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Prevalence and transmission of antimicrobial resistance in *Escherichia coli* isolated from food-producing animals

국내 농장분리 대장균의 항생제 내성유형 및 내성유전자의 전달에 관한 분석

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신 승 원

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By

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A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Microbiology

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Abstract

Prevalence and transmission of antimicrobial resistance in *Escherichia coli* isolated from food-producing animals

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Escherichia coli is a significant reservoir of antimicrobial resistance determinants which can spread pathogenic bacteria to human and animals. *E. coli* strains are able to efficiently exchange genetic mobile material such as integrons, transposomes and plasmid of pathogens. Acquired resistance mechanism mediated by these determinants play on important role in acquisition and dissemination of resistance

mechanism. Thus in order to investigate and analyze the prevalence and transferability of antimicrobial resistance in farm animals, *E. coli* strains isolated from the pathogenic lesions and fecal samples during the year 2009-2015 from beef cattle, pigs and chickens were included in this study.

The first study on the prevalence and characterization of *E. coli* isolated from beef cattle farms showed the diverse patterns of phenotype and genotype in antimicrobial resistance and pathogenicity. The most dominant virulence gene was *f17*. The 152 isolates showed multidrug-resistance. Antimicrobial susceptibility test determined that the most frequent resistance phenotype was streptomycin (63.1%), followed by tetracycline 54.5%), cephalothin (32.8%), and sulphamethoxazole/trimethoprim (16.6%). PCR and sequencing showed the prevalence of associated resistance determinants as follows: *strA-strB* (39.0%, 113/290), *tet*(A) (27.6%, 80/290), bla_{TEM} (23.8%, 69/290), and *sul2* (22.1%, 97/290). PFGE and O serotyping identified that *E. coli* isolates in this study showed the high degree of clonal diversity in genetic relation.

Second study was focused on ampicillin-resistant bovine *E. coli* strains harboring β-lactamases which have possibility to evolve into Extended-spectrum β-lactamase (ESBL) or plasmid-mediated AmpC β-lactamase. In this study, 78 *E. coli* isolates from beef cattle were included in this study. In the disc diffusion test with β-lactams,

38.5% of the isolates showed resistance to ampicillin, amoxicillin, and cephalothin, together. However, none of the isolates had determined to produce ESBL or AmpC β-lactamases by double disc synergy method. All isolates encoded genes for TEM-1-type β-lactamase. In plasmid replicon typing, IncFIB and IncFIA were identified in 71.4% and 41.0% of plasmids, respectively. Of transferable replicon, IncFIB and IncFIA were the most frequent type detected (61.5% and 41.0%, respectively). Based on these results, we might suggest that the transferable plasmids could provide significant effect on the acquisition and dissemination of β-lactam resistance as well as selection pressure although the level of antimicrobial usage in beef cattle is relatively low compared to those in other livestock animals in Korea.

In third study, the prevalence and transferability of resistance in tetracycline-resistant *E. coli* isolates from beef cattle in South Korea were carried out. Among 155 *E. coli* isolates, 146 were confirmed to be resistant to tetracycline. The tetracycline resistance gene *tet*(A) (46.5%) was the most prevalent. Ninety-one (62.3%) isolates were determined to be multidrug-resistant by the disc diffusion method. MIC testing using the principal tetracyclines, revealed that isolates carrying *tet*(B) had higher MIC values than isolates carrying *tet*(A). Conjugation assays showed that 121/155 (82.9%) isolates could transfer a tetracycline resistance gene to a recipient via the IncFIB replicon (65.1%, 95/155). This study suggests that the high

prevalence of tetracycline-resistant *E. coli* isolates in beef cattle might be due to the transferability of tetracycline resistance genes between *E. coli* populations which have survived the selective pressure caused by the use of antimicrobial agents.

In final study, a total of 281 *E. coli* strains isolated from pigs and chickens were investigated for ESBL-production. Fourteen *E. coli* isolates were identified to produce ESBL. The most common CTX-M- and CMY-types were CTX-M-15 (8/14) and CMY-2 (3/14). All ESBL-producing isolates showed resistance to the extent of the fourth-generation cephalosporins, along with multi-drug resistance. A conjugation assay demonstrated that $bla_{\text{CTX-M}}$ and bla_{CMY} genes have the potential to be transferred to non-resistant *E. coli*. The horizontal dissemination of $bla_{\text{CTX-M}}$ and bla_{CMY} genes was mediated mainly by Frep and IncI1 plasmids. PFGE revealed that isolates tested in this study were very diverse, clonally. To our knowledge, this is the first report of *E. coli* isolate possessing $bla_{\text{CMY-6}}$ from chickens in South Korea.

Distribution of resistance determinants in transferable plasmid of *E. coli* investigated in these studies could be critical in the public health. In addition future use of antimicrobial agents for human and veterinary purpose should be limited because of the increase in antimicrobial resistance for *E. coli* in human and farm animals. Thus reasonable use and long-term surveillance are needed for minimizing the emergence and spread of antimicrobial resistance in *E. coli*.

Keywords: *Escherichia coli*, antimicrobial resistance, farm animals, plasmids tetracycline, extended-spectrum β-lactamase, CMY-6

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List of abbreviations

AMC	**Amoxicillin/Clavulanate**
AML, AMX	**Amoxicillin**
AMP	**Ampicillin**
ATCC	**American Type Culture Collection**
ATM	**Aztreonam**
BAP	**Blood agar plate**
BLAST	**Basic Local Alignment Search Tool**
BRICS	**Brazil, Russia, India, China, and South Africa**
C	**Chloramphenicol**
CAZ	**Ceftazidime**
CC-DDDM	**Cefoxitin-Cloxacillin Double Disc Synergy Method**
CDC	**the Center for Disease Control and Prevention**
CEC	**Cefaclor**
CFM	**Cefixime**
CFU	**Colony Forming Unit**
CIP	**Ciprofloxacin**
CLSI	**Clinical and Laboratory Standards Institute**
CN	**Gentamicin**

CPO	**Cefpirome**
CRO	**Ceftriaxone**
CTC	**Chlortetracycline**
CTX	**Cefotaxime**
DDDM	**Double Disc Diffusion Method**
DOX	**Doxycycline**
EFT	**Ceftiofur**
EMB agar	**Eosin Methylene Blue agar**
ENR	**Enrofloxacin**
ESBL	**Extended-Spectrum β-Lactamase**
FEP	**Cefepime**
FFC	**Florfenicol**
FOX	**Cefoxitin**
KAHPA	**the Korea Animal Health Products Association**
KF	**Cephalothin**
LB broth	**Luria-Bertani broth**
MH agar	**Mueller-Hinton agar**
MIC	**Minimum Inhibitory Concentration**
MIN	**Minocycline**

N	**Neomycin**
NA	**Nalidixic acid**
NCBI	**the National Center for Biotechnology Information**
OXY	**Oxytetracycline**
pAmpC	**plasmid-mediated AmpC**
PCR	**Polymerase Chain Reaction**
PFGE	**Pulsed-Field Gel Electrophoresis**
S	**Streptomycin**
STEC	**Shiga toxin-producing *E. coli***
STR	**Streptomycin**
SXT	**Trimethoprim/Sulphamethoxazole**
TBE	**Tris-Borate EDTA**
TE, TET	**Tetracycline**
TSA	**Tryptic Soy Agar**
TSB	**Tryptic Soy Broth**
UPGMA	**the Unweighted Pair Group Method with Arithmetic means**
VTEC	**Verotoxigenic *E. coli***

General introduction

Previous and current researches on antimicrobial resistance in human and farm animals have clearly demonstrated that the extensive use of antimicrobials is the critical factor in selecting resistance. However, information on the actual prevalence and transferability of antimicrobial resistance among the microbes of farm origin has been not much available. Although most *E. coli* strains are considered harmless commensal bacteria of humans and animals, this microbe is a significant reservoir of antimicrobial resistance determinants which can spread pathogenic bacteria to human and animals. Thus, surveillance of antimicrobial resistance among *E. coli* could provide an excellent means for monitoring of antimicrobial resistance in human and food animals. For these reason, our study focused on accessing prevalence and transferability of antimicrobial resistance especially for β-lactams and tetracycline among *E. coli* isolated from farm animals

The prevalence of β-lactam-resistant *Enterobacteriaceae* has increased consistently over the past few decades. *Escherichia coli* (*E. coli*) producing plasmid-mediated AmpC β-lactamases and/or extended-spectrum β-lactamases (ESBLs) has been of particular concern because of their implications in human and food animal health (Livermore, 2012). These strains encode β-lactamases that mediate resistance to β-

lactam antimicrobials included penicillins and extended-spectrum cephalosporins such as 3rd and 4th generation cephalosporins (Carattoli, 2009). Genes encoding β-lactamases are located on mobile genetic elements, mostly plasmids, which can transfer resistance genes horizontally to non-resistant isolates. Thus, these elements are believed to be responsible for the acquisition and dissemination of β-lactam antimicrobial resistance in the bacterial population.

The tetracyclines are one of the most widely used classes of antimicrobial agents in human and veterinary medicine because they have several advantages, which include a broad spectrum of activity, low cost, oral administration, and few side effects (Chopra and Roberts, 2001). After chlortetracycline was introduced into clinical medicine in 1948, many derivatives, such as tetracycline, oxytetracycline, doxycycline, and minocycline, were developed, and today, these derivatives are widely used to treat disease and as growth promoters in the food animal industry. However, the widespread and indiscriminate use of tetracyclines has subjected bacterial populations to selection pressure and increased the prevalence of tetracycline resistance (Chopra and Roberts, 2001; Roberts, 2005).

Thus,to gain more insight into these findings of β-lactams and tetracycline, our study focused on surveillance and molecular characterization of antimicrobial resistance in *E. coli* isolated from cattle, pigs and chickens. As the first part of my study, I focused on assessing the prevalence of general antimicrobial resistance

among the *E. coli* isolated from beef cattle. I looked for the relationship between virulence factors, phenotype and genotype of resistance in *E. coli* isolates from bovine fecal samples. In continuation to my work of characterization of antimicrobial resistance in *E. coli* of farm animals and its impact on animal and human health, I investigated β-lactams' resistances in *E. coli* isolated from cattle, pigs and chickens.

Chapter I

Literature review

After the discovery of penicillin in 1928, antibiotics have been used in human and veterinary medicine for several decades, which have saved human and animal from the threat caused by infectious diseases (Levy and Marshall, 2004). However, the successful use of antimicrobial agent is compromised by the potential development of resistance to that compound from the time it is first employed (Cizman, 2003).

1.1. Antimicrobial resistance

Antimicrobial resistance is the ability of a microorganism to survive and reproduce in the presence of an antimicrobial agent that would normally inhibit or kill this particular kind of microbes. Antimicrobial resistance is one of the many adaptive characteristics that resilient bacterial subpopulations might possess or acquire, enabling them to out-compete and out-survive their microbial neighbors and overcome host strategies aimed against them. This phenomenon is nearly as old as the discovery of antimicrobials themselves, having been described by pioneers like Ehrlich for *trypanosomes* (Ehrlich, 1907) and Fleming for *staphylococci* (Fleming,

1945). What is most alarming today is the rate at which antibiotic resistance often develops and how quickly it spreads across the globe and among different species of bacteria. Furthermore, as a result of sequential, cumulative acquisition of resistance characteristics against different antibiotics, more bacterial pathogens with multiple-drug resistance are being reported worldwide. As a consequence, many bacterial organisms have become resistant to antibiotics which were previously quite efficacious

1.1.1. Resistance mechanism of bacteria

Most antimicrobial agents used for the treatment of bacterial infections may be classified according to their principal mechanism of action. There are 4 major modes of action (Fig. 1.1)

By prevention of the antimicrobial from reaching its target by reducing its ability to penetrate into the cell: Antimicrobial compounds almost always require access into the bacterial cell to reach their target site where they can interfere with the normal function of the bacterial organism. Porin channels are the passageways by which these antibiotics would normally cross the bacterial outer membrane. Some bacteria protect themselves by prohibiting these antimicrobial compounds from entering past their cell walls. For example, a variety of Gram-negative bacteria

reduce the uptake of certain antibiotics, such as aminoglycosides and β lactams, by modifying the cell membrane porin channel frequency, size, and selectivity. Prohibiting entry in this manner will prevent these antimicrobials from reaching their intended targets that, for aminoglycosides and β lactams, are the ribosomes and the penicillin binding proteins (PBPs), respectively.

By expulsion of the antimicrobial agents from the cell via general or specific efflux pumps: To be effective, antimicrobial agents must also be present at a sufficiently high concentration within the bacterial cell. Some bacteria possess membrane proteins that act as an export or efflux pump for certain antimicrobials, extruding the antibiotic out of the cell as fast as it can enter. This results in low intracellular concentrations that are insufficient to elicit an effect. Some efflux pumps selectively extrude specific antibiotics such as macrolides, lincosamides, streptogramins and tetracyclines, whereas others (referred to as multiple drug resistance pumps) expel a variety of structurally diverse anti-infectives with different modes of action.

By inactivation of antimicrobial agents via modification or degradation: Another means by which bacteria preserve themselves is by destroying the active component of the antimicrobial agent. A classic example is the hydrolytic deactivation of the β-lactam ring in penicillins and cephalosporins by the bacterial enzyme called β lactamase. The inactivated penicilloic acid will then be ineffective

in binding to PBPs (penicllin binding proteins), thereby protecting the process of cell wall synthesis.

By modification of the antimicrobial target within the bacteria: Some resistant bacteria evade antimicrobials by reprogramming or camouflaging critical target sites to avoid recognition. Therefore, in spite of the presence of an intact and active antimicrobial compound, no subsequent binding or inhibition will take place

1.1.2. Molecular mechanisms of resistance

Intrinsic Resistance: Intrinsic resistance is the innate ability of a bacterial species to resist activity of a particular antimicrobial agent through its inherent structural or functional characteristics, which allow tolerance of a particular drug or antimicrobial class. This can also be called insensitivity since it occurs in organisms that have never been susceptible to that particular drug. Such natural insensitivity can be due to: i) lack of affinity of the drug for the bacterial target, ii) inaccessibility of the drug into the bacterial cell, iii) extrusion of the drug by chromosomally encoded active exporters, iv) innate production of enzymes that inactivate the drug

Acquired Resistance: Acquired resistance is said to occur when a particular microorganism obtains the ability to resist the activity of a particular antimicrobial

agent to which it was previously susceptible. This can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes or from a combination of these two mechanisms. Unlike intrinsic resistance, characteristics associated with acquired resistance are found only in some strains or subpopulations of each particular bacterial species. Laboratory methods are therefore needed to detect acquired resistance in bacterial species that are not intrinsically resistant. These same methods are used for monitoring rates of acquired resistance as a means of combating the emergence and spread of acquired resistance characteristics in pathogenic and non-pathogenic bacterial species. Acquired resistance results from successful gene change and/or exchange that might involve: mutation or horizontal gene transfer via transformation, transduction or conjugation.

Mutation: A mutation is a spontaneous change in the DNA sequence within the gene that may lead to a change in the characteristics which it codes for. Any change in a single base pair may lead to a corresponding change in one or more of the amino acids for which it codes, which can then change the enzyme or cell structure that consequently changes the affinity or effective activity of the targeted antimicrobials. In prokaryotic genomes, mutations frequently occur due to base changes caused by exogenous agents, DNA polymerase errors, deletions, insertions and duplications. For prokaryotes, there is a constant rate of spontaneous mutation of about 0.0033

mutations per DNA replication that is relatively uniform for a diverse spectrum of organisms. The mutation rate for individual genes varies significantly among and within genes (Gillespie, 2001).

Horizontal Gene Transfer: Horizontal gene transfer, or the process of swapping genetic material between neighboring contemporary bacteria, is another means by which resistance can be acquired. Many of the antibiotic resistance genes are carried on plasmids, transposons or integrons that can act as vectors that transfer these genes to other members of the same bacterial species, as well as to bacteria in another genus or species. Horizontal gene transfer may occur via three main mechanisms: transformation, transduction or conjugation.

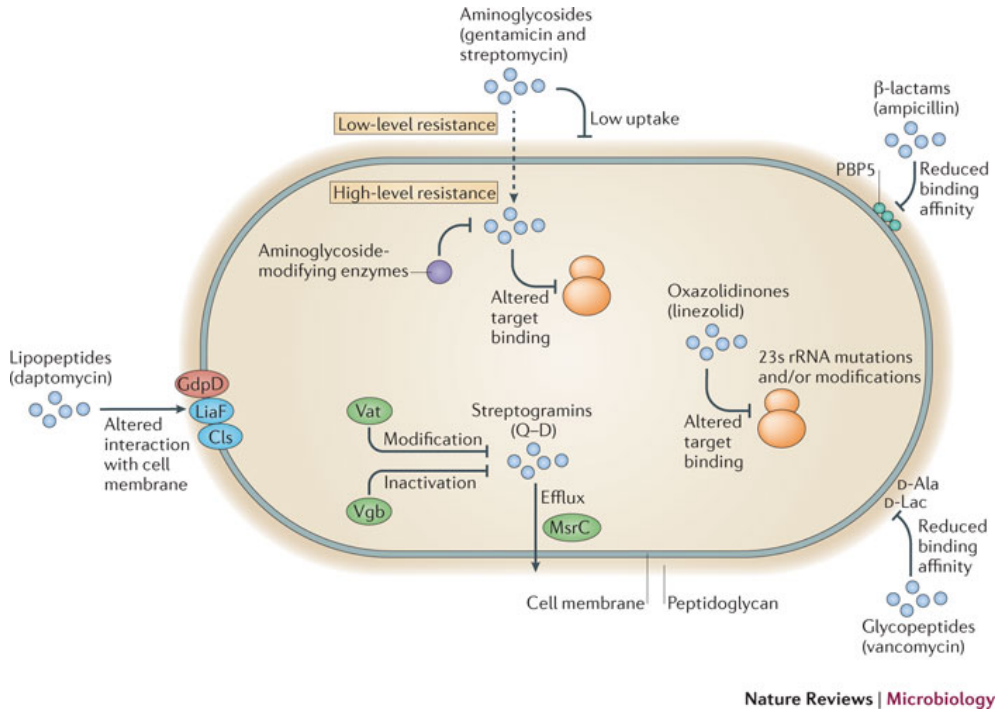

Fig. 1.1. Mechanism of bacterial resistance against antimicrobial agents commonly used. Adapted from Cesar and Barbara, 2012.

1.2. Trend of antimicrobial consumption in food animal

1.2.1. Global trend of antimicrobial consumption

Global consumption of antimicrobials in food animal production was estimated at 63,151 (±1,560) tons in 2010 and is projected to rise by 67%, to 105,596 (±3,605) tons, by 2030. Two thirds (66%) of the global increase (67%) in antimicrobial consumption is due to the growing number of animals raised for food production. The remaining third (34%) is imputable to a shift in farming practices, with a larger proportion of animals projected to be raised in intensive farming systems by 2030. In Asia alone, as much as 46% of the increase in antimicrobial consumption by 2030 is likely due to shifts in production systems. By 2030, antimicrobial consumption in Asia is projected to be 51,851 tons, representing 82% of the current global antimicrobial consumption in food animals in 2010.

In 2010, the five countries with the largest shares of global antimicrobial consumption in food animal production were China (23%), the United States (13%), Brazil (9%), India (3%), and Germany (3%) (Fig. 1.2). By 2030, this ranking is projected to be China (30%), the United States (10%), Brazil (8%), India (4%), and Mexico (2%) (Fig. 1.2). Among the 50 countries with the largest amounts of antimicrobials used in livestock in 2010, the five countries with the greatest projected

percentage increases in antimicrobial consumption by 2030 are likely to be Myanmar (205%), Indonesia (202%), Nigeria (163%), Peru (160%), and Vietnam (157%). China and Brazil are among the largest consumers of antimicrobials currently but are not the countries with the most rapid projected increases in antimicrobial consumption. This indicates that these two countries have already initiated a shift toward more intensified livestock production systems using antimicrobials to maintain animal health and increase productivity. Antimicrobial consumption for animals in the BRICS (Brazil, Russia, India, China, and South Africa) countries is expected to grow by 99% by 2030, whereas their human populations are only expected to grow by 13% over the same period (Van Boeckel *et al.*, 2015).

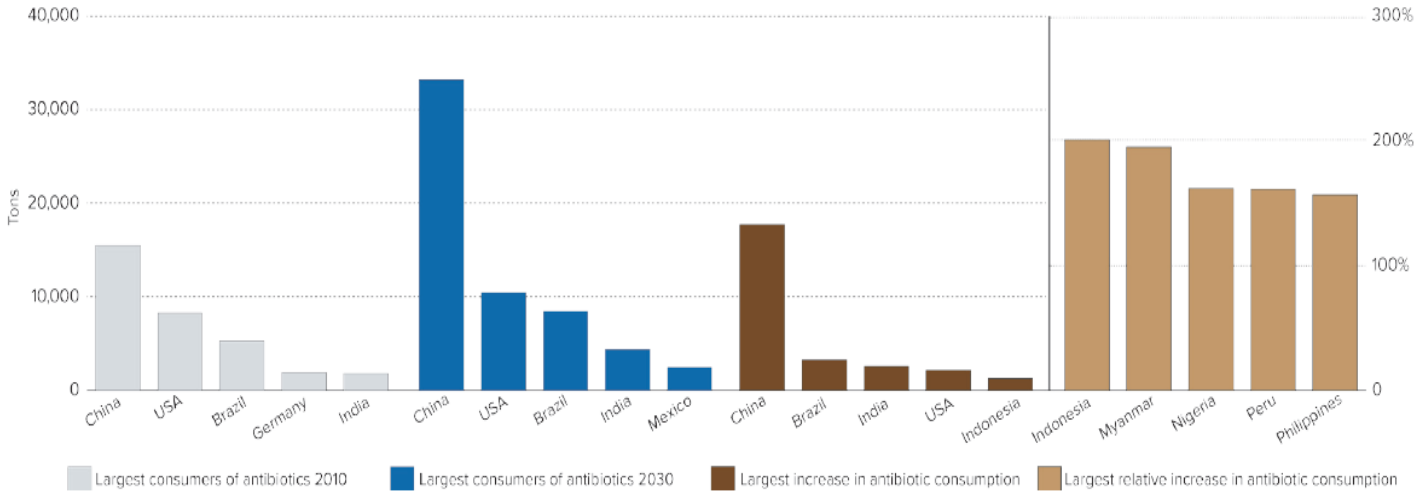

Fig. 1.2. Antibiotic consumption in livestock in high-consuming countries, 2010–2030 (projected for 2030). Adapted from Van Boeckel *et al*. 2015

1.2.2. Trend of antimicrobial consumption in Korea

The trends in the consumptions of antimicrobial drugs in animals were investigated by animal species and antimicrobial agents in Korea from 2003, which has performed by Korea Animal Health Products Association. In total, about 1,500 tons of antimicrobial agents were sold each year during 2003-2007, however, the amounts tend to decrease to around 1,000 tons from 2008 (Fig. 1.3). Furthermore, less than 1,000 tons of antimicrobials were sold from 2011 to 2014 for the four consecutive years and 635 tons of antimicrobials were sold in 2014 which was the lowest since 2003 (Fig. 1.3). The largest volume of antimicrobials was sold for use in pigs (47-57%) followed by poultry (18-24%), fishery (11-26%), and cattle (5-9%). Tetracycline (167 tons) and penicillins (162 tons) were the biggest selling antimicrobial. Overall sale of most of antimicrobials gradually decreased, however, the sales of phenicols and cephalosporins increased by 1.9 and 2.3 times from 2006 to 2014, respectively (Table 1.1).

Table I. Antimicrobial consumption by ingredients in South Korea. Adapted from QIA, 2015

Antimicrobials	Amount of antimicrobial used (AI, ton)											
	'03	'04	'05	'06	'07	'08	'09	'10	'11	'12	'13	'14
Tetracyclines	**724**	**699**	**723**	**630**	**624**	**471**	**288**	**284**	**308**	**282**	**269**	**167**
Penicillins	130	169	229	225	267	171	151	145	155	190	187	162
Sulfonamides	181	164	200	184	183	157	92	117	100	102	75	60
Macrolides	48	49	55	74	75	69	88	91	60	56	41	43
Quinolones	33	45	53	48	57	51	37	46	51	49	50	32
Ionophores	62	57	63	51	59	47	51	36	53	48	55	44
Polypeptides	25	24	34	35	39	44	97	117	57	10	10	8
Phenicols	**10**	**20**	**25**	**28**	**34**	**36**	**55**	**64**	**59**	**83**	**65**	**55**
Aminoglycosides	79	63	72	82	94	73	51	59	46	46	37	35
Pleuromutilins	15	13	18	23	21	20	35	35	22	18	14	13
Lincosamides	10	12	14	18	16	12	6	7	8	9	8	7
Cephems	10	2	2	3	2	3	3	5	6	8	8	8
Streptogramins	4	5	5	5	5	5	8	6	3	1	0.6	1
Orthosomycins	5	4	4	5	5	5	6	4	1	0	0.1	0
Glycolipid	5	3	3	2	2	2	2	2	1	0	0.3	0
Quinoxalines	30	35	16	10	13	18	5	0	0	0	-	0
Others	7	5	36	34	29	27	24	30	26	34	1	2
Total	1,439	1,368	1,553	1,458	1,527	1,211	998	1047	956	936	820	635

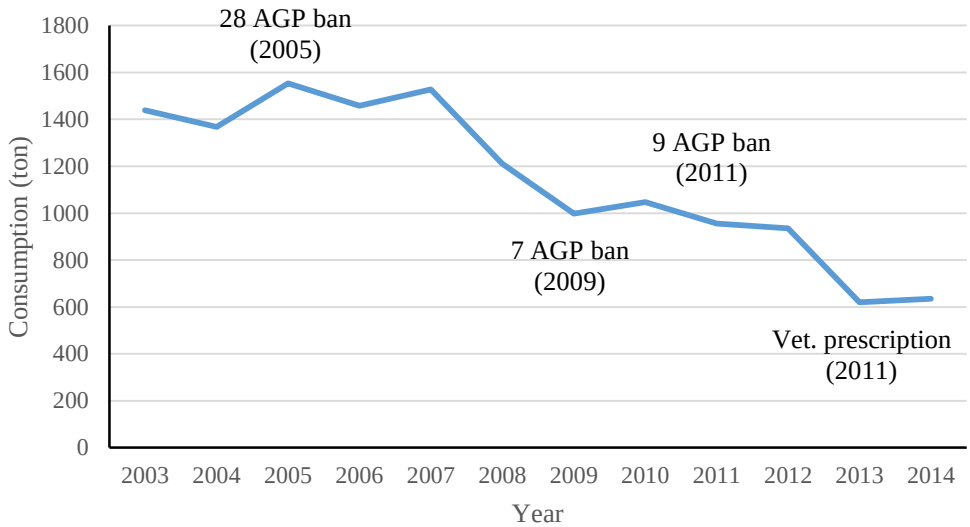

Fig. 1.3. Continuative trend of sales of antimicrobial drugs for food-producing animals in South Korea. Adapted from QIA, 2015

1.3. Prevalence of antimicrobial resistance

Even before penicillin was introduced, resistant strains of bacteria had been detected (Abraham and Chain, 1988). The selection pressure caused by the use of millions of tons of antibiotics over the past 75 years since antibiotics were introduced has made almost all disease-causing bacteria resistant to antibiotics commonly used to treat them. The rapid evolution of bacterial resistance is clear in the case of β-lactamases class of antibiotics. Nearly 1000 resistance-related β-lactamases that inactivate these antibiotics have been identified, a ten times increase since before 1990 (Davies and Davies, 2010).

Resistance has spread worldwide. Antibiotic-resistant *gonorrhoea* emerged in Vietnam in 1967 (Holmes *et al*., 1967), then spread to the Philippines, and finally the USA (Rasnake *et al*., 2005). NDM enzymes, first reported in 2008, are now found worldwide (Nordmann *et al*., 2011). ESBLs are a family of enzymes, produced by Gram-negative bacteria that confer resistance to third- and fourth cephalosporin antibiotics. (Fig. 1.5). The distribution of resistance genes, such as *Enterobacteriaceae* producing extended-spectrum β-lactamase (ESBL), NDM-1, and *Klebsiella pneumoniae* carbapenemase (KPC), indicates the ease with which resistance can spread. Findings of a study (Walsh *et al*., 2011) done in New Delhi showed NDM-1-producing bacteria (including *Shigella boydii* and *Vibrio cholera*) in two (4%) of 50 drinking water samples and 51 (30%) of 171 seepage samples (ie,

water pools in streets or rivulets) suggesting the possibility of acquiring resistance outside health-care facilities.

Quinolone antibiotics in particular are an example of misadventure. These drugs are synthetic and so do not arise in nature, yet 30 years after their widespread introduction resistance is epidemic (Ruiz *et al*., 2012). More specifically, whole genome studies suggest that quinolone resistance was a crucial factor in the evolution of MRSA (Holden *et al*., 2013). Such examples of antibiotic-driven evolution go a long way to explaining present epidemics of resistant health-care associated infections (Ammerlaan *et al*., 2013).

In health-care settings, the spread of a resistant clone can be rapid and have severe consequences for vulnerable hosts. Carbapenem resistance among common *Enterobacteriaceae* has increased sharply over the past decade. In 2012, 4·6% of acute-care hospitals in the USA reported at least one health-care associated infection caused by carbapenem-resistant enterobacteria. The proportion of *Enterobacteriaceae* that were resistant to carbapenems increased from 0% in 2001 to 1·4% in 2010, with most of the increase recorded in *Klebsiella* spp (CDC). Health-care associated infections are also increasingly recognized in low- and middle-income countries (LMICs).

These trends are globally consistent. Data from developing countries suggest that resistance to the WHO recommended regimen of ampicillin and gentamicin in

pathogens causing infections is common: 71% of isolates of *Klebsiella* spp and 50% of *E coli* are resistant to gentamicin (Zaidi *et al*., 2005). Resistance is also a problem in early-onset, presumably maternally acquired, neonatal infections reported from hospital series in developing countries. 60–70% of *E coli* and nearly 100% of isolates of *Klebsiella* spp are ampicillin resistant, and 40–60% are resistant to gentamicin (Waters *et al*., 2011). High rates of ESBL production in *E coli* have restricted the use of second-line treatment with extended-spectrum cephalosporins (Viswanathan *et al*., 2012). Many newborn babies in hospitals in south Asia are now treated with carbapenems as first-line treatment for sepsis or presumed sepsis. Most worrying is the emergence of panresistant untreatable carbapenem-resistant *Enterobacteriaceae* and *Acinetobacter* spp. infections associated with high mortality in neonatal nurseries (Saleem *et al*., 2010).

In Pakistan, the emergence of pan-resistant bacterial isolates such as *Acinetobacter* spp. and carbapenem resistant enterobacteria as causes of health-care associated sepsis in hospitals is rendering these infections untreatable (Saleem et al., 2010; Perry *et al*., 2011; Khan *et al*., 2010). 50–60% of community-acquired Gram negative pathogens such as *E coli* associated with urinary tract infections have become resistant to common oral antibiotics (e.g. amoxicillin, cefixime, and ciprofloxacin), complicating outpatient management.

Between July 2010 and August 2011, 72% of 1294 viable *K pneumoniae* isolates from sentinel sites in South Africa had antibiograms suggestive of ESBL production

(Perovic *et al*., 2012). Compounding this problem is the emergence of several carbapenemase-resistance mechanisms. NDM-1 was first detected in South Africa in September 2011 (Lowman *et al*., 2011), and of 70 carbapenem resistant enterobacteria isolates from private and public hospitals received by the Antimicrobial Resistance Reference Laboratory between May and July 2013, 19 tested positive for NDM-1 (NICD-NHLS, 2012).

In India, *E coli* isolated from urine cultures of pregnant women in their first trimesters in the community showed highest overall resistance to ampicillin, nalidixic acid, and co-trimoxazole, as 75%, 73%, and 59%, respectively, between 2004 and 2007 (Hollyway *et al*., 2009). 30% showed resistance to injectable antibiotics, such as aminoglycosides. In a study of blood stream infections (Datta *et al*., 2012), the proportion of *E coli* producing ESBLs increased from 40% in 2002 to 61% in 2009, and the proportion of *K pneumoniae* with carbapenem resistance increased from 2·4% to 52%. Increasing rates of resistance to colistin and polymyxin B in Gram-negative organisms are being reported from countries around the world, including South Korea (Ko *et al*., 2007), Italy (Capone *et al*., 2013), Greece (Antoniadou *et al*., 2007; Kontopidou *et al*., 2011), and Saudi Arabia (Baadani *et al*., 2013). Moreover, there is some evidence of cross-resistance to colistin and host antimicrobial peptides that are part of the body's immune response (Napier *et al*., 2013). Hospital-acquired MRSA arises worldwide (Fig. 1.5). In high income countries, it is being tackled with a combination of new antibiotics and better hospital

infection control, but community strains of MRSA continue to proliferate (Klein *et al*., 2013). In LMICs such as South Africa, 52% of 1147 S aureus viable isolates from hospitalized bacteremic intensive care unit patients were MRSAs. Gram-positive infections are less common in India, but high rates of MRSA in clinical isolates in various studies in India have been documented as 54·8% (range 32–80%) (Anupurba *et al*., 2003). In Pakistan, rates of MRSA have been fairly consistent since the mid-2000s at roughly 50% (Zafar *et al*., 2011). However, community-acquired MRSA are increasingly reported, and rates range from 5–10% (Malik *et al*., 2009).

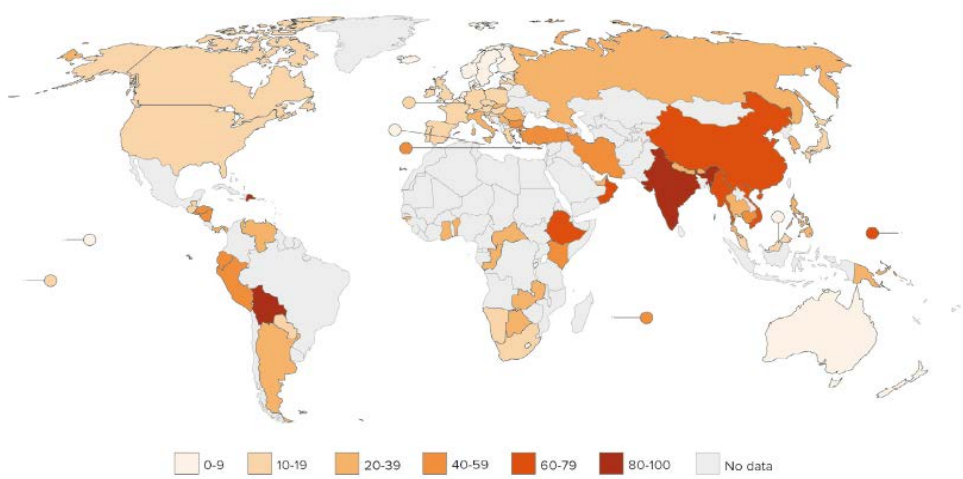

Fig. 1.4. Percentage of extended-spectrum β-lactamase producing *Escherichia coli*, by country. The data were collected from 2011 to 2014. Adapted from CDDEP, 2015.

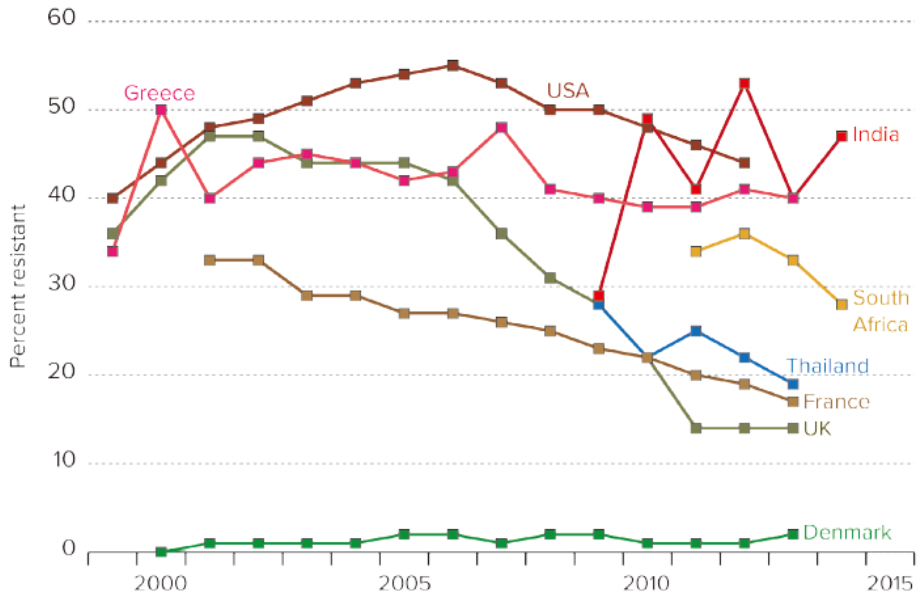

Fig. 1.5. Percentage of *Staphylococcus aureus* isolates that are methicillin resistant (MRSA), by country. Adapted from CDDEP, 2015

1.4. Emerging trends of resistance in *E. coli*

1.4.1. Extended-spectrum β-lactamases (ESBLs)

The most well-known of the newer β-lactamases was first described in 1983 and have been named the extended-spectrum β-lactamases (ESBLs). These enzymes have the ability to hydrolyse the penicillins, cephalosporins and monobactams, but not the cephamycins and carbapenems. ESBLs are inhibited by classical β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Paterson and Bonomo, 2005). Although ESBLs have been identified in a range of *Enterobacteriaceae*, they are most often present in *E. coli* and *K. pneumoniae*. The majority of ESBLs identified in clinical isolates during the 1980s to 90s were of the SHV or TEM types, which evolved from parent enzymes such as TEM-1, -2 and SHV-1. A different type of ESBL, named CTX-M β-lactamases, originated from environmental *Kluyvera* spp, and gained prominence in the early 2000s with reports of clinical isolates of *E. coli* producing these enzymes from Europe, Africa, Asia, South and North America (Pitout *et al*., 2005). Since the middle of 2000's, the prevalence of CTX-M β-lactamases increased significantly in *E. coli* from various parts of the world, and today have become the most wide-spread and common type of ESBL (Pitout *et al*., 2005).

Surveys from several countries worldwide have illustrated an alarming trend of associated resistance to other classes of antimicrobial agents among CTX-M-producing *E. coli* that included trimethoprim-sulfamethoxazole, tetracycline, gentamicin, tobramycin and ciprofloxacin (Pitout *et al.*, 2005). Studies consistently show that infections due to ESBL-producing *Enterobacteriaceae* are associated with a delay in initiation of appropriate antibiotic therapy, which consequently prolongs hospital stays and increases hospital costs (Schwaber and Carmeli, 2007). More importantly, failure to initiate appropriate antibiotic therapy from the start appears to be responsible for higher patient mortality (Ulett *et al.*, 2013).

Currently, the most widespread and prevalent type of CTX-M enzyme among human clinical isolates of *E. coli* is CTX-M-15 (D'Andrea MM *et al*., 2013). In 2008, *E. coli* sequence type (ST) ST131 with CTX-M-15 was simultaneously identified in nine countries, spanning three continents (Coque *et al*., 2008). The intercontinental dissemination of this ST since then, has contributed immensely to the worldwide emergence of fluoroquinolone resistant and CTX-M-15 producing *E. coli* (Peirano and Pitout, 2010). Recent surveys have shown that ST131 accounted for over 50% of fluoroquinolone-resistant or ESBL-producing *E. coli* (Colpan *et al*., 2013; Johnson *et al*., 2010). A recent study from Canada that investigated the molecular epidemiology of ESBLs-producing *E. coli* causing bacteremia over an 11 year period (2000-2010), showed that ST131 was the most common and antimicrobial resistant

sequence type, and the influx of a single pulsotype of ST131, was responsible for a significant increase since 2007 of ESBL-producing *E. coli*.

1.4.2. Plasmid-mediated AmpC-β-lactamase

E. coli possess a chromosomal gene that encodes for an AmpC β-lactamase. Usually, low amounts of these β-lactamases are produced because the AmpC gene is regulated by a weak promoter and a strong attenuator system (Jacoby, 2009). Occasionally, cephamycin and/or cephalosporin-resistant *E. coli* are encountered that produce plasmid-mediated β-lactamases, derived from bacteria with chromosomally encoded AmpC-cephalosporinases (Pitout, 2008). *E. coli* that produce plasmid-mediated or imported AmpC β-lactamases were first reported in the 1980's. These enzymes (e.g. CMY, ACT, FOX, ACT, and DHA types) are derivatives of the chromosomally encoded AmpC cephalosporinases of bacteria such as *Enterobacter* spp., *C. freundii*, *M. morganii*, *Aeromonas* spp. and *Hafnia alvei* and are not inhibited by the classical β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Jacoby, 2009). However, different types of inhibitors such as boronic acid and cloxacillin have the ability to inhibit chromosomal and plasmid-mediated AmpC β-lactamases (Thomson, 2013). Resistance to the fourth generation cephalosporins (e.g. cefepime) are caused by point mutations in AmpC β-lactamases and is called extended-spectrum cephalosporinases (Jacoby, 2009).

The genes are typically encoded on large plasmids containing additional antibiotic resistance genes that are responsible for multi-resistant phenotype, leaving few therapeutic options (Harris and Ferguson, 2012).

A survey from five children's hospitals in China, detected AmpC β-lactamases in 10% of *K. pneumoniae*, in 2% of *E. coli* with an overall increase from 2005 (2.6%) to 9.3% in 2006 (Ding et al., 2008). A multicenter survey from 63 hospitals conducted in the USA detected transferable AmpC β-lactamases in 3.3% of K. pneumoniae isolates at 16 of the 63 sites (25%) with no difference between ICU and non-ICU sites (Moland *et al*., 2006). The SENTRY Antimicrobial Surveillance Program in the USA found plasmid-mediated AmpC b-lactamases in 2% of 1429 *E. coli* isolates from 30 centers; with CMY-2, FOX-5 and DHA-1 being identified (Deshpande *et al*., 2006).

It seems that CMY-2 (stands for active on CephaMYcins) is the most common imported AmpC β-lactamase reported in *Enterobacteriaceae* from different areas of the world (Jacoby, 2009). Jacoby and colleagues found plasmid-mediated AmpC-type resistance in 7 of 75 of ceftazidime resistant *E. coli* from 25 U.S. states; 2 of these isolates produced CMY-2 (Alvarez *et al*., 2004). Mulvey and colleagues studied 232 cefoxitin resistant *E. coli* from 12 different hospitals in Canada and found 25 (11%) strains contained CMY-2 and 51 (22%) had different promoter and attenuator mutations (Mulvey *et al*., 2005). Hospital surveys from Asia, North America and Europe have shown that the DHA types of cephamycinases are mostly

present in *Klebsiella* spp. from Asia, CMY are present in *E. coli* from Asia, North American and Europe while FOX are present in *Klebsiella* spp. from North America and Europe (Jacoby, 2009).

Just like ESBL-producing bacteria, organisms with plasmid-mediated AmpC enzymes have mostly been responsible for nosocomial outbreaks on a worldwide basis especially during the late 1980's and 1990's (Jacoby, 2009). Analysis of these outbreaks had shown that increased length of hospital stay, severity of illness, admission to an intensive care unit (ICU), and previous exposure to antibiotics are associated with infections with plasmid-mediated AmpC β-lactamase producing *Enterobacteriaceae*. In a study reported by Pai et al from Korea, bloodstream infections caused by plasmid-mediated AmpC-producing (i.e. DHA-1 and CMY-1) *K. pneumoniae* had similar clinical features, risk factors and outcomes to those patients infected with TEM- or SHV-related ESBL producers (Pai *et al*., 2004). All the patients that received an extended-spectrum cephalosporin (i.e. cefotaxime, ceftazidime, ceftriaxone) had failed therapy.

A population-based study from the Canada has identified AmpC-producing *E. coli* in 61% of 369 patients with community-associated infections due to cephamycin-resistant isolates and found that women were at five-times higher risk for developing an infection (Pitout *et al*., 2007). PCR showed that 125 (34%) were positive for bla_{CMY} genes and sequencing identified these enzymes to be CMY-2. The study concluded that in this large Canadian region, AmpC-producing *E. coli* is an emerging

pathogen in the community that commonly causes urinary tract infections in older women. This was followed by 2 reports from Washington and Nebraska respectively that showed *Enterobacteriaceae* that produce CMY, ACC and DHA types of AmpC β-lactamases are present in outpatient clinics in the USA (Hanson *et al*., 2008; Qin *et al*., 2008).

1.4.3. Metallo-β-lactamases (MBLs)

The production of MBLs of the IMP and VIM types, have mostly been detected in *P. aeruginosa* and remain relatively rare in members of the *Enterobacteriaceae* except for *K. pneumoniae* and *E. coli* present in Mediterranean Europe (VIM-producing *K. pneumoniae* in Greece, Italy and Spain), and Taiwan and Japan (IMP-producing *E. coli*) (Bushnell *et al*., 2013). IMP and VIM types of MBLs are often associated with class 1 integrons that contain various gene cassettes that often render isolates resistant to various groups of antimicrobial agents.

Recently, a new type of metallo-β-lactamase (MBL), named NDM, was described in *K. pneumoniae* and *E. coli* recovered from a Swedish patient who was hospitalized in New Delhi, India. MBLs have the ability to hydrolyse a wide variety of β-lactams, including the penicillins, cephalosporins and carbapenems, but not the monobactams, and are inhibited by metal chelators such as EDTA (Johnson and Woodford, 2013).

The majority of NDM-1-producing bacteria are broadly resistant to various drug classes and also carry a diversity of other resistance mechanisms (e.g. to aminoglycosides and fluoroquinolones), which leaves limited treatment options.

From their original detection in 2008, NDM-1–carrying *Enterobacteriaceae* have been identified in more than 70 countries in all regions (Fig. 1.6). Kumarasamy and colleagues (Kumarasamy *et al*., 2010), provide compelling evidence that NDM-producing *Enterobacteriaceae* (mostly *K. pneumoniae* and *E. coli*) are widespread in India and Pakistan. They also found that many UK patients infected with NDM-producing bacteria had recently traveled to India to undergo several types of medical procedures. Recent reports from the subcontinent (including India, Pakistan and Bangladesh) show that the distribution of NDM β-lactamases among *Enterobacteriaceae* are widespread through these countries (Castanheira *et al*., 2011a; Castanheira *et al*., 2011b; Lascols *et al*., 2009).

Since 2011, NDM-1-positive bacteria have been reported worldwide (Johnson and Woodford, 2013). Most are *Enterobacteriaceae* including *E. coli* from patients hospitalized in 2009 and 2010 with an epidemiological link to the Indian subcontinent. Recent findings suggest that the Balkan states and the Middle East might act as secondary reservoirs for the spread of NDM-1, which may or may not initially have reached these countries from the Indian subcontinent (Johnson and Woodford, 2013). *Enterobacteriaceae* with NDM-1 have been recovered from many clinical settings, reflecting the disease spectra of these opportunistic bacteria,

including hospital and community-onset urinary tract infections, septicemia, pulmonary infections, peritonitis, device-associated infections and soft tissue infections. NDM-1-positive bacteria have been recovered from the gut flora of travelers returning from the Indian subcontinent and undergoing microbiological investigation for unrelated diarrheal symptoms (Leverstein-Van Hall *et al*., 2010). There is also widespread environmental contamination by NDM-1-positive bacteria in New Delhi (Walsh *et al*., 2011).

There is no evidence that *E. coli* that produce NDM are more virulent than other isolates, however recent studies described presence of NDM β-lactamases in the very successful *E. coli* ST131 with an identical virulence genotype than ST131 that produce CTX-M β-lactamases (Pitout, 2008). Of interest, ST131 with VIM, KPC, OXA-48 carbapenemases have also recently been described (Johnson and Woodford, 2013). Antibiotics such as colistin, tigecycline and fosfomycin show the best activity against NDM-producing bacteria (Akova *et al*., 2012).

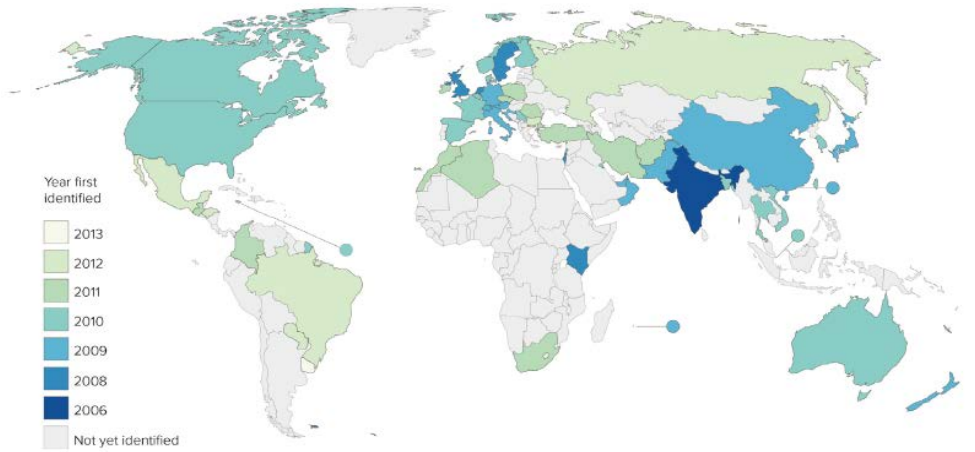

Fig. 1.6. Spread of New Delhi metallo-β-lactamase-1: first detection Adapted from Johnson and Woodford, 2013

1.5. Plasmid mediated transfer of antimicrobial resistance among *E. coli*

There are plasmid families that are largely prevalent and also plasmids prevalently associated with specific resistance genes. The IncFII, IncA/C, IncL/M, and IncI1 plasmids showed the highest occurrence among typed resistance plasmids (Table 1.2)

1.5.1. Plasmids carrying ESBLs in *E. coli* of animal origin

IncF plasmids carrying the *bla*$_{\text{CTX-M-15}}$ gene are not exclusive to clone ST131, since they were identified in other *E. coli* sequence types (ST405, ST354, ST28, and ST695). Plasmids belonging to the IncL/M family were responsible for the spread of CTX-M-3 in Poland, since common plasmid scaffolds were identified in eight species in 15 hospitals (Baraniak *et al*., 2002; Marcade *et al*., 2009). IncL/M plasmids carrying the *bla*$_{\text{CTX-M-3}}$ gene were also reported in other Eastern European countries and in France, Belgium, and Korea, and very often, the aminoglycoside resistance gene *armA* has been co-localized on the same IncL/M plasmid as the *bla*$_{\text{CTX-M-3}}$ gene. The spread of *bla*$_{\text{CTX-M-9}}$ in clinical *E. coli* and *S. enterica* serovar Virchow was

largely due to the dissemination of plasmids in the IncHI2 group, although this gene has been found sporadically with other plasmid families. Plasmids of the IncHI2 group were also associated with the *bla*$_{\text{CTX-M-2}}$ gene in France and Spain. The IncHI2 prototypic plasmid was first identified in *Serratia marcescens* in the United States in 1969, but at that time, this plasmid did not contain any *bla*$_{\text{CTX-M}}$ genes or integrons; thus, these resistance determinants probably represent a recent acquisition into novel IncHI2 plasmid derivatives (Gilmour *et al*. 2004).

IncI1 plasmids were associated with the spread of several other ESBL genes. *E. coli* producing CTX-M-1 was identified in 10.7% of poultry fecal samples collected in 2005 from 10 slaughterhouses located in seven districts in France, and the *bla*$_{\text{CTX-M-1}}$ gene was located on IncI1 plasmids in all the isolates (Girlich *et al*. 2007). Recently, the *bla*$_{\text{CTX-M-1}}$ gene was associated with IncI1 in *E. coli* isolated from human patients in different parts of France, suggesting a potential link between animals and humans for the dissemination of this gene variant in this country (Marcade *et al*., 2009). However, the IncI1 plasmids are so recurrent in *Enterobacteriaceae* that a further typing scheme has been proposed by using plasmid multilocus sequence typing (MLST) (García-Ferna´ndez *et al*. 2009). Besides the IncI1 plasmids, the *bla*$_{\text{CTX-M-1}}$ gene was also identified on plasmids belonging to the IncN group in human clinical strains of *E. coli* and *K. pneumoniae* from France and Spain and in pigs and farm personnel from Denmark (Diestra *et al*. 2009; Marcade *et al*., 2009; Moodley and Guardabassi 2009). The finding that IncI1 and IncN are

both involved in the transmission of the *bla*$_{CTX-M-1}$ gene suggests an animal reservoir for this ESBL gene variant, since either IncN or IncI1 plasmid type has been demonstrated to be highly prevalent in *E. coli* of the avian fecal flora (Johnson *et al*., 2007). The spread of *bla*$_{CTX-M-1}$-carrying plasmids in animals could be sustained by the use of expanded-spectrum cephalosporins in veterinary medicine. In vivo experiments demonstrated the selection and proliferation of indigenous CTX-M-1-producing *E. coli* in the intestinal flora of pigs treated with amoxicillin, ceftiofur, or cefquinome, and such effects persisted for a period longer than the withdrawal time required for these antimicrobials (Cavaco *et al*., 2008).

1.5.2. Plasmids carrying AmpC β-lactamases in *E. coli*

The majority of the *bla*$_{CMY-2}$ plasmids identified in *E. coli* in the United States were categorized in the IncA/C group. IncA/C-positive strains were isolated from beef, chicken, turkey, and pork and were found in samples from different regions of the United States, revealing that this common plasmid backbone is broadly disseminated among resistant zoonotic pathogens associated with agriculture in this country (Winokur *et al*., 2001). Interestingly, repA/C replicons occurred in only 1.0% of *E. coli* obtained from healthy humans not exposed to antimicrobials and were absent in fecal flora from healthy birds (Johnson *et al*., 2007). Therefore, the occurrence of

IncA/C plasmids seems advantageous in bacterial populations that are under antimicrobial selective pressure, likely related to the use of ceftiofur in veterinary medicine (Winokur *et al*., 2001). Several *bla*$_{CMY}$ gene variants were also associated with the IncI1 plasmid family. As previously mentioned, IncI1 plasmids are widespread in *E. coli* animal strains (17.4% and 41% in avian commensal and pathogenic *E. coli* strains, respectively), again suggesting that the dissemination of this gene could occur in the intestinal tract of animals (Johnson *et al*., 2007).

1.5.3. Plasmid-mediated carbapenem resistance in *E. coli*

The 1998–2004 global SENTRY survey found only rare examples of MBL genes (*bla*$_{IMP-1}$, *bla*$_{IMP-11}$, and *bla*$_{VIM-1}$) among *Enterobacteriaceae* isolates (Deshpande *et al*., 2006). The *bla*$_{IMP-4}$ gene was recognized in Australian *Enterobacteriaceae* from Sydney in 2003 to 2006 and caused outbreaks in Melbourne in 2004 and 2005. IncL/M plasmids were identified in 22 of 23 Sydney isolates over 3 years, while IncA/C plasmids were detected in all Melbourne isolates. *K. pneumoniae* isolates carrying the *bla*$_{VIM-1}$ gene and *E. coli* isolates carrying *bla*$_{VIM-1}$ and *bla*$_{CMY-13}$ genes, randomly collected from five different hospitals in Athens and Piraeus from 2001 to 2003 and representative of the VIM-1-producing isolates circulating in Greece, were all assigned to the IncN group, indicating the spread of an epidemic plasmid

associated with the emergence of the *bla*$_{VIM-1}$ gene in that country (Carattoli *et al*. 2006). Four MBL-producing species (*K. pneumoniae, K. oxytoca*, *Enterobacter cloacae*, and *E. coli*) have been described in Spain. The strains showed different *bla*$_{VIM-1}$ genetic environments, and the gene was located on different plasmid scaffolds. A 60-kb conjugative plasmid belonging to the IncI1 group was observed in the *K. pneumoniae* clone and in *E. coli*, while plasmids belonging to the IncH12 group were found among *E. cloacae* isolates.

1.5.4. Plasmids conferring quinolone and/or aminoglycoside resistance

Quinolone resistance in *Enterobacteriaceae* is usually the result of chromosomal mutations, leading to alterations in target enzymes or drug accumulation. More recently, plasmid-mediated quinolone resistance (PMQR) has been reported by the acquisition of the *qnr, qepA, and aac(6')-Ib-cr* genes (Poirel *et al* ,2008). Very often, PMQR is associated with ESBLs and/or aminoglycoside resistance genes on the same plasmid, and the spread of such multidrug-resistance plasmids among *Enterobacteriaceae* strains has a potential impact on the empirical management of complicated urinary tract infections (Paterson, 2006). High-level resistance to aminoglycosides mediated by the production of 16S rRNA methylase has been increasingly reported among various gram negative pathogens. Six plasmid-encoded

16S rRNA methylases have been identified, as follows: *rmtA* to *rmtD*, *armA*, and *npmA* (Doi and Arakawa, 2007). As previously mentioned, the dissemination of *armA* in clinical isolates from Europe has been associated with IncL/M plasmids co-localizing with the $bla_{\text{CTX-M-3}}$ gene (Bercot *et al*., 2008; Bogaerts *et al*., 2007; Galimand *et al*. 2005), while *armA* was identified on an IncN plasmid in animals from Spain (Gonzalez-Zorn *et al*. 2005). The *rmtB* gene was prevalently associated with IncA/C plasmids, which co-localized with the $bla_{\text{CTX-M-14}}$ gene (Kang *et al*., 2008; Kang *et al*., 2009). The prevalent plasmid families carrying *armA* were IncA/C and IncHI2 until 1998, but after 2001. The fully sequenced IncF plasmid pIP1206 was identified in *E. coli* in France and carried the *rmtB* and *qepA* genes, with the latter gene conferring resistance to hydrophilic fluoroquinolones by efflux. pIP1206 carried two copies of the repFII replicon and two additional replicons of the repFIA and repFIB types. The *qepA*, *rmtB* genes and the *qepA2* gene variant were recently identified on IncF plasmids in *Enterobacter aerogenes* from Korea and also in *E. coli* from France (Cattoir *et al*., 2008; Park *et al*., 2009). The *qnrA1* gene was located within a *sul1*-type integron often associated with the $bla_{\text{VEB-1}}$ gene. The *qnrB4* and *qnrB6* genes associated with *armA* and ESBL genes were identified in *E. col* in Korea, located on particular IncF plasmids, carrying the replicon FIIAs, similar to *Salmonella* virulence plasmids (Tamang *et al*., 2008).

Table 1.2. Major plasmid families and associated resistance genes in antimicrobial resistant *E. coli* isolated worldwide from animal. Adapted from Carattoli *et al*. 2009

Replicon	No. of plasmid	Resistance genes
F	331	aac(6')*-Ib-cr*, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-1-2-3-9-14-15-24-27}}$, *bla*$_{\text{DHA-1}}$, *bla*$_{\text{SHV-2-5-12}}$, *bla*$_{\text{TEM-1}}$, *armA, rmtB, qepA, qepA2, qnrA1, qnrB2, qnrB4, qnrB6, qnrB19, qnrS1*
A/C	317	*bla*$_{\text{CMY-2-4}}$, *bla*$_{\text{CTX-M-2-3-14-15-56}}$, *bla*$_{\text{SHV-2-5-12}}$, *bla*$_{\text{TEM-3-21-24}}$, *bla*$_{\text{IMP-4-8-13}}$, *bla*$_{\text{VIM-4}}$, *bla*$_{\text{VEB-1}}$, *armA, rmtB, qnrA1*
L/M	270	*aac(6')-Ib-cr*, *bla*$_{\text{CTX-M-1-3-15-42}}$, *bla*$_{\text{TEM-3-10}}$, *bla*$_{\text{SHV-5}}$, *bla*$_{\text{IMP-4-8}}$, *armA, qnrA1, qnrB1, qnrB2, qnrB4, qnrS1*
I1	146	*bla*$_{\text{CMY-2-7-21}}$, *bla*$_{\text{CTX-M-1-2-3-9-14-15-24}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{TEM-1-3-52}}$, *bla*$_{\text{VIM-1}}$, *armA, rmtB, mphA, qnrA1*
HI2	90	*bla*$_{\text{CTX-M-2-3-9-14}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{VIM-1}}$, *armA, qnrA1, qnrS1*
N	70	*bla*$_{\text{KPC-2}}$, *bla*$_{\text{CTX-M-1-3-15-32-40}}$, *bla*$_{\text{VIM-1}}$, *qnrA3, qnrB2, qnrB19, qnrS1, armA*

Chapter II

Antimicrobial resistance, virulence gene and PFGE-profiling of *Escherichia coli* isolates from cattle farms

Abstract

To estimate the prevalence of *Escherichia coli* (*E. coli*) with potential pathogenicity in cattle farm in the South Korea, a total of 290 *E. coli* isolates were isolated from cattle farms over a period of 2 years in South Korea. These were examined for phenotypic and genotypic characteristics, including antimicrobial susceptibility, serotype, and gene profiles of virulence and antimicrobial resistance. The most dominant virulence gene was *f17* (26.2%), followed by s*tx2* (15.9%), *ehxA* (11.0%), s*tx1* (8.3%), *eae* (5.2%), and *sta* (4.1%). 15.9% of the STEC isolates possessed *eae*. All isolates except for one showed resistance to one or more antimicrobials, with 152 isolates exhibiting multidrug-resistance. The most prevalent resistance phenotype detected was streptomycin (63.1%), followed by tetracycline (54.5%), neomycin (40.3%), cephalothin (32.8%), amoxicillin (30.0%), ampicillin (29.7%), and

sulphamethoxazole/trimethoprim (16.6%). The associated resistance determinants detected were *strA-strB* (39.0%), *tet*(E) (80.0%), *tet*(A) (27.6%), *aac(3)-IV* (33.1%), *aphA1* (21.4%), *bla*$_{TEM}$ (23.8%), and *sul2* (22.1%). When investigated by O serotyping and PFGE molecular subtyping, the high degree of diversity was exhibited in *E. coli* isolates. These results suggest that *E. coli* isolates from South Korean cattle farms are significantly diverse in terms of virulence and antimicrobial resistance. In conclusion, the gastroinstestinal flora of cattle could be a significant reservoir of diverse virulence and antimicrobial resistance determinants, which is potentially hazardous to public health.

Keywords: *Escherichia coli*, prevalence, virulence, antimicrobial resistance, serotyping, PFGE

Introduction

Although *Escherichia coli* (*E. coli*) is usually a non-pathogenic member of the gastroinstestinal flora of the host, some strains may cause diseases that represent a hazard to the public health and the food-producing animal industry. Bacterial pathogenicity is determined by the presence of virulence factors which are mostly encoded by genes located in chromosomes and/or plasmids. Therefore, it is very

important to distinguish the pathogenic *E. coli* from gastroinstestinal flora by detection of the virulence factors, such as toxins, fimbriae and non-fimbrial adhesion molecules. Antimicrobial agents have been used as preventive measures against bacterial infections in the food-producing animal industry, with beneficial effects in decreasing morbidity and mortality (Berge *et al.*, 2009). However, the intensive and indiscriminate usage of antimicrobials may induce the emergence and dissemination of antimicrobial resistance, not only in pathogenic bacteria but also in commensals. Also, until 2011, copious amounts of various antimicrobial agents were used as feed supplements in the livestock industry in South Korea. Furthermore, until 2013, antimicrobial agents could be used without a veterinarian's prescription. These factors may have accelerated the emergence and dissemination of antimicrobial resistance. Antimicrobial resistance genes have been considered as one of virulence factors (Davies and Davies, 2010). Moreover, commensal *E. coli* isolates, resistant to several antimicrobials, may constitute an important reservoir of antimicrobial resistance determinants, which may be transferred via transmissible plasmids intra and/or inter species (Wright, 2007). Thus *E. coli* strains isolated from healthy animals need to be assessed for the prevalence of resistance in animal populations, and these results can be used in several monitoring programs (Franklin *et al.*, 2001). Pathogenic *E. coli* strains isolated from animals can be transmitted from animals to humans through the food chain and give rise to severe disease in humans. Therefore it is important that the pathogenic *E. coli* strains are distinguished from

gastroinstestinal flora in food-animals by identification of virulence factors. Serotyping has been widely used for the differentiation of *E. coli* pathogenicity, and it is of value due to the common association of some serotypes with calf diarrhea (Acres, 1985). However, the pathogenicity of *E. coli* strains cannot be determined only by serotyping because there are many factors to decide the virulence of *E. coli*. According to the Center for Disease Control and Prevention (CDC), pathogenic *E. coli* isolates are classified as enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (CDC, 2001). ETEC isolates can cause diarrhea in newborn calves by the expression of fimbriae (F5, F41, and F17) and enterotoxin (STa and LT) (Nagy and Fekete, 1999). STEC isolates, producing Stx1, Stx2, or both, known also as verotoxigenic *E. coli* (VTEC), are the significant causatives of diarrhea in calves (Nagy and Fekete, 1999). In humans, STEC can cause hemorrhagic colitis (HC) and the hemolytic uremic syndrome (HUS) (Nataro and Kaper, 1998). EPEC and STEC isolates, carrying eae genes, produce the adhesion protein intimin to initiate attachment to the enterocyte and effacement of the microvillus border (Jerse *et al.*, 1990). Although several researches on antimicrobial resistance or virulence factors in pathogenic bacteria have been carried out in South Korea (Kang *et al.*, 2005; Lim *et al.*, 2007), there has been no report showing the relationship between antimicrobial resistance and virulence factors in bacteria isolated on cattle farms. Based on current knowledge,

the genotypic and phenotypic prevalence of virulence factors and of antimicrobial resistance were investigated in enteric *E. coli* isolated from cattle farms in South Korea. Also, their genetic relationships were compared, using the macrorestriction profiling (PFGE). This information may give a new horizon to develop a new preventive measure against foodanimal originated *E. coli* infection.

Materials and Methods

Bacterial isolates

A total 290 *E. coli* strains were isolated from 830 fecal samples collected from beef cattle on eight farms from six different provinces (Asan, Anyang, Buyeo, Chilgok, Namyangju, and Gwangju) between 2011 and 2012. These farms were ceftificated by HACCP (Harzard Analysis Critical Control Point) for their hygeine. The samples were obtained from cattle rectum and pats on the shed. The samples were placed at 4–8°C and delivered to the laboratory within 24 h. The fecal samples were plated onto eosin methylene blue (EMB) agar, and MacConkey agar for selection, and were incubated at 37°C for 18 h. From each sample, three to five colonies that were suspected to be *E. coli* were sub-cultured onto a blood agar plate (BAP). Isolates were confirmed as *E. coli* by a standard biochemical test (Indole,

Methyl Red, Voges-Proskauer, and citrate utilization tests) and by the Vitek2 system (bioMérieux, France). The confirmed *E. coli* isolates were stored in tryptic soy broth (TSB), with 20% glycerol, at -70°C to await further analysis. The reference *E. coli* strains for virulence factor were O9:K35 ($K99^+$ and $F41^+$), O141:K85ab ($987P^+$ and STa^+) and O15:H11 (LT^+) which were kindly provided by the Animal and Plant Quarantine Agency, Anyang, Republic of Korea. In addition, the *E. coli* strains EC192 ($Stx1^+$, $Stx2^+$, $Intimin^+$, and $EhxA^+$) isolated in this study were used for reference.

Antimicrobial susceptibility test

The resistance against 15 antimicrobials was tested by the disk diffusion test. The following antimicrobial compounds were used: enrofloxacin (ENR), 5 μg (Bayer, Germany); ampicillin (AMP) 10 μg; amoxicillin (AML), 10 μg; streptomycin (S), 10 μg; gentamicin (CN), 10 μg; neomycin (N), 30 μg; tetracycline (TE), 30 μg; nalidixic acid (NA), 30 μg; ciprofloxacin (CIP), 5 μg; cephalothin (KF), 30 μg; ceftiofur (EFT), 30 μg; ceftazidime (CAZ), 30 μg; chloramphenicol (C), 30 μg; florfenicol (FFC), 30 μg; sulfamethoxazole/trimethoprim (SXT), 25 μg, which contains 1.25 μg of trimethoprim and 23.75 μg of sulfamethoxazole (Oxoid, England). All antimicrobial resistance tests were performed on Mueller-Hinton (MH) agar, and data were classified as susceptible or resistant, based on the Clinical and

Laboratory Standards Institute (CLSI) guidelines. *E. coli* ATCC 29522 was included as the quality control strain.

Detection of virulence and antimicrobial resistance genes

Bacterial genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, USA), and following the manufacturer's instructions. The concentration of DNA was measured using a Nanodrop N-1000 spectrophotometer (Thermoscientific, USA). PCR assay for virulence genes: All isolates were analyzed for nine different virulence genes. The multiplex polymerase chain reactions (m-PCR) were used to detect toxins (Stx1, Stx2, STa, and LT), adhesions (F5, F17, F41, and Intimin) and enterohemolysin, and were carried out as described in earlier studies (Schmidt *et al.*, 1995; Franck *et al.*, 1998; Van Bost *et al.*, 2001; Lopez-Saucedo *et al.*, 2003), with some modifications. The primers and PCR conditions used in this study are shown in Table 2.1.

PCR assay for antimicrobial resistance genes

Genes encoded for aminoglycoside resistance (*aadA*, *aadB*, *aphA1*, *aphA2*, *strA-strB*, and *aac(3)-IV*), β-lactam resistance (*ampC*, bla_{TEM}, bla_{OXA}, and bla_{SHV}), tetracycline resistance (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G)), phenicol

resistance (*cat*, *cmlA*, and *floR*) and sulfonamide resistance (*sul1*, *sul2*, and *sul3*) were screened as described by previous study (Karczmarczyk et al., 2011a) with some modifications (Table 2.1). EC137, EC192, and EC277 isolated in this study were used as the positive controls for detection of *aadA*, *aphA1*, *strA-strB*, *aac(3)-IV*, *ampC*, bla_{TEM}, *tet*(A), *tet*(B), *tet*(C), *tet*(E), *cat*, *cmlA*, *floR*, *sul1*, *sul2*, and *sul3*. All PCRs were performed on Veriti thermocycler (Applied Biosystems, USA), and each run included a negative control and an appropriate positive control. The reactions were run in duplicate to confirm results. All PCR products were analyzed by electrophoresis on a 2.0% agarose gel for 1 h at 100 V, and photographed under UV light after staining with ethium bromide. Amplified PCR products of expected sizes were subjected to direct sequencing by an automatic sequencer and dye termination sequencing system (Macrogen Co., Korea). A BLAST search for homologous sequence was performed in the GenBank database at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST).

Serotyping

Out of a total 290 *E. coli* isolates, 53 isolates were selected for serotyping. These isolates showed multidrug-resistance and harbored more than one virulence gene. The O serogroup of *E. coli* isolates was determined using 181 O-antisera (O1-O187)

by a standard slide agglutination test (Orskov and Orskov, 1984). The O-antisera were provided by the Animal and Plant Quarantine Agency, Anyang, South Korea.

Pulsed-field gel electrophoresis (PFGE) profiling

Overall, 53 *E. coli* isolates determined serogroups were analyzed by PFGE, according to a standard protocol of the Center for Disease Control and Prevention (CDC), with some modifications. *E. coli* isolates were incubated on tryptic soy agar (TSA) at 37°C for 18 h. Bacteria were suspended in a cell suspension buffer (100 mM Tris:100 mM EDTA, pH 8.0), and adjusted to OD600 of 1.3–1.4 using a spectrophotometer. The cell suspension (400 μl) was mixed with 20 μl of proteinase K and 400 μl of melted 1% SeaKem Gold Agarose (Lonza, USA). The mixture was dispensed into appropriate wells of a disposable plug mold (Bio-Rad Laboratories, USA). After solidification, the plugs were transferred to 15 ml conical tubes containing 5 ml of cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) and 0.5 mg/ml proteinase K. The plugs were lysed in a 55°C hybridization incubator for 2 h. After lysis, the plugs were washed twice with distilled water, and four times with TE buffer, for 15 min per wash at 55°C hybridization. After washing, the plugs were digested with 50 U of *Xba*I (TaKaRa, Japan) at 37°C for 4 h. The digested plugs were loaded into appropriate wells in a 1% SKG gel. The gel was electrophoresed using a CHEF-MAPPER (Bio-Rad Laboratories) with pulse times

of 2–30 sec at 14°C for 18 h in 0.5× tris-borate EDTA (TBE) buffer at 6 V/cm. Then the gels were stained with ethium bromide, and photographed using the Gel Doc XR system (Bio-Rad Laboratories). Gel images were analyzed using GelCompar II software (Applied Maths, Belgium). PFGE dendrograms were constructed using the unweighted pair group method with arithmetic means (UPGMA) analysis based on Dice coefficients.

Results

Antimicrobial susceptibility results

A total of 205 isolates were resistant to one or more antimicrobials. The prevalence of antimicrobial resistances was as follows: streptomycin (63.1%), tetracycline (54.5%), neomycin (40.3%), cephalothin (32.8%), amoxicillin (30.0%), ampicillin (29.7%), nalidixic acid (19.0%), chloramphenicol (17.9%), sulphamethoxazole/trimethoprim (16.6%), gentamicin (14.5%), enrofloxacin (11.0%), ciprofloxacin (10.0%), florfenicol (7.2%), ceftiofur (1.0%), and ceftazidime (1.0%) (Table 2.2). Two isolates showed resistance against 13 antimicrobials. Among all the isolates, 116 (40.0%) showed resistance against more than three different classes of antimicrobials. The most frequent multidrug-resistance

patterns were [ampicillin-amoxicillin-neomycin-streptomycin-tetracycline] and [ampicillin-amoxicillin-cephalothin-gentamicin-neomycin-streptomycin-tetracycline], both detected in eight of the total 116 multidrug-resistant isolates (6.9%) (Table 2.3).

Prevalence of virulence genes

A total of 290 *E. coli* isolates were analyzed by m-PCR. As shown in Table 2.4, 147 (50.7%) isolates carried more than one virulence factor. The most prevalent virulence gene was *f17* (26.2%). No other virulence gene was detected in the *f17*-positive isolates. The frequencies of virulence genes detected by m-PCR were as follows: s*tx*2 (15.9%), *ehxA* (11.0%), *stx1* (8.3%), *eae* (5.2%), and *sta* (4.1%). None of the strains carried *f5*, *f41* or *lt*. Of 55 *stx*-positive isolates, 9 (16.4%) carried *stx1*, while the *stx*2 gene was detected in 31 (56.4%) of the isolates. Fifteen (27.3%) isolates carried both *stx1* and *stx*2. Also, the *eae* and *ehxA* genes were detected in 10 (17.5%) and 15 (26.3%) isolates, respectively, of the *stx* gene positive isolates.

Prevalence of antimicrobial resistance genes

From twenty-two resistance genes investigatedin the 290 *E. coli* isolates, sixteen were detected. Genes *aphA2*, *aadB*, bla_{OXA}, bla_{SHV}, *tet*(D), and *tet*(G) were not

detected in any *E. coli* isolates. A total of 278 isolates (95.9%) carried more than one resistance gene. The prevalence of antimicrobial resistant genes is shown in Table 2.2. Regarding aminoglycoside resistance, the most prevalent gene was *strA-strB* (39.0%), followed by *aac(3)-IV* (33.1%), *aphA1* (21.4%), and *aadA* (19.7%). The predominant β-lactamase gene was bla_{TEM} (23.8%). Among the ampicillin/amoxicillin resistant isolates, the bla_{TEM} gene was identified in 72.1% (62/86). Additionally the *ampC* was detected in 287 isolates (99.0%). The determinants for phenicol resistance were identified as follows: *cat* gene (7.6%), *floR* gene (7.6%), and *cmlA* gene (4.1%). The *floR* gene encoding chloramphenicol/florfenicol efflux pump was detected in 90.5% (19/21) of the florfenicol resistant isolates. Sulfonamide resistance was attributable to *sul2* genes in 33.4% at the following frequencies: *sul1* (22.1%) and *sul3* (3.8%). The most prevalent tetracycline resistant gene was *tet*(E) (80.0%), followed by *tet*(A) (27.6%), *tet*(B) (26.9%), and *tet*(C) (22.4%). Among the tetracycline resistant isolates, 97.5% of isolates (154/158) carried *tet* genes. On the oher hand, 80.3% (106/132) of tetracycline susceptible isolates were positive for *tet* genes.

Serogroup determination

Overall, 47 of 53 isolates examined were serotyped, and belonged to 20 different O serogroups, while six were nontypeable. Only one isolate was identified as O157.

The majority of isolates (62.3%) were classified in four serogroups, including O7 (22.6%), O101 (13.2%), O15 (7.5%), and O9 (7.5%) (Table 2.5).

Molecular subtyping of *E. coli* isolates using PFGE

PFGE of *Xba*I-digested chromosomal DNA of the 53 serotyped *E. coli* isolates showed 93 different PFGE subtype patterns, with 14–26 discernible bands, ranging from 30 to 600 kb in molecular size (Fig. 2.1). The similarity of PFGE profiles was 46.0%, with *Xba*I as analyzed by the Dice coefficient. However, these PFGE subtypes could be clustered into 26 groups of closely related PFGE subtypes, with more than 60% similarity by the Dice coefficient. 1 to 13 isolates were contained within each group. Although the PFGE profiling analyzed in this study showed a high degree of polymorphism, subgroup 14 showed high similarity (Dice coefficient similarity > 75%).

Discussion

According to the data provided by the Korea Animal Health Products Association (KAHPA), since the use of antimicrobials as feed supplements was banned by the Korean government in July 2011, the quantity of antimicrobials used therapeutically

is showing a growing trend. Therefore, it is necessary to choose the efficient antimicrobials for the reduction of them. Our results can be helpful for the antimicrobial selection.

ETEC strains are the most important agent causing diarrhea in cattle (Nagy and Fekete, 1999). Therefore, for the detection of ETEC, we screened the genes of fimbriae (F5, F41, and F17) and enterotoxins (LT and STa). As shown in the results, we found none of the *f5*, *f41*, and *lt* genes, and a relatively low percentage (4.14%) of *sta* genes. We identified that 75 (25.9%) of a total of 290 *E. coli* isolates carried the gene for F17 fimbriae, although these isolates were negative for other virulence genes. These results are in agreement with those of a previous study (Ghanbarpour and Oswald, 2009), which showed a greater prevalence of F17 fimbriae than of F5 and F41. According to Moon and Burnn (Moon and Bunn, 1993), the predominant distribution of F17 fimbriae could be resulted from the environment and/or from genetic pressures on the *E. coli* isolates, such as vaccination against the F5 and F41 fimbriae.

Over the past three decades, studies on STEC infection in cattle have been carried out and have demonstrated that the prevalence of non-O157 STEC in cattle may vary according to the country (Hussein, 2007). The different prevalence rates of STEC might be due to patterns of shedding STEC, influenced by several factors, such as geographic differences, sampling and detection methods, age of host and seasonal variations (Menrath *et al.*, 2010). Although the results in this study is not enough to

reflect true differences in prevalence rate, we could predict that STEC is widely distributed in South Korean cattle farms.

When analyzed by m-PCR, 46 STEC isolates (15.9%) harbored the *stx*2 gene, 24 isolates carried (8.3%) *stx1* and 15 isolates (5.2%) had both genes. This result differed from a previous report (Bergamini *et al.*, 2007) that showed the dominance of the *stx1* gene in cattle. However, other studies have shown agreement with our study (Zschock *et al.*, 2000). Based on epidemiologic data indicating the significance of the Stx2 toxin in the development of HUS (Bonnet *et al.*, 1998), the dominance of STEC strains carrying *stx*2 gene in cattle might cause a serious risk to public health.

The intimin encoded by the chromosomal gene *eae* may be necessary for the development of the virulence of STEC, providing them with attaching and effacing activity (Jerse *et al.*, 1990). Several authors have described the significant association between the presence of the *eae* gene and the pathogenicity of STEC in causing severe diarrhea (HC) and disease (HUS) (Mainil *et al.*, 1993). The presence of the *eae* gene was detected in 17.5% (10/55) of the STEC isolates, including one O157 bovine isolate in the present study. The percentage of *eae*-positive STEC in this study was higher than that found in healthy cattle (Blanco *et al.*, 1997) or in diarrheic calves (Nguyen *et al.*, 2011).

Enterohemolysin is widespread among STEC strains isolated from calves (Aidar-Ugrinovich *et al.*, 2007). It has been suggested that this virulence factor probably

synergizes the effects of the Shiga toxin, and that it can be used as a diagnostic indicator because the presence of the *ehxA* gene is highly associated with the Shiga toxin (Beutin *et al.*, 1989). The prevalence of enterohemolysin among bovine STEC strains has been reported by several authors (Beutin *et al.*, 1989; Wieler *et al.*, 1992; Aidar-Ugrinovich *et al.*, 2007), with the prevalence of STEC ranging from 51.0% to 70.8%. About 62.5% of STEC isolates harbored the *ehxA* gene in our study, showing good agreement with these studies.

In the *strA-strB* determinants encoding enzymes required for streptomycin resistance (Chiou and Jones, 1995), the detection rates from our study were lower than in a previous report that described these determinants as being common in *E. coli* isolates resistant to aminoglycoside compounds (Karczmarczyk et al., 2011b). Notably, among the *E. coli* isolates resistant to gentamicin, none of isolates harbored the *aadB* determinant conferring resistance to gentamicin, tobramycin and kanamycin. Therefore it might be suggested that resistance to gentamicin in this study resulted from the presence of the *aac(3)-IV* gene, mediating a broad spectrum aminoglycosides resistance that included gentamicin (Vinue *et al.*, 2010). It is interesting to note that resistance genes were largely found in isolates identified as susceptible by phenotype, and this could result from the existence of defective genes, and a reduced expression of these determinants (Karczmarczyk et al., 2011b).

Only bla_{TEM} was detected as a β-lactamase gene in this study, which agreed with previous reports (Karczmarczyk et al., 2011b; Wedley *et al.*, 2011). The prevalence

of genes encoding amoxicillin- and ampicillin-resistance was lower than those of the phenotype to the antimicrobials in our study.This phenomenon may be attributed to the other β-lactamase genes not investigated in this study such as CTX-M or the over-expression of AmpC enzyme by mutations (Bergstrom and Normark, 1979; Tracz *et al.*, 2007).

Although the use of chloramphenicol in food-animals was banned in South Korea, the resistance to chloramphenicol was analyzed and was still identified with a relatively high percentage in our data. It could be resulted from the co-resistance to florfenicol by its similarity of molecular structure. The persistence of chloramphenicol has been reported by other authors (Kang *et al.*, 2005; Lim *et al.*, 2007). The persistence of chloramphenicol resistance could result from the presence of *floR* gene. This gene encodes a specific exporter for both chloramphenicol and florfenicol. Gene *cmlA* codify an exporter specific for chloramphenicol, while the *cat* gene codify for the enzymatic inactivation from chloramphenicol. The data on *sul2* and SXT-positive isolates of our study was consistent with the previous study (Enne *et al.*, 2001), which showed that *sul2* was the most prevalent mechanism for resistance to sulfonamides. Also most *sul2*-positive strains (94.8%) also showed positive association with *strA-strB* gene (Boerlin *et al.*, 2005). The *tet*(E) gene was the predominant tetracycline resistance determinant, detected in 80.0% of isolates, followed by *tet*(A) (27.6%), *tet*(B) (26.9%), and *tet*(C) (22.4%). These results showed a difference in relation to the previous study (Medina *et al.*, 2011), which

showed the dominance of *tet*(A) and *tet*(B) determinants in tetracycline resistance. The difference might be due to the origin of the *tet*(E) gene from the environmental Aeromonas strains (Marshall *et al.*, 1986).

In the O serotyping of the 47 *E. coli* isolates, 21 serogroups were identified. These results showed similarity with a previous study showing 25 different O serogroups in healthy cattle (Kobayashi *et al.*, 2001), even though there were differences in the diversity of the O serogroup distribution, which may be attributed to environmental differences, such as diet, antimicrobials used, sampling period and method, geographical difference (Bettelheim *et al.*, 2005). A total of 31.9% of the isolates serotyped were identified as STEC strains, and 2 of 21 O serogroups (O157 and O174) belonged to the major bovine STEC O groups (Blanco *et al.*, 1993).

A genetic comparison of some isolates was carried out us- ing PFGE in order to understand the correlation between virulence genes, antimicrobial resistance, O serotypes, and regional distribution. Our PFGE analysis showed high diversity. Only 4 PFGE patterns were observed more than once. However, isolates that showed the same PFGE pattern shared similarity in the prevalence of serogroups, virulence genes, antimicrobial susceptibility and antimicrobial resistant genes. But there was no isolate that showed co-identity in genotypes and phenotypes. As shown by subgroups 1 and 3, the isolates displaying serotype O101 had common virulence factors, Stx1 and Stx2. But the regional relationship was not found in these subgroups. On the other hand, *E. coli* isolates belonged to subgroup 14 was

determined to share the same serogroup (O7) except two isolates and the same virulence factor (F17). In the view of regional respect, the isolates in subgroup 14 were originated from closed districts, Buyeo and Asan. However, despite this result, our analysis indicates the existence of diverse strains of *E. coli* in regards to virulence factors, antimicrobials resistance, and O serotypes in South Korean cattle farms.

Our results suggest that diverse determinants of virulence and antimicrobial resistance of *E. coli* are widespread in South Korea. Moreover, these determinants can disseminate into non-pathogenic *E. coli* isolates according pathogenicity to them. This suggests that the normal flora of cattle could be a significant reservoir of diverse virulence and antimicrobial resistance determinants, which is potentially threatening to public health. Therefore, surveillance of virulence and antimicrobial resistance in healthy cattle and their transfer mechanisms need to be pursued in further studies.

Table 2.1. Primers and m-PCR conditions for virulence genes and antimicrobial resistance genes used in this study

Target gene	Nucleotide sequence (5'-3')	PCR condition[a]			Amplicon (bp)	Reference
		Denaturing	Annealing	Extension		
f5[a]	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	94℃ for 30s	50℃ for 45s	70℃ for 90s	314	(Franck *et al.*, 1998)
f41[a]	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT				380	
stx$_1$[a]	TTCGCTCTGCAATAGGTA TTCCCCAGTTCAATGTAAGAT				555	
stx$_2$[a]	GTGCCTGTTACTGGGTTTTTCTTC AGGGGTCGATATCTCTGTCC				118	
sta[a]	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA				190	
eae[a]	ATATCCGTTTTAATGGCTATCT AATCTTCTGCGTACTGTGTTCA				425	
f17[a]	GCAGAAAATTCAATTTATCCTTGG CTGATAAGCGATGGTGTAATTAAC	94℃ for 60s	57℃ for 60s	72℃ for 60s	537	(Van Bost *et al.*, 2001)
lt[a]	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT				450	(Lopez-Saucedo *et al.*, 2003)
ehxA[a]	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA				1551	(Schmidt *et al.*, 1995)
aadB[b]	GAGGAGTTGGACTATGGATT CTTCATCGGCATAGTAAAA	94℃ for 60s	53℃ for 60s	72℃ for 60s	208	(Travis *et al.*, 2006)
aphA2[b]	GATTGAACAAGATGGATTGC CCATGATGGATACTTTCTCG				347	
aphA1[b]	ATGGGCTCGCGATAATGTC CTCACCGAGGCAGTTCCAT				600	(Maynard *et al.*, 2003)
aadA[b]	GTGGATGGCGGCCTGAAGCC AATGCCCAGTCGGCAGCG				525	(Madsen *et al.*, 2000)
strA-strB[b]	ATGGTGGACCCTAAAACTCT CGTCTAGGATCGAGACAAAG		58℃ for 60s		893	(Tamang *et al.*, 2007)
aac(3)-IV[b]	TGCTGGTCCACAGCTCCTTC CGGATGCAGGAAGATCAA				653	(Boerlin *et al.*, 2005)
ampC[b]	CCCCGCTTATAGAGCAACAA TCAATGGTCGACTTCACACC	94 ℃ for 60s	53℃ for 60s	72℃ for 60s	634	(Feria *et al.*, 2002)
bla$_{OXA}$[b]	TATCTACAGCAGCGCCAGTG CGCATCAAATGCCATAAGTG				199	
bla$_{TEM}$[b]	TACGATACGGGAGGGCTTAC TTCCTGTTTTTGCTCACCCA				716	(Belaaouaj *et al.*, 1994)
bla$_{SHV}$[b]	TCAGCGAAAAACACCTTG TCCCGCAGATAAATCACCA				475	(MZali *et al.*, 1996)

tet(A) [b]	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG				210	(Ng *et al.*, 2001)
tet(B) [b]	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG		58℃ for 60s		659	
tet(C) [b]	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC	94 ℃ for 60s		72℃ for 60s	418	
tet(D) [b]	AAACCATTACGGCATTCTGC GACCGGATACACCATCCATC				787	(Ng *et al.*, 2001)
tet(E) [b]	AAACCACATCCTCCATACGC AAATAGGCCACAACCGTCAG		58℃ for 60s		278	
tet(G) [b]	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC				468	
cat[b]	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC				547	(Van *et al.*, 2008)
cmlA[b]	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG	94 ℃ for 60s	55℃ for 60s	72℃ for 60s	698	(Keyes *et al.*, 2000)
floR[b]	TATCTCCCTGTCGTTCCAG AGAACTCGCCGATCAATG				399	
sul1[b]	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG				433	(Kerrn *et al.*, 2002)
sul2[b]	CGGCATCGTCAACATAACCT TGTGCGGATGAAGTCAGCTC	94 ℃ for 60s	57℃ for 60s	72℃ for 60s	721	(Lanz *et al.*, 2003)
sul3[b]	CAACGGAAGTGGGCGTTGTGGA GCTGCACCAATTCGCTGAACG				244	(Kozak *et al.*, 2009)

[a] All PCRs were carried out for 25 cycles
[b] All PCRs were carried out for 30 cycles

Table 2.2. The distributions of antimicrobials resistances in phenotypes and genotypes of *E. coli* isolated from South Korean cattle farms.

Antimicrobial class	Antimicrobials (No. of isolates)	Resistance gene (No. of isolates)
Aminoglycosides	Gentamicin (42)	*aac(3)-IV* (96)
	Neomycin (117)	*aadA* (57)
	Streptomycin (183)	*aadB* (0)
		aphA1 (62)
		aphA2 (0)
		strA-strB (113)
β-Lactams	Ampicillin (86)	*ampC* (287)
	Amoxicillin (87)	bla_{TEM} (69)
	Cephalothin (95)	bla_{OXA} (0)
	Ceftiofur (1)	bla_{SHV} (0)
	Ceftazidime (3)	
Phenicols	Chloramphenicol (52)	*cat* (22)
	Florfenicol (21)	*cmlA* (12)
		floR (22)
Sulfonamide	Sulphamethoxazole/ trimethoprim (48)	*sul1* (64)
		sul2 (97)
		sul3 (11)
Tetracycline	Tetracycline (158)	*tet*(A) (80)
		tet(B) (78)
		tet(C) (65)
		tet(D) (0)
		tet(E) (232)
		tet(G) (0)
Fluoroquinolones	Ciprofloxacin (29)	ND
	Enrofloxacin (32)	
Others	Nalidixic acid (55)	ND

[a] ND, not determined

Table 2.3. Multidrug-resistance patterns of *E. coli* isolates from South Korean cattle farms

Resistance profile	No. of resistant antimicrobials	No. of strains (n=116)
AmpAmlNSTe	5	8 (6.9%)
AmpAmlKfCnNSTe	7	8 (6.9%)
KfNaSTe	4	6 (5.2%)
AmpAmlNSTeSxt	6	6 (5.2%)
AmpAmlKfCCipEnrNaCnNSTeSxt	12	6 (5.2%)
AmpAmlKfCCipEnrNaNSTeSxt	11	5 (4.3%)
KfNSTe	4	4 (3.4%)
KfCnNSTe	5	4 (3.4%)
KfSTe	3	3 (3.4%)

Amp, ampicillin; Aml, amoxicillin; S, streptomycin; N, neomycin; Cn, gentamycin; Kf, cephalothin; Enr, enrofloxacin; Cip, ciprofloxacin; Te, tetracycline; Na, nalidixic acid; Sxt, sulfamethoxazole/trimethoprim.

Table 2.4. The distribution of virulence genes of *E. coli* isolated from South Korean cattle farms.

No. of isolates (%)	No. of genes[a]	Virulence gene								
		f5	***f41***	***f17***	***stx1***	***stx2***	***sta***	***lt***	***eae***	***ehxA***
1 (0.3)	4				+	+			+	+
6 (2.1)					+				+	+
2 (0.7)	3					+			+	+
5 (1.7)					+	+				+
9 (3.1)					+	+				
2 (0.7)					+		+			
2 (0.7)						+	+			
1 (0.3)						+			+	
6 (2.1)						+				+
5 (1.7)									+	+
4 (1.4)							+			+
76 (26.2)				+						
1 (0.3)					+					
20 (6.9)	1					+				
4 (1.4)							+			
3 (1.0)										+
143 (50.0)	0									
Total[b] (%)	290	0 (0)	0 (0)	76 (26.2)	24 (8.3)	46 (15.9)	12 (4.1)	0 (0)	15 (5.2)	32 (11.0)

[a] Number of the virulence genes

[b] Sum of each virulence genes

Table 2.5. The distribution of O serotypes of *E. coli* isolated from South Korean cattle farms.

O serogroup	No. of isolates (%)	O serogroup	No. of isolates
O7	12 (22.6)	O140	1 (1.9)
O101	7 (13.2)	O157	1 (1.9)
O15	4 (7.5)	O165	1 (1.9)
O9	4 (7.5)	O168	1 (1.9)
O136	2 (3.8)	O174	1 (1.9)
O2	2 (3.8)	O182	1 (1.9)
O8	2 (3.8)	O26	1 (1.9)
O88	2 (3.8)	O3	1 (1.9)
O1	1 (1.9)	O45	1 (1.9)
O109	1 (1.9)	ND[a]	6 (11.3)
O14	1 (1.9)		

[a] ND, not determined

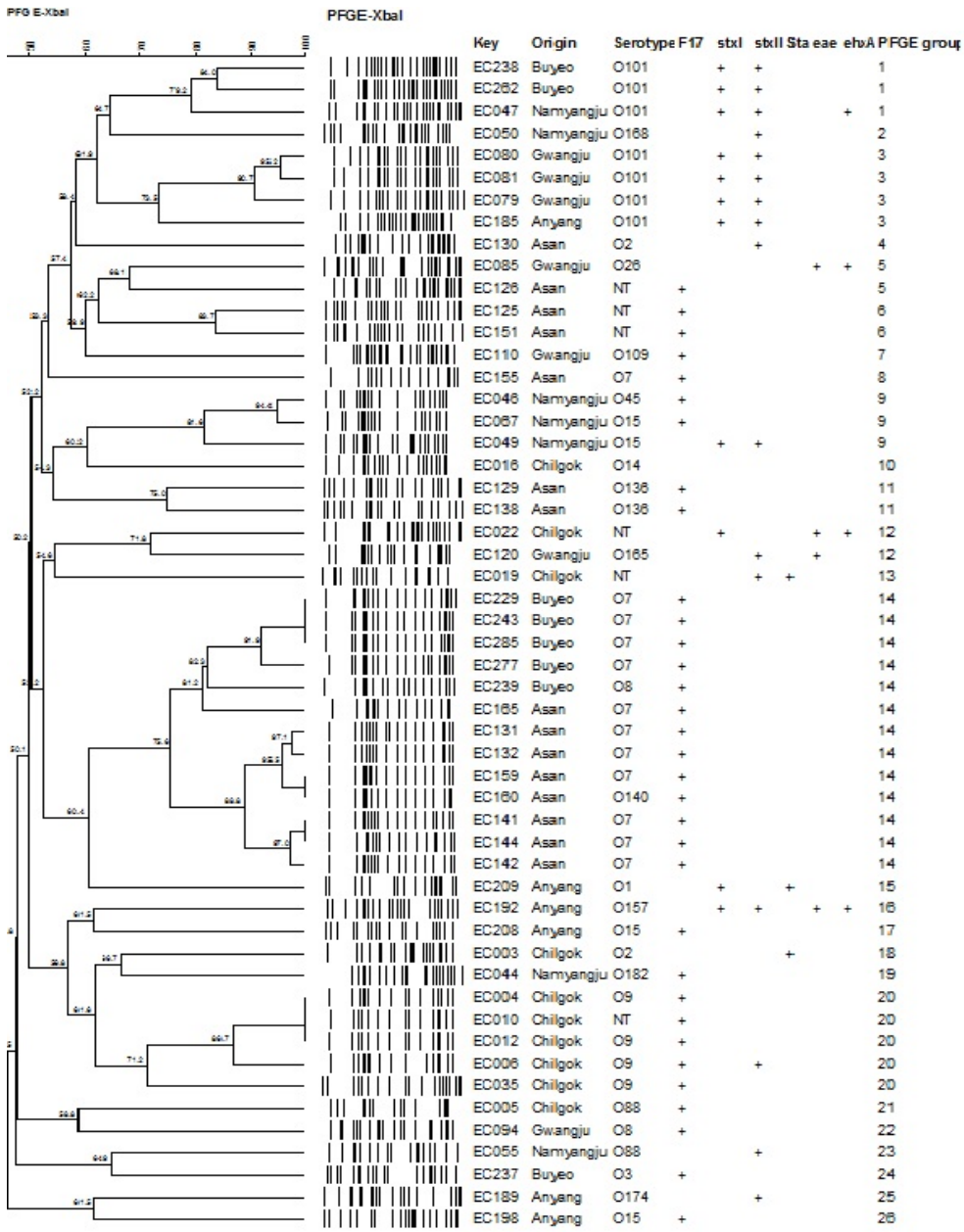

Fig. 2.1. *Xba*I-PFGE dendrogram showing the prevalence of the 53 *E. coli* isolates from South Korean cattle farms

Chapter III

Profiling of antimicrobial resistance and plasmid replicon types in β-lactamase producing *Escherichia coli* isolated from beef cattle

Abstract

In this study, 78 *Escherichia coli* isolated from Korean beef cattle farms were investigated for the production of extended-spectrum β-lactamase (ESBL) and/or AmpC β-lactamase. In the disc diffusion test with ampicillin, amoxicillin, cephalothin, ceftiofur, cefotaxime, ceftazidime, and cefoxitin, 38.5% of the isolates showed resistance to all of ampicillin, amoxicillin, and cephalothin. The double disc synergy method revealed that none of the isolates produced ESBL or AmpC β-lactamases. DNA sequencing showed that all isolates encoded genes for TEM-1-type β-lactamase. Moreover, 78.2% of the isolates transferred the TEM-1-type β-lactamase gene via conjugation. In plasmid replicon typing of all donors, IncFIB and IncFIA were identified in 71.4% and 41.0% of plasmids, respectively. In transconjugants, IncFIB and IncFIA were the most frequent type detected (61.5% and 41.0%, respectively). Based on these results, we might suggest that the

transferable plasmids could provide significant effect on the acquisition and dissemination of β-lactam resistance as well as selection pressure although the level of antimicrobial usage in beef cattle is relatively low compared to those in other livestock animals in Korea. Moreover, to reduce selection pressure and dissemination of β-lactamase, the long-term surveillance of antimicrobial use in domestic beef cattle should be established.

Keywords: β-lactamase, antimicrobial resistance, *Escherichia coli*, plasmid replicon typing

Introduction

The prevalence of β-lactam-resistant *Enterobacteriaceae* has increased consistently over the past few decades. *Escherichia coli* (*E. coli*) producing plasmid-mediated AmpC β-lactamases and/or extended-spectrum β-lactamases (ESBLs) has been of particular concern because of their implications in human and food animal health (Livermore, 2012). These strains encode β-lactamases that mediate resistance to β-lactam antimicrobials included penicillins and extended-spectrum cephalosporins such as 3rd and 4th generation cephalosporins (Carattoli, 2009). Genes encoding β-lactamases are located on mobile genetic elements, mostly plasmids, which can

transfer resistance genes horizontally to non-resistant isolates. Thus, these elements are believed to be responsible for the acquisition and dissemination of β-lactam antimicrobial resistance in the bacterial population.

The incidence of resistance to extended-spectrum β-lactam antimicrobials has increased in Korea (Bradford, 2001; Lee *et al.*, 2013). Most studies that have been performed to date have focused on the characterization of β-lactamases in human clinical isolates (Pai *et al.*, 1999; Pai *et al.*, 2001; Jeong *et al.*, 2003; Jeong *et al.*, 2004; Song *et al.*, 2006). However, there is little information available regarding the prevalence and characteristics of plasmid-mediated AmpC β-lactamases and ESBLs among *E. coli* isolates in the Korean veterinary industry (Rayamajhi *et al.*, 2008; Lim *et al.*, 2009; Tamang *et al.*, 2012; Tamang *et al.*, 2013b). Furthermore, β-lactamases-producing *E. coli* isolated from beef cattle have rarely been reported in Korea.

Enteric bacteria, especially *E. coli*, derived from livestock animals are potentially infectious pathogens and reservoirs for β-lactamase genes; accordingly, investigations of these microorganisms are necessary for public health. In view of the risk of spreading ESBL and AmpC β-lactamase resistance determinants among *E. coli* isolates, it is important to elucidate the mechanism by which resistance is transferred between isolates. Thus, in the present study, we investigated antimicrobial resistance profiles and plasmid replicon types of ampicillin-resistant

E. coli isolates recovered from the feces of beef cattle with the goal of investigating the transfer of β-lactamase genes and antimicrobial resistance to non-resistant *E. coli*.

Materials and Methods

Bacterial isolates

A total of 290 *E. coli* strains were isolated from feces collected from beef cattle during 2011–2012 (Shin *et al.*, 2014). Briefly, *E. coli* isolates of this study were isolated from 830 fecal samples collected from healthy beef cattle on eight farms from six different provinces in South Korea. The fecal samples were collected from rectum and pats of cattle and plated onto MacConkey agar (BD, USA) for selection, then incubated at 37°C for 18 h. From each sample, three to five colonies suspected of being *E. coli* were sub-cultured onto blood agar plates. Isolates were confirmed as *E. coli* by a standard biochemical test and by the Vitek2 system (bioMérieux, France).

Antimicrobial susceptibility test

For selection of β-lactam-resistant *E. coli*, all isolates were screened by plating on MacConkey agar plates containing ampicillin (16 µg/mL) because the MIC value of ampicillin for *E. coli* was above or at the breakpoint (≥ 32 µg/mL) for ampicillin resistance (CLSI, 2013). Overall, a total of 78 *E. coli* isolates were selected for characterization of β-lactamases in this study. All 78 *E. coli* isolates were tested using antimicrobial-containing discs according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013). The following antibiotics were tested: ampicillin (AMP), 10 µg; amoxicillin (AMX), 20 µg; cephalothin (CF), 30 µg; ceftiofur (EFT), 30 µg; cefoxitin (FOX), 30 µg; cefotaxime (CTX), 30 µg; and ceftazidime (CAZ), 30 µg (Oxoid, UK). The minimum inhibitory concentrations (MICs) of the isolates were also determined by the micro-broth dilution method using the same antibiotics. The MIC test was conducted according to the recommendations of the CLSI (CLSI, 2013). The breakpoint of ceftiofur (MIC ≥ 8 µg/mL) was used based on the results of a previous study (Donaldson *et al.*, 2006), because the CLSI guidelines do not include a MIC breakpoint of cefiofur for *E. coli* of bovine origin. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms in the antimicrobial susceptibility tests and ESBL and/or AmpC β-lactamases in the phenotypic screening test.

Screening and phenotypic identification of ESBLs and AmpC β-lactamases

A double disc diffusion method (DDDM) was performed with cefotaxime (30 µg)/cefotaxime-clavulanate (30 µg/10 µg) (BD, USA) and ceftazidime (30 µg)/ceftazidime-clavulanate (30 µg/10 µg) (BD, USA) to detect ESBL production according to CLSI guidelines (CLSI, 2013). Similarly, plasmid-mediated AmpC β-lactamase production was screened by the cefoxitin-cloxacillin double disc synergy method (CC-DDSM) using cefoxitin (30 µg)/cefoxitin-cloxacillin (30 µg/10 µg) (Himedia, India), as described in a previous study (Tan *et al.*, 2009).

Detection of β-lactamase-encoding genes

PCR amplification of genes of the ESBL (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, and *bla*$_{CTX-M}$) and plasmid-mediated AmpC was carried out as previously described (Feria *et al.*, 2002; Perez-Perez and Hanson, 2002; Batchelor *et al.*, 2005; Rayamajhi *et al.*, 2008). The primers used to detect β-lactamases in this study are shown in Table 3.1. The DNA templates used in this study were prepared by the boiling method. In all PCR amplifications, distilled water was used as a negative control. A positive control organism was not used in this assay as all DNA products were sequenced by a dye-termination sequencing system using an automatic sequencer (Macrogen, Korea). Homologous sequence searches were performed against the GenBank database using the BLAST tool of the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST).

Conjugation assay

To determine the transferability of the β-lactamase-encoding genes, a conjugation assay was conducted. A mixed broth culture mating method in a previous study (Rayamajhi *et al.*, 2008) was applied with sodium azide-resistant *E. coli* J53AzR as a recipient strain, with some modifications. Single colonies of donor and recipient isolates were incubated in tryptic soy broth (TSB) (BD) and grown at 37°C for 20 h. The donor and recipient strains were grown in TSB for 8 hrs, after which the cultures were mixed at a ratio of 1: 2 and incubated at 37°C for 20 h. Transconjugants were selected on Mueller-Hinton (MH) agar (BD) supplemented with ampicillin (100 µg/mL) and sodium azide (200 µg/mL). The conjugation frequency of each isolate was calculated as the number of CFU transconjugants per CFU donor. In addition, transfer of the genes was confirmed by PCR amplification of specific genes in the transconjugants.

Typing of plasmid replicons

For typing plasmid replicons, PCR was performed using DNA extracted from all donor and transconjugant strains. The primers used in this study targeted 18 different replicons (Table 3.2), as described previously (Johnson *et al.*, 2007).

Results

Antimicrobial susceptibility results

Resistance to ampicillin and amoxicillin was observed in all isolates, and 30 isolates (38.5%) were resistant to cephalothin. None of the isolates showed resistance to any of the extended-spectrum β-lactams used in the test (ceftiofur, ceftazidime, cefotaxime, and cefoxitin) (Table 3.3). The MIC values of the different β-lactams tested for the 78 *E. coli* isolates are shown in Table 3.3. All isolates were highly resistant to ampicillin (MIC > 1024 µg/mL) and amoxicillin (MIC > 1024 µg/ml). Cephalothin resistance (MIC ≥ 32 µg/mL) was detected in 32 isolates (41.0%). None of the isolates was resistant to ceftiofur (MIC ≤ 4 µg/mL), ceftazidime (MIC ≤ 8 µg/mL), cefotaxime (MIC ≤ 2 µg/mL), or cefoxitin (MIC ≤ 8 µg/mL) (Table 3.3). However, intermediate resistance to ceftiofur (MIC = 4 µg/mL), ceftazidime (MIC = 8 µg/mL), and cefotaxime (MIC = 2 µg/mL) was detected in 39.7%, 17.9%, and 46.2% of the isolates, respectively. The resistance patterns of the isolates were [AMP-AMX] (61.5%) and [AMP-AMX-CF] (38.5%).

Screening of ESBL and AmpC β-lactamase production

None of the isolates were positive for ESBL or AmpC β-lactamase production. In the MIC test, none of the isolates were resistant to cefotaxime, ceftazidime, or cefoxitin, even though 36 (46.2%), 14 (17.9%), and 4 (5.1%) of the *E. coli* isolates showed intermediate MIC values against cefotaxime (MIC, 2 µg/mL), ceftazidime (MIC, 8 µg/mL), and cefoxitin (MIC, 8 µg/mL), respectively (Table 3.3).

Molecular characterization of β-lactamase-encoding genes

All 78 *E. coli* isolates harbored a TEM-type gene. None of the genes encoding the ESBLs (bla_{SHV}, bla_{OXA}, and bla_{CTX-M}) or pAmpC β-lactamases were found in any of the isolates. Sequence analysis identified TEM-1-type β-lactamase in all isolates.

Transferability of β-lactamase resistance and plasmid replicon analysis

Plasmid replicon typing and conjugal transferability of plasmids revealed that the bla_{TEM-1}, gene for β-lactamase resistance was transferred in 59 (75.6%) of the isolates (Table 3.4). The transfer frequency of the isolates ranged from 1.29×10^{-6} to 9.22×10^{-4}. Plasmid replicon typing of the transconjugants was performed to identify the transfer of plasmids in *E. coli* carrying the TEM-1 gene. The prevalence of the plasmid replicon type of the donor isolates was as follows: IncFIB (71.8%); IncFIA (41.0%); IncP (34.6%); Frep (29.5%); IncY (29.5%); IncI1 (28.2%); IncN (15.4%);

IncB/O (10.3%) and IncHI1 (1.3%). Among the 10 plasmids detected from the isolates, the main plasmid for the horizontal dissemination of $bla_{\text{TEM-1}}$ in *E. coli* isolated from beef cattle was the IncFIB (Table 3.4). Plasmid replicon typing revealed that all donor isolates exhibited 32 different replicon combinations. The most frequent combination was [FIA-FIB-Y], which was detected in eight isolates (Table 3.4). For transconjugants, a total of five classes of replicon were detected. IncFIB and IncFIA were the most frequently detected replicons, being found either alone or in combination at ratios of 61.5% and 41.0%, respectively. The prevalence of the remaining plasmid replicons of transconjugants was as follow: IncI1 (17.9%); Frep (16.7%) and IncB/O (5.1%). PCR revealed that all 59 transconjugants harbored TEM-1-type β-lacatamase transferred from the donors.

Discussion

In the present study, the extremely high resistance to ampicillin (MIC > 1024 μg/mL resistance, 100%) and amoxicillin (MIC > 1024 μg/mL resistance, 100%) of these *E. coli* isolates might have been caused by selection pressures from their excessive use in beef cattle farms over the last decade (QIA, 2013). Additionally, the use of β-lactam antimicrobials, such as penicillins and cephems, has increased gradually (QIA, 2013). In addition, the antimicrobial resistance to cephalothin of the

E. coli isolates used in this study was high. A total of 32 (41.0%) isolates showed resistance to cephalothin (MIC ≥ 32 μg/mL), and this resistance was much higher than that of *E. coli* (1.0%) in a previous national report (QIA, 2013). A considerable number of isolates exhibited intermediate resistance to cefotaxime (n = 36), ceftiofur (n = 31), and ceftazidime (n = 14), although none of the isolates in this study were identified as resistant to these compounds (Table 3.3). *E. coli* isolates showing intermediate resistance to these compounds may acquire resistance to β-lactams by selection pressure if they are exposed to continuous use of antimicrobials.

In this study, no ESBL- and/or AmpC β-lactamase-producing *E. coli* isolates were detected, which is consistent with the results of a previous study showing a low prevalence (< 2%) of β-lactamase-producing *E. coli* isolates (Lim *et al.*, 2009, Tamang *et al.*, 2012, Tamang *et al.*, 2013a). Although recent reports indicated that there are various types of ESBL- and AmpC β-lactamase-producing *Enterobacteriaceae* (Pai *et al.*, 2001, Jeong *et al.*, 2003, Paterson *et al.*, 2003, Huang *et al.*, 2005, Hu *et al.*, 2008), only TEM-1-type β-lactamase was detected in the present study. These findings suggest that less third- and fourth-generation cephalosporins might be used in the production of Korean beef cattle than in the human population and production of other livestock. In the present study, PCR and sequencing results revealed that all ampicillin-resistant isolates were only associated with TEM-1-type β-lactamase, which is known to be widely distributed in Korea (Pai *et al.*, 2004, Rayamajhi *et al.*, 2008). These results are in agreement with those

of a previous study, which showed that most of the ampicillin-resistant *E. coli* harbored the TEM-1 β-lactamase gene as the only plasmid-mediated β-lactamase (Cooksey *et al.*, 1990).

Continuous selective pressure exerted by β-lactams is an important reason for occurrence of ESBL- and AmpC β-lactamase determinants (Helfand and Bonomo, 2005). Similarly, genetically non-resistant strains might be able to acquire resistance plasmids, either randomly or specifically, due to constant antimicrobial use, leading to widespread occurrence of resistance plasmids (Petit *et al.*, 1990). Replicon typing of the transconjugant of *E. coli* isolates revealed that the IncFIA and IncFIB plasmids, which are commonly found in the fecal flora of humans and animals, were most frequently detected (Couturier *et al.*, 1988). We found that strains that carried F plasmid (IncFIB, IncFIA and Frep) and I1 either alone or combination had transferred the TEM-1-type β-lactamase. These results suggest that $bla_{\text{TEM-1}}$ gene, a primitive type of β-lactamase encoding gene, is harbored by these kind of plasmids and associated with old type β-lactams such as ampicillin and amoxicillin (Johnson and Nolan, 2009). Two isolates that carried IncB/O did not transfer TEM-1-type β-lactamase to the recipients.

When compared to other veterinary studies, our results are unusual as no resistance to cephems was found and only one kind of β-lactamase was detected. Although these results might be consistent with the decreasing amount of β-lactamases in beef cattle, the transferable plasmids remained among *E. coli* could provide significant

chance on the acquisition and dissemination of β-lactam resistance. Therefore continuous exposure to antimicrobials could increase selection pressure for β-lactamases, which presents a critical risk to human and animal health. Thus, the use of β-lactam antimicrobials such as extended-spectrum cephalosporin should be restricted. In addition, monitoring the use of antimicrobials and assessment of antimicrobial resistance mechanisms in the bacteria of beef cattle could reduce selection pressure and may help enhance treatment for both humans and animals.

Table 3.1. Primers for the detection of β-lactamase genes used in this study

β-lactamase targeted	Primers	Sequence	Product Size (bp)	Annealing Temp (°C)	Reference
TEM	TEM-F	TCG GGG AAA TGT GCG	1074	62	(Rayamajhi *et al.*, 2008)
	TEM-R	TGC TTA ATC AGT GAG GCA CC			
SHV	SHV-F	GCC GGG TTA TTC TTA TTT GTC GC	1016	62	
	SHV-R	ATG CCG CCG CCA GTC A			
OXA	OXA-F	TAT CTACAG CAG CGC CAG TG	199	53	(Donaldson *et al.*, 2006)
	OXA-R	CGC ATC AAA TGC CAT AAG TG			
MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOX-F	GCT GCT CAA GGA GCA CAG GAT	520	64	(Perez-Perez and Hanson, 2002)
	MOX-R	CAC ATT GAC ATA GGT GTG GTG C			
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CIT-F	TGG CCA GAA CTG ACA GGC AAA	462	64	
	CIT-R	TTT CTC CTG AAC GTG GCT GGC			
DHA-1, DHA-2	DHA-F	AAC TTT CAC AGG TGT GCT GGG T	405	64	
	DHA-R	CCG TAC GCA TAC TGG CTT TGC			
ACC	ACC-F	AAC AGC CTC AGC AGC CGG TTA	346	64	
	ACC-R	TTC GCC GCA ATC ATC CCT AGC			
MIR-1T, ACT-1	EBC-F	TCG GTA AAG CCG ATG TTG CGG	302	64	
	EBC-R	CTT CCA CTG CGG CTG CCA GTT			
FOX-1 to FOX-5b	FOX-F	AAC ATG GGG TAT CAG GGA GAT G	190	64	
	FOX-R	CAA AGC GCG TAA CCG GAT TGG			
CTX-M universal	CTXMU-F	CGA TGT GCA GTA CCA GTA A	585	60	(Batchelor *et al.*, 2005)
	CTXMU-R	TTA GTG ACC AGA ATC AGC GG			

Table 3.2. Primers for analysis of plasmid replicon types used in this study

Replicons	Target sites	Primer sequence		Annealing Temp (°C)	Product size (bp)
		Direction	Sequence (5' to 3')		
T	*repA*	F	TTG GCC TGT TTG TGC CTA AAC CAT	60	750
		R	CGT TGA TTA CAC TTA GCT TTG GAC		
P	Iterons	F	CTA TGG CCC TGC AAA CGC GCC AGA AA	60	534
		R	TCA CGC GCC AGG GCG CAG CC		
A/C	*repA*	F	GAG AAC CAA AGA CAA AGA CCT GGA	60	465
		R	ACG ACA AAC CTG AAT TGC CTC CTT		
FIC	*repA*2	F	GTG AAC TGG CAG ATG AGG AAG G	60	262
		R	TTC TCC TCG TCG CCA AAC TAG AT		
B/O	RNAI	F	GCG GTC CGG AAA GCC AGA AAA C	60	159
		R	TCT GCG TTC CGC CAA GTT CGA		
Y	*repA*	F	AAT TCA AAC AAC ACT GTG CAG CCT G	60	765
		R	GCG AGA ATG GAC GAT TAC AAA ACT TT		
FIB	*repA*	F	GGA GTT CTG ACA CAC GAT TTT CTG	60	702
		R	CTC CCG TCG CTT CAG GGC ATT		
FIA	Iterons	F	CCA TGC TGG TTC TAG AGA AGG TG	60	462
		R	GTA TAT CCT TAC TGG CTT CCG CAG		
FIIA	*repA*	F	CTG TCG TAA GCT GAT GGC	60	270
		R	CTC TGC CAC AAA CTT CAG C		
W	*repA*	F	CCT AAG AAC AAC AAA GCC CCC G	60	242
		R	GGT GCG CGG CAT AGA ACC GT		
K/B	RNAI	F	GCG GTC CGG AAA GCC AGA AAA C	60	160
		R	TCT TTC ACG AGC CCG CCA AA		
L/M	RepA,B,C	F	GGA TGA AAA CTA TCA GCA TCT GAA G	60	785
		R	CTG CAG GGG CGA TTC TTT AGG		
HI2	Iterons	F	TTT CTC CTG AGT CAC CTG TTA ACA C	60	644
		R	GGC TCA CTA CCG TTG TCA TCC T		
N	*repA*	F	GTC TAA CGA GCT TAC CGA AG	60	559
		R	GTT TCA ACT CTG CCA AGT TC		
HI1	*parA-parB*	F	GGA GCG ATG GAT TAC TTC AGT AC	60	471
		R	TGC CGT TTC ACC TCG TGA GTA		
X	oriɣ	F	AAC CTT AGA GGC TAT TTA AGT TGC TGA T	60	376
		R	TGA GAG TCA ATT TTT ATC TCA TGT TTT AGC		
Frep	RNAI/ *repA*	F	TGA TCG TTT AAG GAA TTT TG	60	270
		R	GAA GAT CAG TCA CAC CAT CC		
I1	RNAI	F	CGA AAG CCG GAC GGC AGA A	60	139
		R	TCG TCG TTC CGC CAA GTT CGT		

Table 3.3. Antimicrobial susceptibility of 78 *Escherichia coli* isolates to β-lactam antimicrobial agents

Antimicrobials	Phenotype of Disc Diffusion method			MIC (µg/mL)												
	R (%)	I (%)	S (%)	< 0.5	1	2	4	8	16	32	64	128	256	512	1024	> 1024
Ampicillin	100	0	0													78
Amoxicillin	100	0	0													78
Cephalothin	38.5	61.5	0					36	10	18	12	2				
Ceftiofur	0	0	100	4	32	11	31									
Ceftazidime	0	0	100	3	3	4	54	14								
Cefotaxime	0	0	100	2	40	36										
Cefoxitin	0	0	100		3	59	12	4								

MIC, minimum inhibitory concentration; R, resistant; I, intermediate; S, susceptible.

Table 3.4. Profile of plasmid replicon typing and transferability of 78 *Escherichia coli* isolates

Number of replicons	Donor replicon	Number of strains	Transferability	Transfer frequency	Replicon of transconjugant	Transfer of β-lactamase
1	B/O	1	+	2.52×10^{-4}	B/O	
	FIB	1	+	4.01×10^{-5}	FIB	TEM-1
	I1	2	+ (2/2)	$3.68\times10^{-5} \sim 6.81\times10^{-5}$	I1	TEM-1
	N	2	−			
	Frep	2	+ (1/2)	3.14×10^{-4}	Frep	TEM-1
	FIA	5	+ (4/5)	$9.09\times10^{-6} \sim 1.01\times10^{-4}$	FIA	TEM-1
	P	5	−			
2	P-FIA	1	+	3.91×10^{-5}	FIA	TEM-1
	P-I1	1	−			
	FIB-I1	1	+	9.81×10^{-6}	FIB-I1	TEM-1
	FIB-Y	1	+	3.27×10^{-5}	FIB	TEM-1
	FIB-Frep	1	+	7.71×10^{-6}	FIB	TEM-1
	FIA-FIB	2	+ (2/2)	$2.91\times10^{-5} \sim 7.11\times10^{-5}$	FIA-FIB	TEM-1
	FIB-N	3	−			
3	B/O-P-FIB	1	+	2.52×10^{-5}	B/O-FIB	TEM-1
	P-FIA-FIB	1	+	4.45×10^{-5}	FIA-FIB	TEM-1
	P-FIB-Y	1	+	3.62×10^{-5}	FIB	TEM-1
	P-FIB-Frep	1	+	9.22×10^{-4}	FIB	TEM-1
	FIB-Y-Frep	1	+	8.32×10^{-5}	FIB	TEM-1
	FIB-I1-Frep	2	+ (2/2)	$6.24\times10^{-6} \sim 3.33\times10^{-5}$	FIB-I1, FIB-Frep	TEM-1
	FIB-Y-I1	5	+(5/5)	$1.29\times10^{-6} \sim 5.24\times10^{-4}$	FIB, I1, FIB-I1, FIA-FIB-I1	TEM-1
	FIA-FIB-Frep	5	+ (5/5)	$8.24\times10^{-6} \sim 4.48\times10^{-5}$	FIA, FIB, FIA-FIB-Frep,	TEM-1
	P-FIB-I1	6	+ (5/6)	$9.24\times10^{-6} \sim 3.31\times10^{-5}$	FIB-I1	TEM-1
	FIA-FIB-Y	8	+ (8/8)	$9.57\times10^{-6} \sim 1.44\times10^{-5}$	FIA-FIB	TEM-1
4	B/O-FIB-Frep-N	1	+	2.56×10^{-5}	B/O-Frep	TEM-1
	FIA-FIB-Y-HI1	1	+	2.78×10^{-5}	FIA-FIB	TEM-1
	P-FIB-I1-Frep	2	+ (2/2)	$3.01\times10^{-5} \sim 1.19\times10^{-4}$	FIB, FIB-I1-Frep	TEM-1
	B/O-P-FIB-Frep	2	+ (1/2)	5.78×10^{-4}	B/O	
	B/O-P-I1-Frep	2	+ (2/2)	4.27×10^{-5}~7.79×10^{-5}	I1-Frep	TEM-1
	P-FIA-FIB-Frep	3	+ (3/3)	3.33×10^{-5}~1.91×10^{-4}	FIA-FIB-Frep	TEM-1
	FIA-FIB-Y-N	6	+ (6/6)	$2.52\times10^{-6} \sim 4.49\times10^{-5}$	FIA-FIB	TEM-1
5	B/O-P-FIB-I1-Frep	1	+	5.62×10^{-4}	FIB-Frep	TEM-1
0	None	1				

Chapter IV

Prevalence of antimicrobial resistance and transfer of tetracycline resistance genes in *Escherichia coli* isolates from beef cattle

Abstract

The aim of this study was to investigate the prevalence and transferability of resistance in tetracycline-resistant *Escherichia coli* isolates recovered from beef cattle in South Korea. A total of 155 *E. coli* isolates were collected from feces in South Korea, and 146 were confirmed to be resistant to tetracycline. The tetracycline resistance gene *tet*(A) (46.5%) was the most prevalent, followed by *tet*(B) (45.1%) and *tet*(C) (5.8%). Strains carrying *tet*(A) plus *tet*(B) and *tet*(B) plus *tet*(C) were detected in two isolates each. In terms of phylogenetic grouping, 101 (65.2%) isolates were classified as phylogenetic group B1, followed in decreasing order by D (17.4%), A (14.2%), and B2 (3.2%). Ninety-one (62.3%) isolates were determined

to be multidrug-resistant by the disk diffusion method. MIC testing using the principal tetracyclines, namely, tetracycline, chlortetracycline, oxytetracycline, doxycycline, and minocycline, revealed that isolates carrying *tet*(B) had higher MIC values than isolates carrying *tet*(A). Conjugation assays showed that 121 (82.9%) isolates could transfer a tetracycline resistance gene to a recipient via the IncFIB replicon (65.1%). This study suggests that the high prevalence of tetracycline-resistant *E. coli* isolates in beef cattle is due to the transferability of tetracycline resistance genes between *E. coli* populations which have survived the selective pressure caused by the use of antimicrobial agents.

Keywords: *Escherichia coli*, tetracycline resistance, *tet*, beef cattle

Introduction

Antimicrobial resistance in humans and animals is considered a problem worldwide. Resistance to antimicrobial agents impedes the effective prevention and treatment of infectious disease, and thus, many governments have planned and implemented national programs for monitoring resistance in humans and animals (Cizman, 2003, Aarestrup, 2004, Lee *et al.*, 2011, Tadesse *et al.*, 2012). Surveillance data show that the inadequate selection and extensive use of antimicrobials result in the emergence

and spread of resistant bacteria, particularly multidrug-resistant bacteria, and increase resistance to newer compounds, such as tetracycline-class antimicrobials (Levy and Marshall, 2004).

The tetracyclines are one of the most widely used classes of antimicrobial agents in human and veterinary medicine because they have several advantages, which include a broad spectrum of activity, low cost, oral administration, and few side effects (Chopra and Roberts, 2001). After chlortetracycline was introduced into clinical medicine in 1948, many derivatives, such as tetracycline, oxytetracycline, doxycycline, and minocycline, were developed, and today, these derivatives are widely used to treat disease and as growth promoters in the food animal industry. However, the widespread and indiscriminate use of tetracyclines has subjected bacterial populations to selection pressure and increased the prevalence of tetracycline resistance (Chopra and Roberts, 2001, Roberts, 2005). Tetracycline resistance is generally caused by the acquisition of a tetracycline resistance (*tet*) gene, as these genes are associated with primary resistance mechanisms, which involve active efflux pumps, ribosomal protection, and enzyme inactivation (Koo and Woo, 2011). To date, more than 40 different resistance genes have been identified (Roberts, 2005). In Gram-negative bacteria, the most important mechanism involves the efflux pump system, which is encoded by tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(G) (Chopra and Roberts, 2001).

Although most *Escherichia coli* strains are considered harmless commensal bacteria of the gastrointestinal tracts of humans and animals, pathogenic strains that can cause several intestinal and extraintestinal infections exist. Surveillance of *E. coli* isolates is also considered to provide an excellent means of monitoring antimicrobial resistance in food and the environment because of the wide range of hosts of *E. coli* and because it easily acquires resistance (Erb *et al.*, 2007). Thus, the degrees of resistance in commensal and pathogenic *E. coli* strains provide indicators of antimicrobial selection in their environment, and tetracycline-resistant *E. coli* strains could be used for surveillance for tetracycline resistance in humans and animals. Studies have reported tetracycline-resistant *E. coli* strains in various environments (Sengelov *et al.*, 2003, Karami *et al.*, 2006, Tuckman *et al.*, 2007, Gow *et al.*, 2008, Koo and Woo, 2011), but only a small number of studies have been conducted in animals. The aim of this study was to determine the prevalence of tetracycline-resistant *E. coli* isolates in South Korean beef cattle and determine the phenotypes and genotypes of these isolates with a view toward investigating the transferabilities of tetracycline resistance determinants between *E. coli* isolates.

Materials and Methods

Bacterial strains

In total, 290 *E. coli* strains were isolated from feces collected from clinically healthy beef cattle during 2011 and 2012 (Shin *et al.*, 2014). *E. coli* isolates that showed resistance and intermediate resistance to tetracycline were obtained by culture on MacConkey agar plates containing tetracycline at a concentration of 8 μg/ml (the MIC of tetracycline for *E. coli* indicating tetracycline resistance is ≥16 μg/ml) (CLSI, 2013). As a result, 155 *E. coli* isolates were selected for analysis. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms in antimicrobial susceptibility tests and MIC tests.

Antimicrobial susceptibility test

The *E. coli* isolates were tested for susceptibility by the disk diffusion method in accordance with the guidelines issued by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013). The antimicrobial disks (Oxoid, Basingstoke, United Kingdom) used in this study included ampicillin (10 μg), streptomycin (25 μg), gentamicin (10 μg), chloramphenicol (C, 30 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), and tetracycline (30 μg) disks.

Detection of tetracycline resistance genes

All 155 tetracycline-resistant isolates were tested by multiplex PCR for the presence of the *tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(G) genes, as described previously (Ng *et al.*, 2001). Bacterial DNA for PCR was obtained by suspending colonies of bacteria grown on tryptic soy broth (TSB) in 500 µl of ultrapure water and boiling at 100°C for 10 min. The oligonucleotide primers used in this study are shown in Table 4.1. The PCRs included a negative and a positive control, and reactions were run in duplicate to confirm the results. Sequence alignments were performed by use of a search of the GenBank database via the National Center for Biotechnology Information website with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic grouping

The phylogenetic tree described by Clermont *et al*. was used to classify all *E. coli* isolates into one of four phylogenetic groups, that is, groups A, B1, B2, and D (Clermont *et al.*, 2000). Triplex PCR was used to determine the phylogenetic groupings by targeting two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TspE4.C2) (Clermont *et al.*, 2000). The result of phylogenetic typing was used to compare the pattern of antimicrobial resistance and the *tet* gene distributions among the *E. coli* isolates tested in this study.

Determination of MICs of principal tetracyclines

To investigate the phenotypic characteristics of tetracycline-resistant isolates, the MIC values of the principal tetracycline antibiotics, tetracycline, chlortetracycline, oxytetracycline, doxycycline, and minocycline, were determined using the broth dilution method (CLSI, 2013). All antimicrobials used in this study were tested in 2-fold dilutions from 1 to 2,048 μg/ml. MIC tests were conducted in triplicate for each sample.

Conjugation assay and plasmid replicon typing

To determine the transferability of tetracycline resistance, conjugation assays were conducted on tetracycline-resistant isolates using the broth mating method. *E. coli* J53 Azr was used as the recipient strain, and tetracycline-resistant isolates served as the donors (Wang *et al.*, 2004). Eight-hour cultures of recipient and donor cells grown in Luria-Bertani (LB) broth at 37°C were mixed with each other at a ratio of 1:1, and the mixture was incubated for 20 h. To identify resistance carried by plasmids, 100 μl aliquots of these mixtures were spread onto tryptic soy agar (TSA) plates containing tetracycline (8 μg/ml) and sodium azide (200 μg/ml) and incubated at 37°C for 20 h. PCR was used to confirm that the transconjugants carried the *tet* gene of their donors. Multiplex PCR was conducted on all donors and

transconjugants to type the plasmid replicons, as described previously (Johnson *et al.*, 2007).

Statistical analysis

Data were analyzed using IBM SPSS Statistics, version 21, software (SPSS Inc., Chicago, IL). The distributions of the *tet* genes were analyzed using the chi-square test. To compare the different *tet* genes and MIC values, survival analysis was carried out using the Kaplan-Meier method, and the curves so obtained were compared using the logrank test. P values of < 0.05 were considered statistically significant.

Results

Antimicrobial resistance profile

Among 155 *E. coli* isolates, 146 (94.2%) isolates were resistant to tetracycline, as determined using the disk diffusion method. The tetracycline-resistant isolates detected in this study showed concurrent resistance to streptomycin (82.2%), ampicillin (45.3%), nalidixic acid (32.8%), chloramphenicol (28.8%), trimethoprim-

sulfamethoxazole (25.3%), ciprofloxacin (10.3%), and gentamicin (5.5%) (Table 4.2). Of these 146 tetracycline-resistant *E. coli* isolates, 91 (62.3%) were multidrug-resistant. The most frequent combination of multidrug-resistance was tetracycline-streptomycin-ampicillin, which was detected in 20 (13.7%) isolates. Five (3.4%) isolates in phylogenetic group B2 showed resistance to streptomycin; resistance to no other antimicrobial was found (Table 4.2).

Phylogenetic classification

Of the 155 *E. coli* isolates, 101 (65.2%) isolates were classified as phylogenetic group B1; 27 (17.4%) were classified as group D, which is associated with pathogenic bacteria; 22 (14.2%) were classified as group A; and 5 (3.2%) were classified as group B2, the phylogenetic lineage associated with virulent extraintestinal strains (Table 4.3). Prevalence of tetracycline resistance determinants. All 155 isolates carried at least one of the *tet* genes examined. PCR detection of single tet determinants showed that 142 (91.6%) isolates carried *tet*(A) or *tet*(B) only: 72 (46.5%) harbored *tet*(A) only, and 70 (45.1%) isolates harbored *tet*(B) only. *tet*(C) was detected in 11 (7.1%) isolates. Four (2.6%) isolates contained two *tet* genes: *tet*(A) plus *tet*(B) in two (1.3%) isolates and *tet*(B) plus *tet*(C) in two (1.3%) isolates. *tet*(D) and *tet*(G) were not detected. The distributions of *tet*(A) and *tet*(B) in the

phylogenetic groups were not significantly different (chi-square test, $P < 0.05$) (Table 4.3).

MIC values of tetracycline-class antimicrobials

The MIC distributions of tetracycline, chlortetracycline, oxytetracycline, doxycycline, and minocycline for each group of isolates containing the same *tet* genes are shown in Table 4.4. The MIC values of all tetracyclines for isolates susceptible by the disk diffusion method were higher than the breakpoint (MIC > 16 μg/ml). The MIC of chlortetracycline (range, 1,024 to 2,048 μg/ml) was much higher than the MICs of the four other tetracyclines. Resistance to minocycline (MIC > 16 μg/ml) was observed for 35 (22.6%) isolates, and the genomes of 34 of these isolates encoded only the *tet*(B) resistance determinant. In fact, the average MICs for isolates containing the *tet*(B) gene were higher than those for isolates harboring the *tet*(A) gene (Fig. 4.1). Furthermore, the differences in the MICs between isolates containing *tet*(A) or *tet*(B) were greater for doxycycline and minocycline than the other three tetracyclines (Fig. 4.1).

Conjugative transfer of plasmid-mediated tetracycline resistance genes

Of the 146 tetracycline-resistant isolates, 121 (82.9%) isolates were found to transfer the *tet* gene to the recipient strain in conjugation assays. Transfer frequencies ranged from 1.26 X 10^{-8} to 9.26 X 10^{-6} CFU/ml. For 121 isolates possessing *tet*(A) or *tet*(B), the transconjugants possessed the same tet gene as their donors. Interestingly, for isolates containing *tet*(A) plus *tet*(B) or *tet*(B) plus *tet*(C), the transconjugants carried only the *tet*(B) gene. Plasmid replicon typing revealed that the most frequent replicon in the transconjugants was IncFIB, which was found in 95 (65.1%) isolates, and this was followed by Frep (45.2%), IncI1 (25.3%), IncP (24.7%), IncFIA (19.2%), and IncY (17.1%). The results of the conjugation assay with *E. coli* isolates included in phylogenetic groups B2 and D are shown in Table 4.5. The tetracycline resistance gene was successfully transferred for all except two isolates in these phylogenetic groups. IncFIB was the most frequent plasmid replicon detected in transconjugants of these groups (Table 4.5).

DISCUSSION

In the present study, all tetracycline-resistant isolates carried either *tet*(A) or *tet*(B), suggesting that these genes are important for the development of tetracycline resistance. Actually, *tet*(A) and/or *tet*(B), encoding efflux mechanisms, has been reported to be the most common tetracycline resistance determinant in *E. coli* isolates

from humans and animals in many countries (Karami *et al.*, 2006; Tuckman *et al.*, 2007; Ahmed *et al.*, 2010; Schwaiger *et al.*, 2010; Hu *et al.*, 2013). Previous studies conducted in cattle disagree: some have reported that the *tet*(A) determinant is dominant in *E. coli* isolates recovered from cattle (Guerra *et al.*, 2003; Sharma *et al.*, 2008; Karczmarczyk et al., 2011b), whereas others found *tet*(B) to be dominant (Sawant *et al.*, 2007; Walk *et al.*, 2007; Mirzaagha *et al.*, 2011). In the present study, the prevalences of *tet*(A) and *tet*(B) were almost equal at 46.5% and 45.1%, respectively, which is consistent with other reports that showed a similar distribution pattern for the tet gene in *E. coli* isolates recovered from animals (Guerra *et al.*, 2003; Momtaz *et al.*, 2012). The degree of resistance to tetracycline is associated with the presence of *tet*(B) (Gow *et al.*, 2008). In the present study, MIC testing showed that *E. coli* isolates carrying only *tet*(B) appeared to have higher MIC values for tetracycline, chlortetracycline, oxytetracycline, doxycycline, and minocycline, which concurs with previous reports (Blake *et al.*, 2003; Tuckman *et al.*, 2007; Gow *et al.*, 2008). Furthermore, we found that the MIC values for isolates carrying *tet*(B) were significantly higher for doxycycline and minocycline. These results are consistent with those of a previous study, in which *tet*(B) was found to confer resistance to expanded-spectrum tetracyclines, including minocycline and doxycycline (Huys *et al.*, 2005).

In a previous study, *tet*(C) was frequently identified in *E. coli* isolates recovered from a commercial beef processing plant (Aslam and Service, 2006). However, we

found *tet*(C) in only nine strains isolated from beef cattle, and those isolates showed susceptibility, but with low MIC values, to tetracycline, which concurs with the findings of previous studies (Chalmers *et al.*, 2010; Koo and Woo, 2011). Interestingly, the prevalences of *tet*(C) in *E. coli* isolates recovered from animals was reported to be higher than the prevalences of *tet*(C) in *E. coli* isolates recovered from meat and meat products (Koo and Woo, 2011), which suggests that some processing stages may reduce tetracycline resistance in *E. coli*.

Several studies have described *E. coli* isolates carrying more than two *tet* genes (Lanz *et al.*, 2003; Sengelov *et al.*, 2003; Bryan *et al.*, 2004). In South Korea, 40% of *E. coli* strains isolated from cows and pigs in slaughterhouses were found to have two different *tet* genes (Cho, 2008), and in the present study, four *E. coli* isolates were found to carry more than two *tet* genes. Although the prevalence of isolates containing both *tet*(A) and *tet*(B) in the present study was lower than that reported in previous studies (Lanz *et al.*, 2003; Sengelov *et al.*, 2003), we found two isolates harboring *tet*(B) and *tet*(C), which is the first report of this combination in *E. coli* strains isolated from beef cattle in South Korea. However, this conflicts with the findings of a previous study, in which *tet*(C) was always found with *tet*(A) (Maynard *et al.*, 2003). Our study also showed that two isolates that carried more than one *tet* gene did not have higher MIC values than isolates that harbored one *tet* gene. This phenomenon was described in a previous study, in which it was proposed that the

acquisition of more than one *tet* gene is caused by strong selective pressure rather than a selective advantage (Bryan *et al.*, 2004).

The long-term use of tetracycline confers resistance to other antimicrobial agents by *E. coli*. This phenomenon, called coselection, could be the result of *tet* genes being located on the same mobile genetic elements, such as plasmids, transposons, or integrons, as other resistance genes (Gophna *et al.*, 2003). In the present study, many isolates were resistant to tetracycline and other antimicrobials, and 62.3% of tetracycline-resistant isolates exhibited multidrug-resistance. Thus, coselection has important implications, as it means that tetracycline resistance has contributed much to the increased prevalence of multidrug-resistance in *E. coli*.

Phylogenetic groups B2 and D are associated with pathogenicity, whereas strains of groups A and B1 are classified as nonpathogenic commensal strains (Clermont *et al.*, 2000; Cocchi *et al.*, 2007). In the present study, most isolates were classified as group B1 (65.2%). This is consistent with the results of other studies that found that bovine *E. coli* isolates most frequently belong to group A and/or B1 (Houser *et al.*, 2008; Karczmarczyk et al., 2011b). Twentyseven isolates (17.4%) were classified as group D, even though they were cultured from clinically healthy cattle in this study.

Conjugative transfer is the most common mechanism for the delivery of antimicrobial resistance between Gram-negative isolates because plasmid conjugation can occur at a high frequency and transfer resistance genes (Sunde and Norstrom, 2006). In the present study, most tetracycline-resistant isolates (82.9%)

exhibited conjugative transfer, which means that most *tet* genes are carried and transferred by conjugative plasmids. Therefore, we presume that the horizontal transfer of *tet* genes provides an effective mechanism for the widespread distribution of tetracycline resistance in bacterial populations and explains the high prevalence of tetracycline-resistant *E. coli* isolates.

In South Korea, although the use of tetracyclines as feed additives was entirely banned in July 2011, in 2013, about 40% of bovine *E. coli* isolates were found to be resistant to tetracycline (QIA, 2013). Accordingly, we propose that the high prevalence of tetracycline resistance in *E. coli* is probably due to the horizontal transfer of *tet* determinants from *E. coli* isolates carrying *tet* genes which have survived selective pressure caused by the use of tetracycline derivatives. We hope that these findings can be utilized as basic data for epidemiologic studies and studies to assess the risk of tetracycline resistance.

Table 4.1 Primers used in this study

Target gene	Primer	Sequence	Amplicon Size (bp)	Accession No.	Reference
tet(A)	TetA-F	GCTACATCCTGCTTGCCTTC	210	X61367	(Ng *et al.*, 2001)
	TetA-R	CATAGATCGCCGTGAAGAGG			
tet(B)	TetB-F	TTGGTTAGGGGCAAGTTTTG	659	J01830	
	TetB-R	GTAATGGGCCAATAACACCG			
tet(C)	TetC-F	CTTGAGAGCCTTCAACCCAG	418	J01749	
	TetC-R	ATGGTCGTCATCTACCTGCC			
tet(D)	TetD-F	AAACCATTACGGCATTCTGC	787	L06798	
	TetD-R	GACCGGATACACCATCCATC			
tet(G)	TetG-F	GCTCGGTGGTATCTCTGCTC	468	S52437	
	TetG-R	AGCAACAGAATCGGGAACAC			
chuA	ChuA-F	GACGAACCAACGGTCAGGAT	279	HQ284193	(Clermont *et al.*, 2000)
	ChuA-R	TGCCGCCAGTACCAAAGACA			
yjaA	Yja-F	TGAAGTGTCAGGAGACGCTG	211	HQ284194	
	Yja-R	ATGGAGAATGCGTTCCTCAAC			
TspE4C2	TspE4C2-F	GAGTAATGTCGGGGCATTCA	152	HQ284195	
	TspE4C2-R	CGCGCCAACAAAGTATTACG			

Table 4.2. Resistances of 146 tetracycline-resistant *E. coli* isolates in different phylogenetic groups to other antimicrobials

Phylogenetic groups	No. of strains showing antimicrobial resistance (%)						
	AMP	GN	STR	C	SXT	NA	CIP
Total	66 (45.3)	8 (5.5)	120 (82.2)	42 (28.8)	37 (25.3)	48 (32.8)	15 (10.3)
A	14 (9.6)	3 (2.1)	15 (10.3)	7 (4.8)	7 (4.8)	6 (4.1)	5 (3.4)
B1	43 (29.5)	4 (2.7)	75 (51.4)	33 (22.6)	26 (17.8)	24 (16.4)	10 (6.8)
B2			5 (3.4)				
D	9 (6.2)	1 (0.7)	25 (17.1)	2 (1.4)	4 (2.7)	18 (12.3)	

AMP, ampicillin; GN, gentamicin; STR, streptomycin; NA, nalidixic acid; C, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; CIP, ciprofloxacin.

Table 4.3. Distributions of tetracycline resistance genes in *E. coli* isolates in the four identified phylogenetic groups

Phylogenetic groups	No. of isolates (%)	No. of tetracycline resistance genes (%)				
		tet(A)	*tet*(B)	*tet*(C)	*tet*(A)+(B)	*tet*(B)+C)
Total	155 (100)	72 (46.5)	70 (45.1)	9 (5.8)	2 (1.3)	2 (1.3)
A	22 (14.2)	6 (3.9)	9 (5.8)	5 (3.2)	-	2 (1.3)
B1	101 (65.2)	41 (26.5)	54 (34.8)	4 (2.6)	2 (1.3)	-
B2	5 (3.2)	-	5 (3.2)	-	-	-
D	27 (17.4)	25 (16.1)	2 (1.3)	-	-	-

Table 4.4. MICs of tetracycline antimicrobials for *E. coli* isolates with different tetracycline resistance genes

Antimicrobial	Gene profile	No. of strain	Average (μg/ml)	MIC (μg/ml)											
				1	2	4	8	16	32	64	128	256	512	1024	2048
Tetracycline	*tet*(A)	72	200.0							3	27	42			
	tet(B)	70	245.9							1	4	65			
	tet(C)	9	23.1					5	4						
	tet(A)+(B)	2	256.0									2			
	tet(B)+(C)	2	256.0									2			
Chlortetracycline	*tet*(A)	72	1365.3											48	24
	tet(B)	70	1682.3											25	45
	tet(C)	9	170.7								6	3			
	tet(A)+(B)	2	1536											1	1
	tet(B)+(C)	2	1536											1	1
Oxytetracycline	*tet*(A)	72	384.0									36	36		
	tet(B)	70	479.1									9	61		
	tet(C)	9	49.8						4	5					
	tet(A)+(B)	2	512.0										2		
	tet(B)+(C)	2	384.0									1	1		
Doxycycline	*tet*(A)	72	17.3		1		7	54	10						
	tet(B)	70	42.5					4	41	25					
	tet(C)	9	5.8		6	2			1						
	tet(A)+(B)	2	32.0						2						
	tet(B)+(C)	2	32.0						2						
Minocycline	*tet*(A)	72	3.3		39	28	4	1							
	tet(B)	70	13.7		1	6	29	24	10						
	tet(C)	9	1.1	8	1										
	tet(A)+(B)	2	6.0			1	1								
	tet(B)+(C)	2	8.0				2								

Table 4.5. Characterization and transferability of resistance in *E. coli* isolates classified into phylogenetic groups B2 and D

Strain	Phylogeny	Resistance Phenotype[a]	Resistance gene	MIC[b] (μg/ml)					Plasmid replicon type[c]	Transconjugants		
				TET	OXY	CTC	DOX	MIN		Transferability	*tet* genes	Replicon type
60	B2	TE, S	*tet*(B)	256	512	1024	32	8	FIB, Y, I1, Frep	+	*tet*(B)	FIB, I1, Frep
61	B2	TE, S	*tet*(B)	256	512	1024	32	16	FIB, Y, I1, Frep	-	-	-
62	B2	TE, S	*tet*(B)	256	512	1024	32	8	FIB, Y, I1	+	*tet*(B)	FIB, I1
64	B2	TE, S	*tet*(B)	256	512	1024	32	8	FIB, Y, I1	+	*tet*(B)	FIB, I1
68	B2	TE, S	*tet*(A)	256	512	1024	32	8	FIB, Y, I1, Frep	-	-	-
90	D	TE, S, AMP	*tet*(A)	256	512	2048	32	4	P, FIA, FIB, Frep	+	*tet*(A)	FIA, FIB, Frep
106	D	TE, S	*tet*(B)	256	512	2048	32	16	Frep	+	*tet*(B)	Frep
123	D	TE, NA	*tet*(A)	256	512	1024	16	2	FIB, Frep	+	*tet*(A)	FIB, Frep
124	D	TE, CN, SXT, C, S, NA, AMP	*tet*(B)	256	512	2048	64	8	FIA, FIB, Frep	+	*tet*(B)	FIB, Frep
127	D	TE, S, AMP	*tet*(A)	256	512	2048	32	4	P, I1	+	*tet*(A)	I1
128	D	TE, AMP	*tet*(A)	256	512	2048	16	4	FIB, I1	+	*tet*(A)	Frep, I1
133	D	TE, S, NA	*tet*(A)	256	512	1024	2	2	FIB, Frep	+	*tet*(A)	Frep
135	D	TE, S, NA	*tet*(A)	256	512	1024	8	2	FIB	+	*tet*(A)	FIB
136	D	TE, S, NA	*tet*(A)	256	512	1024	16	2	FIB, Frep	+	*tet*(A)	FIB
147	D	TE, S, NA	*tet*(A)	256	512	1024	16	2	FIB	+	*tet*(A)	FIB
148	D	TE, S, NA	*tet*(A)	256	512	1024	8	2	FIB, Frep	+	*tet*(A)	FIB, Frep
152	D	TE, S, NA	*tet*(A)	256	512	1024	16	2	FIB, Frep	+	*tet*(A)	FIB, Frep
153	D	TE, S, NA	*tet*(A)	256	512	1024	16	2	FIB, Frep	+	*tet*(A)	FIB, Frep
156	D	TE, S, NA	*tet*(A)	256	512	1024	16	2	FIB, Frep	+	*tet*(A)	FIB
162	D	TE, S, NA	*tet*(A)	128	256	1024	32	2	FIB, Frep	+	*tet*(A)	FIB, Frep
163	D	TE, S, NA	*tet*(A)	128	256	1024	16	2	FIB, Frep	+	*tet*(A)	FIB, Frep
164	D	TE, S, NA	*tet*(A)	128	256	1024	16	2	FIB, Frep	+	*tet*(A)	FIB, Frep
167	D	TE, S, NA	*tet*(A)	128	256	1024	16	2	FIB, Frep	+	*tet*(A)	FIB
172	D	TE, S, NA	*tet*(A)	128	256	1024	16	2	FIB, Frep	+	*tet*(A)	FIB
173	D	TE, S, NA	*tet*(A)	128	256	1024	16	2	FIB, Frep	+	*tet*(A)	FIB, Frep
174	D	TE, S, NA	*tet*(A)	128	256	1024	16	2	FIB, Frep	+	*tet*(A)	FIB, Frep
175	D	TE, S, NA	*tet*(A)	256	512	1024	8	2	FIB, Frep	+	*tet*(A)	FIB
177	D	TE, S, AMP	*tet*(A)	64	256	2048	16	4	P, FIA, FIB, Frep	+	*tet*(A)	FIB, Frep
178	D	TE, S, AMP	*tet*(A)	64	256	2048	16	4	P, FIA, FIB, Frep	+	*tet*(A)	FIB, Frep
192	D	TE, SXT, C, S, AMP	*tet*(A)	256	512	1024	16	2	P, FIB, Frep	+	*tet*(A)	FIB, Frep
194	D	TE, SXT, S, AMP	*tet*(A)	128	256	1024	16	2	Frep	+	*tet*(A)	Frep
198	D	TE, SXT, S, AMP	*tet*(A)	128	256	1024	32	2	Frep	+	*tet*(A)	Frep

a TE, tetracycline; S, streptomycin; GN, gentamicin; SXT, sulfamethoxazole-trimethoprim; C, chloramphenicol; NA, nalidixic acid; AMP, ampicillin.

b TET, tetracycline; OXY, oxytetracycline; CTC, chlortetracycline; DOX, doxycycline; MIN, minocycline.

c FIB, IncFIB replicon; I1, IncI1 replicon; P, IncP replicon, FIA, IncFIA replicon; Y, IncY replicon.

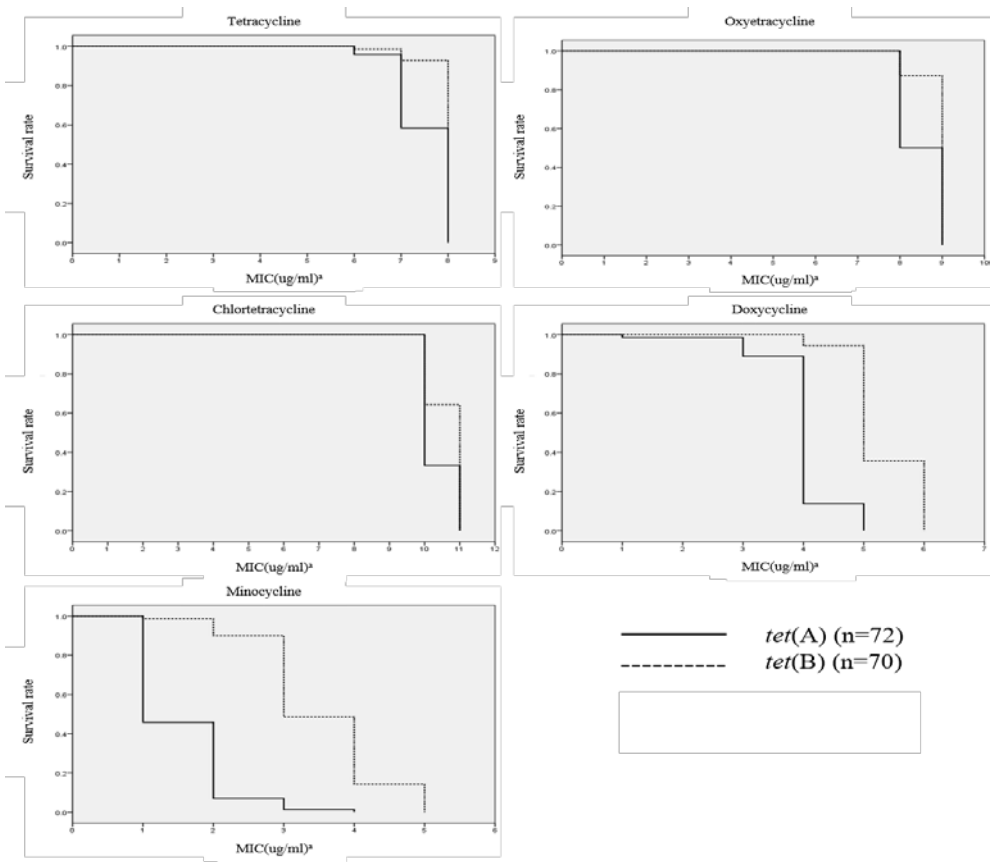

Fig. 4.1. Survival curves (obtained by the Kaplan-Meier method) of *E. coli* isolates harboring *tet*(A) or *tet*(B) for resistance to the tetracycline family of antimicrobials. The survival rates of the *E. coli* isolates are compared with the MIC values of the five tetracyclines (tetracycline, chlortetracycline, oxytetracycline, oxycycline, and minocycline). Full and dotted lines, survival rates of *tet*(A)-carrying and *tet*(B)-carrying strains, respectively. [a], the MIC values of the five etracyclines were log transformed (base 2).

Chapter V

Prevalence and characterization of CTX-M- and CMY-Type extended-spectrum β-lactamase producing *Escherichia coli* isolates from pigs and chickens

Abstract

Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* has disseminated rapidly worldwide and constitutes a serious threat to public health. A total of 281 *Escherichia coli* strains isolated from pigs and chickens between 2009 and 2015 in South Korea were investigated for ESBL-production. ESBL phenotypes were identified in 14 *E. coli* isolates; ten and three ESBL-producing isolates carried only $bla_{\text{CTX-M}}$ and bla_{CMY} genes, respectively, and one isolate harbored both genes. The most common CTX-M- and CMY-types were CTX-M-15 (n=8) and CMY-2 (n=3). We also detected ESBL-producing isolates harboring $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{CMY-6}}$, $bla_{\text{DHA-1}}$, and $bla_{\text{TEM-1}}$ genes. All ESBL-producing isolates showed

resistance to the extent of the fourth-generation cephalosporins, along with multi-drug resistance. MIC test determined that isolates that had produced CTX-M-15 showed higher MIC values than those that produced CTX-M-14 and CTX-M-65. A conjugation assay demonstrated that *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ genes have the potential to be transferred to *E. coli* J53 AzR. The horizontal dissemination of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ genes was mediated mainly by Frep and IncI1 plasmids. PFGE revealed that isolates tested in this study were very diverse, clonally. To our knowledge, this is the first report of *bla*$_{\text{CMY-6}}$ in *E. coli* strains that have been isolated from chickens in South Korea. The emergence of this CMY-6 ESBLs in a population of poultry suggests that extensive screening with long-term surveillance is necessary for the dissemination of ESBL from chicken to human.

Key words: swine, poultry, extended-spectrum β-lactamase (ESBL), CTX-M, CMY, conjugative transfer, public health

Introduction

Extended-spectrum-β-lactamases (ESBLs) are emerging enzymes that confer resistance to extended-spectrum cephalosporins, which are detected most commonly in Enterobacteriaceae (Livermore, 1995). The mechanism of β-lactamases is to

provide protection from the lethal effect of β-lactam class of antimicrobials on cell wall synthesis (Sanders and Sanders, 1992). The vast majority of ESBLs belongs to TEM- and SHV-type families, which are common plasmid mediated β-lactamase of E.coli. The family of CTX-M type β-lactamases, which is a derivative of TEM or SHV, is increasingly being reported in gram-negative bacteria that consist of more than 170 CTX-M subtypes (http://www.lahey.org/studies/webt.asp) and are classified in accordance to the subgroups (CTX-M group 1, 2, 8, and 9) (Bradford, 2001; Bonnet, 2004). CTX-M enzymes possess a clinical significance of the high levels of hydrolytic activity against cefotaxime. Similarly, various groups of plasmid-mediated AmpC (pAmpC) β-lactamases have been reported, and the most common one is CMY-type β-lactamase produced by *E. coli* (Philippon *et al.*, 2002). These enzymes typically confer antimicrobial resistance to cephamycins.

Food-producing animals may play an important role, serving as a reservoir of antimicrobial resistance determinants in bacterial populations, as they transfer resistant bacteria or mobile resistance determinants from food-producing animals to human. Previous studies have generally focused on the presence and transmission of ESBLs and pAmpC β-lactamase in *E. coli* isolated from clinical cases (Saladin *et al.*, 2002; Bonnet, 2004; Kim *et al.*, 2005; Rodriguez-Bano and Navarro, 2008). In South Korea, Lim *et al*. had reported for the first time the presence of $bla_{\text{CTX-M}}$ gene in *E. coli* strain isolated from diseased animals in 2009 (Lim *et al.*, 2009). Moreover, several studies have reported *E. coli* strains producing ESBLs and pAmpC β-

lactamase (Tamang *et al.*, 2011; Tamang *et al.*, 2012; Lim *et al.*, 2015). Recently, we reported extended-cephalosporin resistance among *Enterobactericae* isolates from farm animals in South Korea (Rayamajhi *et al.*, 2011), and hypothesized that ESBL genes would be present in isolates from pigs and chickens in South Korea. However, still limited reports on ESBL-producing *E. coli* strains have been published in food-producing animal populations, which relied on data by national surveillance (Lim *et al.*, 2009; Tamang *et al.*, 2011; Tamang *et al.*, 2012). Therefore, this study aims to investigate the prevalence of ESBL-producing *E. coli* isolates recovered from pigs and chickens that had been collected in South Korea between 2009 and 2015 and also to analyze their phenotypes and genotypes.

Materials and Methods

Bacterial strains

A total of 281 non-duplicate isolates of *E. coli* were collected in this study. Among the 281 isolates, 206 isolates were isolated from necropsied pigs with clinical signs of digestive and respiratory disorders between 2009 to 2015, and 75 isolates of avian pathogenic *E. coli* collected from 2011 to 2015 were kindly provided by Chung Ang Vaccine Laboratory (CAVAC, Korea). These isolates were re-confirmed by using a

biochemical test (IMViC test) and/or Vitek®2 system (bioMérieux, Marcy l'Etoile, France). *E. coli* J53 AzR was used as a recipient for a conjugation assay (Rayamajhi *et al.*, 2011). *E. coli* ATCC 25922 was used as the quality control strains in antimicrobial susceptibility tests and MIC tests. *Salmonella* serotype Braenderup strain (H9812) was selected as the universal size standard (Hunter *et al.*, 2005).

Antimicrobial susceptibility test and detection of ESBL-producing isolates

E. coli isolates were tested for antimicrobial susceptibility by a disc diffusion assay, proposed by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013). The antimicrobial agents (Oxoid, Basingstoke, UK) were as follows: ampicillin (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), and gentamicin (10 μg). The results of antimicrobial susceptibility tests were interpreted in accordance to the guidelines set forth by CLSI (CLSI, 2013). After interpretation of the results, a double-disc synergy method was performed for all isolates showing resistance to ampicillin, ceftazidime, and cefotaxime to determine the production of ESBL, as previously described (CLSI, 2013). Briefly, antimicrobial discs (BD, Franklin Lakes, NJ) containing ceftazidime/clavulanate (30/10 μg) and cefotaxime/clavulanate (30/10 μg) were placed with ceftazidime and cefotaxime on a plate inoculated by the resistant isolates. After overnight incubation at 37°C, the

ESBL production was determined by the difference of zone diameter, which indicated the inactive effect of clavulanate to the test agents. Then, the ESBL-producing isolates were further screened for profiling antimicrobial resistance to the β-lactam class. The following agents (Oxoid, Basingstoke, UK) were included: amoxicillin/clavulanic acid (30 μg), cephalothin (30 μg), cefaclor (30 μg), ceftriaxone (30 μg), cefixime (5 μg), cefpirome (30 μg), cefepime (30 μg), ertapenem (30 μg), and imipenem (10 μg). Moreover, a MIC value of ceftazidime, cefotaxime, ceftriaxone, aztreonam, and cefoxitin was determined by a micro-broth dilution method (CLSI, 2013). All antimicrobial agents used in tis study were tested in 2-fold dilutions from 0.25 to 2,048μg/ml. Antimicrobial susceptibility test and MIC tests were performed in triplicate for each sample.

Detection of β-lactamase determinants

For all ESBL-producing isolates, PCR amplification with primers that target *bla*$_{\text{TEM}}$ (Rayamajhi *et al.*, 2008), *bla*$_{\text{SHV}}$ (Rayamajhi *et al.*, 2008), *bla*$_{\text{CTM-M}}$ (Batchelor *et al.*, 2005), and pAmpC β-lactamase genes (Dallenne *et al.*, 2010) were conducted. For positive isolates of *bla*$_{\text{CTX-M}}$, further PCRs were performed to confirm *bla*$_{\text{CTX-M}}$ genes, using the *bla*$_{\text{CTX-M}}$ group specific primers for the CTX-M-1, CTX-M-2, CTX-M-8, and CTX-M-9 group (Jeong *et al.*, 2005). DNA templates for PCR were obtained by centrifugation of the suspending colonies of isolates grown on a tryptic soy broth

(TSB) in 500µl of distilled water after boiling at 100°C for 10 min. Sequence analyses and comparison with known sequences were performed by searching the GenBank database via the National Center for Biotechnology Information website, with a BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic grouping and plasmid replicon typing

All ESBL-producing isolates were classified into one of the four phylogenetic groups (A, B1, B2, and D) by a phylogenetic grouping PCR method (Clermont *et al.*, 2000). A multiplex PCR was used to determine the phylogenetic groupings by targeting two genetic determinants (*chuA* and *yjaA*) and an anonymous DNA fragment (TSPE4.C2). The result of phylogenetic typing was used to compare the pattern of antimicrobial resistance and the β-lactamase gene distributions among the ESBL-producing *E. coli* isolates tested in this study. A multiplex PCR was performed on all isolates to type the plasmid replicons, as described previously (Carattoli *et al.*, 2005).

Pulse-field gel electrophoresis (PFGE)

For 14 ESBL-producing isolates, PFGE of *Xba*I digested genomic DNA was carried out according to a standard protocol of the Center for Disease Control and Prevention (CDC), using a CHEF MAPPER apparatus (Bio-Rad Laboratories, Hercules, CA), as previously described (Shin *et al.*, 2014). Gel images were analyzed using a GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). Clustering was constructed using an unweighted pair group method with arithmetic means (UPGMA) analysis based on the Dice similarity index.

Conjugation assay

To determine the transferability of β-lactamase resistance genes, conjugation assays were conducted on ESBL-producing isolates using the broth-mating method. *E. coli* J53 AzR was used as the recipient strain, and ESBL-producing isolates served as the donors, as previously described (Shin *et al.*, 2015). The PCR method used above was conducted to determine whether the transconjugants carried the β-lactamase resistance gene of their donors. In addition, for all transcnjugants, antimicrobial susceptibility test, MIC test, and plasmid replicon typing were performed to determine the characteristics of transconjugants as conducted to the donors..

Results

Antimicrobial resistance profiling of ESBL-producing isolates

Among the 281 *E. coli* isolates, 20 of them showed resistance and/or intermediate resistance to ceftazidime and/or cefotaxime, as determined by the disc diffusion method. Of these 20 isolates, 14 were confirmed to produce ESBLs by the double-disc synergy test. ESBL-producing isolates detected in this study showed a concurrent resistance to nalidixic acid (n=12), tetracycline (n=11), ciprofloxacin (n=9), gentamicin (n=8), and trimethoprim/sulfamethoxazole (n=7) (Table 5.1). As a result, 10 isolates showed multi-drug resistance to be resistant to at least three classes of antimicrobial agent.

The prevalence of antimicrobial resistance to β-lactam agents was as follows: ampicillin (n=14); cephalothin (n=14); cefaclor (n=14); cefotaxime (n=14); ceftriaxone (n=14); cefixime (n=13); ceftazidime (n=11); cefpirome (n=7); cefepime (n=4); and amoxicillin/clavulante (n=4). None of the isolates were resistant to ertapenem and imipenem (Table 5.1).

The MIC distribution of ceftazidime, cefotaxime, ceftriaxone, aztreonam and cefoxitin for ESBL-producing isolates are shown in Table 5.1. There are various MIC values of all agents, ranging from 0.25 to 2,048 μg/ml. The MIC values of all agents for the isolates that showed a resistance by the disc diffusion method were

higher than the breakpoint of each agent (ceftazidime ≥ 16 μg/ml, cefotaxime ≥ 4 μg/ml, ceftriaxone ≥ 4 μg/ml, aztreonam ≥ 16 μg/ml, cefoxitin ≥ 32 μg/ml). The average MIC value of cefotaxime and ceftriaxone is much higher than that of the other three agents. Resistance to cefoxitin (MIC ≥ 32 μg/ml) was detected in four isolates, and these isolates had plasmid-mediated AmpC β-lactamase encoding genes (CMY-2 or CMY-6) in common (Table 5.1).

Prevalence of β-lactamase encoding genes

A total of 11 ESBL-producing *E. coli* isolates that harbored the $bla_{\text{CTX-M}}$-type genes were included in the CTX-M-1 or CTX-M-9 group. A sequencing analysis of the $bla_{\text{CTX-M}}$-positive isolates identified that eight isolates carried $bla_{\text{CTX-M-14}}$, two isolates harbored $bla_{\text{CTX-M-14}}$, and one isolate had $bla_{\text{CTX-M-65}}$ (Table 5.1). Among these 11 $bla_{\text{CTX-M}}$-positive isolates, six isolates co-carried $bla_{\text{TEM-1}}$, one isolate each co-harbored $bla_{\text{CMY-2}}$, and $bla_{\text{DHA-1}}$. None of the isolates was positive for the bla_{SHV} gene. Of the three isolates that were negative for $bla_{\text{CTX-M}}$, two isolates showed to produce CMY-2, and one isolate to produce CMY-6 (Table 5.1).

Profile of phylogenetic grouping and plasmid replicon typing

Of the 14 ESBL-producing *E. coli* isolates, ten were classified into the non-virulent groups A (n=7) and B1 (n=3), and four isolates into groups B2 (n=2) and D (n=2), which are associated with virulent bacteria (Table 5.1). Plasmid replicon typing revealed that four types of plasmid were detected in 14 ESBL-producing isolates. Incompatibility Frep was the most frequent replicon type detected in the 13 ESBL-producing isolates. This was followed by IncFIB (n=12), IncI1 (n=6), and IncN (n=2) (Table 5.1).

PFGE analysis

All 11 CTX-M-producing strains and three CMY-producing strains showed PFGE profiles with a low similarity (< 70% similarity), which suggests that these isolates are unlikely to be derived from a single clone of *E. coli*. However, EC085 and EC092 strains isolated from pigs in the same year and carrying both $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ genes showed a high genetic homogenicity with 89.7% of similarity (Fig. 5.1).

Conjugation assay

The horizontal transfer of the phenotypes and genotypes of the ESBL-producing isolates to the recipient strains (*E. coli*J53 AzR) by the conjugation assay was found in nine transconjugants. The characteristics of transconjugants are shown in Table

4.2. For nine trasnsconjugants, PCR analysis for β-lactamase genes identified the transfer of *bla*$_{\text{CTX-M-15}}$ (n=4), *bla*$_{\text{TEM-1}}$ (n=4), *bla*$_{\text{CTX-M-14}}$ (n=1), *bla*$_{\text{CMY-2}}$ (n=1), and *bla*$_{\text{CMY-6}}$ (n=1) genes to the recipient strain as detected in their donors. The antimicrobial susceptibility test on β-lactams of nine transconjugants determined the transfer of β-lactam-resistance. All transconjugants were resistant to ampicillin, and all but EC096-Tc were resistant to cephalothin. The two transconjugants producing only TEM-1-type β-lactamase were resistant to ampicillin and/or cephalothin. The other seven transconjugants producing CTX-M-type or CMY-type β-lactamase showed resistance to cefaclor and ceftriaxone. EC085-Tc and EC240-Tc that carried the *bla*$_{\text{CTX-M-15}}$ gene were resistant to cefpirome, which is included in the fourth-generation cephalosporin. In addition to the transfer of β-lactams resistance, resistance to non-β-lactams was also determined in four isolates. The resistance to gentamicin was commonly identified in four transconjugants. The MIC test for transconjugants revealed that the isolates producing only TEM-1-type β-lactamase were susceptible to five antimicrobial agents. Three transconjugants harboring *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CMY-6}}$ and *bla*$_{\text{CMY-2}}$ gene showed resistance to cefoxitin with high MIC values, ranging from 512 to 1024 μg/ml. The plasmid replicon typing results for nine transconjugants revealed four different replicon types as their donors. Among these, three of nine transconjugants contained more than one type of replicon. The replicon type Frep was the most frequent replicon detected in five transconjugant, followed by IncI1 (n=4), IncFIB (n=3), and IncN (n=1).

Discussion

In the present study, the phenotypic and genotypic characteristics of ESBL-producing *E. coli* strains that had been isolated from pigs and chickens between 2009 and 2015 in South Korea were investigated. From 206 isolates that originated in necropsied pigs, nine isolates (4.36%) were ESBL-producing *E. coli* strains. From 75 isolates of avian pathogenic *E. coli*, five isolates (6.67%) produced ESBLs. A total of 14 isolates (4.98%) were identified as ESBL-producing *E. coli* from 281 *E. coli* isolates. This prevalence is similar with previous studies that reported *E. coli* isolates from pigs in Denmark (Agerso and Aarestrup, 2013) and from chickens in Japan (Hiroi *et al.*, 2012). In contrast, a high prevalence of ESBL-producing *E. coli* isolates was reported for *E. coli* isolates from pigs and chickens in Hong Kong (Ho *et al.*, 2011), Netherland (Dierikx *et al.*, 2013), and South Korea (Lim *et al.*, 2015). With respect to the β-lactamase type produced, out of 281 *E. coli* isolates tested, 11isolates (3.91%) produced CTX-M-type β-lactamase and three isolates (1.07%) produced CMY-type β-lactamase. One isolate (EC051) produced a combined type of β-lactamases (CTX-M-15 and CMY-2). Although a similar frequency of CTX-M-type β-lactamase producing *E. coli* isolates was observed in *E. coli* strains isolated from pigs and cattle in South Korea (Lim *et al.*, 2009; Tamang *et al.*, 2013a), the prevalence of CTX-M-type lactamase described in previous reports is much higher than that of our study (Ho *et al.*, 2011; Zheng *et al.*, 2012; Dierikx *et al.*, 2013).

The ESBL-producing *E. coli* isolates investigated in this study carried a various type of β-lactamase genes. Three different CTX-M type- (*bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, and *bla*$_{\text{CTX-M-65}}$) and two different CMY type- (*bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CMY-6}}$) β-lactamase genes were detected. These resistance variants have been reported previously for *E. coli* strains isolated from food-producing animals in Asia (Lim *et al.*; 2009, Ho *et al.*, 2011; Hiroi *et al.*, 2012; Zheng *et al.*, 2012; Hiki *et al.*, 2013). The *bla*$_{\text{CTX-M-15}}$, which is the most dominant CTX-M-type β-lactamase detected globally (Nicolas-Chanoine *et al.*, 2008), was also identified most frequently in this study. Similarly, the *bla*$_{\text{CMY-2}}$ detected in this study has previously been reported for *E. coli* strains isolated from food-producing animals in Europe, the United States, and South Korea (Winokur *et al.*, 2001; Rayamajhi *et al.*, 2008; Liebana *et al.*, 2013). In South Korea, many studies reported the prevalence of *bla*$_{\text{CTX-M-14}}$ gene in *E. coli* from pigs, cattle, and chickens and *Salmonella* spp. from chicken (Rayamajhi *et al.*, 2011; Tamang *et al.*, 2011; Tamang *et al.*, 2013a; Tamang *et al.*, 2013b, Tamang *et al*., 2014). Furthermore, in this study, we identified the *bla*$_{\text{CMY-6}}$ gene in one isolate from chicken. This variant has previously been reported for *E. coli* strains from only clinical cases in South Korea (Yoo *et al.*, 2010). Thus, to our knowledge, this study is the first report of an *E. coli* strain carrying *bla*$_{\text{CMY-6}}$ from chicken in South Korea. This findings suggest that the emergence of *bla*$_{\text{CMY-6}}$ gene in *E. coli* strains isolated from chicken may constitute a potential risk to public health with increased consumption of chicken in South Korea.

In this study, the MIC test for five β-lactam agents showed that the average MICs of each agent for isolates that produced CTX-M-15 was higher those that produced CTX-M-14 and CTX-M-65, especially in cefotaxime and ceftriaxone (data not shown). Although the number of samples tested was low to have statistical significance, this data is similar with a previous study which reported that the CTX-M group 1 showed higher MIC values than the CTX-M group 9 in third- and fourth-generation cephalosporins (Tarnberg *et al.*, 2011). Meanwhile, our results revealed that the isolates producing CMY-2 or CMY-6 β-lactamase had higher MICs of cefoxitin (ranging 512 to 1024 μg/ml) than those without CMY-type β-lactamase (ranging 4 to 16 μg/ml), which is in agreement with the previous studies that showed the effectiveness of CMY-type β-lactamase onto cefoxitin-resistance (Yan *et al.*, 2000, Winokur *et al.*, 2001). Therefore, we suggest that the isolates that produced both CTX-M-15 and CMY-2 could have high MIC values in cefotaxime, ceftriaxone and cefoxitin. The susceptibility test for β-lactam resistance determined that all ESBL-producing *E. coli* isolates showed extensive resistance to the extent of the fourth-generation cephalosporins. This may have resulted from an increasing trend in the amount of usage of cephems, such as ceftiofur, annually from 2006 (QIA, 2014). Usually, CMY-type β-lactamase confers resistance to 1st-, 2nd-, and 3rd-generation cephalosporins, but not to 4th-generation cephalosporins (Liebana *et al.*, 2013). Interestingly, in this study, all isolates producing CMY-2 and/or CMY-6 showed resistance to 4th-generation cephalosporins.

In this study, nine isolates transferred the β-lactamase-encoding genes by the conjugation assay to the recipient strain. The *bla*$_{\text{CTX-M-65}}$ and *bla*$_{\text{DHA-1}}$ genes did not transfer to the recipient strains, which indicate that these genetic determinants may not be located in the plasmid that transferred the *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CMY-6}}$, and *bla*$_{\text{TEM-1}}$ genes. Furthermore, our results indicate that the horizontal dissemination of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CMY}}$ genes in the *E. coli* strains tested in this study is due mainly to Frep and IncI1 plasmids, respectively. Some β-lactamase-encoding genes are located within the mobile genetic elements associated with other resistance genes, which confer resistance to the antimicrobials that could be extensively used in humans and animals (e.g. fluoroquinolones and aminoglycosides), which could also play an important role in the co-selection of these resistance genes (Brinas *et al.*, 2005). Similarly, in this study, four isolates co-transfer gentamicin resistance with β-lactams to the recipient strain. Interestingly, the two isolates only transferred the *bla*$_{\text{TEM-1}}$ gene that had lost their ESBL-producing phenotype, and showed an increased susceptibility to β-lactams in the disc diffusion test and MIC test.

In this study, three *E. coli* isolates harboring *bla*$_{\text{CTX-M}}$ genes and one isolate harboring *bla*$_{\text{CMY}}$ genes belonged to the virulent phylogenetic groups B2 and D. These findings may constitute concerns regarding the transfer of CTX-M- or CMY-producing *E. coli* isolates that belong to the virulent phylogenetic groups from animals to humans, because humans share the same environment and remain in close contact with them (Hammerum *et al.*, 2014). Furthermore, molecular typing by

PFGE showed that CTX-M- and CMY-producing *E. coli* strains were clonally very diverse, which suggests that the spread of the $bla_{\text{CTX-M}}$ and bla_{CMY} genes in *E. coli* strains among pigs and chicken may mainly result from horizontal transmission, rather than clonal expansion from a single clone of *E. coli*. In general, there was no significant correlation between the phylogenetic groups and the groupings of strains in each cluster by *Xba*I-digested PFGE.

In conclusion, our results describe the recent dissemination of ESBL-producing *E. coli* strains among pigs and chickens in South Korea. Although the presence of extended-spectrum cephalosporin resistance in animals is very low, the spread of ESBLs genes in these strains could arise among animal species, as well as humans, which can lead to treatment failures in both veterinary and human medicines. To our knowledge, this is the first report of $bla_{\text{CMY-6}}$ gene in *E. coli* strains from chicken in South Korea. More studies are needed with a better long-term surveillance to trace the evolution and dissemination of CTX-M- and CMY-type β-lactamase between different food-producing animals.

Table 5.1. Profile of antimicrobial resistance and resistance gene of ESBL-producing *Escherichia coli* isolated form pig and chicken

Strain	Origin	Phylo-genetic group	β-lactamase genes		Plasmid replicon	Transfer	Antimicrobial resistance		MIC values (μg/mℓ)				
			CTX-M type	Other β-lactamase			β-lactams	Others	CAZ	CTX	CRO	ATM	FOX
EC010	Pig	A	CTX-M-14	TEM-1	Frep, FIB, N	Positive	AMP, KF, CEC, CTX, CRO, CFM	NA, TE, SXT, GN	4	128	512	8	8
EC035	Pig	A	CTX-M-15	-	Frep, FIB	Positive	AMP, KF, CEC, CAZ, CTX, CRO, CFM, CPO, FEP	CIP, NA, TE, SXT, GN	64	1024	1024	256	16
EC050	Pig	D	CTX-M-15	TEM-1	Frep, FIB	Positive	AMP, KF, CEC, CAZ, CTX, CRO, CFM, CPO, FEP	GN	256	2048	2048	256	8
EC051	Pig	A	CTX-M-15	CMY-2	Frep, FIB	Positive	AMP, AMC, KF, CEC, CAZ, CTX, CRO, CFM, CPO, FEP	CIP, NA, GN	512	512	1024	256	512
EC065	Pig	B2	CTX-M-15	DHA-1	Frep	Negative	AMP, KF, CEC, CAZ, CTX, CRO, CFM	CIP, NA, TE, SXT, GN	32	128	256	32	16
EC085	Pig	A	CTX-M-15	TEM-1	Frep, I1	Positive	AMP, KF, CEC, CAZ, CTX, CRO, CFM, CPO	CIP, NA, TE	128	1024	1024	128	16
EC092	Pig	A	CTX-M-15	TEM-1	Frep, FIB, I1	Negative	AMP, KF, CEC, CAZ, CTX, CRO, CFM, CPO	CIP, NA, TE, SXT, GN	128	512	1024	128	16
EC096	Pig	A	CTX-M-15	TEM-1	Frep, FIB, I1, N	Positive	AMP, KF, CEC, CAZ, CTX, CRO, CFM, CPO	CIP, NA, TE, SXT, GN	32	2048	2048	64	8
EC105	Pig	B1	CTX-M-65	TEM-1	Frep, FIB, I1	Negative	AMP, KF, CEC, CTX, CRO, CFM	CIP, NA, TE, SXT, GN	2	512	512	16	8
EC240	Chicken	D	CTX-M-15	-	Frep, FIB	Positive	AMP, KF, CEC, CAZ, CTX, CRO, CFM, CPO, FEP	-	32	1024	1024	64	8
EC243	Chicken	B1	-	CMY-2	Frep, FIB	Negative	AMP, AMC, KF, CEC, CAZ, CTX, CRO, CFM	NA, TE,	256	64	128	32	512
EC260	Chicken	A	-	CMY-6	FIB, I1	Positive	AMP, AMC, KF, CEC, CAZ, CTX, CRO, CFM	CIP, NA, TE	512	128	256	128	1024
EC262	Chicken	B2	-	CMY-2	Frep, FIB, I1	Positive	AMP, AMC, KF, CEC, CAZ, CTX, CRO, CFM	NA, TE	1024	128	256	128	512
EC264	Chicken	B1	CTX-M-14	-	Frep, FIB	Negative	AMP, KF, CEC, CTX, CRO	CIP, NA, TE, SXT, GN	2	256	256	4	4

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; KF, cephalothin; CEC, cefaclor; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CFM, cefixime; CPO, cefpirome; FEP, cefepime; CIP, ciprofloxacin; NA, nalidixic acid; TE, tetracycline; SXT, trimethoprim/sulfamethoxazole; GN, gentamicin

Table 4.2. Characteristics of transconjugants of ESBL-producing *Escherichia coli* isolates

Strain	Donor strains	Transferred β-lactamase genes		Plasmid replicon	Antimicrobial resistance		MIC values (μg/mℓ)				
		CTX-M type	Other β-lactamase		β-lactams	Others	CAZ	CTX	CRO	ATM	FOX
EC010-Tc	PEC510	CTX-M-14	TEM-1	Frep, FIB, N	AMP, KF, CEC, CTX, CRO, CFM	-	4	64	64	8	16
EC035-Tc	PEC574	CTX-M-15	TEM-1	Frep, FIB	AMP, KF, CEC, CAZ, CTX, CRO, CFM	GN	64	512	512	128	8
EC050-Tc	PEC590	-	TEM-1	Frep	AMP, KF	GN	2	0.25	1	1	4
EC051-Tc	PEC591	CTX-M-15	-	Frep, FIB	AMP, KF, CEC, CAZ, CTX, CRO, CFM	GN	64	512	512	128	16
EC085-Tc	PEC705	CTX-M-15	-	I1	AMP, KF, CEC, CAZ, CTX, CRO, CFM, CPO	-	128	1024	1024	256	16
EC096-Tc	PEC716	-	TEM-1	I1	AMP	SXT, GN	2	0.25	1	1	4
EC240-Tc	AEC34	CTX-M-15	-	Frep	AMP, KF, CEC, CAZ, CTX, CRO, CFM, CPO	-	128	1024	512	8	1024
EC260-Tc	AEC54	-	CMY-6	I1	AMP, AMC, KF, CEC, CAZ, CTX, CRO, CFM	-	512	32	128	32	512
EC262-Tc	AEC56	-	CMY-2	I1	AMP, AMC, KF, CEC, CAZ, CTX, CRO, CFM	-	256	128	128	64	1024

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; KF, cephalothin; CEC, cefaclor; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CFM, cefixime; CPO, cefpirome; SXT, trimethoprim/sulfamethoxazole; GN, gentamicin; ATM, aztreonam; FOX, cefoxitin

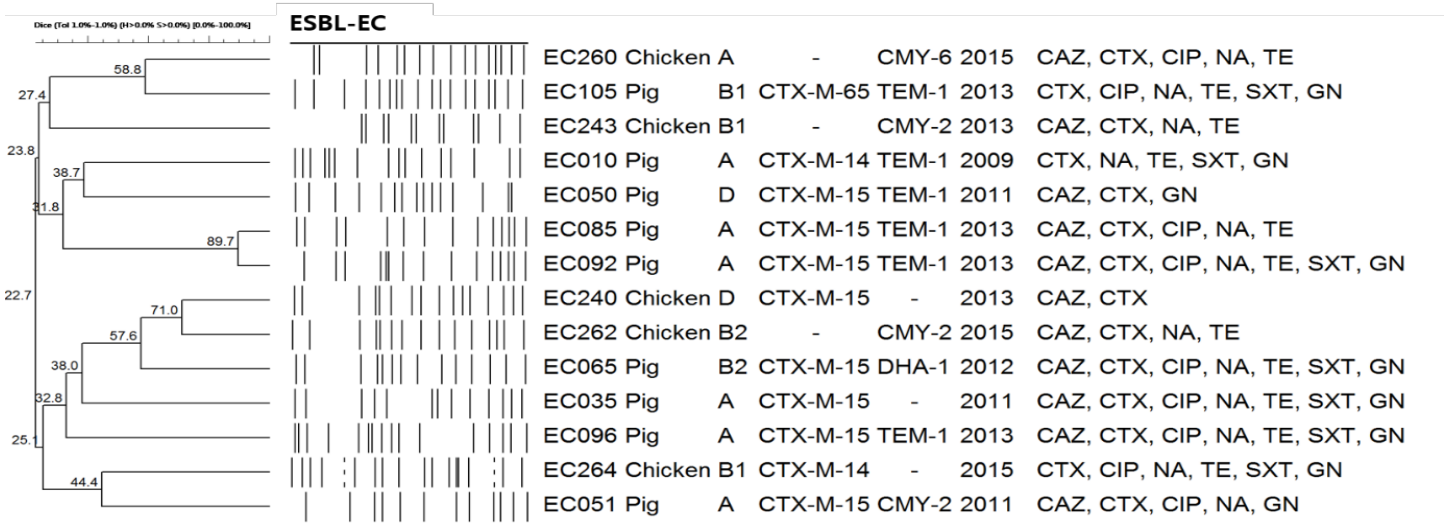

Fig. 5.1 Dendrogram generated showing the cluster analysis of *Xba*I digested PFGE patterns of ESBL-producing *E. coli* strains isolated from pigs and chickens. Similarity analysis was performed by using the Dice coefficient, and clustering was done by the unweighted-pair group method using average linkages (UGPMA). For 14 ESBL-producing *E. coli* strains, details given include the strain, origin, phylogenetic groups of each strain, β-lactamase encoding genes, the sampled year of each strain, and antimicrobial resistance profiles in order.

General conclusions

This study showed that plasmid mediated resistance in *E. coli* is on rise and are conferring resistance to important antimicrobial used in farm animal. In first study, our results suggested that diverse determinants of virulence and antimicrobial resistance of *E. coli* were widespread in *E. coli* strains isolated from cattle farms in South Korea. Moreover, these determinants could disseminate into non-pathogenic *E. coli* isolates according pathogenicity to them. This suggests that the normal flora of cattle could be a significant reservoir of diverse virulence and antimicrobial resistance determinants, which is potentially threatening to public health. In second study, when compared to other veterinary studies, our results were unusual as no resistance to cephems was found and only one kind of β-lactamase was detected. These results suggested that the present selection pressure of antimicrobial use on β-lactamases in beef cattle might be relatively low in comparison to other livestock in Korea. However, increased exposure to antimicrobials could increase selection pressure for β-lactamases, which presented a critical risk to human and animal health. In third study, we proposed that the high prevalence of tetracycline resistance in *E. coli* was probably due to the horizontal transfer of *tet* determinants from *E. coli* isolates carrying *tet* genes which have survived selective pressure caused by the use of tetracycline derivatives. In final study, our results described the recent dissemination of ESBL-producing *E. coli* strains among pigs and chickens in South

Korea. Although the presence of extended-spectrum cephalosporin resistance in animals was very low, the spread of ESBLs genes in these strains could arise among animal species, as well as humans, which can lead to treatment failures in both veterinary and human medicines. To our knowledge, this is the first report of *bla*$_{\text{CMY-6}}$ gene in *E. coli* strains from chicken in South Korea. More studies are needed with a better long-term surveillance to trace the evolution and dissemination of CTX-M- and CMY-type β-lactamase between different food-producing animals.

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국문초록

국내 농장분리 대장균의 항생제 내성유형 및 내성유전자의 전달에 관한 분석

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수의학과 수의미생물학 전공

대장균은 항생제 내성 유전체를 사람과 동물의 해로운 미생물에게 전달하는 중요한 전달매체이다. 대장균은 병원체들과 인테그론, 트랜스포좀 그리고 플라스미드와 같은 유동성 유전물질을 효과적으로 교환할 수 있다. 이러한 유전체들에 의해 획득한 내성기전은 내성의 획득과 전파에 있어서 중요한 역할을 한다. 따라서 산업동물 내의 항생제 내성의 분포와 전달성에 대해 조사 및 분석하기 위해 2009 년부터 2015 년까지 비육우, 돼지 그리고 닭의 병변과 분변으로부터 분리한 대장균이 이번 연구에 포함되었다.

비육우에서 분리한 대장균의 분포와 특성에 관한 첫 번째 연구는 표면적으로 및 유전적으로 다양한 유형의 항생제 내성 및 병원성을

보여주었다. 19% (55/290)의 균주들이 Shiga 독소를 분비하는 대장균(STEC)으로 판명되었다. 가장 많이 발견된 병원성 유전자는 *f17* (26.2%, 76/290)이었다. 152 주의 균주들이 다재 항생제 내성을 보였다. 항생제 감수성 검사에서 가장 많은 내성률을 보인 항생제는 streptomycin (63.1% 95/290)이었으며, tetracycline (54.5%, 158/290), cephalothin (32.8%, 95/290), 그리고 sulphamethoxazole/trimethoprim (16.6%, 48/290)가 그 뒤를 이었다. PCR 및 sequencing 결과는 다음과 같은 연관 내성 유전자의 분포를 보여주었다; *strA-strB* (39.0%, 113/290), *aac(3)-IV* (33.1%, 96/290), *aphA1* (21.4%, 62/290), *tet*(A) (27.6%, 80/290), *tet*(B) (78/290, 26.9%), bla_{TEM} (23.8%, 69/290), *sul2* (22.1% 97/290). PFGE 와 O 혈청형 분석 결과 이번 연구에서 분리된 대장균은 유전적으로 클론 다양성의 정도가 매우 높게 나타났다.. 이러한 결과들은 비육우에서 분리된 대장균 균주들의 병원성 및 항생제 내성이 매우 다양함을 암시하였다.

다음 연구는 ESBL 이나 plasmid-mediated AmpC (pAmpC) 베타락탐효소로 진화할 가능성이 있는 베타락탐효소를 가지고 있는 ampicillin 내성 소 유래 대장균에 관한 것이었다. 이 연구에서는 비육우로부터 분리된 78 주의 대장균에 대하여 ESBL 또는 pAmpC 베타락탐효소의 생성에 대해 조사하였다. 베타락탐계 항생제를 이용한 디스크확산법에서 38.5%의 균주들이 ampicillin, amoxicillin 그리고

cephalothin 에 내성을 보였다. Double disc synergy method 결과 ESBL 이나 pAmpC 베타락탐효소의 생성을 보이는 균주는 없었다. DNA 염기서열분석 결과 모든 균주가 TEM-1 형의 베타락탐효소를 암호화하였다. 제공균주의 플라스미드 레플리콘형 분석에서는 IncFIB 와 IncFIA 이 각각 71.4%와 41.0% 를 차지하였다. transconjugant 에서는 IncFIB 와 IncFIA 가 각각 61.5%와 41.0%가 발견되었다. 이 연구는 국내 비육우군에서 베타락탐효소에 대한 항생제의 선택적압박이 다른 가축군에 비해 상대적으로 낮음을 시사하였다.

세번째 연구에서 국내 비육우에서 분리된 tetracycline 내성 대장균의 분포와 항생제 전달성에 대해 조사하엿다. 155 주의 대장균 중 146 주가 tetracycline 에 대해 내성인 것으로 나타났다. tetracycline 내성유전자 *tet*(A)가 가장 많이 발견되었으며 (46.1%, 72/155). *tet*(B) (45.1, 70/155)와 *tet*(C) (5.8%, 9/155)가 그 뒤를 이었다. 계통학적 분석에서 65.2% (101/155)의 균주가 그룹 B1 에 속하였으며, D (17.4%), A (14.2%) 그리고 B2 (3.2%)가 그 뒤를 이었다. 91 주 (62.3%)의 균주가 디스크확산법 결과 다재 내성이었다. 중요 tetracycline 계 항생제, tetracycline, chlortetracycline, oxytetracycline, doxycycline 그리고 minocycline 을 이용한 MIC 검사결과 *tet*(B)를 지닌 균주가 *tet*(A)를 지닌 균주에 비해 더 높은 MIC 수치를 나타냈다. Conjugation 실험결과 82.9% (121/155)의 균주가 tetracycline 내성

유전자를 수령 균주에 전달하였으며, IncFIB 형의 플라스미드가 가장 많이 이용되었다 (65.1% 95/155). 이 연구는 비육우 유래 tetracycline 내성 대장균의 높은 분포가 항생제로부터 살아남아, 대장균집단에 의한 내성유전자의 전파를 통해 이루어진다는 것을 시사하였다.

마지막 연구에서는 2009 년부터 2015 년까지 돼지와 닭에서 분리된 총 281 주의 대장균을 대상으로 ESBL 생성성을 조사하였다. 14 주의 균주에서 ESBL 생성성이 확인되었다; 각각 10 주 및 3 주의 ESBL 생성 대장균에서 $bla_{\text{CTX-M}}$ 및 bla_{CMY} 유전자가 홀로 발견되었고, 한 주의 대장균에서 두 유전자 모두 발견되었다. 가장 많이 발견된 CTX-M 형 및 CMY 형은 CTX-M-15 (8/14) 과 CMY-2 (3/14)이었다. 또한 $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-65}}$, $bla_{\text{CMY-6}}$, $bla_{\text{DHA-1}}$ 그리고 bla_{TEM} 유전자를 지닌 ESBL 생성 균주들이 발견되었다. 모든 ESBL 생성 균주들은 4 세대 cephalosporin 항생제에 까지 내성을 나타냈으며, 모두 다재 내성이었다. MIC 검사결과 CTX-M-15 을 분비하는 균주들이 CTX-M-14 와 CTX-M-65 를 분비하는 균주들에 비해 높은 MIC 수치를 나타내었다. Conjugation 실험 결과 $bla_{\text{CTX-M}}$ 과 bla_{CMY} 유전자는 모두 수령 균주에게 전달되었다. $bla_{\text{CTX-M}}$ 과 bla_{CMY} 유전자의 수평 전파에는 Frep 과 IncI1 형 플라스미드가 매개하였다. PFGE 실험 결과 이 실험에 사용된 균주들은 매우 다양한

클론들이 분포한다는 것을 보여주었다. 또한 이 결과는 국내최초로 닭에서 분리된 대장균에서 *bla*$_{CMY-6}$ 가 분포한다는 것을 밝혔다.

이러한 연구 결과, 대장균 유래 전달성 플라스미드와 염색체 내에 존재하는 항생제 내성 유전자는 공공위생에 치명적일 수 있다. 게다가 사람과 축산동물 내 대장균의 항생제 내성이 증가하므로 사람과 수의학적 목적으로 사용되는 항생제의 사용이 제한되어야 한다는 의견을 뒷받침하였다. 따라서 대장균 내 항생제 내성의 출현과 전파를 최소화하기 위해 항생제의 합리적인 사용과 내성에 관한 장기적인 조사가 필요하였다.

핵심어 : 대장균, 항생제 내성, 축산동물, 플라스미드, tetracycline, ESBL, CMY-6

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