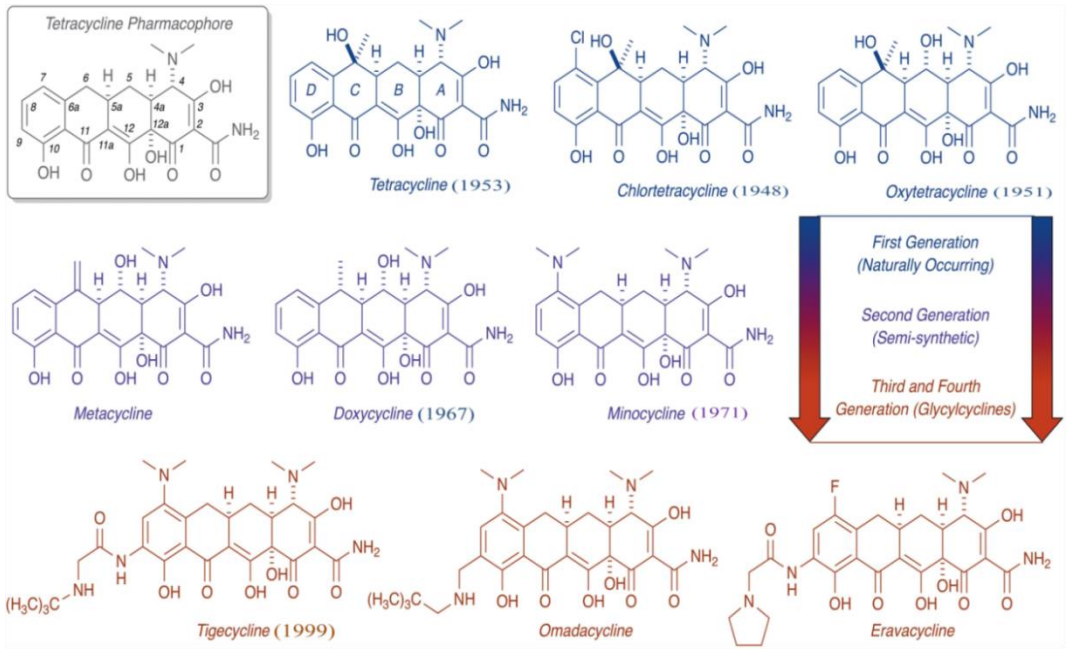


Figure 10. Generations of commercially available tetracycline class and their chemical structures (Adopted from Markley and Wencewicz, 2018).

6.2.2. Chemical nature

Tetracycline molecules composed of a linear fused tetracyclic scaffold with rings, designated A, B, C, and D, to which a collection of functional groups are bonded. 6-deoxy-6- demethyltetracycline is the simplest tetracycline to display detectable antibacterial activity which may be regarded as the minimum pharmacophore (Chopra and Roberts, 2001). Chemical modifications at C7 and C9 positions of the tetracycline D-ring considered as the best approach for better antimicrobial activity and have led to the discovery of minocycline and more recently tigecycline (Jones

and Petersen, 2005; Olson *et al.*, 2006). Although a lot of studies have been undertaken on the chemical biology of tetracycline antibiotics, their structural–activity relationships are not yet clearly understood (Zakeri and Wright, 2008).

6.2.3. Mechanism of action

Tetracyclines, as one of the bacteriostatic antibiotics, are protein synthesis inhibitors by preventing the association of aminoacyl- transfer RNA (tRNA) with the bacterial mRNA-ribosome complex. They preferentially interact with a highly conserved 16S-ribosomal RNA target in the 30S ribosomal subunit and interfere with the docking of aminoacyl-tRNA to halt protein translation (Pioletti *et al.*, 2001; Brodersen *et al.*, 2000). Particularly, the first nucleotide of the anticodon of the tRNA contacts with ring C and D of tetracycline to interact with the third base of the mRNA's A-site codon (Nguyen *et al.*, 2014). The mechanism of tetracyclines uptake by Gram-negative bacteria achieved by passive diffusion via the OM porins (OmpC and OmpF) (Mortimer and Piddock, 1993; Thanassi *et al.*, 1995).

6.2.4. Mechanisms of tetracycline resistance

Widespread application of the tetracyclines for decades in human and veterinary medicine resulted not only in the drastic decrease in the efficacy of these agents but

also substantial bacterial resistance (Chopra and Roberts, 2001; Hawkey, 2008). There are four primary mechanisms by which bacteria can acquire resistance for tetracyclines have been reported to date (Table 2) (Nguyen *et al.*, 2014; Sun and Xiao, 2017).

Table 2. Tetracycline resistance mechanisms and associated resistance genes (Adopted from Roberts, 2012; Zhanel *et al.*, 2016).

Tetracycline resistance mechanism	Tetracycline resistance genes	
	Gram-negative bacteria	Gram-positive bacteria
Efflux pump	*tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetI, tetJ, tetK, tetL, tetY, tet30, tet31, tet34, tet35, tet39, tet41, tet42*	*tetK, tetL, tetV, tetZ, tetAP, tetAB, tet33, tet38, tet40, tet45, otrB, otrC, tcr3*
Ribosomal protection protein	*tetM, tetO, tetQ, tetS, tetW, tet36, tet44*	*tetM, tetO, tetP, tetQ, tetS, tetT, tetW, tetZ, tetB(P), tet32, tet36, otrA*
Drug degradation	*tetX, tet34, tet37*	
rRNA mutations	A926T, G927T, A928C, ΔG942	G1058C

Efflux proteins: The efflux proteins are one of the most investigated of the Tet proteins which encodes for transport proteins (efflux pumps) that mediates for energy-dependent efflux of tetracyclines out of the bacteria cytoplasm (Chopra, 2001). The *tet* coding genes belong to the major facilitator superfamily (MFS) which comprises of more than 300 individual proteins (Paulsen *et al.*, 1996). In

Gram-positive and Gram-negative bacteria active drug efflux genes [*tet(A)–tet(D)* and *tet(K)–tet(L)*] were widely reported (Roberts, 1994; Chopra and Roberts, 2001; Tuckman *et al.*, 2007; Linkevicius *et al.*, 2016).

Most of the efflux pump genes are linked with conjugative transposons, integrons gene cassettes and mobile plasmids (Chopra, 2001). Second generation tetracyclines, such as minocycline and doxycycline, are less affected by most tetracycline efflux pumps (Nguyen *et al.,* 2014). The work of Hirata *et al.* (2004) suggested that *E. coli* strains carrying plasmids coding for various tetracycline efflux pump and multidrug transporter genes demonstrated that tigecycline, a third generation glycylcyclines, was not recognized by the *tet* efflux pump genes. Tigecycline has long side chain that blocks' binding to most efflux proteins and transporters, because of this it overcomes tetracycline resistance via efflux mechanisms (Someya *et al.,* 1995).

The frequently reported *tet* genes varied among different investigators, however *tet(A)* and/or *tet(B)* are regarded as the most prevalent tetracycline resistance determinant in *E. coli* strains from animals and humans in many countries. For instance the works of Karczmarczyk *et al.* from Ireland (2011), Shin *et al.* from Korea (2015), and Iweriebor *et al.* from South Africa (2015) demonstrated that *tet(A)* was the predominant tetracycline resistance mechanisms observed in *E. coli* strains originated from cattle. Whereas, Mirzaagha *et al.* (2011), Sawant *et al.* (2007), and Bryan *et al.* (2004) reported the dominant tetracycline resistance

determinant was *tet(B)*. In contrast, Srinivasan *et al.* (2007) depicted that among tetracycline resistance genes, *tet(C)* was reported in the highest frequency followed by *tet(A)* in *E. coli* isolates from dairy cattle. This is all an indication of a considerable variation of tetracycline resistance determinant of *E. coli* isolates. In addition it is a common phenomenon that *E. coli* isolates could harbor more than one *tet* gene (Belaynehe et al., 2018A).

Ribosomal protection proteins: RPPs are proteins found in the cytoplasm to protect bacterial ribosomes from the effect of tetracyclines and confer resistance to minocycline and doxycycline (Chopra and Roberts 2001). However, tetracyclines having side chains at the C-9 position of the D-ring, like tigecycline, generally retain antibacterial and inhibitory activities even in the presence of RPPs (Grossman *et al.,* 2012; Jenner *et al.,* 2013). In general there are twelve RPPs known so far (Nguyen *et al.,* 2014; Warburton *et al.,* 2016), these includes, *tet(M), tet(O), tetB(P), tet(Q), tet(S), tet(T), tet(W), tet(32), tet(36), tet(44), otr(A)* and the mosaic *tet* genes. Among the RPPs, the best defined and the most common are *tet(M)* and *tet(O)* having 75% sequence similarity to each other (Grossman, 2016). These genes are widely distributed both in Gram-positive and –negative bacterial populations mainly carried on mobile genetic elements (Roberts, 2012), however, RPPs, such as, *tet(M) and tet(O)*, are more frequently observed in Gram-positive bacteria such as *Streptococcus* spp and *S. aureus* (Grossman, 2016; Sun and Xiao, 2017).

***Enzymatic inactivation*:** The *tetX* and *tet37* genes are tetracycline resistance determinants encoding tetracycline-modifying enzymes that award resistance to tetracyclines through modification of the drug (Yang *et al.*, 2004). The *tetX* gene has only been found and first identified in obligate anaerobic bacteria of the genus *Bacteroides.* This enzyme inactivates tetracycline in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen (Chopra and Roberts, 2001). At present no report has been issued on new glycylcyclines, such as tigecycline, resistant isolates harboring *tetX*, since the enzymatic reaction is severely impaired with tigecycline (Nguyen *et al.*, 2014). In contrast to other antibiotic resistance mechanisms, the impact of enzymatic antibiotic inactivation has a lasting effect by permanently neutralizing the antibiotic challenge thereby reduces extracellular and intracellular antibiotic concentrations (Markley and Wencewicz, 2018).

Mutation in the 16S rRNA: Mutation of drug target often caused in reduced affinity for the drug, and this is a common resistance mechanism for antibiotics targeting ribosome (Wilson, 2009). Most of those drugs, including tetracyclines, interact solely with rRNA; thus, rRNA mutation can directly (or indirectly) determine the conformation of the drug-binding site which contributes to high level of resistance (Wilson, 2014). Ribosomal mutations in the 16S rRNA conferring resistance to tetracyclines was first detected in *Propionibacterium acnes.* Detail investigation of several strains of *P. acnes* demonstrated to contain a G-to-C mutation at position 1058 (Ross *et al.*, 1998).

Chapter I

Occurrence of aminoglycoside-modifying enzymes among isolates of *Escherichia coli* exhibiting high levels of aminoglycoside resistance isolated from Korean cattle farms

Abstract

This study investigated 247 *Escherichia coli* isolates collected from four cattle farms to characterize aminoglycoside-modifying enzyme (AME) genes, their plasmid replicons and transferability. Out of 247 isolates a high number of isolates (total 202; 81.78%) were found to be resistant to various antibiotics by disc diffusion. Of the 247 strains, 139 (56.3%) were resistant to streptomycin, and other antibiotic resistances followed as tetracycline (12.15%), ampicillin (7%), chloramphenicol (5.7%) and trimethoprim-sulfamethoxazole (0.8%). Among 247 isolates B1 was the predominant phylogenetic group identified comprising 151 isolates (61.1%), followed by groups A (27.9%), D (7%) and B2 (4%). Out of 139 isolates investigated for AME, 130 (93.5%) isolates carried at least one AME gene.

aph3″-1a and *aph3″-1b* (46%) were the principal genes detected, followed by *aac3-IVa* (34.5%). *ant2″-1a* was the least detected gene (2.2%). Nine (6.5%) strains carried no AME genes. Twelve (63.2%) among 19 isolates transferred an AME gene to a recipient and *aph3′-1a* was the dominant transferred gene. Transferability mainly occurred via the IncFIB replicon type (52.6%). Pulsed-field gel electrophoresis typing demonstrated a higher degree of diversity with 14 distinct cluster types. This result suggests that commensal microflora from food-producing animals has a tremendous ability to harbor and transfer AME genes, and poses a potential risk by dissemination of resistance to humans through the food chain.

Key words: *Escherichia coli*, antibiotics resistance, aminoglycoside-modifying enzyme genes, plasmid replicons, pulsed-field gel electrophoresis analysis, transferability

Introduction

Escherichia coli, a gram-negative bacterium, is a common inhabitant of the gastrointestinal tract found in both domestic animals and humans that has a great potential to acquire and transfer genes for resistance carried in another bacterium and found in the environment (Sun *et al.,* 2010; Tadesse *et al.,* 2012). Isolates from food animals are responsible for food-borne infection and serve as an important reservoir of transmissible resistance genes in humans (Neidhardt and Curtiss, 1996; Winokur *et al.,* 2001; Sayah *et al.,* 2005; Marshall and Levy, 2011).

The clinical significance of aminoglycosides decreased following the introduction of fluoroquinolones and expanded-spectrum β-lactams, which was associated with reduced interest in the investigation of the microbiological aspects of those antibiotics and their mechanisms of resistance (Fernández-Martínez *et al*., 2015). Aminoglycoside antibiotics block protein synthesis by targeting the A site or recognition site located in the 16S rRNA of the bacterial 30S ribosomal subunit where codon–anticodon accuracy is assessed (Davis *et al.,* 2010).

Even though clinical applications of aminoglycosides have not completely halted, the ever-increasing resistance to all major antimicrobial drugs has once again led to an interest in these compounds, particularly their application in the treatment of severe infections by gram-negative bacteria (Livermore *et al.,* 2011; Lindemann *et al.,* 2012; Almaghrabi *et al*., 2014; Fernández-Martínez *et al.,* 2015).

Bacteria have been furnished with various resistance mechanisms to cope with aminoglycosides, the most common being chemically modifying aminoglycosides by aminoglycoside-modifying enzymes (AMEs). AMEs are a large family of enzymes consisting of three subclasses categorized according to the type of chemical modification they impart on their aminoglycoside substrates: *O*-phosphotransferases (*aphs*), *O*-nucleotidyltransferases (*ants*) and *N*-acetyltransferases (*aacs*) (Garneau-Tsodikova and Labby, 2016). Beside AME genes other mechanisms of resistance include change in the bacterial membrane permeability for aminoglycoside antibiotics and increased efflux of aminoglycosides from bacterial cell to the exterior environment. Additionally, in the last few decades, 16S rRNA methyltransferase (16S-RMTase) production has emerged as a mechanism of high-level aminoglycoside resistance among *Enterobacteriaceae* strains (Wachino and Arakawa, 2012).

The extensive application of antibiotics to food-producing animals creates antibiotic residues in foods and the selection pressure of resistant organisms (Simonsen *et al.,* 2004). Consequently, the transfer of plasmids conferring antimicrobial resistance from antibiotic-treated animals to humans has long been suspected, and the resulting animal-to-human transfer of resistance genes was confirmed from the findings of recent investigations employing the whole-genome sequencing approach (Harrison *et al.,* 2013; Laxminarayan *et al.,* 2013). Accordingly, many of these AMEs are encoded on plasmids, transposons and integrons, which give them high mobility and readily facilitate the spreading of

resistance (Becker and Cooper, 2013). The genes coding for those enzymes have the capability to evolve continually; moreover, the large number of mobile elements where they are situated have resulted in a high adaptability of these enzymes enabling them to exploit new antimicrobials as substrate and also efficient dissemination among bacteria giving rise to a multidrug resistance (Ramirez and Tolmasky, 2010).

Knowledge of the molecular characteristics associated with a higher degree of resistance to aminoglycosides in commensal *E. coli* strains would be of profound significance to clinical practice, infection control measures and treatment options in veterinary and human medicine. Moreover, the limited information available regarding AMEs production in *Enterobacteriaceae* isolates resistant to aminoglycoside antibiotics indicates the need for further work (Fernández-Martínez *et al.,* 2015). Hence, the purpose of this work was to investigate the pattern of aminoglycoside resistance in *E. coli* isolates obtained from four different cattle farms. Subsequently, the distribution and transferability of AMEs from the isolates were investigated.

Materials and Methods

Bacterial strains

A total of 247 *E. coli* isolates from 405 fecal samples were collected from four different beef cattle farms located in four different cities (Pyeongchang, Anyang,

Yangpyeong, and Cheonan) between 2014 and 2015, of which 139 *E. coli* isolates phenotypically displaying streptomycin resistance (a representative aminoglycoside antibiotic) were used for further analysis. Isolates were confirmed through amplification of the 16S rRNA gene using polymerase chain reaction (PCR) (Sabat *et al.,* 2000).

Antimicrobial susceptibility test

The following antibiotics were tested by disc diffusion: ampicillin (10 μg), tetracycline (30 μg), streptomycin (10 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), amoxicillin-clavulanic acid (30 μg), chloramphenicol (30 μg) and trimethoprim-sulfamethoxazole (25 μg). MIC values for five major aminoglycoside antibiotics, namely, streptomycin, gentamicin, neomycin, tobramycin and kanamycin (Sigma-Aldrich, St. Louis, MO, USA) were assessed by the microbroth dilution method on cation-adjusted Mueller–Hinton broth (Oxoid, Basingstoke, UK). MIC_{50} and MIC_{90} values are also calculated as the value at which 50% and 90% of the strains within a test population are inhibited, respectively. Clinical and Laboratory Standards Institute (CLSI) procedures and interpretation guidelines were used. Antibiotic solutions were prepared for each antibiotic at levels ranging from 0.125 μg ml^{-1} to 256 μg ml^{-1}. *E. coli* ATCC 25922 strain was used as a quality control organism and all antimicrobial susceptibility tests were carried out in triplicate for each sample.

Phylogenetic typing

Escherichia coli isolates were assigned to four phylogenetic groups, namely, A, B1, B2 and D, as described previously (Clermont *et al.*, 2000). A multiplex PCR assay, established on the amplification of two genes, namely, *chuA* and *yjaA* and the DNA fragment (TspE4C2), were employed to classify phylogenetic groups.

Molecular detection of AMEs-encoding genes

For PCR amplification template DNA was obtained by adding a suspension of individual colonies from overnight growth in tryptic soy agar in 200 µl of ultrapure water and boiled for 10 min at 100℃. The PCR cycling conditions were as follows: one cycle of initial denaturation at 94℃ for 5 min, followed by 30 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 1 min, and then an additional extension of 72℃ for 10 min. The primer pairs used in this work are summarized in Table 3. Standardized sequencing (Macrogen, Seoul, Korea) was performed followed by sequence alignment using a search in the GenBank database of the National Center for Biotechnology Information website of the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR-based plasmid replicon typing

Escherichia coli isolates were tested for the presence of 18 plasmid replicons (B/O, FIC, A/C, P, T, K/B, W, FIIA, FIA, FIB, Y, I1, Frep, X, HI1, N, HI2 and L/M) using a simplified version of three multiplex PCR panels as described

previously (Johnson *et al.,* 2007B). Similarly, multiplex PCRs were performed on transconjugants to type plasmid replicons.

Conjugation assay

The transferability of AMEs genes between donors and recipient strains was evaluated using conjugation assay by the broth mating technique. For this experiment, 19 representative strains among the 139 aminoglycoside-resistant isolates were selected based on their high MIC value (MIC ≥256 256µg/ ml) for one of the tested aminoglycoside antibiotics. Conjugation was conducted in Luria-Bertani (LB) broth with *E. coli* J53 Az^r (azide resistant) strain (derived from *E. coli* K-12 strain from a stool sample of a hospitalized human patient) as a recipient and aminoglycoside-resistant isolates as a donor strain. Overnight broth cultures of both the recipient and donor were added to 4 ml of freshly prepared LB broth at a donor:recipient ratio of 1:1 (Wang *et al.,* 2003). To determine if resistance to aminoglycoside was co-transferred, 100 µl of the aliquots were plated in duplicates on tryptic soy agar plates with and without streptomycin. Confirmation was made by PCR if the transconjugants acquired the AME genes of their donors. The efficiency of conjugation was also calculated, which represents the ratio of number of CFU ml^{-1} of transconjugants to the number of CFU ml^{-1} of donors grown on tryptic soy agar.

Pulsed-field gel electrophoresis analysis

To investigate clonal relationships among *E. coli* strains harboring AMEs genes, pulsed-field gel electrophoresis (PFGE) analysis was carried out using a protocol previously established by the Centers for Disease Control and Prevention (CDC). Genomic DNA was digested on 50 U *XbaI* (TaKaRa, Japan) for 2 h and analysis was made using the GelCompar II software, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). A PFGE dendrogram was generated based on Dice coefficients of similarity using the unweighted-pair group method with arithmetic means.

Statistical analysis

MS-Excel 2010 computer program was used to store the generated data and also to construct graph for distribution of resistant isolates. IBM SPSS/Statistics, version 24, was used to perform data analysis. The prevalence and the percentage of quantitative data were analyzed by descriptive statistics. The Mann–Whitney test for continuous variables was used for comparison between groups of aminoglycoside antibiotics. *P* values represent differences in median MIC for respective aminoglycosides between the gentamicin-susceptible and -resistant isolates. *P* values <0.05 were considered statistically significant.

Results

Antibiotics susceptibility testing

Overall, 247 *E. coli* isolates were obtained from the 405 fecal samples. A high number of isolates (total 202; 81.78%) were found to be resistant to various antibiotics. Among them, the majority were found to be resistant to streptomycin (139; 56.3%), and other antibiotic resistances followed as tetracycline (30; 12.15%), ampicillin (17; 7%), chloramphenicol (14; 5.7%) and trimethoprim-sulfamethoxazole (2; 0.8%). Out of 139 streptomycin-resistant isolates interpreted from the results of disc diffusion tests, 138 (99.3%) were resistant to streptomycin by microbroth dilution, with MIC values ranging from 32 μg ml^{-1} to 256 μg ml^{-1}. Additionally, 21 isolates (15.1%) were resistant to neomycin (MIC ≥ 32 μg ml^{-1}) and 14 were resistant to gentamicin (MIC ≥ 16 μg ml^{-1}). Moreover, resistance to kanamycin and tobramycin was observed for nine (6.5%) and eight (5.8%) isolates, respectively.

Significantly higher median kanamycin MICs were observed for neomycin-resistant isolates in comparison to neomycin-susceptible isolates ($P = 0.001$; Table 4); however, there was no significant statistical difference in median MIC values for gentamicin, streptomycin and tobramycin between neomycin-resistant and -susceptible strains.

Analysis of phylogenetic groups

Most of the investigated isolates were categorized as phylogenetic groups B1 and A, with 151 (61.1%) isolates assigned to group B1 and 69 (27.9%) isolates falling under group A, followed by groups D and B2, each consisting of 17 (7%) and 10 (4%) isolates, respectively. Higher number of isolates resistant to ampicillin, chloramphenicol, tetracycline and streptomycin were categorized under B1 phylogroup (Figure 11).

Molecular characterization of AME gene-harboring isolates

Among the 139 isolates, 130 (93.5%) isolates were shown to contain at least one AME gene that conferred resistance to different antimicrobial drugs. The most common AME genes were *aph3"-1a* and *aph3"-1b* (64 strains; 46%), followed by *aac3IVa* (48 strains; 34.5%), *aac3IIa* (31 strains; 22.3%), *aac6´-1b* (21 strains; 15.1%), *ant3"-1a* and *aph3´-1a* (10 strains each; 7.2%) and only three isolates carried *ant2´-1a*. Fifty-four isolates harbored only one gene, 45 isolates two genes, 18 isolates three genes and 12 isolates four genes. Only a single isolate harbored five AME genes (*aac3IVa*, *aac6´-1b*, *ant3´-1a*, *aph3´-1a* and *aph3"-1a*). None of the evaluated AME genes were detected in nine (6.5%) isolates (Table 5).

AME gene transferability

Among 19 isolates, 12 (63.2%) were established to transfer the AME genes to the recipient *E. coli* strain by broth mating assay. The conjugation efficiency of the

isolates was between 4.92×10^{-3} and 1.82×10^{-4}.

Seven of the transconjugants (36.8%) carried only one of the investigated AME genes and three (15.8%) harbored two of the AME genes. The co-carriage of conjugative resistance by *aac6´-1b* and *aph3"-1a* was the predominant combination (three transconjugants; 15.8%), followed by *aph3"-1a* and *aph3´-1a* (two transconjugants; 10.5%). One transconjugant co-carried three genes (*aac6´-1b*, *aph3"-1a* and *aph3´-1a*). The predominantly transferred AME gene was *aph3"-1a* (seven transconjugants; 36.8%), followed by four isolates with the *aac6´-1b* gene (Table 6).

Characterization of plasmid replicons

A summary of the plasmid replicons observed from the 19 *E. coli* strains is given in Table 6. Overall, of the 18 known *E. coli* Inc groups, seven different Inc groups were identified. IncFIB was the predominant Inc group, found in 10 (52.6%) isolates. Other identified Inc groups include IncFrep (42.1%), IncI1 (31.6%), IncB/O (26.3%) and IncFIC (21.1%). Furthermore, only one type of replicon was detected in four isolates (21.1%), otherwise all of the isolates contained at least two Inc groups. Additionally, plasmid replicons in transconjugants showed that AME genes were primarily borne by IncFIB plasmids (26.3%), followed by IncI1 (15.8%). Only a single IncP group was found in the transconjugants (Table 6).

Genetic relatedness of aminoglycoside-resistant isolates

The genetic relationship among high levels of aminoglycoside-resistant *E. coli* strains carrying the most frequently detected AME genes was assessed on their *XbaI*-digested DNA fragments as depicted in Figure 12. Fourteen to thirty DNA fragments were observed from 19 *E. coli* isolates when investigated. Fourteen clusters were noticed when applying a 70% cut-off band pattern similarity, while a 50% cut-off band pattern similarity resulted in four clusters with little similarity to one another. In addition, between one and three isolates were detected within each PFGE group. Two isolates belonging to PFGE sub-cluster 4 showed high similarity with a Dice coefficient similarity of >90% (Figure 12).

Discussion

In this study, the aminoglycoside resistance mechanism, transferability potential of strains and plasmid replicons were described in multidrug-resistant *E. coli* isolates. Among the 139 aminoglycoside-resistant isolates, 138 (99.3%) were resistant to streptomycin by microbroth dilution test. This is presumed to be the result of selection pressure from their excessive and imprudent application in cattle farms over the last decade (Han *et al.*, 2011). *Escherichia coli* isolates have a chromosomal flexibility that facilitates their adaptation to various selective pressures that can exist in a wide environmental situation (Touchon *et al.*, 2009). Description of a correlation between MICs of one aminoglycoside with another is

crucial to assess whether there is a potential for cross resistance or not. Of note, the MICs of kanamycin-resistant isolates were significantly higher in neomycin-resistant isolates compared to neomycin-susceptible isolates ($P = 0.001$). Isolates harboring *aph3′-1a* were resistant to both neomycin and kanamycin as this AME gene specifically determines resistance to those aminoglycosides, and hence these two aminoglycoside antibiotics maybe affected by the same type of AME gene (Ramirez and Tolmasky, 2010). Furthermore, in South Korea, neomycin constitutes roughly about 50% of the total aminoglycoside antibiotics used in food-producing animals, which makes cross resistance possible with kanamycin (NARMP, 2004–2008).

Commensal *E. coli* strains generally belong to the B1 and A phylogroups, while pathogenic strains usually belong to the B2 and D phylogenetic groups of *E. coli*, which possesses virulence associated genes than commensal strains (Clermont *et al.,* 2000). The finding of this study also depicted that a considerable number of strains (61.1%) were classified under the B1 phylogenetic group, whereas the least commonly observed phylogroup was B2 (4%). These findings are in agreement with those of previously conducted studies in France, Ireland and the United States, where most commensal strains of bovine origin belonged to lineages B1 or A (Russo and Johnson, 2000; Duriez *et al.,* 2001; Karczmarczyk *et al.,* 2011). These findings imply that commensal strains also have the potential to harbor resistance genes and are crucial to the dissemination of antimicrobial resistance.

As demonstrated in the results section, the *E. coli* isolates collected from Korean

cattle farms displayed a remarkable AME genes diversity. Overall, eight major AMEs variants were characterized that can be associated with different degrees of resistance to aminoglycoside antibiotics. Out of 139 *E. coli* isolates, 130 harbored at least one AME gene. It can be anticipated that the remaining nine isolates could have carried a resistance mechanism other than AMEs, since those isolates were resistant to one of the aminoglycoside drugs investigated. This is an indication of the existence of a variety of resistance mechanisms whereby bacteria inactivate aminoglycoside antibiotics, for instance, modifying the target binding site where aminoglycoside binding occurs either via mutation of the 30S ribosomal subunit or methylation of the bacterial 16S rRNA by methyltransferases that transform nucleotides in the binding site. Furthermore, decreasing the concentration of antibiotics inside the bacteria via modifying membrane permeability to achieve efflux of the drugs and proteolysis of mistranslated proteins that occur due to the presence of aminoglycosides by proteases are other resistance mechanisms that confer resistance to aminoglycoside antibiotics (Gad *et al.*, 2011; Becker and Cooper, 2013).

In a previous study, *aac6′-Ib* was the most frequently detected acetyltransferase and was found to be causative for resistance to amikacin and other aminoglycoside antibiotics encountered in several Gram-negative bacteria belonging to the family *Enterobacteriaceae* (Becker and Cooper, 2013). Nevertheless, *aac6′-1b* was found to be not the prominent AME gene and harbored by only 15.1% of the isolates. This gene was mainly detected as a defective gene cassette or as a gene cassette

within class 1 integrons. Similar to the results of previous studies *aph3′-1a* and *aph3′-1b* were the most prevalent AME genes, with the exception of a high diversity among the *aph3′* subtypes, since *aph3′* has a high potential for recombination, which makes this gene naturally variable (Shin *et al.*, 2014; Woegerbauer *et al.*, 2015).

In our study, *ant2′-1a* was the least common AME type, which differed from the results of several other studies conducted in France (71%), Japan (50%) and the United States (81%), where this type of AME is widespread among aminoglycoside-resistant Gram-negative bacteria (Witchitz, 1981; Miller *et al.*, 1997). The frequency of detection of any given AME varies according to the type of on the type of aminoglycoside most frequently used in a particular country. Indeed, in a previous study (Vakulenko and Mobashery, 2003) gentamicin and amikacin accounted for 80% and 10% of all aminoglycosides used in the USA, respectively, while amikacin was the most common aminoglycoside in Japan. Following a ban on the use of antibiotics as feed additives since 2003, an overall gradual decrease in the sale of antibiotics for food-producing animals was observed in South Korea. However, the increased incidence of AME genes among *E. coli* isolates may be attributed to the extensive use of aminoglycoside antibiotics in the past years (APQA, 2012).

A considerable number of isolates that harbored the *aac3Iva* gene was detected, which encodes resistance mainly to tobramycin and gentamicin. Similarly, this AME gene was isolated from animals and humans in Belgium and Korea, where

aac3IV is carried by various plasmid replicons (Frep, B/O, I1, FIC and FIB) (Pohl *et al.,* 1993; Choi *et al.,* 2011). These results indicate the need for careful application of veterinary antimicrobial agents to safeguard against cross-resistance with antimicrobials for human use (Maron *et al.,* 2013).

Conjugation experiments revealed that plasmid transfer of high-level aminoglycoside resistance to the recipient strain (*E. coli* J53 Az^r) was successful for 12 of the 19 selected isolates. Among the isolates transferred by conjugation experiments, in one of the isolates none of the investigated AME genes were detected. This implies a possibility for co-transfer of a resistance mechanism other than AMEs that confers resistance to aminoglycosides. Many previous studies also showed that the AME genes co-transfer with other genes, such as 16S rRNA methyltransferase-encoding genes, which are responsible for the high level of resistance to various aminoglycosides (Wachino and Arakawa, 2012; Becker and Cooper, 2013). Although the majority of AME genes were borne by conjugative plasmids, some resistance genes could also be carried by chromosomes in the case of the *aac6´* gene (Becker and Cooper, 2013).

This study reports that carriage of AMEs is mainly by the IncFIB (52.6%) plasmid group. Similarly, previous research has suggested that IncFIB was the major incompatibility group in *E. coli* isolates from South Korean cattle farms (Shin *et al.,* 2015). Likewise, other studies have shown that, regardless of the origin of *E. coli* isolates, IncFIB plasmids were the predominant type identified (Johnson *et al.,* 2007B). Furthermore, the investigated AMEs-harboring isolates transferred

their plasmids in mating experiments and IncFIB (26.3%) was the predominant replicon in these transconjugants. The efficient transfer of plasmids carrying resistance genes suggests that this might play a major role in the spread of drug resistance to other clinically relevant pathogenic *E. coli* (Villa *et al.,* 2010; Carattoli, 2013; Ben Sallem *et al.,* 2014; Sidjabat *et al.,* 2014; Skurnik *et al.,* 2015).

Replicon typing revealed only one isolate with an IncP group; however, this strain carried the predominant resistance genes (*aph3′′-1b* and *aac6′-1b*) and had the ability to transfer its resistance genes to a recipient. Resistance to aminoglycoside mediated by IncP as described previously is also of considerable interest since its proven efficiency of conjugative transfer and capability of replicating in a broad range of hosts (Sen *et al.,* 2012; Popowska and Krawczyk-Balska, 2013). Certain plasmid replicons were found to be associated with multiple drug resistance; however, some plasmid replicon groups, such as IncF plasmids, can be carried regardless of resistance genes (Johnson *et al.,* 2007; Carattoli *et al.,* 2012).

PFGE analysis revealed a huge genetic diversity in the majority of isolates, whereas some displayed a clonal relationship. For instance, EC252 and EC581 had a similar antimicrobial resistance pattern (S, KAN, NEO, C, TE), the same AME genes profile (*aac3IIa, aph3′-1a, aph3″-1a, aph3″-1b*) and high genetic similarity (>90%) with each other. These findings are similar to those of previous studies in Korea that showed a noticeable genetic heterogeneity, regardless of the origin of isolates (Shin *et al.,* 2014).

Conclusion

The present study revealed that the attribute of resistance that emerged from commensal microorganisms of food-producing animal origin can be present at high density, making possible horizontal resistance gene transfer between isolates from a single species and/or between species. Moreover, this high resistance level in livestock herds generally reflects the overall antimicrobial pressure in these herds and the potential to readily exchange antibiotic-resistant genes. Plasmid-mediated aminoglycoside resistance genes carried by commensal *E. coli* could ultimately confer resistance to pathogenic organisms, posing a direct risk to human health.

Table 3. Primers used to characterize aminoglycoside modifying enzyme (AME) genes.

Gene	Primer name	Primer sequence	Annealing temperature (°C)	Product size (bp)	GenBank accession no.	Reference
Aac(6')-1b	Aac6'-IbF	TTG CGA TGC TCT ATG AGT GGC TA	60	482	M21682	(Galani *et al.,* 2012)
	Aac6'-IbR	CTC GAA TGC CTG GCG TGT TT				
Aac(3)-IIa	Aac3-IIaF	GGC AAT AAC GGA GGC GCT TCA AAA	55	563	X13543	(Aggen *et al.,* 2010)
	Aac3-IIaR	TTC CAG GCA TCG GCA TCT CAT ACG				
Aac(3)-IVa	Aac3-IVaF	TCG GTC AGC TTC TCA ACC TT	55	314	X01385	(Fernández-Martínez *et al.,* 2015)
	Aac3-IVaR	GAT GAT CTG CTC TGC CTG TG				
Ant(2'')-1a	Ant2"-1aF	ACG CCG TGG GTC GAT GTT TGA TGT	55	572	X04555	(Aggen *et al.,* 2010)
	Ant2"-1aR	CTT TTC CGC CCC GAG TGA GGT G				
Aph(3')-1a	Aph3'-1aF	CGA GCA TCA AAT GAA ACT GC	55	624	V00359	(Aggen *et al.,* 2010)
	Aph3'-1aR	GCG TTG CCA ATG ATG TTA CAG				
Ant(3")-1a	Ant3"-1aF	TCG ACT CAA CTA TCA GAG G	55	245	X02340	(Miró *et al.,* 2013)
	Ant3"-1aR	ACA ATC GTG ACT TCT ACA GCG				
Aph(3")-1a	Stra-F	TCA ACC CCA AGT CAG AGG GT	55	804	EF646764	This work
	Stra-R	CTG AAG GAA CCT CCA TTG AA				
Aph(3")-1b	Strb-F	GTG GCT TGC CCC GAG GTC ATC A	55	612	M28829	(Miró *et al.,* 2013)
	Strb-R	CCA AGT CAG AGG GTC CAA TC				

Table 4. *In vitro* susceptibility to common aminoglycosides antibiotics, stratified by susceptibility pattern to Neomycin

	Neomycin resistant (n=21)*					Neomycin susceptible (n= 58)					
	MIC (μg ml^{-1})				Resistance rate[d]	MIC (μg ml^{-1})				Resistance rate[d]	
Aminoglycoside	Median	Range	MIC_{50}	MIC_{90}		Median	Range	MIC_{50}	MIC_{90}		P value[b]
Kanamycin	32	(16 to >256)	32	>256	14 (3/21)	16	(4 to 32)	16	32	NA[c]	0.001
Gentamycin	4	(2 to 16)	4	8	5 (1/21)	4	(1 to16)	4	8	5 (3/58)	NS (0.831)
Streptomycin	64	(32 to 256)	64	>256	100 (21/21)	32	(32 to >256)	32	64	100 (58/58)	NS (0.320)
Tobramycin	8	(1 to 16)	8	16	24 (5/21)	4	(2 to 16)	4	8	28 (16/58)[a]	NS (0.097)

[a] Tobramycin resistance rate includes both resistant and intermediate strains.

[b]P values were computed by using the Mann Whitney U test. NS, not statistically significant.

[c]NA, not applicable; No strain was resistant to kanamycin among the neomycin susceptible group.

[d]Numbers in the bracket represent the number with resistance/number tested in each stratum.

* Only neomycin resistant and susceptible isolates were used for comparison, whereas, intermediately resistant isolates were excluded.

Table 5. Aminoglycoside modifying enzymes (AMEs) detected in selected isolates

Isolate	Phylogenetic group	Farm ID	*AMEs genes detected in the isolates	Resistance spectrum
EC51	B2	A	*Aac6'-1b,Aph3''-1a, Aph3''-1b*	S, AMP, TE
EC311	A	A	*Aph3''-1b*	KAN, S, NEO, AMP,SXT
EC341	A	A	*Aac3Iva, Aac6'-1b, Ant3''-1a, Aph3'-1a, Aph3''-1a*	S, NEO, C, TE
EC141	B1	B	*Aac3IIa, Aph3''-1a, Aph3''-1b*	S, TE
EC151	B1	B	*Aac3Iva, Ant3''-1a, Aph3''-1a, Aph3''-1b*	S, C, SXT, TE
EC252	A	C	*Aac3IIa, Aph3'-1a, Aph3''-1a, Aph3''-1b*	S, KAN, NEO, C, TE
EC471	A	C	*Aac3IIa, Ant3''-1a*	S
EC581	B1	C	*Aac3IIa, Aph3'-1a, Aph3''-1a, Aph3''-1b*	S, KAN, NEO, C, TE,
EC1181	A	D	*Aac6'-1b, Aph3'-1a, Aph3''-1a, Aph3''-1b*	S, KAN, TOB, NEO, TE
EC1372	D	D	*Aph3''-1a, Aph3''-1b*	S, TE
EC1641	A	D	*Aph3''-1a, Aph3''-1b*	KAN, S, GEN, AMP, TE
EC1701	B1	D	*Aac3Iva, Aac6'-1b*	KAN, S, GEN
EC1711	A	D	*Aac3Iva*	KAN, S, GEN, TOB
EC1941	B1	D	ND	KAN, S, GEN
EC2062	B1	D	ND	S, GEN, NEO, TOB
EC2422	B1	D	ND	KAN, S, GEN
EC2581	B1	D	*Ant2''-1a*	S, NEO, TOB
EC2691	B1	D	ND	S, NEO, TOB
EC3041	B1	D	*Aac3IVa*	S, NEO, TOB

*ND, Not determined

Table 6. AME genes, replicon typing and transferability of aminoglycoside resistance isolates

Isolate	Replicon type	Transferability	Conjugation efficiency	Transconjugants	
				AMEs	Plasmid Inc. Groups
EC51	P, I1, FIB	+	$5.86x10^{-4}$	*Aac6'-1b, Aph3''-1b*	P, I1
EC311	FIC, FIB	+	$1.17x10^{-3}$	*Aph3''-1b*	FIC, FIB
EC341	Frep	+	$1.82x10^{-4}$	*Aac6'-1b, Aph3''-1a*	Frep
EC141	B/O, FIB	+	$1.33x10^{-3}$	*Aph3''-1a*	FIB
EC151	B/O	-	-	-	-
EC252	FIA, FIB	+	$9.53x10^{-4}$	*Aph3''-1a*	FIA, FIB
EC471	FIB, I1	-	-	-	-
EC581	FIA, FIB	+	$1.08x10^{-3}$	*Aph3''-1a, Aph3''-1b*	FIA, FIB
EC1181	B/O, Frep, FIB	+	$1.56x10^{-3}$	*Aac6'-1b, Aph3''-1a, Aph3'-1a*	B/O
EC1372	Frep	-	-	-	-
EC1641	FIC	+	$4.92x10^{-3}$	*Aph3''-1a*	FIC
EC1701	B/O, I1, Frep	+	$9.9x10^{-4}$	*Aac6'-1b*	I1
EC1711	I1, Frep	-	-	-	-
EC1941	ND	-	-	-	-
EC2062	FIC, FIB	-	-	-	-
EC2422	FIB, Frep	-	-	-	-
EC2581	I1, Frep	+	$7.37x10^{-4}$	*Ant2''-1a*	Frep, I1
EC2691	I1, Frep	+	$2x10^{-3}$	ND	ND
EC3041	B/O, FIC, FIB	+	$1.61x10^{-3}$	*Aac3IVa*	B/O, FIB

ND, Not determined

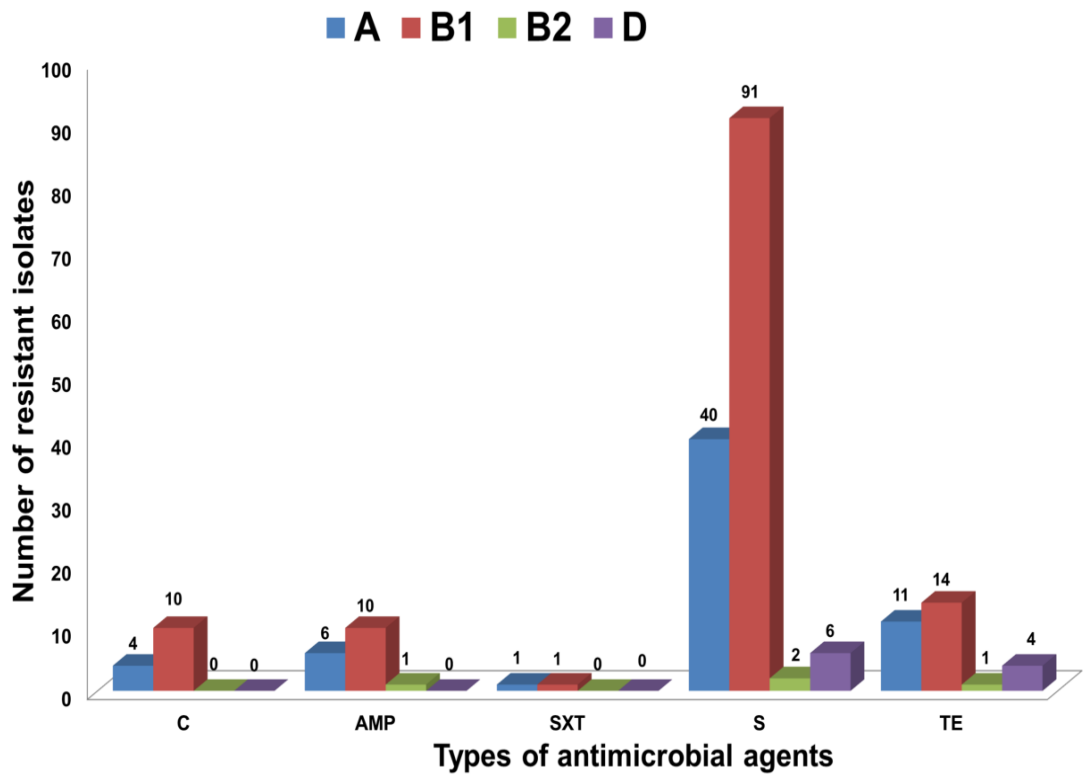


Figure 11. Distribution of antimicrobial resistance among 247 *E. coli* isolates in different phylogenetic groups to other antimicrobials. C, chloramphenicol; AMP, ampicillin; SXT, trimethoprim-sulfamethoxazole; S, streptomycin; TE, tetracycline.

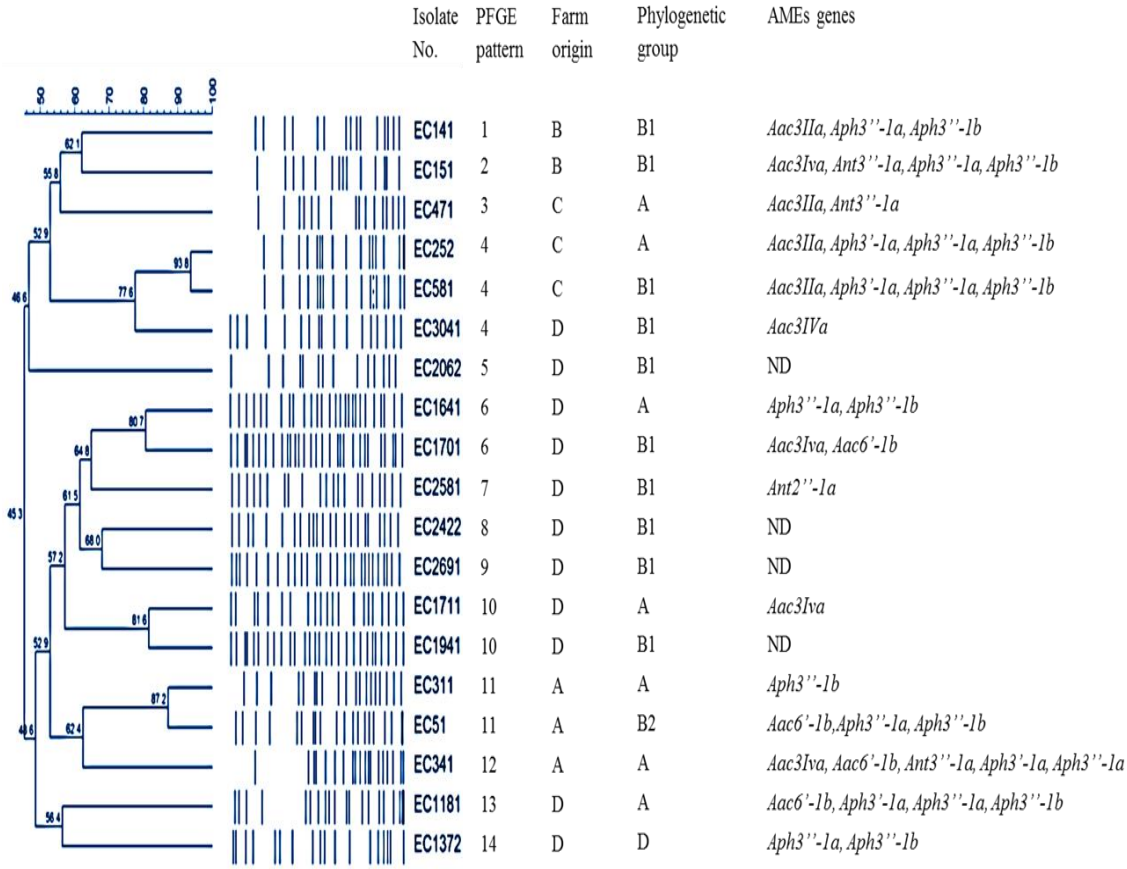


Figure 12. Pulsed-field gel electrophoresis of *XbaI*-digested DNA of *E. coli* isolates with a high level of aminoglycoside resistance, containing aminoglycoside-modifying enzymes, isolated from cattle. The dendrogram was produced with Applied Maths version 6.6 software analysis.

Chapter II

Interrelationship between tetracycline resistance determinants, phylogenetic group affiliation and carriage of class 1 integrons in commensal *Escherichia coli* isolates from cattle farms

Abstract

Carriage of antibiotic-resistant foodborne pathogens by food production animals is one of many contributors to treatment failure in health care settings, and it necessitates an integrated approach to investigate the carriage of resistant pathogens harboring integrons in food-producing animals. *Escherichia coli* isolates with reduced susceptibility to tetracycline antibiotics (n= 92) were tested for associations between carriage of class1 integrons, phylogenetic group affiliation and tetracycline resistance determinants using the MIC method, PFGE analysis, PCR and sequencing. Phylogroups B1 and A were the most common (58.7 and 19.6%, respectively), followed by groups D (20.7%) and B2 (1.1%). All isolates carried at least one of the *tet* genes examined. In addition, 88 (95.7%) of all

tetracycline-resistant isolates carried *tet(A)* or *tet(B)*, while 47 (51.1%) and 41 (44.6%) harbored only *tet(A)* or *tet(B)*, respectively. Likewise, isolates harboring these genes had a higher chance ($P<0.05$) of carrying class 1 integrons. Of the tested isolates, 38 (41.3%) carried the *intI1* gene. Classical integrons with complete genes (*sul1* and *qacEΔ1*) at the 3′-CS were recognized in 27 isolates. PCR screening and subsequent sequencing demonstrated that 84.2% (32/38) of the *intI1*-positive isolates harbored resistance gene cassettes. Overall, seven gene cassettes were identified, either solely or combined with another gene cassette. The most common gene was *aadA1* (10 isolates), followed by a combination of *aadA1-dfrA1* (seven isolates), *aadA1-dfrA12* (six isolates) and *aadA1-aadA2-dfrA12* (three isolates). Genetic typing using PFGE showed minimum clonal relatedness with 28 different clusters and 12–25 discernible DNA fragments. This study brings new insight into the relationships between the presence of integrons, phylogenetic group association and characteristics of tetracycline antibiotic resistance determinants in commensal *E. coli* strains.

Keywords: *E. coli*, class 1 integrons, tetracycline resistance, phylogenetic group, PFGE

Introduction

The spread and emergence of resistance to antimicrobial drugs among bacteria has been observed over the past several decades, and this constraint has been a constant impediment to effective infectious disease therapy for as long as antibiotics have been used (Mazel, 2006). In many cases, multidrug resistance was determined to be associated with transmissible plasmids, and the importance of integrons in the acquisition of resistance genes constitute the major vector of multidrug resistance in Gram-negative and, to a lesser extent, in Gram-positive bacteria (Cambray *et al.,* 2010; Ponce-Rivas *et al.,* 2012; Escudero *et al.,* 2015).

Over the last few years, rigorous exploration of the diversity of integrons in natural environments has indicated that they are more than just a curious feature of antibiotic-resistant pathogens but that they play a more general and crucial role in the genomic evolution and adaptation of bacteria (Gillings, 2014). To date, five mobile integron classes have been described and characterized based on variations in the *intI* sequences. However, class 1 integrons are ubiquitous and the most frequently encountered among clinical and commensal isolates; therefore, they have been the focus of numerous studies (Mazel, 2006; Cambray *et al.,* 2010; Deng *et al.,* 2015).

The basic structure of class 1 integrons includes two conserved segments (CSs) that are usually separated by a variable region that includes mobile cassettes containing antibiotic resistance genes. The 5′-CS carries an integrase class 1 (*intI1*)

gene encoding an integrase enzyme and a recombination site (*attI1*), whereas *qacEΔ1* and *sul1*, which confer resistance to quaternary ammonium compounds and sulfonamides, respectively, are localized at the 3′-CS (Mazel, 2006; Deng *et al.*, 2015; Recchia and Hall, 1995; Carattoli, 2001). The site-specific recombination system between *attI* and *attC* has enabled a diverse array of resistance determinants to be drawn by individual class 1 integrons (Mazel, 2006; Cambray *et al.*, 2010; Stokes *et al.*, 2006).

The coding regions of the gene cassettes have no promoters; however, most cassettes encode various antimicrobial resistance genes, with more than 130 distinguishable resistance genes having been found to date (Deng *et al.*, 2015). The majority of class 1 integrons harbors an aminoglycoside adenyltransferase gene (*aadA*) and a dihydrofolate reductase gene (*dfr*), which confers resistance to streptomycin and spectinomycin, and trimethoprim, respectively (Singh *et al.*, 2017; Mazel *et al.*, 2000).

Tetracycline has been used in human and veterinary medicine and as a growth promoter in animal husbandry. The major mechanisms of tetracycline resistance in *Enterobacteriaceae* are mediated through one of several mechanisms; namely, efflux pump activity, ribosomal protection, and enzymatic inactivation. The predominant genes that confer tetracycline resistance via efflux pump activity are *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)*, and *tet(G)*. Indiscriminate application of tetracyclines in food-producing animals enhances multidrug resistance due to antibiotic selective pressure induced by the presence of high environmental

concentrations of the antibiotics. This selective pressure ultimately leads to increased prevalence of tetracycline resistance via the *tet* genes and promotes the dissemination of mobile genetic elements in bacteria (Koo and Woo, 2011; Karami *et al.,* 2006).

E. coli isolates belong to four major phylogenetic groups, A, B1, B2, and D, and strains from the B2 phylogenetic group happen to be the least resistant to antimicrobials. Moreover, there is a tendency towards lower integron carriage among phylogroup B2 (Johnson *et al.,* 2003). Nevertheless, due to certain factors, such as the level of resistance to antimicrobials, the site of the infection and geographical location, there is variation in the prevalence of different phylogenetic groups (Bukh *et al.,* 2009). Isolates in the phylogenetic groups B1 and D tend to harbor class 1 integrons, and a previous report also showed that *intI*-positive B2 strains were the least prevalent (Skurnik *et al.,* 2005). There are various observations on the interrelationship of different phylogroups and integron carriage for environmental, human and animal isolates raising the hypothesis that the two phenomena are connected and indicating that various genetic elements are involved in strains with different phenotypes. Characterization of this association will help to better understand the infection process and will reflect the possible different survival strategies of *E. coli* phylogroups under different circumstances.

Antimicrobial resistant bacteria derived from animals seriously compromises public health by causing food-borne infections and raises a food safety issue globally. The effects of such bacteria are not only limited to food safety but also

pose occupational hazards for animal handlers, meat inspectors and veterinarians. In particular, carriage of antibiotic-resistant foodborne pathogens by food-production animals is one of many contributors to treatment failure in health care settings, and it establishes the need for a detailed and thorough investigation of the carriage of such antibiotic-resistant pathogens harboring integrons in food-producing animals (Koo and Woo, 2011). Furthermore, integrons are not only limited to pathogenic organisms but have also been isolated from bacteria recovered from environmental samples and healthy animals (Petersen *et al.*, 2000). Similarly, the lack of sufficient and current information describing the association between antibiotic resistance and phylogenic groups with respect to integron carriage in commensal *E. coli* isolates of cattle from Korea necessitates further research. Therefore, in the present study, the role of integrons and their associated diverse gene cassettes in mediating antimicrobial resistance in commensal *E. coli* isolates recovered from cattle were investigated. Moreover, the relationship between class 1 integron carriage with respect to phylogroups and patterns of tetracycline resistance was examined.

Materials and Methods

Selection of bacterial strains for the study

In total, 247 commensal *E. coli* isolates obtained by our research group between 2014 and 2015 from fecal samples from 405 tested animals at four healthy beef

cattle farms located in four different cities in South Korea (Pyeongchang, Anyang, Yangpyeong, and Cheonan) were used in the present study (Belaynehe *et al.*, 2017). The beef farms consisted of different age groups of cattle, such as weaned calves, bulls and steers. Since the farms are intensive, cattle were kept in confinement in a conventional housing system. Generally, in the study farms antimicrobial agents are used mainly to treat individual cases. The overall hygiene of the farms was in a good condition. Fecal samples were freshly collected from the rectum of each cattle and a single bacterial isolate was recovered per animal. All bacterial strains were routinely cultured in tryptic soy broth (TSB) (Oxoid, Basingstoke, UK) for 18 h at 37℃. Among the 247 isolates, 92 *E. coli* isolates demonstrating resistance or decreased susceptibility by microbroth dilution assays to any of the tetracycline antibiotics referred to below were selected for further investigation.

Susceptibility testing

Phenotypic characterization for all isolates was performed using the disc diffusion method, and the following antibiotic discs were analyzed in this study: tetracycline (TE, 30 μg), streptomycin (S, 10 μg), chloramphenicol (C, 30 μg), ampicillin (AMP, 10 μg), amoxicillin-clavulanic acid (AMC, 30 μg), ciprofloxacin (CIP, 5 μg), nalidixic acid (NA, 30 μg) and trimethoprim-sulfamethoxazole (SXT, 25 μg) (Sigma-Aldrich, St. Louis, MO, USA). The MICs for oxytetracycline, doxycycline, tetracycline, minocycline and tigecycline were determined using

cation-adjusted Mueller-Hinton broth (Oxoid, Basingstoke, UK). All susceptibility testing was performed according to the procedures and an interpretive criterion specified by the Clinical Laboratory Standards Institute (CLSI), and E. coli ATCC 25922 was used as a quality control strain (CLIS, 2013).

Phylogenetic group determination

E. coli phylogenetic groups (A, B1, B2 and D) were investigated by amplifying two genes and a DNA fragment using multiplex PCR as previously described (Clermont *et al.*, 2000).

Analysis of antimicrobial resistance genes

PCR amplification to investigate the tetracycline resistance-encoding genes was conducted for all isolates. The following genes, encoding the tetracycline efflux mechanism, were investigated as previously described: *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)* and *tet(G)* (Koo and Woo, 2011; Lanz *et al.*, 2003; Levy *et al.*, 1999; Zhao and Aoki, 1992; Kerrn *et al.*, 2002). Furthermore, genes conferring resistance to sulfonamide antibiotics (*sul1*, *sul2* and *sul3*) and genes conferring chloramphenicol/florfenicol resistance (*cat1*, *cmlA* and *floR*) were also analyzed. PCR amplification of the resistance genes was conducted using the primers presented in Table 7.

Detection and characterization of class 1 integrons and their gene cassettes

Total DNA was extracted by boiling a suspension of overnight-cultured bacterial cells [grown on tryptic soy agar plates (TSA) at 37°C for 10 min] in 200 μl of sterile RNase/DNase-free distilled water. All *E. coli* isolates were PCR screened for the presence of *IntI1* gene-encoding class 1 integrons. Further testing was performed on the integron positive isolates for the presence of gene cassettes in the variable region (*var1*), and the *sul1* and *qacEΔ1* genes in the 3′-CS. All primers and PCR conditions are presented in Table 1. Gel purification of all PCR products was conducted using PCR quick-spin PCR product purification kits (iNtRON Biotechnology, USA), after which the samples were sequenced (Macrogen Co., Seoul, Korea). Following sequencing, the gene cassettes within the variable regions of the class 1 integrons were determined by using BLAST (Basic Local Alignment Search Tool) searches of the NCBI database (National Center for Biotechnology Information).

Clonal relationships among integron positive strains

Determination of the genetic relationship between the integron positive isolates was accomplished by pulsed-field gel electrophoresis (PFGE) analysis according to the protocols and criteria previously established by the Centers for Disease Control and Prevention (CDC) using *XbaI* as the restriction enzyme. Briefly, following 18 to 20 h growth on TSA at 37℃, genomic DNA was digested with 50 U *XbaI* (TaKaRa, Japan) for 2 h at 37℃, then the DNA fragments were subsequently

separated on a 1.0% SeaKem Gold agarose gel (Lonza, USA) in 0.5× Tris-borate-EDTA (TBE) buffer using a CHEFMapper gel apparatus (Bio-Rad Laboratories, California, USA). The conditions for electrophoresis were as follows: pulse time, 2–30s at 14℃; run time, 18 h; voltage, 6 V/cm. Analysis of the image was performed by using the Bionumerics software (Applied Maths, Belgium).

Statistical Analysis

All experiment data are stored in Excel 2010, and the susceptibility testing was analyzed using IBM SPSS/Statistics, version 24. The association between the *tet* genes and the presence of class 1 integron gene was analyzed by Fisher's exact test or Pearson's χ^2 test, contingent on cell frequencies. The median MICs for the respective tetracycline antibiotics between the isolates with and without *Intl1* were analyzed using the Mann-Whitney test. A $P < 0.05$ was considered to indicate statistical significance.

Results

Antimicrobial resistance phenotypes

The resistance percentages to the tested antibiotics were as follows: streptomycin, 84 (91.3%); nalidixic acid, 36 (39.1%); ampicillin, 35 (38%); chloramphenicol, 28 (30.4%); trimethoprim-sulfamethoxazole, 24 (26.1%); ciprofloxacin, 11 (12%) and amoxicillin-clavulanic, 2 (2.2%) (Figure 13). The MIC range for the 92 tetracycline

resistant isolates was >256 µg/ml to 16 µg/ml, and their MIC_{50} and MIC_{90} values were 128 and 256 µg/ml, respectively. Oxytetracycline resistance was identified in all isolates (MIC range >256 µg/ml–32 µg/ml), of which 49 isolates were highly resistant (MIC ≥256 µg/ml). Moreover, 80 strains (87%) were resistant to doxycycline, 41 (44.6%) to minocycline and none to tigecycline. Significantly higher median oxytetracycline MICs were observed for isolates with class 1 integrons than for isolates without class 1 integrons (P< 0.006; Table 8); however, there were no significant statistical differences in the median MIC values for tetracycline, doxycycline, minocycline and tigecycline between class 1 integron-positive and integron-negative strains.

E. coli phylogenetic groups

Of the 92 isolates, phylogenetic groups B1 and D were the most common (54 isolates; 58.7% and 19 isolates; 20.7%, respectively), followed by group A, which was assigned to 18 isolates (19.6%). Group B2 was rare, occurring in only 1 isolate. The number of integron-positive and integron-negative isolates across the phylogenetic groups was compared, and phylogenetic group D (16 isolates) was the most prevalent among the *intI1*-positive isolates, whereas B1 (40 isolates) was most prevalent among the *intI1*-negative isolates. Our results showed an association between the presences of class 1 integrons and affiliation with phylogenetic groups D and B1 (P<0.01). The frequencies of integron-negative and integron-positive strains for in A and B2 phylogenetic groups were similar, with no

statistically significant differences (Figure 14).

Characterization of antimicrobial resistance genes

All isolates carried at least one of the *tet* genes examined. Overall, 88 (95.7%) of the tetracycline-resistant isolates carried *tet(A)* or *tet(B)*, with 47 (51.1%) and 41 (44.6%) isolates harboring only *tet(A)* or *tet(B)*, respectively. The *tet(C)* and *tet(G)* genes were found in only five (5.4%) and six (6.5%) isolates, respectively, and the *tet(C)* gene was found in isolates that were not harboring integrons. Moreover, eight isolates harbored two *tet* genes, while none of the isolates carried the *tet(D)* or *tet(E)* genes. The distribution of tetracycline resistance genes among the integron-positive and -negative isolates is shown in Table 9. *E. coli* isolates carrying class 1 integrons were more likely to harbor the *tet(A)* gene ($P<0.01$). In addition, the following determinants for chloramphenicol/florfenicol resistance were identified: *cat1* (47.4%), *floR* (50%), and *cmlA* (18.4%). Among the 92 *E. coli* isolates investigated, 28 isolates harboring integrons and eight isolates without integrons (n = 36; 39.1%) had the *sul1* gene. Moreover, the *sul2* and *sul3* genes were identified in 16 (17.4%) and seven (7.6%) isolates, respectively.

Detection of the *intI1* gene and characterization of gene cassettes

Integrase gene-encoding class 1 integrons were detected by PCR in 38 (41.3%) isolates. Resistance to quaternary ammonium compounds and sulfonamides conferred by the *qacEΔ1* and *sul1* genes in the 3′-CS, respectively, was identified

in 36 integron-positive isolates. Among these, 27 contained the entire 3′-CS (*qacEΔ1-sul1*) structure, whereas nonclassical integrons lacking the 3′-CS were found in only two of the 38 *intI1*-positive *E. coli* isolates. Of the 38 isolates, one had only *sul1* in the 3′-CS and eight possessed only *qacEΔ1* in the 3′-CS. The class 1 integron variable regions were amplified in 32 (84.2%) of the 38 *intI1*-positive isolates and their genetic contents were ascertained via PCR amplification of the integron variable regions and subsequent full sequence analysis. Different lengths of PCR products ranging from ~1–2.5 kb were observed for strains having variable regions. Of these, the predominant cassette amplicons carried by the isolates were 1 kb in 18 strains, 1.5 kb in 10 strains and 2.5 kb in four strains (Table 10).

Overall, seven gene cassettes and eight distinct profiles of gene cassette arrays, namely, *aadA1* (10 isolates), *aadA2* (two isolates), *dfrA12* (two isolates), *aadA1-dfrA1* (seven isolates), *aadA1-dfrA12* (six isolates), *aadA5-dfrA17* (one isolate), *aadA1-aadA2-dfrA12* (three isolates) and *aadA1-aadA5-dfrA5* (one isolate), were described. The 2.5 kb amplicon consists of *aadA1-aadA2-dfrA12* and *aadA1-aadA5-dfrA5* (Table 10).

PFGE analysis of isolates containing class 1 integrons

The genetic relatedness among the multidrug resistant *E. coli* isolates carrying integrons was established based on their *XbaI*-digested chromosomal DNA fragments, and the most commonly detected genotypes are depicted in Table 10 and Figure 15. Several profiles were observed, with 12–25 discernible DNA

fragments from 38 *intI1* positive isolates when analyzed by the Dice coefficient method. When an 80% cut-off band pattern similarity was used, 28 different PFGE clusters were observed, whereas 26 clusters were detected when a 70% cut-off band pattern similarity was applied. Strong relationships (>90% similarity) were encountered in six clusters constituting 12 isolates sharing the same antibiotics resistance spectrum and resistance gene pattern. For instance, isolates EC174 and EC175 had more than 97% band pattern similarity, as well as the same antibiotic resistance pattern (TE-S-NA), integron gene cassette arrays (*aadA1-dfrA12*), and resistance genes [*tet(A)*, *sul1* and *floR*].

Discussion

Our study investigated the association of class 1 integron carriage, phylogenetic group affiliation and different tetracycline antibiotics resistance patterns in commensal *E. coli* strains isolated from cattle farms in Korea. All 92 *E. coli* isolates were significantly resistant to tetracycline and oxytetracycline. These findings indicate a widespread application of these antibiotics either for therapeutic purposes or as a supplement for promoting growth, and this continuous exposure to tetracyclines has led to a higher percentage of tetracycline-resistant *E. coli* isolates. The findings of our work were comparable with other observations where a high prevalence of resistance to antimicrobials commonly used with livestock, such as tetracycline and streptomycin were observed in commensal *E. coli* isolated from food-producing animals in South Korea. For instance, Lim *et al.* (2007) observed

tetracycline (30.5%) as the most frequently observed resistance in *E. coli* isolates of cattle origin, and Kang *et al.* (2005) showed that *E. coli* isolates recovered from swine with diarrhea were highly resistant to streptomycin (99.0%) and tetracycline (97.1%); furthermore, the work of Shin *et al.* (2014) also demonstrated that the most prevalent resistance phenotype observed was streptomycin (63.1%), followed by tetracycline (54.5%). Tetracycline antibiotics have long been the single most commonly used class of antimicrobial in livestock, accounting for around 50% of the total amount of antimicrobial consumption both in the USA (USFDA, 2015) and Korea (KFDA, 2004), and it is, therefore, not surprising to observe tetracycline resistance as the most frequently observed resistance class of antimicrobial in *E. coli* isolates. As indicated by the animal and plant quarantine agency of Korea (APQA) (APQA, 2012), although gradually decreasing since 2003, tetracyclines still comprise the predominant antibiotics sold for veterinary use.

The investigated isolates were recovered from clinically healthy animals; accordingly, B1 (58.7%), which is commonly associated with nonpathogenic commensal strains, was the common phylogroup classified. Accordingly, only a single isolate was classified into phylogenetic group B2, which is normally linked with pathogenicity (Carlos *et al.*, 2010). Moreover, no isolates categorized as B2 carried the *intI1* gene, which is similar to the results of a previous study that demonstrated that the B2 phylogroup has a lower tendency to harbor integrons than other phylogroups (Skurnik *et al.*, 2005). In the present study, significant differences in the numbers of isolates with and without integrons were observed

(P< 0.01) in isolates categorized under phylogroups D and B1. These agree with those of a previous study that demonstrated that strains associated with phylogroup A tend to carry integrons less often than those associated with D phylogroup (Singh *et al.*, 2017; Wu *et al.*, 2011). In contrast, affiliation with a specific phylogenetic group was not linked to the presence of integrons in *E. coli* strains recovered from river water (Koczura *et al.*, 2013). This variability is likely because of ecological differences among the sites from which the *E. coli* isolates were recovered that may influence their ability to harbor integron genes.

In the present study, the *tet(A)* gene was the predominant resistance determinant, followed by the *tet(B)* gene. There is general agreement regarding the widespread importance of the link between the *tet(A)* and *tet(B)* genes and resistance to tetracycline antibiotics in *Enterobacteriaceae* as reported by multiple investigators (Koo and Woo, 2011; Thaker *et al.*, 2010; Shin *et al.*, 2015). In this study, isolates having more than one *tet* gene were also observed in 8.7% of the strains, which is a common phenomenon in *E. coli* isolates of cattle origin. Previous studies have shown similar results, in which 3.5% (Marshall *et al.*, 1983), 5.4% (Sengeløv *et al.*, 2003) and 22.2% (Bryan *et al.*, 2004) of isolates had two *tet* genes, with only a slight difference in the total number of isolates used between the studies. The acquisition of more than one *tet* gene by a given strain is attributed to powerful selection pressures due to the high level of tetracycline in the environment rather than to a special selective advantage conferred by the *tet* genes (Koo and Woo, 2011).

There are varying accounts of which *tet* gene is most frequently reported in different countries. For example, Karami *et al.* (2006) reported that *tet(B)* was the most frequently observed (51%) among commensal *E. coli* strains from Sweden, while Shin *et al.* (2015) and Dessie *et al.* (2013) reported that *tet(A)* accounted for 46.5% and 63.2% of all *tet* genes detected in Korea, respectively. A significantly higher frequency of the *tet(A)* gene ($P<0.01$) was also observed in isolates with integrons, demonstrating an association between *tet(A)* carriage and presence of class 1 integrons. This observation has previously been reported by others, who found that *intI1* and *tet(A)* coexisted on the same large transferable plasmid or other genetic elements in *E. coli*, validating an established association between tetracycline resistance genes and class 1 integrons (Sunde and Norström, 2006; Boerlin *et al.*, 2005). *sul1* was identified in 39.1% of isolates; since it is commonly linked to integrons and transposons as a component of the 3′-CS, previous studies have similarly reported it among bacteria of the family *Enterobacteriaceae* (Vinué *et al.*, 2008).

In the present study, 41.3% of *E. coli* isolates harbored *intI1* gene-encoding class 1 integrons. A comparable result regarding the prevalence of class 1 integrons was previously reported in Korea and other countries; for instance, 40% of the *E. coli* isolates carried class 1 integrons in Lithuania (Povilonis *et al.*, 2010), 49.8% in Italy (Cavicchio *et al.*, 2015), and 27% in the United States (Shaheen *et al.*, 2010), as well as 44% of the commensal *E. coli* isolates from poultry in Korea (Kang *et al.*, 2005). Non-classical integrons lacking the normal 3´-CS were detected in only two

class 1 integron-positive isolates. Similar observations were made for *intI1*-positive *E. coli* isolates that originated from food, animals, and healthy humans (Sáenz *et al.*, 2010). Moreover, 32 (84.2%) of the 38 *intI1*-positive isolates had variable regions containing gene cassettes. Overall, our analysis showed that the *aad* and *dfr* families comprise the majority of class 1 integron gene cassettes, similar to the results reported for *E. coli* originating from beef cattle (Wu *et al.*, 2011). In the present study, *aadA1-dfrA1* was the most commonly detected combination, which is in agreement with previous reports on isolates recovered from clinical and healthy animals, humans and food samples (Sáenz *et al.*, 2004; Povilonis *et al.*, 2010; Kang *et al.*, 2005; Cocchi *et al.*, 2007). Furthermore, 27 (71.1%) of the cassette arrays contained the *aadA1* gene, either alone or in combination with other gene cassette arrays that encode aminoglycoside adenyltransferases, which confer resistance to streptomycin/spectinomycin (Koczura *et al.*, 2013). When comparison between environmental and clinical isolates from the same region was made, commensal *E. coli* isolates from animals mostly carried a single gene cassette, whereas clinical *E. coli* isolates from humans had multigene cassettes (Kang *et al.*, 2005). In addition, between one and three gene cassette arrays were found in a single isolate, which is a distinguishing feature of class 1 integrons in which no more than 6 gene cassettes are carried in the variable region (Escudero *et al.*, 2015).

Based on the results of the *XbaI*-PFGE, the *E. coli* isolates carrying class 1

integrons could be categorized into 28 and 26 different PFGE cluster groups when 80% and 70% cut-off band pattern similarities were applied, respectively. In this study, *E. coli* isolates carrying integrons showed a high degree of polymorphism. This diverse clonal relationship resulted from the horizontal transfer of resistance genes between different strains, rather than a dissemination of a single clonal strain, as previously described (Sawant *et al.*, 2007).

Conclusion

Due to their carriage of resistant genes and class 1 integrons, commensal *E. coli* isolates have a significant implication in public health through their ability to disseminate antibiotic resistant genes via contamination of the food chain. A positive association was observed between isolates harboring the *intI1* and *tet(A)* genes, confirming that isolates containing the *tet(A)* gene are more likely to carry class 1 integrons. Likewise, affiliation with phylogroup D was positively associated with the presence of class 1 integrons. Further detailed investigation of the class 1 integron genetic content should be conducted to provide a more complete understanding of the molecular mechanisms responsible for multidrug resistance in *E. coli* strains. Moreover, the interrelation of integron related resistance genes with other factors should be studied by integrating environmental and veterinary factors and factors associated with the food chain. Accordingly, the resulting advances could have a profound effect on clinical practice, infection control measures and treatment options, both in veterinary and human medicine.

Table 7. Primers used for the PCR detection of resistance genes

Primer name	Target gene	Nucleotide sequence	Annealing temperature (℃)	Amplicon size (bp)	Reference
TetA-F	*tet(A)*	GGCGGTCTTCTTCATCATGC	55	502	(Lanz *et al.*, 2003)
TetA-R		CGGCAGGCAGAGCAAGTAGA			
TetB-F	*tet(B)*	CATTAATAGGCGCATCGCTG	55	930	(Lanz *et al.*, 2003)
TetB-R		TGAAGGTCATCGATAGCAGG			
TetC-F	*tet(C)*	GCTGTAGGCATAGGCTTGGT	55	888	(Lanz *et al.*, 2003)
TetC-R		GCCGGAAGCGAGAAGAATCA			
TetD-F	*tet(D)*	GAGCGTACCGCCTGGTTC	55	780	(Koo and Woo, 2011)
TetD-R		TCTGATCAGCAGACAGATTGC			
TetE-F	*tet(E)*	AAACCACATCCTCCATACGC	55	278	(Levy *et al.*, 1999)
TetE-R		AAATAGGCCACAACCGTCAG			
TetG-F	*tet(G)*	GCTCGGTGGTATCTCTGCTC	55	468	(Zhao and Aoki, 1992)
TetG-R		AGCAACAGAATCGGGAACAC			
Sul1-F	*sul1*	CGGCGTGGGCTACCTGAACG	57	433	(Kerrn *et al.*, 2002)
Sul1-R		GCCGATCGCGTGAAGTTCCG			
Sul2-F	*sul2*	CGGCATCGTCAACATAACCT	57	721	(Lanz *et al.*, 2003)
Sul2-R		TGTGCGGATGAAGTCAGCTC			
Sul3-F	*sul3*	CAACGGAAGTGGGCGTTGTGGA	57	244	(Kozak *et al.*, 2009)
Sul3-R		GCTGCACCAATTCGCTGAACG			
Cat-F	*Cat*	GGT GAG CTG GTG ATA TGG	55	209	(Orman *et al.*, 2002)
Cat-R		GGG ATT GGC TGA GAC GA			
Flor -F	*flor*	CAC GTT GAG CCT CTA TAT	55	868	(Sáenz *et al.*, 2004)
Flor -R		ATG CAG AAG TAG AAC GCG			
CmlA -F	*cmlA*	TGT CAT TTA CGG CAT ACT CG	55	455	(Sáenz *et al.*, 2004)
CmlA -F		ATC AGG CAT CCC ATT CCC AT			
Var1-F	*var1*	GGCATCCAAGCAGCAAG	55	Variable	(Levesque *et al.*, 1995)
Var1-R		AAGCAGACTTGACCTGA			
qacEΔ1 F	*qacEΔ1*	ATCGCAATAGTTGGCGAAGT	60	225	(Wan and Chou, 2015)
qacEΔ1 R		CAAGCTTTTGCCCATGAAGC			
IntI1-F	*intI1*	GGGTCAAGGATCTGGATTTCG	60	483	(Vali *et al.*, 2007)
IntI1-R		ACATGCGTGTAAATCATCGTCG			

Table 8. Susceptibility to various tetracycline antibiotics stratified by the presence or absence of class 1 integrons

Antimicrobial agents	*Intl1* present					*Intl1* Absent					P value
	MIC (µg/ml)				Number (%) of resistant isolates	MIC (µg/ml)				Number (%) of resistant isolates	
	Range		MIC_{50}	MIC_{90}		Range		MIC_{50}	MIC_{90}		
Tetracycline	64	>256	128	256	38 (41.3)	16	>256	128	256	54 (58.7)	0.244
Doxycycline	4	128	16	64	36 (39.1)	4	64	32	64	44 (47.8)	0.975
Oxytetracycline	64	>256	>256	>256	38 (41.3)	32	>256	256	>256	54 (58.7)	**P<0.006**
Minocycline	4	64	8	32	14 (15.2)	2	64	16	32	28 (30.4)	0.267
Tigecycline	0.25	2	1	1	-	0.25	8	0.5	1	1 (1.1)	0.054

*Number indicated in bold represent significance difference (P< 0.05)

Table 9. Association between integron-positive and integron-negative *E. coli* isolates and the frequencies of *tet* genes

tet genes	Class 1 integron presence		P value
	intI1 positive	*intI1* negative	
tet(A)	26	21	**P< 0.01**
tet(B)	12	30	**0.023**
tet(C)	-	5	0.054
tet(G)	1	5	0.205
tet(A)+ tet (B)	-	1	0.399
tet(A)+ tet (C)	-	1	0.399
tet(B)+ tet (G)	1	5	0.205

*Numbers indicated in bold represent significance difference (P< 0.05)

Table 10. Characterization of *E. coli* isolates harboring class 1 integrons and description of their associated gene cassettes

Isolates No.	3'CS	Cassette amplicons (bp)	Other resistance gene pattern	Integron gene cassette arrays	PFGE pattern	Resistance pattern
EC151	*qacEΔ1-sul1*	1500	*tetA, sul1, sul2, cat1*	*aadA1-dfrA1*	B	TE-S-C-SXT
EC139	*qacEΔ1-sul1*	1500	*tetB, tetG, sul1, sul2, cat1, floR*	*aadA1-dfrA1*	A	TE-S-C-AMP-SXT-CIP-NA
EC143	-	1500	*tetA, sul3, cmlA, cat1*	*aadA1-dfrA12*	U	TE-S-AMP-SXT-CIP-NA
EC147	*qacEΔ1-sul1*	1000	*tetA, sul1*	*aadA2*	H	TE-S-NA
EC152	*qacEΔ1-sul1*	1000	*tetA, sul1*	*aadA1*	H	TE-S-AMP-SXT-NA
EC153	*qacEΔ1-sul1*	2500	*tetA, sul1*	*aadA1-aadA2-dfrA12*	H	TE-S-NA
EC155	qacEΔ1-sul1	-	*tetB, sul1, sul2, cat1*	-	S	TE-S-C-SXT-CIP-NA
EC156	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1*	H	TE-S-AMP-NA
EC157	*qacEΔ1-sul1*	1500	*tetB, sul1, sul2, cat1*	*aadA5-dfrA17*	G	TE-S-AMP-SXT-CIP-NA
EC159	*qacEΔ1-sul1*	1500	*tetB, sul1, sul2, cat1*	*dfrA12*	R	TE-S-C-AMP-SXT-CIP-NA
EC160	*qacEΔ1-sul1*	1500	*tetB, sul1, sul2, cat1*	*dfrA12*	R	TE-S-C-AMP-SXT-CIP-NA
EC161	-	-	*tetB, sul3, cmlA*	-	Q	TE-S-C
EC162	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1*	N	TE-S-NA
EC163	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1-dfrA1*	L	TE-S-NA
EC164	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1*	M	TE-S-SXT-NA
EC165	*qacEΔ1-sul1*	1000	*tetB, sul1, sul2, cat1, floR*	*aadA1*	T	TE-S-C-AMP-SXT-CIP-NA
EC166	*qacEΔ1-sul1*	-	*tetB , sul1, sul2, cat1*	-	G	TE-S-C-AMPCIP-NA
EC167	*qacEΔ1-sul1*	2500	*tetA, sul1, floR*	*aadA1-aadA2-dfrA12*	K	TE-S-NA
EC172	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1*	J	TE-S-NA

EC173	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1*	J	TE-S-NA
EC174	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1-dfrA12*	I	TE-S-NA
EC175	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1-dfrA12*	I	TE-S-NA
EC176	*qacEΔ1*	1000	*tetB, sul2, sul3, cmlA, floR*	*aadA1*	W	TE-S-C
EC177	*qacEΔ1-sul1*	1000	*tetA, sul1, floR*	*aadA1*	Y	TE-S-AMP
EC178	*qacEΔ1-sul1*	2500	*tetA, sul1, floR*	*aadA1-aadA5-dfrA5*	Y	TE-S-AMP-NA
EC179	*qacEΔ1*	2500	*tetB, sul2, sul3, cml1, floR*	*aad1-aadA2-dfrA12*	O	TE-S-C
EC180	*qacEΔ1*	1000	*tetB, sul2, sul3, cml1, floR*	*aadA2*	P	TE-S-C
EC181	*qacEΔ1-sul1*	1500	*tetB, sul1, sul2, cat1*	*aadA1-dfrA1*	D	TE-S-C-AMP-SXT-CIP-AMC-NA
EC185	*qacEΔ1-sul1*	1500	*tetA, sul1, sul2*	*aadA1-dfrA1*	Z	TE-S-AMP-SXT-AMC-NA
EC191	*qacEΔ1*	1000	*tetA, sul3, cml1, cat1, floR*	*aadA1-dfrA12*	A2	TE-S-C-SXT
EC194	*qacEΔ1-sul1*	1500	*tetA, sul1, cat1*	*aadA1-dfrA1*	F	TE-S-AMP-SXT
EC198	*qacEΔ1-sul1*	1500	*tetA, sul1, cat1*	*aadA1-dfrA1*	F	TE-S-AMP-SXT
EC209	*qacEΔ1*	1000	*tetA, sul3, cml1, cat1, floR*	*aadA1-dfrA12*	X	TE-S-C-SXT
EC230	*qacEΔ1*	-	*tetA, sul2, cat1*	-	E	TE-S-C-AMP-SXT-NA
EC231	*qacEΔ1*	-	*tetA, sul2, cat1*	-	E	TE-S-AMP
EC254	*qacEΔ1-sul1*	1000	*tetA, sul1, cat1*	*aadA1-dfrA12*	V	TE-S-*AMP*
EC258	*sul1*	-	*tetA, sul1*	-	A1	TE-AMP-SXT
EC262	*qacEΔ1*	1000	*tetA, sul2, cat1, floR*	*aadA1*	C	TE-S-C-AMP

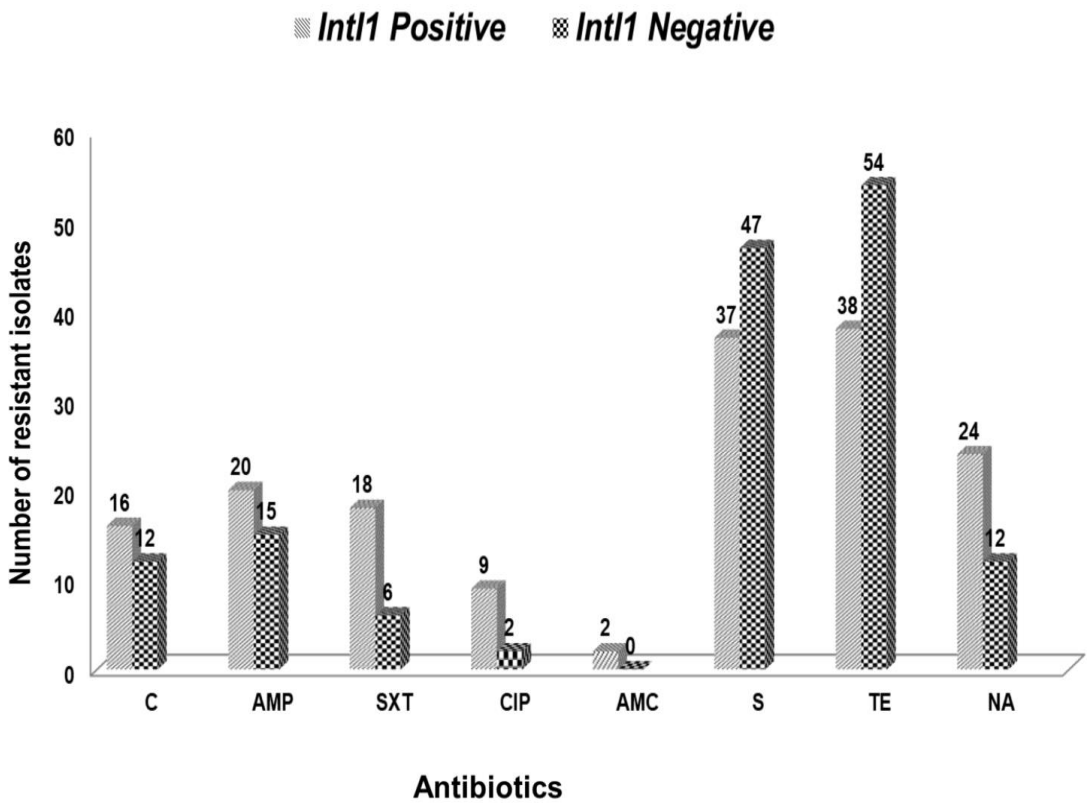


Figure 13. Different classes of antibiotics with respect to their presence or absence in class 1 integrons. C, chloramphenicol; AMP, ampicillin; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; AMC, amoxicillin-clavulanic; S, streptomycin; TE, tetracycline; NA, nalidixic acid.

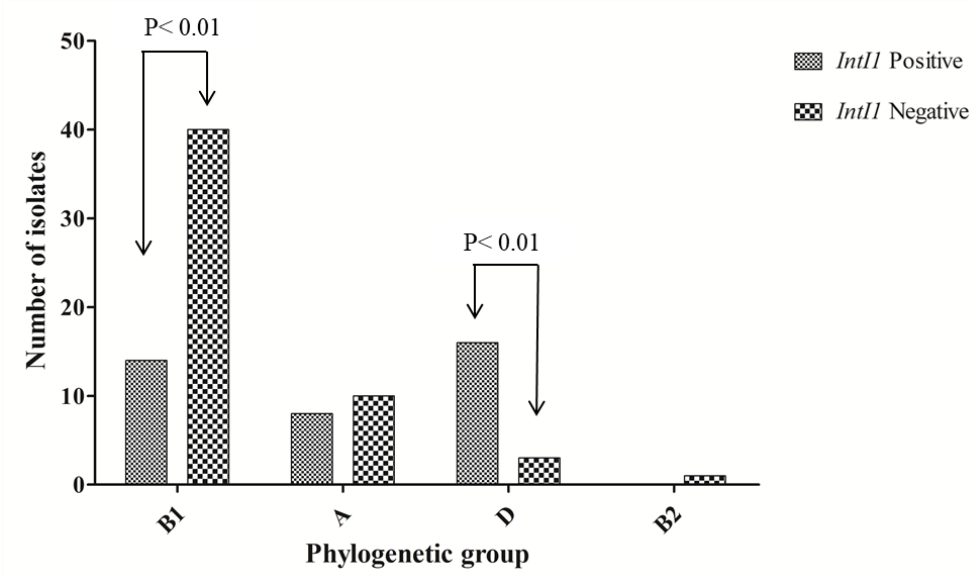
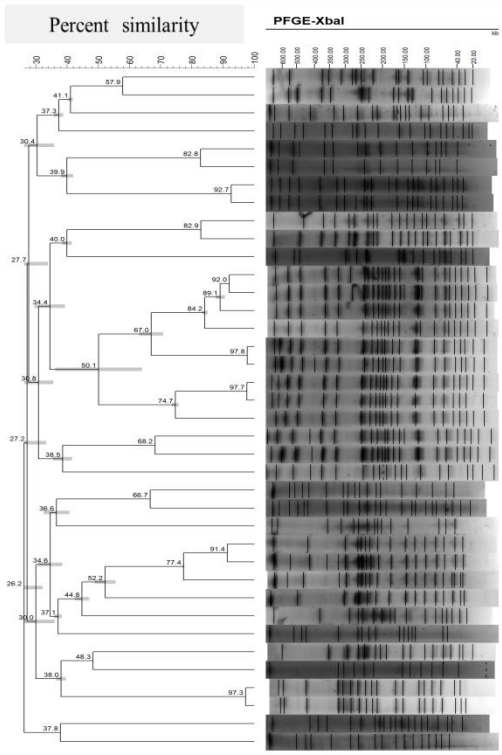


Figure 14. Association between phylogenetic group and isolates carrying class 1 integrons



Isolates No.	Resistant genes profile	PFGE pattern	Resistance pattern
EC139	*tetB, tetG, sul1, sul2, cat1, floR*	A	TE-S-C-AMP-SXT-CIP-NA
EC151	*tetA, sul1, sul2, cat1*	B	TE-S-C-SXT
EC262	*tetA, sul2, cat1, floR*	C	TE-S-C-AMP
EC181	*tetB, sul1, sul2, cat1*	D	TE-S-C-AMP-SXT-CIP-AMC-NA
EC230	*tetA, sul2, cat1*	E	TE-S-C-AMP-SXT-NA
EC231	*tetA, sul2, cat1*	E	TE-S-AMP
EC194	*tetA, sul1, cat1*	F	TE-S-AMP-SXT
EC198	*tetA, sul1, cat1*	F	TE-S-AMP-SXT
EC157	*tetB, sul1, sul2, cat1*	G	TE-S-AMP-SXT-CIP-NA
EC166	*tetB , sul1, sul2, cat1*	G	TE-S-C-AMPCIP-NA
EC191	*tetA, sul3, cml1, cat1, floR*	A2	TE-S-C-SXT
EC152	*tetA, sul1*	H	TE-S-AMP-SXT-NA
EC153	*tetA, sul1*	H	TE-S-NA
EC147	*tetA, sul1*	H	TE-S-NA
EC156	*tetA, sul1, floR*	H	TE-S-AMP-NA
EC174	*tetA, sul1, floR*	I	TE-S-NA
EC175	*tetA, sul1, floR*	I	TE-S-NA
EC172	*tetA, sul1, floR*	J	TE-S-NA
EC173	*tetA, sul1, floR*	J	TE-S-NA
EC167	*tetA, sul1, floR*	K	TE-S-NA
EC163	*tetA, sul1, floR*	L	TE-S-NA
EC164	*tetA, sul1, floR*	M	TE-S-SXT-NA
EC162	*tetA, sul1, floR*	N	TE-S-NA
EC179	*tetB, sul2, sul3, cml1, floR*	O	TE-S-C
EC180	*tetB, sul2, sul3, cml1, floR*	P	TE-S-C
EC161	*tetB, sul3, cmlA*	Q	TE-S-C
EC159	*tetB, sul1, sul2, cat1*	R	TE-S-C-AMP-SXT-CIP-NA
EC160	*tetB, sul1, sul2, cat1*	R	TE-S-C-AMP-SXT-CIP-NA
EC155	*tetB, sul1, sul2, cat1*	S	TE-S-C-SXT-CIP-NA
EC165	*tetB, sul1, sul2, cat1, floR*	T	TE-S-C-AMP-SXT-CIP-NA
EC143	*tetA, sul3, cmlA, cat1*	U	TE-S-AMP-SXT-CIP-NA
EC254	*tetA, sul1, cat1*	V	TE-S-***AMP***
EC176	*tetB, sul2, sul3, cmlA, floR*	W	TE-S-C
EC209	*tetA, sul3, cml1, cat1, floR*	X	TE-S-C-SXT
EC177	*tetA, sul1, floR*	Y	TE-S-AMP
EC178	*tetA, sul1, floR*	Y	TE-S-AMP-NA
EC185	*tetA, sul1, sul2*	Z	TE-S-AMP-SXT-AMC-NA
EC258	*tetA, sul1*	A1	TE-AMP-SXT

Figure 15. Genetic relatedness of *E. coli* isolates with class 1 integrons indicated by *XbaI*-digested chromosomal DNA

Chapter III

Genomic analysis of highly prevalent plasmid harboring *mcr-1* and *mcr-3* genes in colistin resistance *Escherichia coli* isolates from food-producing animals in South Korea

Abstract

The aim of this study was to describe the prevalence, genetic mechanism, and transferability of plasmid-mediated colistin resistance among commensal *E. coli* isolates recovered from fecal samples of healthy food-producing animals in South Korea. In total 636 commensal *E. coli* isolates recovered between 2014 and 2017 were used in this study. MIC testing was conducted according to CLSI and EUCAST protocols. Detection of *mcr-3 and mcr-1* was performed using PCR. Conjugation experiments were performed using broth mating method. ResFinder2.1 and PlasmidFinder was used for *in silico* analysis. Plasmid p17S-208 was analyzed using WGS on a PacBioRSII platform. Of the 636 investigated *E.*

coli isolates, 9 (1.4%) were resistant to colistin, of which *mcr-3* and *mcr-1* gene was detected in 2 (MIC of 4 mg/ml) and 3 isolates (MICs 4 mg/ml to 8 mg/ml), respectively. All isolates harboring *mcr-1* and *mcr-3* genes were MDR, and confirmed to transfer their genes to a recipient *E. coli J53* with conjugation efficiencies of the isolates lay between $8.6x10^{-3}$ and $1.9x10^{-4}$. The WGS reads of p17S-208 contained 241 predicted ORFs. p17S-208 (260,339bp), encoding *mcr-3* gene, showed 99% nucleotide identity with plasmid pWJ1 from a swine *E. coli* strain containing *mcr-3*. ResFinder and plasmidfinder tool demonstrated the presence of resistant genes to various antibiotic agents and a typical IncHI2-type backbone. Plasmid MLST showed that p17S-208 belonged to ST3. Comparison of the p17S-208 plasmid's deduced amino acid sequence depicted 35% identity with *mcr-2* isolated from Belgium (pKP37-BE) and *mcr-1* from South Korea (pEC28), whereas 100% identity with *mcr-3* from China (pWJ1). In Conclusion, care when applying colistin for therapeutic purposes has to be encouraged and regular WGS-based surveillance to early detection of *mcr-3* genes, and to describe novel *mcr* genes.

Keywords: *E. coli*, *mcr-1* gene, *mcr-3* gene, colistin resistance, healthy-animals transferability, IncHI2-ST3-type plasmid

Introduction

The increase in colistin-resistant *Enterobacteriaceae* is a worldwide concern. Since it was first described in China in 2015 (Liu *et al.*, 2016), the plasmid-mediated *mcr-1* gene has been gaining huge attention worldwide, and the awareness and the extent of the problem also increased gradually. Following the first report, another *mcr*-variant of the plasmid-mediated colistin resistance called *mcr-2* gene was identified in *E.coli* strain recovered from swine and cattle in Belgium (Xavier *et al.,* 2016), and in June 2017, *mcr-3* was detected on the 261 kb plasmid (IncHl2) of *E. coli* isolated from swine in China (Yin *et al.,* 2017). This third variant showed nucleotide sequence similarities of 45% and 47% with previously detected *mcr-1* and *mcr-2* genes, respectively. Quite recently, a novel *mcr-4* gene in *E. coli* strains recovered from pigs was detected in Europe (Carattoli *et al.,* 2017).

Excessive use of colistin in veterinary medicine has resulted in the emergence of colistin resistance; for example, 6–16 tons of colistin was annually applied to food-producing animals between the years 2005 and 2015 (Lim *et al.,* 2016). Currently, colistin is the main therapeutic choice used to treat infections in pigs, and it has emerged as a critical antibiotic for treating illnesses caused by multidrug-resistant Gram-negative bacteria. There is a need for strict regulation on the use of colistin in veterinary medicine to reduce the danger of transferable *mcr* genes to humans via the food chain (Liu *et al.,* 2016).

Thus, early detection of colistin resistance is mandatory. Furthermore, it is also important to note that genetic analysis of plasmid-mediated *mcr* genes, coding for phosphoethanolamine transferase, by employing whole-genome sequencing (WGS)-based surveillance are clearly warranted in assisting early detection. Herein, we report the first detection of the *mcr-3* gene variant in South Korea and describe the molecular characteristics of *mcr-1* and *mcr-3* harboring *E. coli* isolates recovered from healthy animals. Furthermore, to understand the genetic backgrounds, *mcr-3*-harboring *E. coli* isolate was further subjected for an elaborated WGS analysis.

Materials and Methods

Bacterial isolation

A total of 636 commensal *E. coli* isolates recovered from fecal samples obtained from clinically healthy animals (341 from cattle, 265 from swine, and 30 from chickens) during a nationwide surveillance study conducted between 2014 and 2017 on antimicrobial susceptibility. Briefly, one sample was collected from each animal at farms that normally do not use antibiotics as feed additives, other than for a therapeutic purpose. Fecal samples were freshly collected from cattle, swine, and chickens and *E. coli* isolates were obtained by culturing samples on MacConkey and Eosin methylene blue agar. Isolate identities were confirmed by amplifying the 16S rRNA gene.

Antimicrobial resistance profiling of *E. coli* isolates

MIC testing was conducted according to the recommendation of the Clinical Laboratory Standards Institute (CLSI, 2015), and resulting MIC values were interpreted using CLSI and European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2017) breakpoints. The following antibiotics were tested by the micro-broth dilution method on cation adjusted Mueller–Hinton broth (Oxoid, Basingstoke, UK); CL, colistin; LEV, levofloxacin; OXY, oxytetracycline; MIN, minocycline; TOB, tobramycin;CF, cephalothin; NEO, neomycin; STR, streptomycin; AMP, ampicillin; TET,tetracycline; CIP, ciprofloxacin and DOX, doxycycline (Sigma-Aldrich, St. Louis, MO, USA) .

PCR detection of colistin resistance genes

PCR amplification to investigate the *mcr-1* and *mcr-3* encoding genes was conducted on isolates exhibiting colistin resistance using previously employed primer pairs and PCR conditions (Liu *et al.*, 2016; Yin *et al.*, 2017). The primer pairs used were *mcr-1F* (5'- CGGTCAGTCCGTTTGTTC-3') and *mcr-1R* (5'-CTTGGTCGGTCTGTAGGG-3'), and *mcr-3F* (5′-TTGGCACTGTATTTTGCATTT-3′), and *mcr-3R* (5′-TTAACGAAATTGGCTGGAACA-3′), which generate 315- and 542-bp fragments, respectively. The PCR cycling conditions were as follows: 5 min at 94℃ for initial denaturation followed by 30 cycles of 30 sec at 94℃, 30 sec at 55℃ and 1 min at 72℃ and then final extension for 10 min at 72℃.

Conjugation analysis

The transferability of colistin resistant genes between donors (*mcr-1* and *mcr-3* positive isolates) and recipient bacteria [*E. coli* J53 AZ^R (azide resistant)] was assessed by conjugation experiments using the broth mating method (Wang *et al.*, 2003).

Whole genome sequencing (WGS)-based analysis

Identification of known antimicrobial resistance genes in the next-generation sequencing data was performed on ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder), and PlasmidFinder (https://cge.cbs.dtu.dk/services) was used to detect plasmid replicons in the sequences (Zankari *et al.*, 2012; Carattoli *et al.*, 2014). To identify the colistin resistance mechanism and characterize the genetic environment surrounding the colistin resistance gene, WGS was performed. The *mcr-3*-harboring plasmid, p17S-208, was recovered from swine in 2017. The PacBio RS II platform was used to perform the sequencing (Pacific Biosciences, Menlo Park, CA). This was followed by assembly of sequence reads using the de novo assembly method in the HGAP.2 assembler. Finally, putative gene coding sequences from the assembled contig were annotated by using Blastall 2.2.26 and Glimmer v3.02. Ribosomal and transfer RNAs were predicted by using RNAmmer 1.2 and tRNAscan-SE, respectively. *mcr* gene harboring colistin-resistant isolates underwent molecular typing following the protocols specified at the E. coli multilocus sequence typing (MLST) website of the Warwick database

(http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The nucleotide sequences of the *mcr-3* gene identified in this study were compared with representative sequences from the National Center for Biotechnology Information (NCBI) by using the neighbor-joining method. Comparative evolutionary analyses were conducted in MEGA6 (Alikhan *et al.,* 2011).

Results

Susceptibility testing and PCR detection of resistance genes in *E. coli* isolates

Of the 636 investigated E. coli isolates, 9 (1.4%) were resistant to colistin, and the *mcr-3* gene was detected in 2 (MIC of 4 mg/ml) of these 9 isolates, whereas the *mcr-1* gene was detected in 3 isolates (MICs 4 mg/ml to 8 mg/ml). For isolates harboring the *mcr-1* gene (isolated between 2015 and 2016) colistin MIC values ranging from 4 mg/ml to 8 mg/ml and belonged to phylogenetic groups A and B, whereas *mcr-3* (isolated between 2016 and 2017) harboring isolates both had a colistin MIC value of 4 mg/ml and affiliated to D and A phylogenetic groups (Table 11).

DNA sequencing of the PCR fragments was performed, with following BLASTN against the GenBank database of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results revealed 99% and 98% sequence identities with previously deposited *mcr-1* (GenBank accession no.KY964067.1) and *mcr-3* (GenBank accession no.KY924928.1) gene

sequences, respectively. All isolates harboring *mcr-1* and *mcr-3* genes were MDR, resistance for at least 3 antimicrobial agents, and harboring various antimicrobial resistant genes which confers resistance to different classes of antimicrobials (Table 11).

Conjugation assay

All the 5 isolates that harbored *mcr-1* or *mcr-3* were capable of transferring their genes to the recipient *E. coli J53* AZ^R strain as indicated by broth mating assay. The conjugation efficiencies of the isolates lay between $8.6x10^{-3}$ and $1.9x10^{-4}$ (Table 12).

Genetic analysis of plasmid harboring mcr-3 gene

The WGS reads of the plasmid contained 241 predicted ORFs and the GC content of the contigs was 46.24% (Figure. 16). In the p17S-208 plasmid, the plasmid-mediated colistin resistance gene, *mcr-3*, is located between 21,566 and 23,191 (length 1,626 bp). The 260,339bp plasmid (p17S-208) encoding *mcr-3* showed 99% nucleotide identity and 83% coverage with plasmid pWJ1 from a swine *E. coli* strain containing *mcr-3* (KY924928). Potential ORFs were identified in the sequences flanking the *mcr-3* gene of plasmid p17S-208, and most of the translated amino acids that encode for proteins were located downstream of the *mcr-3* gene.

ResFinder server demonstrated the presence of genes resistant to various

antibiotic agents such as aminoglycoside [*aph (3")-1b*, *aph (6)-1d* and *aph (3')-1a*], colistin (*mcr-3*), phenicol (*floR*) and sulphonamide (*sul2*). The *in silico* plasmid finder tool, used to predict the plasmid sequences on *de novo* assembled contigs, demonstrated that p17S-208 exhibited a typical IncHI2-type backbone. BLASTn local alignments at the GenBank database of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed 100% sequence identity with the previously deposited reference plasmid IncHI2-R478 (GenBank accession no. BX664015), while plasmid MLST on the pMLST-1.4 server at the center of genetic epidemiology (https://cge.cbs.dtu.dk/services/pMLST-1.4) demonstrated that the plasmid belonged to sequence type ST3. In addition, fimH allele could not be detected in either pWJ1 or p17S-208 when using FimTyper (https://cge.cbs.dtu.dk/services/FimTyper). Sequence typing showed that two (DCA4245 and15S-103) of the three colistin-resistant *mcr-1*-harboring *E. coli* isolates had the same sequence types (ST10), while the ST for the remaining isolate (15S-123) was ST4398. The other *mcr-3*-harboring isolate (16S-251) was assigned to ST1.

Our comparison of the p17S-208 plasmid's *mcr-3* gene deduced amino acid sequence with 28 other bacterial species' deduced amino acid sequences of putative phosphoethanolamine transferase depicted that p17S-208 had 94% query coverage with 35% identity to amino acids found in plasmid pKP37-BE isolated from a swine ST10 E. coli isolate containing *mcr-2* in Belgium (SBV31106.1) and in an IncI2 pEC28 *E. coli* containing *mcr-1* isolated from a hospitalized patient from

South Korea (ASD54270.1). On the other hand, *mcr-3* of pWJ1 had 32.5% and 31.7% deduced amino acid similarity with the *mcr-1* and *mcr-2* deduced amino acid sequences, respectively. Furthermore, the *mcr-3* gene detected in this study had 98% query coverage and 100% identity with that of amino acid sequences found in the pWJ1 plasmid (ASF81896.1) of *E. coli* origin (Figure. 17).

Discussion

The increase in colistin-resistant *Enterobacteriaceae* is a worldwide concern. The emergence of the variants of *mcr* gene has attracted considerable attention because it confers resistance to the last resort used to treat serious infections caused by carbapenem resistant *Enterobacteriaceae*. The first plasmid-mediated *mcr-1* gene was described in China in 2015 (Liu *et al.,* 2016). Since then other variants, *mcr-2, mcr-3,* and *mcr*-4 continue to be reported worldwide (Xavier *et al.,* 2016; Yin *et al.,* 2017; Carattoli *et al.,* 2017).

Although, to the best of our knowledge, this is the first report of the *mcr-3* gene in South Korea, the *mcr-1* gene has been previously detected in human and livestock (Lim *et al.,* 2016; Kim *et al.,* 2017). The emergence of colistin resistance resulted due to excessive consumption of colistin in veterinary medicine. In the years 2005 to 2015 on average 6-16 tons of colistin was annually applied in food producing animals. Besides, colistin is the main treatment option used to treat infections in pigs.

A literature review showed that only 3 reports of *mcr-3* in *E. coli* have been issued, *mcr-3* was first described in China in 2017 (Yin *et al.*, 2017), and this was followed by a Danish report on a hospitalized patient who had traveled to Thailand (Roer *et al.*, 2017), and a Spanish report (Hernández *et al.*, 2017) on the isolation of MDR *E. coli* recovered from cattle. Although the prevalence of the *mcr-1* gene is considered to be very low in isolates from healthy animals, a slight increase in the prevalence of this gene has been reported in South Korea mainly among healthy chickens (Lim *et al.*, 2016). However, in our report all isolates carrying the *mcr-1* gene were from pigs, which might have been due to the investigation of fewer isolates from chickens or possibly further spread of this gene, which necessitates profound surveillance to determine its prevalence in different animal species. The global spread of the *mcr-1* gene suggests there might be a similar chance for *mcr-3* to spread into different geographical regions (Hernández *et al.*, 2017). Interestingly, all isolates harboring *mcr-1* and *mcr-3* genes were MDR. The isolates showed resistance to at least 3 antimicrobials and carried various antimicrobial resistant genes, which concurs with a previous report (Lim *et al.*, 2016).

The plasmid reported here had 99% nucleotide identity with the first reported *mcr-3* harbored plasmid (pWJ1) from china (Yin *et al.*, 2017). Initially, the described *mcr-3* gene was mapped on a 261 kb plasmid, pWJ1, which had an IncHI2 backbone and 18 known antimicrobial resistance gene markers (Yin *et al.*, 2017). The *in silico* investigated isolate carried resistance for therapeutically

important antimicrobial agents both in veterinary and human medicine. Similarly, previous studies have demonstrated that IncHI2-type plasmids retrieved from *E. coli* isolates from food-producing animals have a major role in spreading antibiotic resistance genes (Fang *et al.,* 2016; Sun *et al.,* 2018). Sequence type ST3 IncHI2 plasmid is reported to possess high genetic plasticity and can fuse through mobile genetic elements to fit itself with other plasmids, thereby attracting various important antimicrobial resistance genes (Fang *et al.,* 2018). Two *mcr-1*-harboring *E. coli* isolates were assigned to ST10. This ST has already been reported in a colistin-resistant *E. coli* that harbored the *mcr-1* gene; that strain was identified from stool samples of travelers returning from India, indicating the importation of a plasmid-mediated *mcr-1* gene (Bernasconi *et al.,* 2016). ST10 is the most prevalent sequence type reported from hospitalized patients in different counties within China (Luo *et al.,* 2017) and from food-producing animals sampled in Europe (El Garch *et al.,* 2017).

The deduced amino acid sequence of the *mcr-3* gene of the p17S-208 plasmid identified in this study had shown similarity with other reported amino acid sequence of *mcr-2* gene from Belgium (Xavier *et al.,* 2016) and *mcr-1* gene from Korea (Kim *et al.,* 2017). On the other hand, *mcr-3* of pWJ1 had 32.5% and 31.7% deduced amino acid similarity with the *mcr-1* and *mcr-2* deduced amino acid sequences, respectively (Yin *et al.,* 2017). These results of evolutionary relationships are an indication of close genetic similarity, and that the *mcr-3* variant might have originated from a previous *mcr* variant through the continuous

evolution of the bacterial genome, while it disseminated among different hosts (Zhang *et al.*, 2018).

Conclusion

It would appear that the extensive application of colistin to food-producing animals has promoted the emergence of plasmid-mediated colistin resistance in *E. coli*. Therefore, care when applying colistin for therapeutic and prophylactic purposes has to be encouraged to limit the further spread of plasmid mediated colistin resistance in *Enterobacteriaceae*. Importantly, one crucial concern is the potential for an isolate to acquire resistance to various antimicrobial agents that are used in human and veterinary medicines. On the other hand, ST3-IncHI2 plasmids co-harboring *mcr* genes and other diverse important antimicrobial resistance genes have been increasing among *E. coli* strains from food-producing animals. Hence, it is highly advised to commence a continuous and regular WGS-based surveillance by implementing an appropriate monitoring program on antimicrobial resistance. In addition, this program should include the pattern of antimicrobial use to trace the early dissemination of *mcr-3* genes, and for that matter to describe novel *mcr* genes.

Table 11. MICs, Phylogrouping and antimicrobial resistant genes for colistin resistant *E. coli* isoltaes of animal origin from South Korea between 2015- 2017

Isolates	Province	Origin	Year of isolation	Phylo-group	MIC µg/ml												*mcr* gene detected	Transferability
					CL*	LEV	OXY	MIN	TOB	CF	NEO	STR	AMP	TET	CIP	DOX		
DCA4245	Chungnam	Pig	2016	B1	4	8	≥256	32	8	≥256	128	256	≥256	64	≥128	32	*mcr-1*	Yes
17S-208	Jeollabuk	Pig	2017	D	4	8	256	16	4	32	32	32	≥256	16	≥128	16	*mcr-3*	Yes
16S-251	Chungnam	Pig	2016	A	4	0.5	256	4	32	16	64	256	≥256	8	≥128	16	*mcr-3*	Yes
15S-103	Gyeonggi	Pig	2015	A	8	8	256	16	32	16	1	256	≥256	64	≥128	16	*mcr-1*	Yes
15S-123	Chungnam	Pig	2015	A	8	0.5	≥256	16	4	32	1	128	≥256	32	≥128	16	*mcr-1*	Yes

* EUCAST interpretation was used

Table 12. Conjugation transfer efficiencies of *mcr-3* and *mcr-1* positive *E. coli* isolates, and colistin MIC values for transonjugants.

Isolate	Transferability	MIC (µg/ml)	Recipient (* *E. coli* J53 AZ^R)	Donor	Transonjugants	Conjugation efficiency
DCA4245	+	4	$1.41x10^8$	$2.2X10^6$	$4.1X10^2$	$1.9X10^{-4}$
17S-208	+	4		$1.7X10^5$	$1.47X10^3$	$8.6X10^{-3}$
16S-251	+	4		$7X10^5$	$4.8X10^2$	$6.9X10^{-4}$
15S-103	+	4		$1.1X10^6$	$4.5X10^2$	$4.1X10^{-4}$
15S-123	+	8		$9.2X10^5$	$1.4X10^3$	$1.5X10^{-3}$

*Colistin MIC value for the recipient strain *(E. coli* J53 AZ^R) was 0.5µg/ml

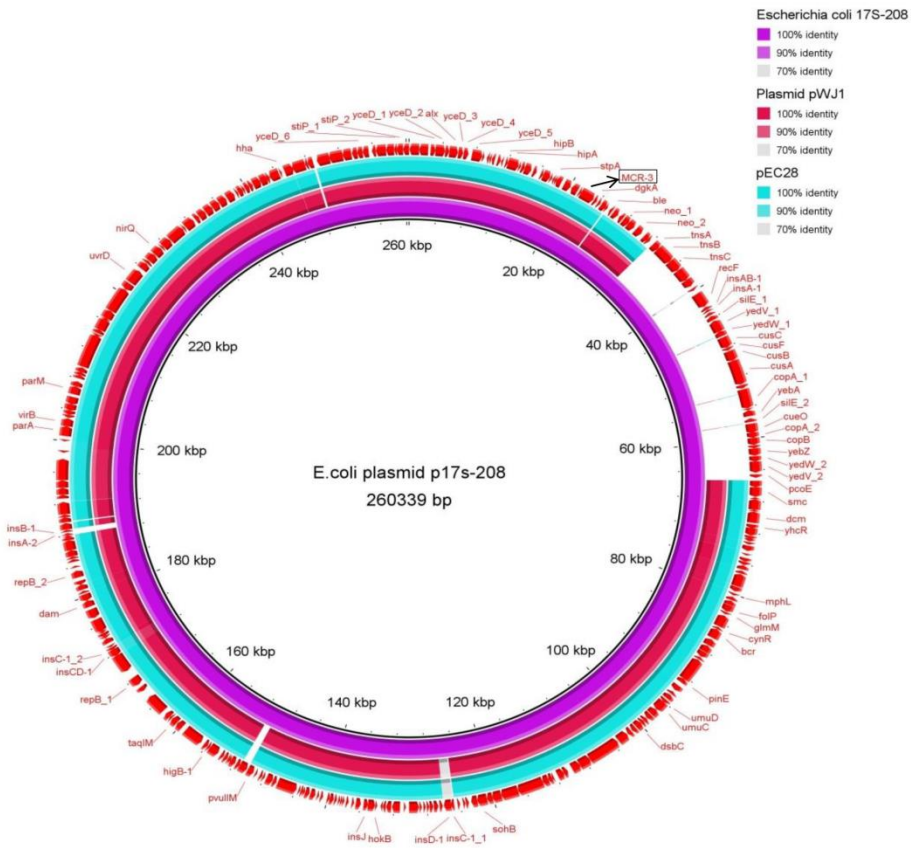


Figure 16. The *mcr-3*-harboring p17S-208 plasmid was compared with plasmids pWJ1 (GenBank accession no. KY924928) from an *E. coli* strain isolated from swine and pEC28 (GenBank accession no. KY405001) in an *E. coli* strain from a hospitalized patient. The BLAST Ring Image Generator (BRIG) was used to perform the comparative analysis. Arrow indicates the location of *mcr-3* gene in the plasmid.

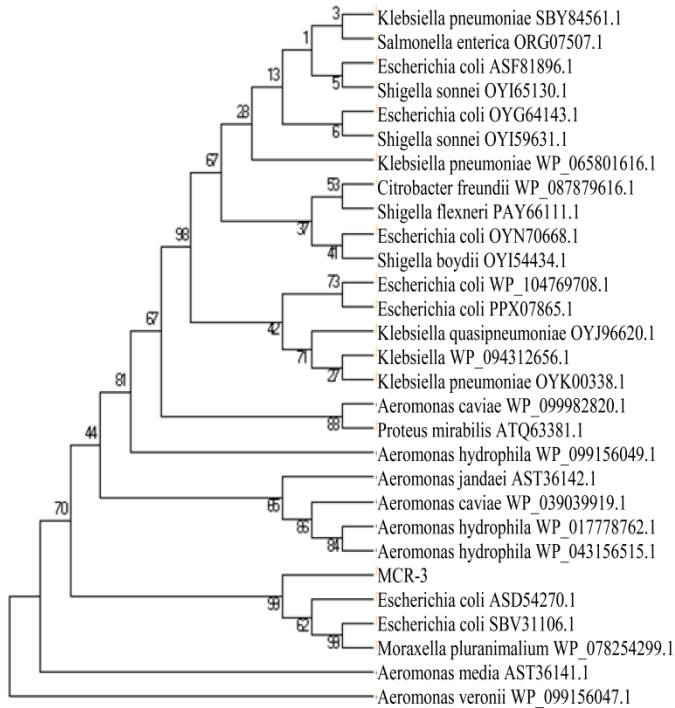


Figure 17. Phylogenetic analysis of colistin resistance *mcr-3* gene. The nucleotide sequence of *mcr-3* (1,626 bp) identified in this study was compared, using the neighbor-joining method, with representative other sequences obtained from the National Center for Biotechnology Information. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. MEGA version 6 was used to conduct the evolutionary analysis.

General conclusions

In this study, the spread and emergence of resistance to antimicrobial drugs among *E. coli* isolates has been observed in food-producing animals. Moreover, the data showed a significant increasing trend in the veterinary antimicrobial consumption which is expressed in the more elevated percentage of resistant isolates detected among the investigated animal population. The results confirm the necessity for a continuous surveillance of antimicrobial resistance for various antimicrobial agents having wide application in veterinary medicine such as aminoglycosides, tetracyclines and colistin. Moreover, it is believed that this practice should not be only a onetime attempt but demands regular monitoring programs. Most of the investigated isolates demonstrated to transfer efficiently their antimicrobial resistance genes to other strain which could be a public health risk. This study has also attempted to cast light on features about the danger of the currently spreading plasmid mediated colistin resistance in *E. coli* isolates from food-producing animals.

Hence, on the basis of the findings the following points are concluded:

1. Transferable aminoglycosides resistance is wide spread and as demonstrated in the results of our work the *E. coli* isolates collected from Korean cattle farms displayed a remarkable AME genes diversity. The result described most of the

AME genes are carried and transmissible via plasmid as different plasmid replicons were described in the investigated multidrug-resistant *E. coli* isolates. Plasmid-mediated aminoglycoside resistance genes carried by commensal *E. coli* could ultimately confer resistance to pathogenic organisms, posing a direct risk to human health.

2. Commensal isolates also takes part in carrying class 1 integrons thereby disseminating antibiotic resistance genes imbedded on their gene cassettes. The study also depicted multiple antibiotic resistance integrons are important contributors to the development of antibiotic resistance in commensal *E. coli* isolates recovered from food-producing animals. It also suggests that tetracycline-resistant *E. coli* in the studied farm animals may be due to *tet(A)* and *tet(B)* genes. Similarly, our study concludes widespread application of tetracycline antibiotics either for therapeutic purposes or as a supplement for promoting growth might contribute to the observed higher percentage of tetracycline-resistance in the investigated *E. coli* isolates. In this study, a positive association was observed between isolates harboring the *intI1* and *tet(A)* genes. Likewise, affiliation with phylogroup D was positively associated with the presence of class 1 integrons.

3. *E. coli* isolates recovered from food-producing animals carried the recently disseminated plasmid mediated colistin resistance genes, namely, *mcr-1* and *mcr-3*. To best of our knowledge, this is the first report of the plasmid mediated *mcr*-3

gene in *E. coli* isolates from swine farms in South Korea. The plasmid reported in this study, p17S-208, belongs to ST3-IncHI2 type plasmid and demonstrated to carry various genotypic resistance determinants for various classes of antimicrobial agents. Moreover, the *mcr-3* gene carried by this plasmid had a close genetic similarity with other *mcr* variants, which suggest it might have originated from a previous *mcr* variant through the continuous evolution of the bacterial genome, while it disseminated among different hosts. The report elucidate the need for reassessing our use of colistin in veterinary medicine for both therapeutic and prophylaxis purpose and the essentiality of a routine WGS- based surveillance and monitoring system to early trace the spread of different variants of *mcr* genes.

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국문초록

국내산업동물유래 *Escherichia coli* 항생제 내성의 분포, 전달성 및 분자유전학적 특성

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수의학과 수의병인생물학 및 예방수의학 전공

항생제 내성 문제는 공중보건학적으로 21 세기에 가장 중요한 이슈 중 하나이며, 항생제 내성 발달은 미생물의 자연 진화 산물이기도 하지만, 항생제 소비와 항생제 내성균 발달 사이에 연관성이 있을 것이라는 연구 결과들이 계속적으로 의학과 수의학 연구분야에서 확인되고 있다. 일반

적으로 축산물로 소비되는 산업 동물이 사람과 동물간에 항생제 내성을 전파를 하는 중요 매개체 역할을 할 것이라 여겨지고 있으며, 다양한 항생제 계열의 내성 패턴들이 병원성 / 공생성 대장균에서도 일반적으로 관찰되고 있다. 이번 연구를 통하여, 국내산업동물에서 분리한 대장균 분리 주들의 항생제 내성 패턴들을 확인하였다.

첫 번째로 국내 소 농장에서 247주의 대장균을 분리하여, aminoglycoside-modifying enzyme (AME) 유전자를 조사하였으며, 그들의 플라스미드 복제물 (plasmid replicons)과 전달성여부를 조사하였다. 이번 연구에서는 다양한 항생제 계열에서 높은 항생제 내성률이 관찰되었고, 특히 streptomycin 내성이 56.3% (139개 분리주)로 가장 많이 관찰되었으나, 주로 비 병원성 균주 (B1, A)에서 확인되었다. 주요 aminoglycoside 내성인자로는 *aph3''-1a, aph3''-1b* 이 확인 되었고, 표현형에서 내성이 확인되었지만, AME 유전자는 확인되지 않았다. Broth 접종을 통한 conjugation 실험에서 내성 유전자 전달유무를 확인한 결과, 63.2%의 분리 주에서 AME 유전자가 전달됨을 확인하였고, *aph3''-1a* 유전자는 IncFIB replicon-type의 주요 내성 유전자인 것으로 확인되었다. PFGE 을 통한 유전자 분석에서는 14개의 클러스터 유형이 확인되었으며, 이는 공생성 대장균들이 AME 유전자의 전파 역할을 하며, 잠재적으

로 공중보건학적인 문제를 일으킬 수 있음을 제시하고 있다.

두 번째로, 대장균의 class 1 integron과 관련된 다양한 유전자 cassette와 항생제 내성 메커니즘을 확인하였는데, tetracycline 내성과 관련하여, 내성 표현형과 class1 integron 운반과의 연관성은 거의 알려진바 없으나, 이번 연구에서 분리된 92개의 모든 분리 주들이 최소 한 개 이상의 tetracycline 내성인자를 가지고 있었고, *tet(A), tet(B)* 유전자가 주요 내성 인자로 확인되었다. *tet* 유전자를 가지고 있는 비율이 class 1 integrons 유전자 (*intI1)*를 가지고 있는 비율에서 높았으며, 다른 표현형과 비교해 봤을 때, *intI1* gene 의 경우, B2 그룹에서 낮은 비율이 관찰되었다. 또한 38개의 분리 주 (41.3%)에서 *intI1* gene을 가지고 있음을 확인하였고, 그 중 27개의 분리주의 3′-CS 에서 *sul1, qacEΔ1*이 확인되었다. Sulfonamide 내성 유전자의 경우 3′-CS의 class 1 integrons이 39.1%의 분리 주에서 확인되었고, 7개의 유전자 cassette가 단독으로 또는 다른 유전자 cassette 함께 확인되었는데, 이러한 내성 유전자들은 다양한 계열의 항생제에 대한 내성 스펙트럼을 형성하게 하며, class 1 integrons의 경우 공생성 대장균에 널리 전파되어 있으며, tetracycline 내성 결정인자 운반과 연관성이 있다. 반면에 병원성 그룹에서 분리한 분리 주에서는 intI1 유전자를 적게 가지고 있는 것으로 확인되었다.

마지막으로 임상적으로 건강한 동물의 분변 샘플에서 획득한, 636개의 공생성 대장균 분리 주에서 플라스미드 유래 콜리스틴 내성율과 유전학적 메커니즘, 내성 유전자의 전달성여부를 조사한 결과, 콜리스틴에 대한 저항성이 9개의 분리주 (1.4%)에서 확인되었으며, 그 중에서 *mcr-3*, *mcr-1* 내성 유전자가 2개, 3개의 분리 주에서 각각 확인되었다. *mcr-1*, *mcr-3* 유전자를 가진 모든 분리주들은 다양한 항생제 내성 유전자를 가지고 있음이 확인되었고, 이는 conjugation 분석을 통한 *E. coli* J53 AZ^R 균주로의 전달 유무로 확인하였다. *mcr-3* 유전자를 가지고 있는 또 다른 분리주인 17S-208를 Whole Genome Sequencing (WGS) 으로 플라스미드를 분석한 결과, plasmid p17S-208 (260,339bp)에서 241개의 predicted ORFs를 확인하였고, 다양한 항생제에 내성을 갖는 유전자들이 확인되었다. *In silico* plasmid finder tool에서는 p17S-208의 경우 전형적인 IncHI2-type backbone을 보여주고 있음을 확인하였고, plasmid MLST 분석결과 ST3에 속하는 것을 확인하였다. p17S-208 플라스미드를 다른 28개의 세균 종의 deduced amino acid 서열과 비교해본 결과, 벨기에의 돼지에서 분리된 p17S-208의 *mcr-2*, 한국의 환자에서 분리된 *mcr-1* 유전자와 35%의 동일성을 갖는 것을 확인하였는데, 반면에 중국에서 분리된 p17S-208의 *mcr-3*과는 100%의 동일성을 보인 것을 확인하였다. 콜리스틴을

치료목적으로 적용할 때는 반드시 주의가 필요하며, 정기적인 WGS기반 조사가 *mcr* 내성 유전자를 조기 발견하고, 새로운 *mcr* 내성 유전자를 확인하는데, 필수적이다.

결론적으로, 항균 내성 박테리아의 문제는 매우 복잡하고 광범위하다. 게다가 저항성유전자가 한 생물체에서 다른 생물체로 퍼질 가능성이 높다. 또한 산업동물에서 기인 한 저항 세균의 확산과 출현을 제한하기 위해 적절한 진단 기술을 사용하는 정기적 인 감시가 보장되어야 한다. 따라서 앞으로의 연구 방향은 다양한 저항 메커니즘의 특성화, 효과적인 신약 개발 및 통합 된 학제 간 접근을 통한 제어 전략에 초점을 맞추어야 할것으로 생각된다.

핵심어: 대장균, AME 유전자, 전달성, class 1 integrons, tetracycline내성, phylogenetic그룹, *mcr* 유전자들, IncHI2-ST3 형 플라스미드

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