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Antimicrobial resistance and possible transmission of *Escherichia coli* between companion animals and related-personnels

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(Major : Veterinary Pathobiology and Preventive Medicine)

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Abstract

Livestocks today are not that much more valuable than they were long ago, we treat our companion animals as if they were far more valuable. In addition, we have seen a huge switch in animal medicine, from a focus on livestocks to a focus on companion animals such as horses, dogs and cats. In the Korean companion animal industry, the market size associated with companion animals is rapidly increasing and estimated to be $5.4 billion by 2020. In addition, more and more Korean people have recognized the importance of horse industry according to the increasing trend of horse-riding. However, limited information is available regarding horse-associated antimicrobial resistant (AR) bacteria in Korea.

As first study, we evaluated the frequency and characterize the pattern of AR *Escherichia coli* (*E. coli*) from healthy horse-associated samples. Thirty of the *E. coli* isolates (21%) showed antimicrobial resistance to at least one antimicrobial agent, and four of the AR *E. coli* (13.3%) were defined as multi-drug resistance. Pulsed-field gel electrophoretic analysis showed the cross-transmissions between horses or horses and environments were detected in two facilities. Although cross-transmission of AR *E. coli* in horses and their environments was generally low, our study suggests a risk of transmission of AR bacteria between horses and humans.

Quinolone (Q) and fluoroquinolone (FQ) are broad-spectrum synthetic antimicrobials used to treat bacterial infections in humans and animals. Since they are very potent antimicrobial agents against Gram-negative bacteria including *E. coli*, these agents have been widely used to treat a range of infections in companion animals. Consequently, (F)Q resistance has markedly increased worldwide, posing a significant threat to the health of animals and humans. In the second study, we investigated the prevalence and the mechanisms of FQ/Q resistance in *E. coli* isolates from companion animals, owners, and non-owners. A total of 27 nalidixic acid (NA)-resistant isolates were identified. Of these, 10 isolates showed ciprofloxacin (CIP) resistance. Efflux pump activity was detected in 18 isolates (66.7%), but this was not correlated with the increased minimum inhibitory concentration (MIC). Target gene mutations in Q resistance-determining regions (QRDRs) were the main cause of (F)Q resistance in *E. coli*. The number of point mutations in QRDRs was strongly correlated with increased MIC (R = 0.878 for NA and 0.954 for CIP). Interestingly, (F)Q resistance mechanisms observed in isolates from

companion animals were the same as those in humans. Therefore, a prudent use of (F)Q in veterinary medicine is warranted to prevent the dissemination of (F)Q-resistant bacteria from animals to humans.

Companion animals such as horses and dogs are considered as one of the reservoirs of AR bacteria that can be cross-transmitted to humans. The inherent risk of any use of antimicrobials to select for AR bacteria poses a relevant risk for public health by spreading of antimicrobial resistance from animals to humans via direct or indirect contacts. However, limited information is available on the possibility of AR bacteria originating from companion animals being transmitted secondarily from owners to non-owners sharing the same space. To address this issue, in the third place, we investigated clonal relatedness among AR *E. coli* isolated from dog owners and non-owners in the same college classroom or household. Of 31 *E. coli*, 20 isolates (64.5%) were resistant to at least one antimicrobial, and 16 isolates (51.6%) were determined as multi-drug resistant *E. coli*. Pulsed-field gel electrophoretic analysis identified three different *E. coli* clonal sets among isolates, indicating that cross-transmission of AR *E. coli* can easily occur between owners and non-owners. The findings emphasize a potential risk of spread of AR bacteria originating from companion animals within human communities, once they are transferred to humans.

Antimicrobial resistance is an urgent global problem. There are increasing concerns about the emergence of multi-drug resistant bacteria in humans, animals and environments. The antimicrobial resistance is a complex phenomenon driven by many factors such as the interaction of humans, animals and environmental sources for

antimicrobial resistance. Our study also showed that they could be not only reservoirs but also transmitters of antimicrobial-resistant bacteria. Therefore, the aims of combating antimicrobial-resistant bacteria and preserving the efficacy of the currently available antimicrobials in human and veterinary medicine as well as in ecological systems should be addressed in an interdisciplinary effort within a "One Health" approaches.

Addressing this urgent threat requires the multifaceted strategies. Elements include strengthened surveillance of antimicrobial usage; improved antimicrobial stewardship in humans and animals; approaches to incentivize new antimicrobials development; increased research on mechanisms of resistance; a prudent use of antimicrobials by veterinarians as well as clinicians.

Keywords: Antimicrobial resistance, *Escherichia coli*, one health, horses, companion animal-owners, non-owners, fluoroquinolone

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CONTENTS

***Escherichia coli* from national horse racetracks and private horse-riding courses in Korea**

Summary of Abbreviation

ABC	ATP binding cassette
AR	Antimicrobial resistant
ATCC	American type culture collection
CLSI	Clinical and laboratory standards institute
DAEC	Diffuse adhering *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended-spectrum beta-lactamases
ETEC	Enterotoxigenic *Escherichia coli*
FQ	Fluoroquinolone
MATE	Multidrug and toxic efflux
MDR	Multidrug resistance
MF	Major facilitator
MICs	Minimal inhibitory concentrations
OST	Organic solvent tolerance assay
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
QRDRs	Quinolone resistance-determining regions

RFLP	Restriction fragment length polymorphism
RND	Resistance nodulation division
SMR	Small multidrug resistance

Literature Review

I. The genus *Escherichia coli*

Escherichia coli (*E. coli*) is a predominant facultative anaerobe Gram-negative bacteria. *E. coli* is the species of the genus *Escherichia* within the family *Enterobacteriaceae* and the tribe *Escherichia* [19]. Most *E. coli* strains are harmless and commonly isolated from the intestinal tract of healthy humans and animals [133]. The harmless strains can benefit their hosts by producing vitamin K2 [18] and by preventing the colonization of pathogenic bacteria such as *Salmonella* spp. within the intestine [74]. However, these bacteria can cause the infection and illness in immunosuppressed hosts. Three clinical symptoms are usually detected from infected hosts with pathogenic *E. coli* strains: i) enteric or diarrheal, ii) sepsis or meningitis and iii) urinary tract infection. The five main categories of pathogenic *E. coli* are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and enteroadherent *E. coli* (EAEC) [108]. ETEC infection is acquired by ingesting contaminated food or water. The bacteria colonize the proximal small intestine, the critical site of host-parasite interactions, where they elaborate heat-labile enterotoxin (LT) or heat-stable enterotoxin (ST). The clinical features of ETEC infection are watery diarrhea, nausea, abdominal cramps and low-grade fever. EIEC can invade and proliferate within epithelial cells and cause eventual death of the cell [39]. The invasive capacity of EIEC is dependent on the presence of large (~140 MDa) plasmids coding for the production of several outer membrane proteins involved in invasiveness [63]. EPEC is an important category of diarrheagenic *E. coli* which has been linked to infant

diarrhea in the developing countries [83]. The hallmark of infections due to EPEC is the attaching-and-effacing (A/E) histopathology, which can be observed in intestinal biopsy specimens from patients or infected animals and can be reproduced in cell culture [12]. The term EHEC was originally coined to denote strains that cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), express shiga-toxin (*stx*), cause A/E lesions on epithelial cells and possess a ca. 60-MDa plasmid [108]. EAEC expresses aggregative adherence (AA) distinguished by prominent autoagglutination of the bacterial cells to each other. The necessary feature of AA was the characteristic layering of the bacteria, best described as a stacked-brick configuration.

E. coli can be cultured from clinical specimens on enrichment or selective media at 37℃ under aerobic conditions. MacConkey and eosin methylene blue agar are the most popular selective media for isolation of *E. coli* [23]. For epidemiological purposes, *E. coli* is often isolated by presumptive visual identification. However, this method sometimes makes an error in identification of *E. coli* strains. Only about 90% of *E. coli* strains have the ability to ferment lactose. Some diarrheagenic *E. coli* strains such as EIEC strains are lactose negative [133]. Unlike other *E. coli* strains, *E. coli* O157:H7, which is the most important EHEC serotype is unable to ferment sorbitol. Therefore, this *E. coli* strain can be differentiated from other strains on the MacConkey agar containing sorbitol instead of lactose.

E. coli can be serotyped on the basis of O (somatic), H (flagella) and K (capsular) antigen profiles [40]. The O, H and K antigens can be found in many of the possible combinations. Theoretically, the possible number of *E. coli* serotypes reaches about

50,000 to 100,000 [142]. Serotyping of *E. coli* was the predominant method for the identification of pathogenic *E. coli* strains prior to the detection of virulence factors. There are many studies about the association of the *E. coli* serotypes with outbreaks of diarrhea in patients [182]. Karmali *et al*. reported that the *E. coli* O157:H7 can cause the HUS which is defined by acute renal injury, thrombocytopenia and microangiopathic haemolytic anaemia in patients [129]. Especially, *E. coli* O157:H7 produces numerous virulence factors, most notably *stx* which is also called verocytotoxin or shiga-like toxin [129]. Shiga toxin can be classified into diverse variants such as *stx1*, *stx2*, *stx2c* and inhibits protein synthesis in endothelial cells [139].

The problem of drug resistance is not restricted to pathogenic bacteria—it also involves the commensal bacterial flora, which may become a major reservoir of resistant strains. Since *E. coli* acquires resistance easily and is commonly found in many different animal species, it is well suited for surveillance studies of antimicrobial resistance [195]. Chromosomal and plasmid-borne integrons have been identified as one of the crucial factors for the development of multidrug resistance in *E. coli* as well as many other bacterial species by harboring and lateral gene transfer of gene cassettes [162, 176]. Most common in resistant *Enterobacteriaceae* are class 1 resistance integrons, which are primarily located on elements derived from Tn5090 such as Tn402 and Tn21. They carry the site-specific tyrosine recombinases *IntI*, often contain *qacED1* and *sul1* conferring resistance to quaternary ammonium compounds and sulfonamides and harbor gene cassettes encoding resistance to ß-lactams, streptomycin–spectinomycin and trimethoprim. Several investigators observed a significant correlation between the

presence of class 1 integrons and multiresistance in Gram-negative isolates [66, 107]. In addition to integron-mediated resistance, antimicrobial resistance may be caused or increased by mutations. *E. coli* is one of several pathogens for which elevated mutation frequencies (*f*) have been described among natural isolates. Baquero *et al*. proposed to differentiate between strong mutators if $f \geq 4 \times 10^{-7}$ and weak mutators if their frequency was $4 \times 10^{-8} \leq f < 4 \times 10^{-7}$ [15].

II. Use of antimicrobials in animals

An antimicrobial is an agent that kills microorganisms or stops their growth. Antimicrobials can be classified according to their function. Agents that kill microbes are called microbicidal, while those that merely inhibit their growth are called biostatic. The use of antimicrobials to treat infection is known as antimicrobial chemotherapy, while the use of antimicrobials to prevent infection is known as antimicrobial prophylaxis.

Antimicrobials are widely used in the treatment and prevention of bacterial infection in livestock (cow, pig and poultry) and companion animals (horse, dog and cat). In livestock, sub-therapeutic doses of these antimicrobials are commonly utilized to promote growth and improve feed efficiency [87]. In cattle, the use of narrow-spectrum antimicrobials is favored in cases of clinical mastitis, with first-choice antimicrobials being the β-lactam antimicrobials used when treating mastitis resulting from streptococci or penicillin when treating mastitis caused by staphylococci [197].

Antimicrobial treatment is one of the most common treatment plans for therapy of bovine mastitis [56]. A standard recommendation for most clinical mastitis is a 3-day intra-mammary treatment of antimicrobials. Cure rates are highly depended on the causal pathogens and other cow factors [16]. In swine, usage of antimicrobials for prevention is a common practice in farms, especially in stressful periods that predispose for infectious diseases. Such periods are the time between birth and first lactation. For the prevention and treatment of bacterial enteritis, especially when the etiological agent is *E. coli*, antimicrobial treatment with penicillins, tetracyclines (chlortetracycline, oxytetracycline), quinolones (enrofloxacin) or aminoglycosides (gentamicin, neomycin) is required [38]. In poultry, antimicrobials used for therapeutic reasons are usually administered through water, in contrast to growth-promoting use, where antimicrobials are added in feed [70]. The most commonly used antimicrobials in livestocks are penicillins (amoxicillin), quinolones (enrofloxacin), tetracyclines (doxycycline and oxytetracycline), macrolides (erythromycin and tylosin), aminoglycosides, the sulfonamide/trimethoprim combination, polymyxins (colistin) and other antimicrobials (tiamulin) [122]. In the United States, antimicrobials are used primarily in swine and poultry production and to a lesser extent in dairy cows, sheep and companion animals [46]. An estimated 14,788 tons of antimicrobials were sold for use in animals in 2013 in the United States, including 4,434 tons of ionophores, a class of antimicrobials used only in veterinary medicine [50]. Rapid income growth in low- and middle-income countries has increased demand for animal protein [34, 154, 184]. This increasing demand is being met by a shift toward intensive livestock production systems that

depend on antimicrobials to keep animals healthy and operate efficiently [175]. According to the survey of Korea animal health products association in 2015, the largest volume of antimicrobials was sold for use in pigs (53%, 481 tons) followed by fishery (22%, 201 tons), poultry (17%, 157 tons) and cattle (8%, 71 tons) [1]. The use of antimicrobials in livestock first started in the 1940s when they were added to feeds used in broiler poultry production [177]. As a result, a chicken weighing greater than 2.27 kg could be produced in less than 50 days in 2010 [93]. In case of swine industry, antimicrobials used in feed improved the daily weight gain in starter pigs by an average of 16.4% and the feed efficiency by 6.9% [31]. In addition to improving feed efficiency, adding antimicrobials to swine feed was found to reduce the mortality rate by 50% in young pigs (2.0% vs 4.3%) [31]. However, it remains unclear why antimicrobials cause livestock to gain weight more quickly. A certain hypothesize that they lead to decreased illness allowing weight to gain faster [47]. In 2011, the use of antimicrobials for the purpose of animal growth promoters was banned in Korea [81]. However, antimicrobial agents can still be added to water, feed and injected into animals with a veterinary prescription on individual farms [121].

A companion animal, as defined by the American society for the prevention of cruelty to animals (ASPCA) is a “domesticated or domestic-bred animals whose physical, emotional, behavioral and social needs can be readily met as companions in the home or in close daily relationship with humans. The ASPCA also specified “species suitable to be companion animals include dogs, cats, horses, rabbits, ferrets, birds, guinea pigs and select other small mammals, small reptiles and fish. A strong human-animal bond exists

for companion animals. In addition, most owners consider their companion animals as parts of their family members. Companion animal ownership, or just being in the presence of a companion animal, can have a positive effect on individuals' mental and physiological health status. Most research addressing health benefits of companion animal ownership or companion animals focuses on reductions in distress and anxiety, decreases in loneliness and depression and increases in exercise [52]. In addition, companion animal owners made about 15% fewer annual doctor visits than companion animal non-owners, even after controlling for gender, age, marital status, income and other variables related to health [67]. Subsequently, they go to great length for medical treatments to their companion animals [199]. Companion animals account for most (65%) of first generation cephalosporins used in veterinary medicine in Denmark [44]. Urinary tract infection (UTI) is a major reason for antimicrobial prescription in small animal such as dog and cat [79]. Suggested first-line antimicrobials for uncomplicated UTIs include amoxicillin, cephalexin or trimethoprim-sulfamethoxazole [140]. In equine medicine, streptomycin is the first-line antimicrobial for Gram-negative bacterial infection in horses [183]. Oxytetracycline, which is the most commonly used antimicrobial, use applied in an injectable form in combination with a sulfa antimicrobial agent to treat bacterial respiratory infections in horses [210]. Furthermore, the antimicrobials used in companion animals are not much different from those in human medicine [57]. Antimicrobials used in both veterinary and human medicine are: penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides,

spectinomycin, lincosamide, macrolides, nitrofuranes, nitroimidazoles, sulfonamides, trimethoprim, polymyxins and quinolones [155].

III-1. Mechanisms of antimicrobial resistance in bacteria

Bacteria can acquire resistance to antimicrobials. This can be mediated by some mechanisms, which fall into three main mechanisms: first, the minimization of intracellular concentrations of antimicrobials; second, the modification of target of antimicrobials; and third, the inactivation of antimicrobials.

(1) Minimization of intracellular concentrations of antimicrobials

Gram-positive bacteria are intrinsically less permeable to many antimicrobials than Gram-negative bacteria as their outer membrane limits a permeation of antimicrobials [91]. Porin proteins present in the outer membrane of Gram-negative bacteria and some Gram-positive bacteria [145]. Unlike other membrane transport proteins, they act as passive channels which molecules can diffuse from high to low concentrations [145]. Reducing the permeability of the outer membrane is commonly associated with a down-regulation of porin proteins or replacement of porin proteins with selective channels. For example, *E. coli* and *Enterobacter* spp. exposed to antimicrobials such as carbapenem, show the emergence of mutations in porin-associated genes as well as in genes which regulate the expression of porin proteins [101]. *E. coli* produces three major trimeric

porins (OmpF, OmpC and PhoE) [33, 136]. These outer membrane proteins are termed classical porins. Despite their 'non-specific' nature, the members of this family can be classified according to a range of selective filters with respect to the charge and size of the solutes and charges in key regions of the porin channels: the OmpF and OmpC families show a slight preference for cations, whereas PhoE selects inorganic phosphate and anions [136]. Activities of β-lactams and fluoroquinolones, which blocks the synthesis of peptidoglycan and disrupt the activity of gyrase and topoisomerase, respectively are strongly affected by the porin channel. Several clinical studies have reported a modification of the porin profile in antimicrobial resistant isolates: resistant *Enterobacteriaceae* can exhibit a shift in the type of porin they express, a reduction in the porin expression level or the presence of a mutated porin [145]. An altered porin phenotype is also commonly associated with the expression of degradative enzymes such as β-lactamases and cephalosporinases, which efficiently confer a high level of β-lactam resistance [137, 146].

Efflux pumps of bacteria actively transport antimicrobials or harmful agents out of the cell. While some efflux pumps such as Tet pump show narrow substrate specificity, most pumps have a wide range of substrate specificity. These are known as multidrug resistance efflux pumps. The resistance nodulation division (RND) pump is a kind of multidrug resistance pump in *E. coli* [150]. Especially, AcrAB-TolC included in RND pump is a major resistance mechanism against antimicrobials in *E. coli* [167]. This pump has three major components: a transporter of the resistance-nodulation division family (AcrB), a periplasmic accessory protein (AcrA) and an outer membrane protein (TolC)

[167]. The substrate profile of the AcrAB-TolC pump includes chloramphenicol, lipophilic β-lactams, fluoroquinolones, tetracycline, rifampin, novobiocin, fusidic acid, nalidixic acid, ethidium bromide, acriflavine, bile salts, short-chain fatty acids, SDS, Triton X-100 and triclosan [49] In *E. coli*, *acrD* and the *acrEF* operon also encode efflux pumps [163] and AcrD has been shown to efflux aminoglycosides [138]. AcrE and AcrF are 80 and 88% similar to AcrA and AcrB, respectively [118].

(2) The modification of targets of antimicrobials

Some antimicrobials bind to their targets which have essential functions in bacteria and kill those bacteria. Alterations of the target structure that prevent efficient antimicrobial bindings, but that still enable the target to conduct a normal function, are called as antimicrobial resistance. For example, the erythromycin ribosome methylase (*erm*) family of genes methylate 16S rRNA and change the binding site, thus preventing the efficient binding of macrolides, lincosamines and streptogramins [96]. The *qnr* gene families (*qnrA*, *qnrB*, and *qnrS*), responsible for quinolone resistance are found on plasmids of bacteria [221]. These genes encode pentapeptide repeat proteins that bind to and protect DNA gyrase (topoisomerase II) and topoisomerase IV proteins from quinolones. The essential function of these two topoisomerase proteins is to relax positive supercoils of DNA and allows replication and transcription can occur continuously [221].

(3) The inactivation of antimicrobials

Many enzymes have been identified that can degrade and modify diverse antimicrobials such as ß-lactams, aminoglycosides and macrolides. Extended-spectrum beta-lactamases (ESBLs) are enzymes that confer resistance to most ß-lactam antimicrobials, including penicillins, cephalosporins and monobactams [77]. They arise by mutations in genes (especially *TEM* and *SHV* genes) that alter the configuration around the active site of TEM and SHV enzymes so as to increase their efficiency with non-hydrolyzable cephalosporins and monobactams [77]. In addition, there are hundreds of variants of *CTX-M* genes which encode ESBLs that show greater activity against cefotaxime than other ß-lactam antimicrobials [20]. Especially, the CTX-M-14 and CTX-M-15 enzymes are the most widely prevalent hydrolytic enzymes worldwide [152].

III-2. Mechanisms of quinolone resistance

Fluoroquinolones (FQs) are broad-spectrum antimicrobials used widely in the treatment of bacterial infections in humans and animals [84]. Resistance to FQs emerged following their widespread use and posed a significant threat to the health of companion animals and humans.

Three major mechanisms of (F)Q resistance have been investigated: i) mutations in genes encoding DNA gyrase and topoisomerase IV; ii) the presence of plasmid-mediated Q resistance (PMQR) genes; and iii) efflux pump activity transporting

antimicrobials or harmful agents out of the bacteria [71].

1. Mutations in DNA gyrase and topoisomerase IV

(F)Qs bind to and inhibit two types of topoisomerases, DNA gyrase and topoisomerase IV which are essential for bacteria replication and transcription. DNA gyrase is composed of two GyrA and two GyrB subunits and topoisomerase IV is composed of two ParC and two ParE subunits, respectively. The main function of DNA gyrase is to catalyse the negative supercoiling of DNA [73] and the main role of topoisomerase IV seems to be associated with decatenating the daughter replicons [36]. However, mutations in topoisomerases protect bacteria from the bactericidal activity of (F)Qs [71]. Mutations in DNA gyrase and topoisomerase IV are commonly identified in quinolone-resistant bacteria [71]. Alterations described in the GyrA of *E. coli* are predominantly in the so-called quinolone-resistance determining region (QRDR) [216], between positions 67 and 106. In DNA gyrase, two amino acids, Ser83 and Asp87 of GyrA are known as the most frequently changeable sites in *E. coli* and the Ser83Trp mutation causes the decreased binding efficacy of (F)Qs to gyrase-DNA complexes [209]. The presence of a single mutation in the above-mentioned positions of the QRDR of *gyrA* usually results in high-level resistance to nalidixic acid, but to obtain high levels of resistance to fluoroquinolones, the presence of additional mutations in *gyrA* and/or in another target such as *parC* is required [166, 192]. Thus it has been proposed that the MIC of nalidixic acid could be used as a generic marker of resistance for the quinolone family in Gram-

negative bacteria [61, 166]. Different amino acid substitutions at the same position result in different quinolone susceptibility levels [32, 193], indicating that the final MIC is a function of the specific substitutions [215]. This fact is probably due to the mechanism of interaction between the quinolones and their targets. It has been suggested that amino acid 83 (numeration for *E. coli*) of GyrA interacts with the radical in position 1 of quinolones, whereas amino acid 87 of GyrA interacts with the radical in position 7 [192]. Thus, different amino acid substitutions at these points would affect in different ways the affinity for the quinolone molecule. In addition, mutations in other positions might affect the whole protein structure, affecting the interaction with quinolones. In GyrB of *E. coli*, substitutions resulting in resistance to quinolones have been described at positions 426 (Asp-426 to Asn) and 447 (Lys-447 to Glu) [217]. Substitutions at position 426 seem to confer resistance to all quinolones, whereas those at position 447 result in an increased level of resistance to nalidixic acid, but a greater susceptibility to fluorinated quinolones. In case of topoisomerase IV, mutations tend to frequently occur in the positions equivalent to Ser80 and Glu84 of ParC in *E. coli* [71]. In *E. coli*, another substitution (Gly-78 to Asp) in ParC has been described both in clinical isolates and laboratory obtained quinolone-resistant mutants [68, 95]. The role of amino acid substitutions in ParE, resulting in the development of quinolone resistance in clinical isolates of Gram-negative microorganisms appears to be irrelevant [45, 165]. Moreover, this mutation only seems to affect the MIC of quinolones in the presence of a concomitant mutation in *gyrA* [21].

2. The presence of PMQR genes

Plasmid-mediated quinolone resistance was first reported in 1998 from a *Klebsiella pneumoniae* clinical isolate in Birmingham, Alabama [123]. This isolate had a plasmid pMG252 encoding a pentapeptide repeat family that protects bacteria from a quinolone binding [185]. The responsible gene was termed *qnr*, later amended to *qnrA*, as additional *qnr* alleles were discovered. Investigation of a *qnrA* plasmid from Shanghai that provided more than the expected level of ciprofloxacin resistance led to the discovery in 2006 of a second mechanism for PMQR: modification of certain quinolones by a particular aminoglycoside acetyltransferase, AAC(6´)-Ib-cr [159]. A third mechanism for PMQR was added in 2007 with the discovery of plasmid-mediated quinolone efflux pumps QepA [144, 213] and OqxAB [65]. The *qnr* gene has been detected with class 1 integrons which are also known as *sul1*-type integrons [201]. *Sul1*-type integrons possess *qacEΔ1* and *sul1* genes that involve a sequence that may act as a recombinase for mobilization of the antimicrobial resistance genes such as *qnr*, *blaCTX-M* and *ampC* [120]. Therefore, the quinolone resistance is usually associated with multi-drug resistance. Several ß-lactamase genes are associated with *qnr*-positive plasmids encoding for the cephalosporinase FOX-5, ß-lactamases SHV-7 and CTX-M-9 and the penicillinase PSE-1 [120]. In cell-free systems QnrA, QnrB and QnrS have been shown to protect *E. coli* DNA gyrase from quinolone inhibition. Qnr proteins with their additional structural features (loops, N-terminal extension) are proposed to bind to gyrase and topoisomerase IV targets in such a way as to destabilize the cleavage complex between enzyme, DNA and quinolone causing

its release, religation of DNA and regeneration of active topoisomerase [189, 211]. AAC(6′)-Ib-cr is a bifunctional variant of a common acetyltransferase active on such aminoglycosides as amikacin, kanamycin and tobramycin but also able to acetylate those fluoroquinolones with an amino nitrogen on the piperazinyl ring, such as ciprofloxacin and norfloxacin [159]. Compared to other AAC(6′)-Ib enzymes, the −cr variant has two unique amino acid substitutions: Trp102Arg and Asp179Tyr, both of which are required for quinolone acetylating activity. Models of enzyme action suggest that the Asp179Tyr replacement is particularly important in permitting π-stacking interactions with the quinolone ring to facilitate quinolone binding. The role of Trp102Arg is to position the Tyr face for optimal interaction [190] or to hydrogen bond to keto or carboxyl groups of the quinolone to fix it in place [127]. QepA is a plasmid-mediated efflux pump in the major facilitator (MFS) family that decreases susceptibility to hydrophilic fluoroquinolones, especially ciprofloxacin and norfloxacin [143, 213]. A *qepA* gene has often been found on plasmids also encoding aminoglycoside ribosomal methylase, RmtB [115, 144].

3. Efflux pump activity

Decreased quinolone uptake may be associated with two factors: an increase in the bacterial impermeability to these antimicrobials or the overexpression of efflux pumps. Efflux pumps are transporters which extrude harmful substrates from cells to the external environment [202]. There are five major families of efflux pump system: major

facilitator (MF), multidrug and toxic efflux (MATE), resistance-nodulation-division (RND), small multidrug resistance (SMR) and ATP binding cassette (ABC) [202]. Most efflux pump systems use the proton motive force except for ABC which utilizes ATP hydrolysis as an energy source [202]. A major efflux pump system in *E. coli* is AcrAB-TolC transporter which is included in the RND family [84]. This pump has three protein components: a trans-membrane spanning integral inner membrane protein (AcrB), a periplasmic lipoprotein (AcrA) and an outer membrane protein (TolC) [167]. Over expression of efflux pumps from mutations within local repressor genes [4, 200] or may result from activation of a regulon regulated by a global transcriptional regulator such as MarA or SoxS of *E. coli* [9, 153]. The broad substrate range of efflux systems is of concern, as often overexpression of a pump will result in resistance to antimicrobials of more than one class as well as some dyes, detergents and disinfectants (including some commonly used biocides). Over-expression of a multidrug resistance efflux pump alone often does not confer high-level, clinically significant resistance to antimicrobials. However, such bacteria are better equipped to survive antimicrobial pressure and develop further mutations in genes encoding the target sites of antimicrobials [85]. It has been shown that fluoroquinolone resistant strains of *E. coli* are selected 1000-fold more readily from *mar* mutants than wild-type bacteria [29], and highly fluoroquinolone resistant *E. coli* contain mutations in genes encoding the target topoisomerase enzymes and have reduced accumulation and increased efflux [200, 203]. Additive increases in MICs of antimicrobials have also been seen after concurrent over-expression of more than one pump of different classes, also resulting in highly resistant *E. coli* [102].

IV. The emergence of antimicrobial resistance in animals

The veterinary use of antimicrobials includes the use on companion animals, livestocks and animals raised in aquaculture. Antimicrobials are vital agents in veterinary medicine and cannot be replaced due to the lack of suitable alternatives such as vaccines [187]. However, the increased use of antimicrobials induces the emergence of antimicrobial resistant (AR) bacteria from animals. Some surveillance studies have shown an increased incidence of development of antimicrobial resistance in bacteria from animals [2]. High resistance rate in indicator *E. coli* from all age group of pigs and their farm environment in Korea [113]. The extensive and long-term use of tetracycline has apparently resulted in high prevalence of tetracycline resistant bacteria in swine and other food animals. In Korea, tetracycline has long been used and was the most commonly used antimicrobials occupying over 50% of total antimicrobials consumption in Korean livestock [5]. The tendency of higher prevalence of resistance of tetracycline in animals was also observed in other countries such as Denmark [42] and Japan [134], although resistance is much higher in Korea. Especially, many studies reported that the antimicrobial resistance rate and incidence of multiple resistances were markedly higher in the young pigs (piglet and nursery) in all antimicrobials than those from adult pigs [99, 125]. The high incidence of resistance noted in piglet and nursery may be a reflection of increased antimicrobial use at that time and may also reflect the increased colonization by pathogens that occurs during postweaning [126, 128]. *Enterobacteriaceae* isolates from chicken cecums were highly resistant to common

antimicrobials such as ampicillin, cephalothin, kanamycin, nalidixic acid, spectinomycin and streptomycin in Korea [181]. Specifically, the overall antimicrobial resistance rates were higher in *E. coli* isolates from chickens compared with commensal *E. coli* isolates from humans [181]. *E. coli* isolates from chicken showed a resistance rate the ranged between 56.9% and 84.7% to ampicillin, chloramphenicol, kanamycin, streptomycin or trimethoprim whereas *E. coli* isolates from humans displayed between 3.6% and 36.5% to the same antimicrobials [82]. However, unlike other livestocks, much less attention has been given to the prevalence and characterization of AR bacteria associated with companion animals such as horse, dog and cat in Korea. The number of people living with dogs and cats has been increasing annually worldwide. According to the 2013 to 2014 American Pet Products Association survey, about 70% of U.S. households include companion animals [114]. In the Korean companion animal industry, the market size associated with companion animals is rapidly increasing and estimated to be $5.4 billion by 2020 [172]. In addition, more and more Korean people have recognized the importance of horse industry according to the increasing trend of horse-riding [88]. Reflecting these figures, as Korea's economy is expanding, the horse industry is in the process that is similar to that of the developed countries. The main facts were as follows: the number of horses raised in Korea in 2014 reached up to 25,819, which was 5.5% more increased than the previous year. The horse businesses increased 9.6% totaled 1,999. The horse-riding facilities were 395 nationwide, 19.3% increased [88].

The possible bacterial transmission routes between animals and humans are numerous. The most probable ways of interaction are summarized in transmission through the food

chain [173]; through direct or indirect contact with people working in close contact with animals, such as farmers and animal health workers [109]; and through manure contaminated environments and aquaculture [149]. In particular, the role of the environment is extremely important, as it can serve as the reservoir of antimicrobial-resistance genes [158]. Companion animals such as horses, dogs and cats are responsible for potential sources of spread of antimicrobial resistance due to the extensive use of antimicrobials in these animals and their close contact with humans [57]. Likewise, the inherent risk of any use of antimicrobials to select for antimicrobial resistant bacteria poses a relevant risk for public health by spreading of antimicrobial resistance from animals to humans via direct or indirect contacts [187]. Direct contact includes a bite, lick or scratch and handling of animal feces, whereas indirect contact can occur by sharing the bed or toilet environment or being bitten by arthropods originating from companion animals [160].

Subsequently, it is needed to strengthen efforts to prevent and control the spread of antimicrobial resistance between companion animals and humans. An antimicrobial resistance is not a new problem and has long been recognized as a threat to effective treatment. For a number of years the priority focus in many countries was tackling healthcare-acquired infections caused by bacteria such as methicillin resistant *Staphylococcus aureus* and *Clostridium difficile*. Globally, the world health organization (WHO) is leading some of this effort through the global antimicrobial resistance surveillance system and the world organization for animal health is tracking some antimicrobial uses in animals [141]. Despite these efforts, antimicrobial resistance has

continued to escalate and the need to accelerate progress has been acknowledged by the WHO. To achieve change at the rate required to impact on antimicrobial resistance requires political will and global action, working across human and animal health sectors through an international partnership, known as the 'One Health' approach. The one health concept means that human, animal and environmental health are closely linked and injudicious use of antimicrobials in one person or animal can harm other people and animals [161]. The concept is not new, having been promoted by Rudolf Virchow and others in the late nineteenth century, and emphasizes the linkages between human, animal and environmental health in today's rapidly changing world [98]. The concept received relatively little attention during much of the twentieth century, but in recent years, a one health movement has generated increased interest, primarily as a result of efforts by the veterinary community [98]. The WHO is well placed to coordinate this action, and the existing tripartite relationship between the WHO, the world organization for animal health and the united nations food and agriculture organization provides a mechanism for collaboration across sectors including through the codex alimentarius [10]. A global one health surveillance is a gold standard being called for, however, there are other immediate smaller-scale initiatives that can prevent to spread antimicrobial resistance; avenue to decrease the demand for antimicrobials from the public and animal-owners; avenue to increase uptake of vaccines; avenue to utilize other non-pharmaceutical disease prevention methods. On both the human and animal health, improved diagnostic tests can decrease unnecessary prescription of antimicrobials. Furthermore, increased one health instruction is needed for veterinarians,

physicians, and other health professionals. Integrated training and increased venues for physician-veterinarian discussion are needed. It is possible that a more integrated, one health–oriented approach will lead to cost savings, but more data are needed to assess the cost effectiveness of such an approach. If it does yield long-term health and economic benefits, economic support by governments and others should follow.

General Introduction

Livestocks today are not that much more valuable than they were long ago, we treat our companion animals as if they were far more valuable. In addition, we have seen a huge switch in animal medicine, from a focus on livestocks to a focus on companion animals. During the past few decades, the veterinary profession has undergone a profound shift in focus from agricultural animals to companion animals in the United States (US) and to a lesser extent in Western Europe [208]. Several demographic and socioeconomic factors have contributed to this shift, including consolidation of the livestock and food production industries [100], urbanization and changing social attitudes [132], a gender shift towards women in veterinary medicine [132, 164] and expanding clinical specialization [117]. In 2016, 71.2% of veterinarians in the US work primarily with companion animals and 6.7% work primarily with livestocks [13]. A companion animal, as defined by the American society for the prevention of cruelty to animals (ASPCA) is a "domesticated or domestic-bred animals whose physical, emotional, behavioral and social needs can be readily met as companions in the home or in close daily relationship with humans. The ASPCA also specified "species suitable to be companion animals include dogs, cats, horses, rabbits, ferrets, birds, guinea pigs and select other small mammals, small reptiles and fish. In the Korean companion animal industry, the market size associated with companion animals is rapidly increasing and estimated to be $5.4 billion by 2020 [172]. In addition, more and more Korean people have recognized the importance of horse industry according to the increasing trend of

horse-riding [88]. Reflecting these figures, as Korea's economy is expanding, the horse industry is in the process that is similar to that of the developed countries. The main facts were as follows: the number of horses raised in Korea in 2014 reached up to 25,819, which was 5.5% more increased than the previous year. The horse businesses increased 9.6% totaled 1,999. The horse-riding facilities were 395 nationwide, 19.3% increased [88].

The prevalence and distribution of antimicrobial resistant (AR) *E. coli* from food and companion animals have been widely investigated and the transmission of AR *E. coli* between those animals and humans has been demonstrated [170, 174]. However, unlike other animals, much less attention has been given to the prevalence and possible cross-transmission of AR *E. coli* associated with horses. According to the report of the Korean Racing Association, the size of the horseback-riding industry is rapidly increasing annually and the estimated number of horse riders was about 420,000 in 2013 [89]. Therefore, we investigated the frequency of AR *E. coli* from horses, and their AR profiles and molecular fingerprints to evaluate the distribution and clonalities of them in horses and horse-associated environments in chapter I.

Quinolone (Q) and fluoroquinolone (FQ) are broad-spectrum antimicrobials used to treat bacterial infections in humans and animals [119, 157]. Since they are very effective antimicrobials against Gram-negative bacteria including *E. coli*, these agents have been widely used to treat a range of infections in human and veterinary medicine. *E. coli* are also considered as major causative agents of bacterial infections. *E. coli* easily acquire antimicrobial resistance by genetic mutation and horizontal gene transfer [17]. The

transmission of AR *E. coli* from animals to humans has been demonstrated [64]. Several studies have investigated multi-factorial (F)Q resistance mechanisms in *E. coli* isolated from humans and food-producing animals [84]. However, few studies have examined the prevalence and the resistance mechanisms of (F)Q-resistant *E. coli* from companion animals [58]. Moreover, there have been no studies investigating the distribution of (F)Q-resistant *E. coli* in companion animals and their owners. Therefore, in this study, we investigated how (F)Q resistance develops and distributes in companion animals and humans that they contact is important for understanding (F)Q resistance trends in veterinary medicine in chapter II.

Companion animals such as horses, dogs and cats are responsible for potential sources of spread of antimicrobial resistance due to the extensive use of antimicrobials in these animals and their close contact with humans [57]. The cross-transmission of AR bacteria or associated antimicrobial resistance genes is frequently occurred between humans and animals via direct or indirect contacts [64]. Likewise, the possibility of cross-transmission of AR bacteria between humans and companion animals has already been investigated [25, 170]. However, those studies were mainly focusing on only the possibility of cross-transmission of AR bacteria between humans and companion animals. Actually, a bacterial transmission (from human to human) is frequently occurred in confined human communities such as schools and households [55]. Nevertheless, there is no study about the possibility of cross-transmission of AR bacteria between companion animal owners and non-owners in human communities. Therefore, in this study, we compared the genetic similarity of AR *E. coli* isolates from owners of

dogs and non-owners sharing a class room or households to identify the possibility of secondary transmission of AR bacteria between humans in confined community in chapter III.

Chapter I

Isolation and characterization of antimicrobial-resistant *Escherichia coli* from national horse racetracks and private horse-riding courses in Korea

I. Introduction

Escherichia coli (*E. coli*) is a predominant facultative anaerobe, Gram-negative and commensal microorganism that is present in the gastrointestinal microflora of humans and animals. Most *E. coli* strains are non-pathogenic and some strains play an important role as a constituent of microflora in intestinal tract of healthy animals. However, pathogenic *E. coli*, such as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffuse adhering *E. coli* (DAEC), cause disease of the gastrointestinal, urinary or central nervous system in humans [133]. The illness is sometimes associated with food poisoning caused by ingestion of contaminated hamburgers at fast-food restaurants.

Resistance to antimicrobials in bacterial strains is considered as a serious threat to public health, particularly in developing countries. In the past 2 decades, it has increased the frequency of isolation of antimicrobial resistant (AR) bacteria, including those resistant to fluoroquinolones and cephalosporins [110]. *E. coli* is sometimes used as a sentinel strain for monitoring antimicrobial resistance in fecal bacteria because it is most commonly cultured from wide range of hosts [43] and easily acquires antimicrobial resistance by genetic mutation or horizontal gene transfer via certain mobile genetic elements, such as transposons, bacteriophages and plasmids [17]. Especially, many *dfr* genes responsible for trimethoprim (TMP) resistance have been found in gene cassettes inserted in integrons [218]. Since many gene cassettes of integrons possess the diverse antimicrobial resistance genes in Gram-negative bacteria, such as *E. coli*, the horizontal

gene transfer by integrons causes the emergence of multi-drug resistant (MDR) bacteria [156].

The prevalence and distribution of AR *E. coli* from food and companion animals have been extensively studied and the transmission of AR *E. coli* between animals and humans has been demonstrated [170, 174]. However, unlike other animals, much less attention has been given to the prevalence and possible cross-transmission of AR *E. coli* associated with horses. According to the report of the Korean Racing Association (KRA), the size of the horseback-riding industry is rapidly increasing annually and the estimated number of horse riders was about 420,000 in 2013 Korea [89]. This may suggest an increased chance of transmission of zoonotic pathogens originated from horses to humans due to the increased number of contacts with horses. Thus, this kind of risk may need to be evaluated in the near future with a concern of the public health.

In the present study, horse-associated *E. coli* were isolated and identified from samples acquired from horses and their environments in national racetracks and private horse-riding courses in Korea during 2013. The frequency of AR *E. coli*, and their AR profiles and molecular fingerprints were determined to evaluate the distribution and clonalities of them in horses and horse-associated environments. The current study provides the first data on the dissemination of AR *E. coli* in horses in Korea that will be invaluable to estimate the potential risk of transmission of AR *E. coli* from horses to humans.

II. Materials and methods

1. Sampling

A total of 3,078 swab and specimen cup samples were collected from 3 national racetracks (Seoul, Busan-Gyeongnam and Jeju race parks) and 14 private horse-riding courses (Gyeonggi-do, *n*=6; Chungcheongnam-do, *n*=1; Jeollabuk-do, *n*=3; Jeollanam-do, *n*=2, Kyongsangbuk-do, *n*=2) in Korea from July to October in 2013. From horses, healthy skin (*n*=645), nasal cavity (*n*=644) and fecal (*n*=637) samples were collected. For the environmental samples, feed box (*n*=646), drinking water (*n*=495) and bedding (*n*=11) samples were obtained. Swab methods were used to collect superficial samples (skin and feed box) and nasal cavity samples as follows: i) healthy skin: a swab was placed on the healthy skin of horse neck region and swept 3 to 5 times along 15 cm of the surface; ii) feed box: the residual feeds were removed and a swab sample was collected from the surface as described above; iii) nasal cavity: a swab was passed into horse's nostril at least 10 cm deep and rotated to absorb nasal secretion. All the swab samples were immediately placed into the individual sterile collection tubes containing Amies transport medium (Yu-Han Lab Tech, Korea). The rest of samples (feces, drinking water and bedding) were aseptically collected and placed into the sterile specimen cups (Medikorea, Korea). All the individual samples were transported to the laboratory on ice within 6 h after collection. On arrival, the samples were immediately processed as described below.

2. Isolation and identification of *E. coli*

All the samples were subjected to the non-selective pre-enrichment step as described below. Briefly, the tip of swab stick (swab samples: skin, nasal cavity and feed box) or 1 g (or 1 ml) of specimen cup sample (feces, drinking water and bedding) was put in 10 ml of buffered peptone water (BPW: BD, USA) and vigorously vortexed. The pre-enrichment medium was incubated at 37°C for 24 h. After incubation, 1 ml of BPW was transferred into 9 ml of Escherichia coli broth (ECB) and incubated at 37°C for 24 h for the selective growth of coliforms or *E. coli*. The culture in ECB was streaked on MacConkey agar (BD, USA) plate and incubated at 37°C for 24 h. Putative *E. coli* colonies were selected according to a standard protocol previously established in our laboratory. For further confirmation, *E. coli* were identified by strain-specific polymerase chain reaction (PCR) targeting the 16s ribosomal 20 RNA region [186].

3. Antimicrobial resistance profiling of *E. coli* isolates

Antimicrobial susceptibility was determined by a standard disk diffusion test [207] with the following antimicrobial disks (BD, Sparks, MD, USA): ampicillin (AM, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), ceftazidime (CAZ, 30 μg), cefotetan (CTT, 30 μg), imipenem (IMP, 10 μg), gentamicin (GM, 10 μg), tetracycline (TE, 30 μg), ciprofloxacin (CIP, 5 μg), nalidixic acid (NA, 30 μg), sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 μg), chloramphenicol (C, 30 μg), aztreonam (ATM, 30 μg), ceftriaxone (CRO, 30 μg), cefotaxime (CTX, 30 μg), amikacin (AN, 30 μg) and streptomycin (S, 10 μg). The interpretation of antimicrobial

resistance, intermediate resistance or susceptibility was done following the Clinical and Laboratory Standards Institute (CLSI) guidelines [207]. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA) was used as a reference strain. The MDR isolates were defined as *E. coli* isolates resistant to three or more different subclasses of the evaluated antimicrobials [92].

To confirm the TMP resistance of candidate *E. coli* isolates, the Minimal Inhibitory Concentrations (MICs) of TMP were determined by the standard agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) [28]. The isolate showing MIC of 16 μg/ml or higher was considered be resistant to TMP. *E. coli* ATCC 25922 was used as a reference strain.

4. Detection of antimicrobial resistance and integrase genes

The isolates showing resistance to AM, S, TE and SXT were PCR screened for the presence of the following antimicrobial resistance genes; AM resistance genes (*SHV* and *TEM*) [151], S resistance genes (*strA-B* and *aadA*) [180], TE resistance genes (*tetA* and *tetB*) [135], sulfamethoxazole (SMX) resistance gene (*sul1*) [188] and TMP resistance genes (*dfrA1*, *A9*, *A7/17* and *A12/13*) [54, 104]. To differentiate *dfrA7* and *dfrA17* genes, the PCR products of *dfrA7/17* genes were digested with *Pst1* restriction enzyme before gel electrophoresis. Since the *Pst1* restriction site is only present in *dfrA17* gene, the two genes can be easily differentiated by the band pattern of restricted PCR fragments (1 vs 2 bands) [104].

The integrase genes were amplified with the PCR primers (*hep35*-TGCGGGTYAARGATBTKGATTT and *hep36*-CARCACATGCGTRTARAT) binding to the conserved regions present outside of integron-encoded integrase genes *intI1*, *intI2* and *intI3* [206]. The class of the integrons was determined by restriction analysis of the PCR fragments (restriction fragment length polymorphism, RFLP). Briefly, the PCR fragments were restricted with *HinfI* restriction enzyme and the band patterns of restricted fragments were analyzed by gel-electrophoresis. *intI1* generates a single band of 491 bp, *intI2*, two bands of 191 and 300 bp and *intI3*, two bands of 119 and 372 bp, respectively [206].

5. Determination of O and H serotypes

The type of O-antigen of each isolate was determined by the slide agglutination method as described by Guinee *et al*. [59] using polyvalent and monovalent antisera (Joongkyeom, Korea). The H-antigen typing was carried out by the test tube method using the bacteria cultured in liquid medium with H2, H4, H7, H11, H16, H19, H21 and H51 antisera as previously described [59].

6. Molecular fingerprinting

The genetic relatedness among the AR *E. coli* isolates was determined by standard pulsed-field gel electrophoresis (PFGE) using CHEF MAPPER (Bio-Rad, Hercules, CA,

USA) following the manufacture's instruction. In brief, the AR *E. coli* isolates cultured overnight in Tryptic Soy Broth (BD, USA) were streaked on Tryptic Soy Agar (BD, USA) and incubated at 37℃ for 14 - 18 h. The bacterial colonies of each isolate were suspended in 0.8% saline and adjusted to 4.0 McF. The suspensions were embedded in 1.0% agarose plugs and lysed by proteinase K (Sigma-Aldrich, USA). The lysed plugs were then digested for 2 h with 50 U of *XbaI* restriction enzyme (New England Biolabs,Waltham, MA, USA) at 37℃. Digested plugs were then placed on 1.0% SeaKem Gold agarose (Lonza, Allendale, NJ, USA) and PFGE was carried out at 6.0 V for 19 h with a ramped pulse time of 6.76 - 35.38 sec in 0.5x Tris-Borate-EDTA (TBE) buffer at 14℃. BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) was used to establish a DNA similarity matrix using the dice coefficient (0.5% optimization, 1.0% tolerance) and the un-weighted pair group method (UPGMA).

III. Results

1. *E. coli* isolation from the horse-associated samples

A total of 143 *E. coli* (4.6%) were isolated from 3,078 horse-associated samples (Table 1). Ninety six isolates (5.0%) were obtained from horses; 51 isolates from fecal samples, 25 isolates from nasal cavity swab samples and 20 isolates from healthy skin swab samples. Forty seven isolates (4.1%) were from facility environments; 19 isolates

from drinking water samples, 25 isolates from feed box swab samples and 3 isolates from bedding samples (Table 1).

2. Phenotypic characterization of antimicrobial resistance of *E. coli* isolates

The number of *E. coli* isolates showing antimicrobial resistance to each antimicrobial is shown in Table 2. Thirty *E. coli* isolates (21%) were resistant to at least one antimicrobial and all isolates were susceptible to CAZ, IMP, CIP, NA, C, CRO, CTX and AN. The antibiogram analysis revealed that the frequencies of AR *E. coli* isolates were 10.5% (15 isolates) and 8.4% (12 isolates) for S and TE, respectively, followed by 6.3% (9 isolates) for SXT. The other 11 isolates resistant to each AM, AMC, GM, ATM and CTT accounted for minor portions in this study. Only 4 isolates were identified as MDR *E. coli*, which showed resistance to more than 3 classes of antimicrobials. The frequencies of antimicrobial resistance to each antimicrobial were higher in *E. coli* isolated from horses than those from environmental samples, except for AMC (Table 2).

3. Detection of the antimicrobial resistance and integrase genes in AR *E. coli* isolates

For the AR *E. coli* isolates belong to the 4 most frequent AR phenotypes (AM, S, TE and SXT), gene-specific PCRs were performed to detect AR genes responsible for their AR phenotypes (Table 3). Among the *E. coli* isolates resistant to AM, 3 isolates (60.0%)

harbored the *TEM* gene. The *strA-B* genes were widely distributed among the S-resistant isolates (86.7%). Interestingly, the *tetA* gene (66.7%) was more prevalent than *tetB* gene (8.3%) in TE-resistant *E. coli* isolates in this study. Only two SXT-resistant *E. coli* isolates (22.2%) harbored the *sul1* gene responsible for SMX resistance. The *dfrA1* gene was the most prevalent TMP resistance gene, followed by *dfrA9* gene (22.2%) and *dfrA17* gene (11.1%) (Table 3). All 30 AR isolates were screened for the presence of integrase genes. Only 4 isolates harbored an integrase gene (all class 1 integrase gene, *intI1*). They were all isolated from horses (3 isolates from horse feces and 1 isolate from nasal cavity). Although only 2 of them were defined as MDR isolates based on the definition in this study, they all carried at least 2 antimicrobial resistance genes (Table 4).

4. Serotyping of *E. coli* isolates

Out of the 143 *E. coli* isolates, only 41 isolates (28.7%) were defined by their serotypes based on the serotyping methods used in this study. The 41 isolates were clustered into 19 serotypes including O28ac (O28ac/H-, 4 isolates) and O148 (O148/H- and O148/H7, 4 and 1 isolates, respectively) (data not shown).

5. Genotyping of AR *E. coli* by PFGE

Since the serotyping method used in this study revealed serotypes from only 3 out of the 30 AR isolates, PFGE analysis was performed for the all AR isolates to determine the genetic relatedness among the AR isolates. Since 2 AR isolates did not show their electrophoresed bands, these were excluded from the analysis. Except for 5 isolates clustered into two clonal sets (Type G and L), all AR isolates showed distinct genotypes which indicated a weak genetic relatedness (Type A to Y) (Fig. 1). In case of type G, two *E. coli* isolates showed the same antibiogram profiles and were isolated from feces of two different horses at the same horse-riding course. In case of type L, three *E. coli* isolates showing slightly different antibiogram profiles were acquired from different sample sources (feces, feed box and nasal cavity) at the same national racetrack (Fig. 1).

IV. Discussion

To date, many studies have evaluated the prevalence of AR bacteria in food and companion animals and the risk of transmission to humans [170, 174]. However, only a few studies have investigated leisure and sports animals, such as horses, for antimicrobial resistant microorganisms. The recent increase in the horse racing and riding industries in Korea [88] indicates an increased possibility of introducing horse-related pathogens into human communities. In this study, we investigated the frequency of antimicrobial resistant *E. coli* isolated from horses and surrounding environments and characterized AR *E. coli* to evaluate the risk of transmission to humans. This is the first

study conducted to isolate and characterize AR bacteria from horses using samples collected nationwide, including from three national horse racetracks in Korea.

E. coli is one of the most commonly isolated bacteria from animal related specimens [43]. However, the isolation rates from horse related samples were generally low in the current study (overall average: 4.6%). *E. coli* are first secreted from the animal intestine by fecal shedding, then spread to other material by contact transmission. Except for the primary source of *E. coli* (feces, 8.0%) and their first contact material (bedding, 27.3%), *E. coli* isolation rates from horses and their surrounding environments were very low (< 4%). These findings indicate that all of the investigated horse related facilities maintain a good hygienic management that minimizes the transmission of fecal contaminants. All of the facilities we visited kept a high level of hygiene by immediate removing dropped feces, frequently replacing bedding and periodically cleaning the facilities.

Out of the 143 *E. coli* isolated, 30 (21%) showed resistance to at least one antimicrobial compound. This frequency was much less that observed for *E. coli* isolated from fecal samples of hospitalized horses (81.7%), but similar to that for healthy horses (24.5%) [7]. When *E. coli* isolated from horses and environments was compared, the AR rate was higher in isolates collected directly from horses. In addition, MDR *E. coli* and *E. coli* carrying class I integrase gene, a gene associated with multi-drug resistance in bacteria, were only isolated from horses. These results are somewhat different from a previous study in which the frequencies of AR *E. coli* from animals were similar to or higher than those from environments in food animal farms [168]. Therefore, the lower AR ratio of environmental isolates in this study might suggest good

hygienic management in the horse facilities minimized fecal contamination of the environment as mentioned above.

The most frequently detected phenotypes of antimicrobial resistance in AR *E. coli* were against S, TE and SXT in the current study. This pattern is very similar to the results from a previous study conducted in northwest England [6], and suggests a relationship with the amount of antimicrobials used in equine medicine. In veterinary medicine, streptomycin is the first-line antimicrobial for Gram-negative bacterial infection in horses [183]. Oxytetracycline, which is the most commonly used antimicrobial, use applied in an injectable form in combination with a sulfa antimicrobial agent to treat bacterial respiratory infections in horses [210]. Similarly, *Enterococcal* spp. isolated from the same samples used in this study also showed high antimicrobial resistance to TE (18.6%, unpublished data). SXT has also been used extensively for oral administration to horses due to the minor side-effects on the normal microflora of the horse intestine [37].

As shown in Table 3, many of the AR *E. coli* isolated in this study harbored corresponding antimicrobial resistance genes (60–100% in each AR group). The AR *E. coli* without the antimicrobial resistance genes may have other kinds of antimicrobial resistance genes not screened in this study, or alternative resistance mechanisms such as a biofilm formation [75]. Consistent with the results of a previous study [6], the TEM gene was most prevalent in AM-resistant *E. coli* isolates. Most AR *E. coli* resistant to S, the most prevalent phenotype of antimicrobial resistance in this study, contained *strA-B* genes (13/15), which are known to confer a higher level of resistance to *E. coli* than the

aadA gene [180]. Our findings indicate that *E. coli* harboring *strA-B* genes are prevalent in healthy horses in Korea. The portion of genes responsible for TE resistance in *E. coli* is different from that in other countries [22]. While the *tetB* gene was the prevalent resistance gene in TE-resistant *E. coli* isolated from horses in the United States [22], most TE-resistant *E. coli* harbored the *tetA* gene in this study. Additionally, all TMP-resistant *E. coli* harbored at least one of the *dfr* genes, which are usually encoded on mobile genetic elements including plasmids or transposons [11]. The most predominant *dfr* gene they harbored was *dfrA1*gene (88.9%, Table 3), which is consistent with the results of a study conducted in northwest England [7]. However, the *dfrA17* gene was reported to be the predominant antimicrobial resistance gene to TMP in *E. coli* isolated from hospitalized horses at the university of Liverpool, UK [6]. Taken together, these results suggest that horse riders and horse-care workers could be transmitters of these AR *E. coli* to other humans.

Class 1 integron, which is known to be an important genetic element carrying TMP resistance genes in *E. coli*, is horizontally transferred by conjugative plasmids [218]. Lee et al. [104] reported that most of the TMP-resistant *E. coli* isolates harbored *dfr* genes encoded in *intI*. Class 1 integron is also known to carry multiple resistance genes in enterobacteria [124]. Similarly, all four *E. coli* isolates carrying the class 1 integron harbored at least two different antimicrobial resistance genes, and two of these were MDR *E. coli* based on the phenotypical definition (resistant to more than three antimicrobials) in this study. These findings indicate that integrase genes are strongly related to multi-drug resistance, as previously suggested [156]. Out of the 143 isolates,

41 were serotyped into 19 different serological groups, while the remaining isolates were un-typed by the anti-sera used in this study (data not shown). The 19 defined serological groups included two clinically important serotypes, O28ac and O148, in humans [148]. O28ac is associated with EIEC, which cause non-bloody diarrhea and dysentery by invading and multiplying within colonic epithelial cells. O148 is related to ETEC, which cause travelers' diarrhea [148]. Yun et al. [220] previously reported the prevalence of O28ac and O148 to be 3.1% and 1.1%, respectively, in *E. coli* isolated from thoroughbred brood mares in Korea. In the current study, the prevalence of the O28ac serotype in *E. coli* isolated from healthy horses and environments was similar (2.8%) to that observed in a study conducted by Yun et al. [220], but the prevalence of O148 serotype was slightly higher (3.5%) than that from thoroughbred brood mares in the previous study (data not shown).

Since serotyping only revealed the serotypes of three out of 30 AR *E. coli* isolates, PFGE analysis was performed for all AR isolates to analyze the clonal relatedness among isolates. Our results revealed no clear evidence of clonal expansion of AR *E. coli* in horses and their environments. However, two types of clones showed cross-transmission between horses or horses and their associated environments within the same facilities. These results indicate that horses could be carriers of AR *E. coli* to environments and other animals. Thus, these findings suggest a potential possibility of transmission of AR *E. coli* from horses to humans via close contact as previously demonstrated between companion animals and humans [57]. In conclusion, this is the first study to isolate and characterize AR *E. coli* from healthy horses and their

environments by nationwide sampling in Korea. The results indicate that all of the investigated racetracks and private horse-riding courses maintain a high level of hygienic management and there was no clonal transmission of AR *E. coli* among horse facilities. However, the *E. coli* isolated from horses showed a considerably high level of antimicrobial resistance, including multidrug resistance. Due to the frequent contact with horses, our study indicates that horse-care workers and riders may be exposed to a potential risk of infection with AR and pathogenic bacteria carried by horses. Therefore, further studies are needed to evaluate the risk of transmission of AR bacteria between horses and horse riders or workers in horse industries.

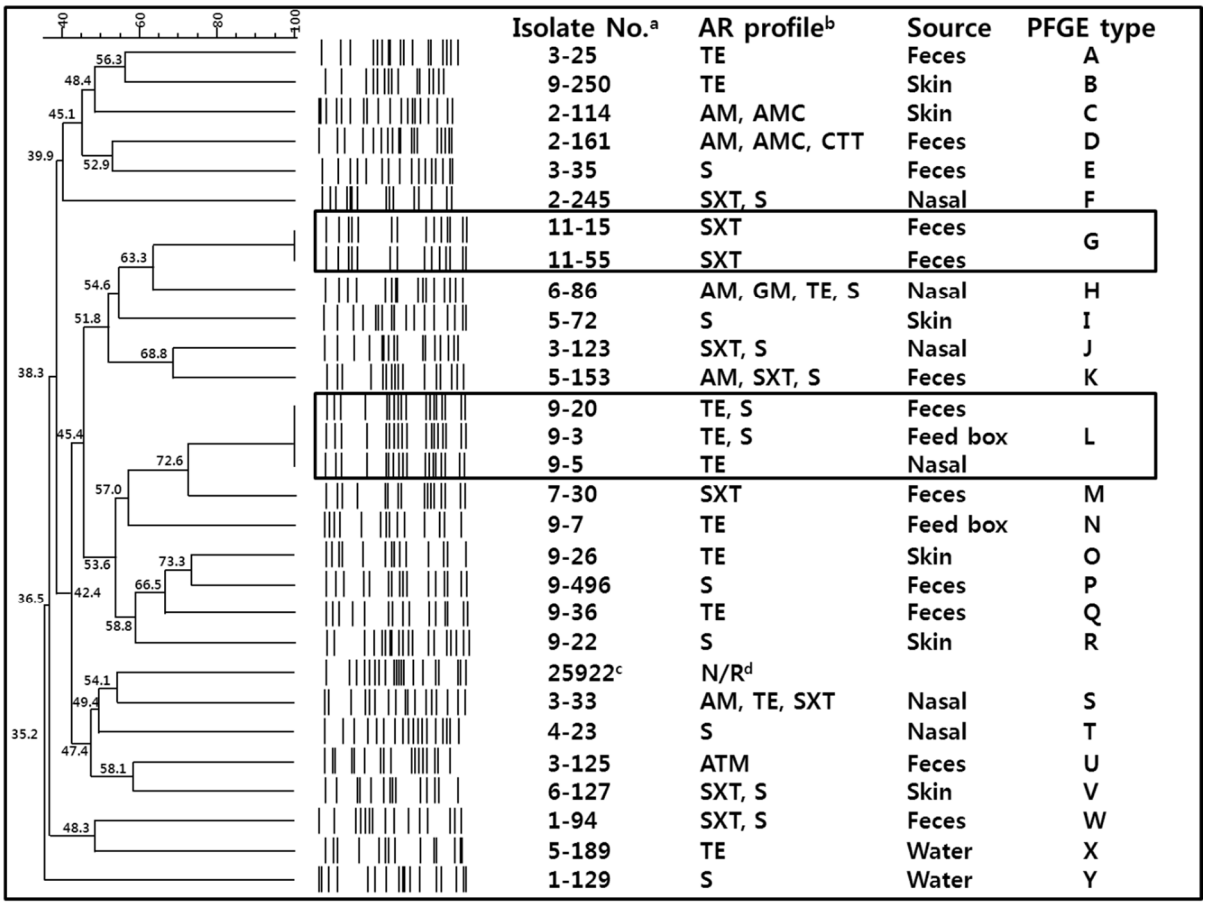


Fig. 1. Pulsed-field gel electrophoresis (PFGE) analysis of antimicrobial-resistant *E. coli* isolates. All the genomic DNAs were digested with *XbaI* followed by standard PFGE analysis (see Materials and Methods). Levels of similarity were determined using Dice coefficient (0.5% optimization, 1.0% tolerance) and the un-weighted pair group method. Individual PFGE patterns are summarized with their isolate ID, antimicrobial resistance profiles, sample sources and PFGE types. TE, tetracycline; SXT, sulfamethoxazole/trimethoprim; AMC, amoxicillin/clavulanic acid; AM, ampicillin; CTT, cefotetan; S, streptomycin; GM, gentamicin; ATM, aztreonam.

[a] Identification number of each *E. coli* isolate was given as a serial number of sampling facility followed by the isolate number in the facility.

[b] Antimicrobial-resistance profiles.

[c] Reference strain for PFGE analysis.

[d] Not resistant to any tested antimicrobials.

Table 1. Prevalence of *E. coli* isolates from horse and environmental samples

Sample sources		**No. of samples**	**No. of *E. coli* isolates (%)**
Horse	**feces**	637	51 (8.0)
	nasal cavities	644	25 (3.9)
	skins	645	20 (3.1)
	sub-total	1,926	96 (5.0)
Environment	**drinking water**	495	19 (3.8)
	feed boxes	646	25 (3.9)
	beddings	11	3 (26.3)
	sub-total	1,152	47 (4.1)
	Total	**3,078**	**143** (4.6)

Table 2. Antimicrobial resistance (AR) profiling of *E. coli* isolated from different samples

Source of *E. coli* isolates		**No. of resistant isolates (%)**								
		AM	**AMC**	**GM**	**TE**	**SXT**	**ATM**	**S**	**CTT**	**MDR**
Horse[a]	feces (*n*=51)	2	1	0	4	5	1	6	1	2
	skin (*n*=20)	1	1	0	2	1	0	3	0	0
	nasal cavity (*n*=25)	2	0	1	3	3	0	4	0	2
	sub-total (*n*=96)	5 (5.2%)	2 (2.1%)	1 (1.0%)	9 (9.4%)	9 (9.4%)	1 (1.0%)	13 (13.5%)	1 (1.0%)	4 (4.2%)
Environ-ment[b]	drinking water (*n*=19)	0	0	0	1	0	0	1	0	0
	feed box (*n*=25)	0	1	0	2	0	0	1	0	0
	bedding (*n*=3)	0	0	0	0	0	0	0	0	0
	sub-total (*n*=47)	0 (0%)	1 (2.1%)	0 (0%)	3 (6.4%)	0 (0%)	0 (0%)	2 (4.3%)	0 (0%)	0 (0%)
Total	***n*=143**	**5 (3.5%)**	**3 (2.1%)**	**1 (0.7%)**	**12 (8.4%)**	**9 (6.3%)**	**1 (0.7%)**	**15 (10.5%)**	**1 (0.7%)**	**4 (2.8%)**

[a,b] Total 30 isolates (21%) were defined as AR *E. coli* from horses (n=25: 13 from feces, 6 from skins, and 6 from nasal cavities, respectively) and environmental samples (n=5: 2 from drinking water and 3 from beddings, respectively). Note that AR *E. coli* showing

resistance to more than one antimicrobial agent were redundantly counted in each antimicrobial resistant test.

AM, ampicillin; AMC, amoxicillin/clavulanic acid; GM, gentamicin; TE, tetracycline; SXT, sulfamethoxazole/trimethoprim; ATM, aztreonam; S, streptomycin; CTT, cefotetan; MDR, multi-drug resistance.

Table 3. Detection of the antimicrobial resistance genes related to the AR phenotypes

AR phenotype	**Ampicillin (*n*=5)**		**Streptomycin (*n*=15)**		**Tetracycline (*n*=12)**		**Sulfamethoxazole/trimethoprim (*n*=9)**				
AR Genes	***SHV***	***TEM***	***strA-B***	***aadA***	***tetA***	***tetB***	***sul1***	***dfrA1***	***dfrA9***	***dfrA17***	***dfrA12/13***
No.	0	3	13	0	8	1	2	8	2	1	0
(%)	(0)	(60.0)	(86.7)	(0)	(66.7)	(8.3)	(22.2)	(88.9)	(22.2)	(11.1)	(0)

SHV and *TEM*, ampicillin resistance genes; *strA-B* and *aadA*, streptomycin resistance genes; *tetA* and *tetB*, tetracycline resistance genes; *sul1*, sulfamethoxazole resistance gene; *dfrA1/A9/A7/A17/A12/A13*, trimethoprim resistance genes.

Table 4. The characterization of 4 *E. coli* isolates harboring integrase gene

Isolate No.	**Class of integrons**	**Sample group**		**Antibiogram**[a]	**Integron-associated genes**[b]
1-94	class 1/*intI1*	horse	feces	SXT and S	*dfrA1* and *strA-B*
3-33	class 1/*intI1*	horse	nasal cavity	AM, TE and SXT	*sul1, dfrA1, dfrA9, tetA* and *TEM*
5-153	class 1/*intI1*	horse	feces	AM, SXT and S	*dfrA1, strA-B* and *TEM*
7-30	class 1/*intI1*	horse	feces	SXT	*dfrA9* and *dfrA17*

[a] AM, ampicillin; TE, tetracycline; SXT, sulfamethoxazole/trimethoprim; S, streptomycin.

[b] *TEM*, ampicillin resistance gene; *strA-B*, streptomycin resistance genes; *tetA*, tetracycline resistance gene; *sul1*, sulfamethoxazole resistance gene; *dfrA1/A9/A17*, trimethoprim resistance genes.

Chapter II

Mechanisms of quinolone resistance in *Escherichia coli* isolated from companion animals, owners, and non-owners

I. Introduction

Quinolone (Q) and fluoroquinolone (FQ) are broad-spectrum synthetic antimicrobials used to treat bacterial infections in humans and animals [119, 157]. Since they are very potent antimicrobial agents against Gram-negative bacteria including *Escherichia coli* (*E. coli*), these agents have been widely used to treat a range of infections in human and veterinary medicine. Consequently, (F)Q resistance has markedly increased worldwide, posing a significant threat to the health of animals and humans [119, 157].

Three major mechanisms of (F)Q resistance have been reported: i) mutations in genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) that are associated with quinolone resistance-determining regions (QRDRs); ii) the presence of plasmid-mediated Q resistance (PMQR) genes; and iii) reduced accumulation of drugs or chemicals due to active efflux pump activity [76]. PMQR genes include members of the *qnr* gene family (*qnrA*, *qnrB*, and *qnrS*) as well as genes encoding FQ-modifying enzyme [*aac-(6')-Ib-cr*] and the efflux pump (*qepA*) [221]. AcrAB-TolC overexpression is a major resistance mechanism against (F)Q that is associated with increased efflux pump activity and contributes to multi-drug resistance (MDR) in *E. coli* [167]. AcrAB-TolC has three components: a transporter of the resistance-nodulation division family (AcrB), a periplasmic accessory protein (AcrA), and an outer membrane protein (TolC) [167].

E. coli are usually commensal bacteria in humans and animals. They are also considered as major causative agents of bacterial infections. *E. coli* easily acquire

antimicrobial resistance by genetic mutation and horizontal gene transfer [17]. The transmission of antimicrobial-resistant *E. coli* from animals to humans has been demonstrated [170]. Several studies have investigated multi-factorial (F)Q resistance mechanisms in *E. coli* isolated from humans and food-producing animals [84]. However, few studies have examined the prevalence and the resistance mechanisms of (F)Q-resistant *E. coli* from companion animals [58], and most of these have been limited to elucidating one or two of the above-mentioned (F)Q resistance mechanisms. Moreover, there have been no studies investigating the distribution of (F)Q-resistant *E. coli* in companion animals and owners. Clarifying how (F)Q resistance develops and distributes in companion animals and the humans that they contact is important for understanding (F)Q resistance trends in veterinary medicine. To this end, the present study examined the frequency of nalidixic acid (NA)-resistant *E. coli* isolated from companion animals and owners, and investigated the three basic mechanisms of (F)Q resistance in these isolates relative to those obtained from non-owners.

II. Materials and methods

1. Sampling

Sampling was carried out with informed consent from owners of companion animals and other human subjects. A total of 104 anal swab samples were collected from four

local veterinary clinics, one veterinary teaching hospital, and one local university in Seoul, South Korea between April 2010 and November 2012. The sampling procedures that were used have been previously described [60]. Swab samples were obtained from 49 dogs, 4 cats, 14 dog owners, 3 cat owners and 34 non-owners (Table 1). People living with and without companion animals at the time of sampling were designated as owners and non-owners, respectively. Owners were selected from among visitors of four local veterinary clinics and a veterinary teaching hospital; non-owners were selected from among freshman students at a university. All protocols and procedures were approved by the institutional review board at the Seoul National University (IRB No. 1208/001-004).

2. Isolation of NA-resistant *E. coli* from swab samples

E. coli isolation and confirmation was carried out as previously [26]. After isolating and identifying *E. coli* from swab samples, 30-μg NA antimicrobial disks (BD Biosciences) were used to select NA-resistant isolates according to Clinical and Laboratory Standards Institute (CLSI) standards [27].

3. Antimicrobial resistance profiling of NA-resistant *E. coli* isolates

The susceptibility of 27 NA-resistant isolates to other antimicrobials was characterized using the following antimicrobial disks (BD Biosciences): ampicillin (AM,

10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), ceftazidime (CAZ, 30 μg), cefotetan (CTT, 30 μg), imipenem (10 μg), gentamicin (10 μg), tetracycline (30 μg), ciprofloxacin (CIP, 5 μg), sulfamethoxazole/trimethoprim (1.25/23.75 μg), chloramphenicol (C, 30 μg), aztreonam (ATM, 30 μg), ceftriaxone (CRO, 30 μg), and cefotaxime (CTX, 30 μg). Susceptibility or resistance to antimicrobials was determined according to CLSI standards [27]. *E. coli* ATCC 25922 was used as a reference strain (American Type Culture Collection; Manassas, VA, USA). MDR isolates were defined as isolates showing resistance to more than three different classes of antimicrobials [92]. NA-resistant *E. coli* isolates showing inhibition zone diameters of ≤ 25 mm against CRO were selected for the confirmation test of extended spectrum β-lactamase (ESBL) production [27]. Isolates were determined as ESBL-producing *E. coli* by the disk diffusion method using CAZ, CAZ/clavulanic acid (CAZ/CL, 30/10 μg), CTX, and CTX/CL (30/10 μg); those showing resistance to CAZ and/or CTX in combination with an increase in inhibition zone diameter of ≥ 5 mm for CAZ/CL and/or CTX/CL were defined as ESBL-producing *E. coli* [196]. In addition, the presence of the *bla*CTX-M gene in ESBL-producing isolates was determined by PCR using CTX-M universal primers [112].

4. Determination of minimum inhibitory concentrations (MICs) of NA and CIP

MICs of NA and CIP were determined for 27 NA-resistant *E. coli* isolates by the

broth microdilution method according to the CLSI standards [27], with *E. coli* ATCC 25922 used as a reference strain.

5. Detection of PMQR genes and mutations in QRDRs

The 27 NA-resistant *E. coli* isolates were screened by PCR for the presence of the following PMQR genes: *aac(6')-Ib-cr*, *qepA*, *qnrA*, *qnrB*, and *qnrS* [24, 212, 219]. The *cr* variant of the *aac(6')-Ib* gene was identified by direct sequencing of the amplified *aac(6')-Ib* gene [147]. Mutations in DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV genes (*parC* and *parE*) of the NA-resistant isolates were identified using specific primers [14, 48, 191, 214]. The wild-type *E. coli* K-12 sequence (GenBank accession no. U00096) was used as a reference [84].

6. Organic solvent tolerance (OST) assay

The activity of the AcrAB-TolC efflux pump system in NA-resistant *E. coli* isolates was measured by the OST assay [205]. For efficiency-of-plating assays [205], cultures of isolates in logarithmic growth phase were diluted to an optical density of 0.2 at a wavelength of 530 nm, and 100-μL aliquots were spread onto Luria-Bertani (LB) agar, which was then overlaid with a mixture of hexane and cyclohexane (3:1 v/v). The plates were sealed and incubated for 24–36 h at 30°C. The number of colonies was counted in

triplicate and colony growth was recorded as confluent (++, ≥ 100 colonies), visible (+, < 100 colonies), or none (−). *E. coli* ATCC 25922 was used as a reference strain.

7. Evaluation of the effect of each (F)Q resistance mechanism on MICs of NA and CIP

To assess the relevance of the three (F)Q resistance mechanisms to the increase in (F)Q resistance in *E. coli*, we evaluated the frequencies of point mutations in the QRDR region and PMQR genes as well as efflux pump activity in 27 NA-resistant *E. coli* isolates. To assess the effects of point mutations and efflux pump activity, an additional comparison was made by determining Pearson's correlation coefficient (R) [3]. The strength of efflux pump activity was graded based on the result of OST test described above (−, 0; +, 1; ++, 2). R values were calculated between the number of point mutations or strength of efflux pump activity and MICs of NA or CIP using SPSS software (SPSS Inc., Chicago, IL, USA) [3].

III. Results

1. Isolation of NA or CIP resistant *E. coli* from companion animals and humans

A total of 63 *E. coli* isolates (60.6%) were collected from 104 anal swab samples. Of these, 27 isolates (42.9%) were determined as NA-resistant *E. coli*. Ten isolates (15.9%) were resistant to CIP, all of which also showed resistance to NA (Table 1). Overall, *E. coli* isolation and (F)Q resistance rates were higher in non-owners than in owners (Table 1).

2. Susceptibility of NA-resistant *E. coli* isolates to other antimicrobials

Of the 27 NA-resistant *E. coli* isolates, 23 (85.2%) were resistant to at least one additional antimicrobial (data not shown) and 20 (74.1%) were identified as MDR (Table 2). The antibiogram analysis revealed that more than half of NA-resistant isolates were also resistant to AM (17/27, 63.0%), AMC (17/27, 63.0%) and TE (14/27, 51.9%). Ten of the NA-resistant isolates showed resistance to CIP (10/27, 37.0%); interestingly, these were all identified as MDR (Table 2). On the contrary, resistance against CAZ, CTT, C, ATM, CRO, and CTX was relatively low. In addition, 3 of the 27 NA-resistant isolates (11.1%) were identified as ESBL-producing *E. coli* harboring the *bla*CTX-M gene and were obtained from two dogs and a non-owner (data not shown).

3. Determination of MICs

The MICs of NA for the 27 NA-resistant *E. coli* ranged from 128 to > 1024 μg/mL. Ten isolates showed much higher MICs of NA (> 1024 μg/mL) than the others, and only

these isolates showed resistance to CIP (MICs of CIP, 16–256 μg/mL). The remaining isolates showed low MICs of CIP, ranging from 0.125 to 1 μg/mL (Table 3).

4. Analysis of mutations in QRDRs and detection of PMQR genes

Mutations in QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes of the 27 NA-resistant *E. coli* isolates were analyzed. Mutations were identified as nucleotide alterations in QRDRs responsible for changes in amino acid sequences in the protein products; silent mutations were excluded from the analysis. Mutations were detected in the *gyrA* gene in all NA-resistant isolates (Table 3); 10/27 (37.0%) had double amino acid substitutions (S83L and D87N); 16/27 (59.3%) had a single amino acid substitution (S83L); and 1/27 (3.7%) had a single D87N substitution. Mutations in the *parC* gene were detected in 10/27 isolates (37.0%); nine (33.3%) had a single S80I mutation and one had double mutations of S80I and E84G. However, no mutations were found in the *gyrB* gene. Eleven isolates (40.7%) had mutations in codons 355, 416, 458, or 477 of the *parE* gene (Table 3).

Of interest, all 10 isolates with double amino acid substitutions in GyrA had point mutations in both *parC* and *parE* genes, and only these isolates were resistant to CIP. While *parC* mutations were detected only in these 10 isolates among the 27 NA-resistant *E. coli*, *parE* mutations were found in those 10 isolates and an additional isolate with a single amino acid substitution in the *gyrA* gene (no. P123; Table 3). Of the 10 CIP-resistant isolates, five originating from humans had an amino acid substitution in

only codon 416 of *parE* (L416F). In contrast, the other five CIP-resistant *E. coli* isolates from dogs had amino acid substitutions in codon 355 or 458 of *parE* (I355T or S458A) (Table 3).

The PMQR gene was detected in only one of the 27 isolates (no. K73), which harbored the *aac(6')-Ib-cr* gene encoding a CIP-modifying enzyme (data not shown).

5. Measurement of efflux pump activity

Efflux pump activity in 27 NA-resistant *E. coli* isolates was measured with the OST assay [200, 205]. A total of 14 (51.9%) and four (14.8%) isolates showed confluent and visible growth, respectively, on the organic solvent mixture (Table 3).

6. Relative contribution of each (F)Q resistance mechanism to increases in MIC

We analyzed the correlations between efflux pump activity or target mutations and MICs of NA or CIP in the 27 NA-resistant isolates, and found that MICs of both NA and CIP were highly correlated with the number of point mutations in the QRDR (R = 0.878 and 0.954, respectively; Fig. 1). However, efflux pump activity was not correlated with either NA MIC (R = −0.239) or CIP MIC (R = −0.169) in NA-resistant *E. coli* isolates (Fig. 1).

IV. Discussion

Many epidemiological studies have reported that companion animals in households are potential sources of transmissible bacteria such as *E. coli* and *Salmonella* spp. (reviewed in [35]). However, the isolation and antimicrobial resistance rates of *E. coli* were not higher in owners then in non-owners. Another study reported a lower risk of MDR *Staphylococci* carriage in nursing home residents living with companion animals than in those living without companion animals [53]. These findings may suggest that although companion animals can be sources of bacterial infections in households, they do not always negatively affect the hygienic status of their owners.

(F)Q resistance is closely associated with MDR in *E. coli* [178]. We found that about 74% of NA-resistant *E. coli* isolates were MDR. Cross-antimicrobial resistance between Q and β-lactams frequently occurs in *E. coli* and *Klebsiella* spp. due to the extensive use of antimicrobials against these bacteria in human and veterinary medicine [51]. The mechanism underlying the association between MDR and Q resistance is currently unclear. In our study, the MDR rate was higher in CIP-resistant than in NA-resistant *E. coli* isolates (100% vs. 74.1%). As shown in previous [58, 69, 84] and as well as in the current study, CIP resistance is strongly correlated with multiple mutations in QRDRs.

All 27 NA-resistant *E. coli* had at least one target mutation in the QRDRs (100%) in this study. Low or high efflux pump activity was observed in 18 isolates (66.7%), but only one (3.7%) harbored a PMQR gene. Mobile (F)Q resistance gene transfer by plasmids has been demonstrated, and high detection rates of PMQR genes in (F)Q-

resistant *E. coli* isolated from human and animal specimens have been reported [119, 130]. However, other studies have reported low detection rates or an absence of PMQR genes in FQ-resistant *E. coli* from animals [58, 84]. The results of the present study are consistent with the latter findings, and highlight the low prevalence of PMQR in Korea. The presence of PMQR genes is closely associated with that of genes encoding ESBL in *E. coli* isolates [80]. Accordingly, the isolate harboring *aac-(6')-Ib-cr* (no. K73) was determined as an ESBL-producing *E. coli* strain carrying the *bla*CTX-M gene in this study.

We found no correlation between increased efflux pump activity and the increase in MICs of NA and CIP in NA-resistant *E. coli*. When MICs in 15 isolates harboring the same single amino acid substitution in GyrA (S83L) were compared to exclude other mutational variables in QRDRs (Table 3), MICs in isolates with high OST were not higher than those in isolates with no OST (average MICs: NA, 277 vs. 299 μg/mL and CIP, 0.33 vs. 0.33 μg/mL). Efflux pumps reduce the concentration of substrates within cells via active transport, and an increase in their activity has been reported to contribute to reduced (F)Q susceptibility [157]. However, our findings indicate that efflux pump activity did not contribute to resistance against (F)Q in these resistant isolates. If mutations in QRDRs lead to sufficiently high levels of (F)Q resistance in *E. coli*, the activity of the efflux pump may not further increase the MICs of (F)Q [3]. Similar findings were reported in other Gram-negative bacteria such as *Klebsiella pneumoniae* and *Campylobacter* spp. [30, 169]. In contrast, the number of mutations in QRDRs was strongly correlated with increases in the MICs of both NA and CIP (Fig. 1). Particularly,

in QRDRs, (F)Q resistance in clinical *E. coli* isolates is more closely associated with mutations in the *gyrA* gene, whereas those in *gyrB*, *parC*, and *parE* genes are less important in the establishment of (F)Q resistance [72]. We found that all NA-resistant isolates in this study had amino acid alterations in GyrA (S83L and/or D87N). A single mutation in the *gyrA* gene has been linked to low FQ resistance in *E. coli* [216], while high FQ resistance was found to be acquired via accumulation of mutations in QRDRs [69]. In particular, FQ resistance was related to double amino acid substitutions in GyrA with or without mutations in *parC* and *parE* genes [58, 84, 86]. In the present study, all CIP-resistant isolates from both humans and animals had double point mutations in *gyrA* concurrently with target mutations in *parC* and *parE* genes. These results indicate that (F)Q resistance mechanisms in *E. coli* at animal hospitals are similar to those observed in humans.

Mutations in the *parC* or *parE* gene have been reported to be closely related to secondary mutations in the *gyrA* gene [21, 86]. In consistent, all mutations in *parC* or *parE* were detected in isolates with double amino acid substitutions, except in one case. Among the four amino acid substitutions in ParE identified in this study, three have been previously reported (I355T, L416F, and S458A) [84, 131]. However, the amino acid substitution (L477M) found in a cat isolate (no. P123) is a novel finding. Interestingly, the patterns of *parE* mutations observed in NA-resistant *E. coli* isolates were distinct in each species. L477M and L416F were present only in cat and human isolates, respectively. All I355T and S458A substitutions were only found in dog isolates, and they were in conjunction with amino acid substitutions in both GyrA and

ParC. Taken together, the CIP-resistant isolates from humans were distinct from those obtained from dogs and cats in terms of the position of *parE* gene mutations.

In conclusion, this is the first study to examine the mechanisms of (F)Q resistance in NA-resistant *E. coli* isolates from companion animals and their owners as compared to those from non-owners. The rates of *E. coli* isolation and (F)Q resistance were not higher in owners than in non-owners, which may suggest that persons living with companion animals are not always at a higher risk of bacterial infections than those living without companion animals. The prevalence of PMQR genes was very low and efflux pump activity was not found to contribute alone to the acquisition of high-level (F)Q resistance. Target site alterations in QRDRs appeared to be the most important mechanism contributing to high-level (F)Q resistance in *E. coli* of both animal and human origins in Korea. Since the (F)Q resistance mechanisms in companion animal isolates are the same as those found in human isolates, prudent use of (F)Q by veterinarians is warranted to prevent the development and dissemination of (F)Q-resistant bacteria. To this end, a continuous monitoring process in veterinary hospitals may be needed to identify the trends and dissemination of (F)Q resistance in companion animals and their owners.

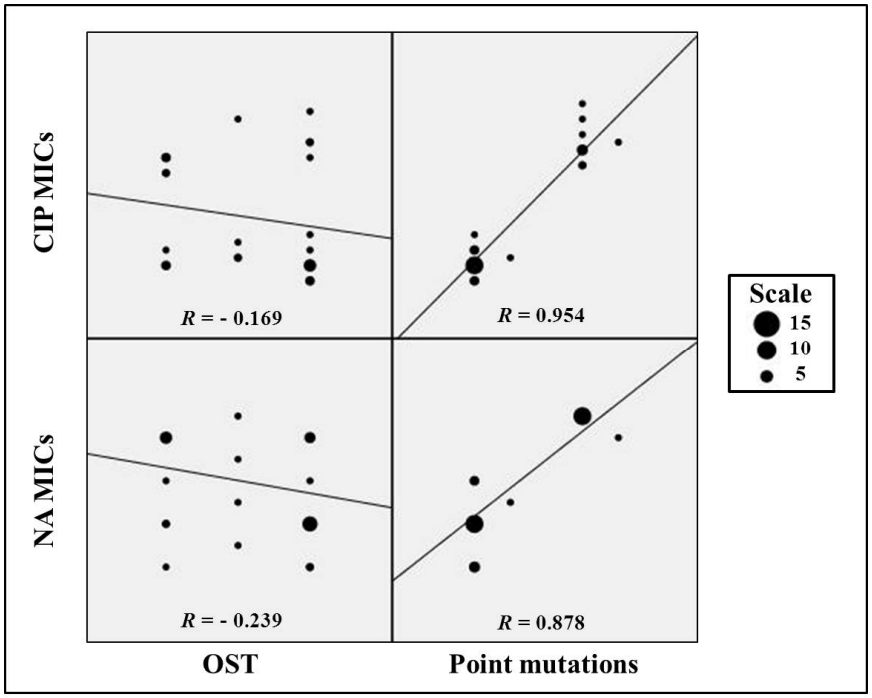


Fig. 1. Correlations between organic solvent tolerance (OST) or number of target mutations and minimum inhibitory concentrations (MICs) of nalidixic acid (NA) and ciprofloxacin (CIP) among 27 NA-resistant *E. coli* isolates. The size of the closed circle in each dot plot represents the number of NA- or CIP-resistant *E. coli* isolates. The scale box located on the right side of graphs shows three different-sized closed circles with the corresponding number of NA- or CIP-resistant isolates. The gradient of the trend line in each dot plot represents positive or negative correlation between two variables. *R*: correlation coefficient.

Table 1. Prevalence of NA- or CIP-resistant *E. coli* isolates from anal samples

Sample sources	No. of samples	No. of *E. coli* isolates (%)[a]	No. of NA-resistant *E. coli* isolates (%)[b]	No. of CIP-resistant *E. coli* isolates (%)[c]
Companion animals	53	32 (60.4)	13 (40.6)	5 (15.6)
Owners	17	9 (52.9)	3 (33.3)	1 (11.1)
Non-owners	34	22 (64.7)	11 (50.0)	4 (18.2)
Total	**104**	**63 (60.6)**	**27 (42.9)**	**10 (15.9)**

[a]The percentage indicates the frequency of *E. coli* isolation from the anal samples.

[b,c]The percentage indicates the frequency of NA- or CIP-resistant isolates from the collected *E. coli* isolates. Note that all CIP-resistant isolates also showed resistance against NA.

Table 2. Additional antimicrobial resistance profiling of 27 NA-resistant *E. coli* isolates

Source of NA-resistant *E. coli* isolates		**No. of resistant isolates (%)**												
		AM[a]	**AMC**	**CAZ**	**CTT**	**GM**	**TE**	**CIP**	**SXT**	**C**	**ATM**	**CRO**	**CTX**	**MDR**
Humans	Owners (*n*=3)	1	1	0	0	1	1	1	1	0	0	0	0	2
	Non-owners (*n*=11)	9	9	0	0	2	7	4	5	0	0	1	1	10
	sub-total (*n*=14)	10 (71.4)[b]	10 (71.4)	0 (0.0)	0 (0.0)	3 (21.4)	8 (57.1)	5 (35.7)	6 (42.9)	0 (0.0)	0 (0.0)	1 (7.1)	1 (7.1)	12 (85.7)
Companion animals	Dogs (*n*=12)	7	7	3	4	3	6	5	3	2	2	3	3	8
	Cat (*n*=1)	0	0	0	0	0	0	0	1	0	0	0	0	0
	sub-total (*n*=13)	7 (53.8)	7 (53.8)	3 (23.1)	4 (30.8)	3 (23.1)	6 (46.2)	5 (38.5)	4 (30.8)	2 (15.4)	2 (15.4)	3 (23.1)	3 (23.1)	8 (61.5)
Total	***n*=27**	**17 (63.0)**	**17 (63.0)**	**3 (11.1)**	**4 (14.8)**	**6 (22.2)**	**14 (51.9)**	**10**[c] **(37.0)**	**10 (37.0)**	**2 (7.4)**	**2 (7.4)**	**4 (14.8)**	**4 (14.8)**	**20 (74.1)**

[a]Abbreviations: AM, ampicillin; AMC, amoxicillin/clavulanic acid; CAZ, ceftazidime; CTT, cefotetan; GM, gentamicin; TE, tetracycline; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; C, chloramphenicol; ATM, aztreonam; CRO, ceftriaxone; CTX, cefotaxime; MDR, multi-drug resistance.

[b]The frequency of *E. coli* isolates showing resistance to each used antimicrobial is shown in the parenthesis.

[c]All the 10 CIP-resistant isolates were determined as MDR

Table 3. Determination of MICs and characterization of 27 NA-resistant *E. coli* isolates

Isolate No.	Source	NA MIC (μg/mL)	CIP MIC (μg/mL)	Mutations in the QRDRs[a]				Growth in presence of organic solvent[c]
				GyrA	ParC	GyrB	ParE	mixture of hexane and cyclohexane (3:1 [vol/vol])
P127	Owner	>1024	32	S83L D87N	S80I	–[b]	L416F	–
P128	Owner	256	0.5	S83L	–	–	–	–
P143	Owner	256	0.25	S83L	–	–	–	++
P97	Non-owner	256	0.25	S83L	–	–	–	+
P98	Non-owner	128	0.25	S83L	–	–	–	–
P102	Non-owner	>1024	32	S83L D87N	S80I	–	L416F	–
P103	Non-owner	>1024	32	S83L D87N	S80I	–	L416F	–
P108	Non-owner	256	0.25	S83L	–	–	–	++
P109	Non-owner	512	1	S83L	–	–	–	++
P99	Non-owner	>1024	16	S83L D87N	S80I	–	L416F	–
P100	Non-owner	256	0.25	S83L	–	–	–	++
P154	Non-owner	>1024	16	S83L D87N	S80I	–	L416F	–
P144	Non-	128	0.25	S83L	–	–	–	+

	owner							
P150	Non-owner	512	0.25	S83L	–	–	–	–
K132	Dog	256	0.125	S83L	–	–	–	++
R14	Dog	256	0.5	S83L	–	–	–	++
K161	Dog	>1024	256	S83L D87N	S80I	–	S458A	++
K48	Dog	>1024	64	S83L D87N	S80I E84G	–	I355T	++
K56	Dog	>1024	64	S83L D87N	S80I	–	S458A	++
K73	Dog	>1024	128	S83L D87N	S80I	–	S458A	+
K154	Dog	256	0.25	S83L	–	–	–	++
R56	Dog	128	0.125	D87N	–	–	–	++
K168	Dog	>1024	32	S83L D87N	S80I	–	S458A	++
J11	Dog	512	0.5	S83L	–	–	–	+
P129	Dog	256	0.25	S83L	–	–	–	++
K328	Dog	128	0.125	S83L	–	–	–	++
P123	Cat	256	0.25	S83L	–	–	L477M	–
ATCC 25922	Control	8	0.008	–	–	–	–	–

[a]The identified codon sites of mutations in NA-resistant *E. coli* isolates are indicated.

[b]No mutations were found in the target gene.

[c]Scoring: ++, confluent growth (≥ 100 colonies); +, visible growth (< 100 colonies); –, no growth.

Chapter III

Probable secondary transmission of antimicrobial-resistant *Escherichia coli* between people living with and without companion animals

I. Introduction

The number of people living with companion animals has been increasing annually worldwide. According to the 2013 to 2014 American Pet Products Association survey, about 70% of U.S. households include companion animals [114]. In the Korean companion animal industry, the market size associated with companion animals is rapidly increasing and estimated to be $5.4 billion by 2020 [172]. Additionally, most owners consider their companion animals as family members and go to great lengths for their medical treatment [199]. As such, the use of antimicrobials in companion animals is increasing, which has resulted in the emergence and spread of antimicrobial-resistant (AR) bacteria. Companion animals are often considered as one of the reservoirs of AR bacteria that could be transferrable to their owners through direct or indirect contact [25, 170]. Direct contact includes a bite, lick or scratch and handling of animal feces, whereas indirect contact can occur by sharing the bed or toilet environment or being bitten by arthropods originating from companion animals [160].

AR bacteria in companion animals can be cross-transmitted to humans [25, 170]. Bacterial transmission among humans frequently occurs in confined environments, such as schools and households [55]; indeed, the spread of hemolytic uremic syndrome and bloody diarrhea caused by infection with a same clone of Shiga toxin-producing *Escherichia coli* (*E. coli*) has been reported in these environments [94, 106, 116]. Likewise, owners of companion animals could spread AR bacteria originating from their companion animals to other persons via close contact. However, there have been no

studies investigating this possibility. We addressed this in the present study by comparing the genetic similarity of AR *E. coli* isolates from owners of dogs and non-owners sharing a classroom or household to determine the risk of secondary transmission of AR bacteria between humans.

II. Materials and methods

1. Sampling

All study participants provided written, informed consent for their participation. All protocols and procedures were approved by the institutional review board at the Seoul National University (IRB No. 1208/001-004). A total of 48 anal samples were collected from owners of dogs and non-owners at a college classroom and households located in Seoul, Korea, from April in 2010 to November in 2012. We used the sampling method described in previous studies [60, 97]. Owner samples (n=14) were collected from 11 undergraduate students as well as three of their family members; non-owner samples (n=34) were collected from 28 undergraduate students sharing the classroom with 11 owner students as well as six of their family members. Samples were placed in individual collection tubes containing Amies transport medium (Yu-Han Lab Tech, Seoul, Korea) and transported to our laboratory on ice within 6 hr of collection.

2. *E. coli* isolation and identification

For non-selective enrichment of microorganisms in samples, the swabs were mixed by vortexing in 10 ml buffered peptone water (BD Biosciences, Franklin Lakes, NJ, U.S.A.) and incubated at 37°C for 24 hr [198]. One milliliter aliquot of culture was inoculated in 9 ml *E. coli* broth and incubated at 37°C for 24 hr. The cultures were streaked on MacConkey agar plates and incubated at 37°C for 24 hr to isolate coliform bacteria, including *E. coli* [90]. Pink colonies suspected as *E. coli* were selected according to a standard protocol previously established in our laboratory [26]. Strain-specific PCR targeting 16S ribosomal RNA was carried out to confirm the bacterial species as *E. coli* [186]. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, U.S.A.) was used as a positive control strain.

3. Antimicrobial susceptibility tests

Antimicrobial susceptibility was tested by the standard disk diffusion method according to Clinical and Laboratory Standard Institute guidelines [207]. The antimicrobial disks (BD Biosciences) used in this study were as follows: ampicillin (AM, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), aztreonam (ATM, 30 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), cefotetan (CTT, 30 μg), ceftriaxone (CRO, 30 μg), chloramphenicol (30 μg), ciprofloxacin (CIP, 5 μg), imipenem (IMP, 10 μg), gentamicin (GM, 10 μg), nalidixic acid (NA, 30 μg),

sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 μg) and tetracycline (TE, 30 μg). Resistance, intermediate resistance and susceptibility to antimicrobials were established as described by Clinical and Laboratory Standards Institute guidelines [207]. *E. coli* ATCC 25922 was used as a reference strain. Multidrug resistance (MDR) was defined as resistance to three or more different subclasses of antimicrobial [92].

4. Detection of integrase genes in *E. coli* isolates

To determine the association between MDR and the presence of mobile genetic elements, integrase genes responsible for horizontal gene transfer, were detected in all *E. coli* isolates. Briefly, the integrase genes were amplified by PCR using the common integrase primer set, hep35 (5'-TGCGGGTYAARGATBTKGATTT-3') and hep36 (5'-CARCACATGCGTRTARAT-3'). For positive isolates, PCR fragments were first digested with HinfI restriction enzyme (New England Biolabs, Ipswich, MA, U.S.A.) and analyzed by gel-electrophoresis. The class of integron was determined based on the number and size of DNA bands as previously described [206].

5. Molecular fingerprinting

To investigate cross-transmission of AR *E. coli* between owners and non-owners, the genetic relatedness of AR *E. coli* isolates was evaluated by standard pulsed-field gel electrophoresis (PFGE) using CHEF MAPPER (Bio-Rad, Hercules, CA, U.S.A.) [78].

Briefly, isolates cultured overnight in tryptic soy broth (BD Biosciences) were streaked on tryptic soy agar (BD Biosciences) plates and incubated at 37°C for 14–18 hr. The turbidity of bacterial suspensions was adjusted to 4.0 McFarland, and cells were embedded in 1.0% agarose plugs that were lysed with proteinase K prepared as a 20 mg/ml stock solution (Sigma-Aldrich, St. Louis, MO, U.S.A.), followed by digestion for 2 hr with 50 U *XbaI* (New England Biolabs) at 37°C. Digested plugs were then placed on 1.0% SeaKem Gold agarose (Lonza, Allendale, NJ, U.S.A.), and PFGE was carried out at 6.0 V for 19 hr with a ramped pulse time of 6.76–35.38 sec in 0.5× Tris-Borate-EDTA buffer at 14°C. BioNumerics software (Applied Maths, Sint-MartensLatem, Belgium) was used to analyze DNA restriction patterns using the dice coefficient (0.5% optimization and 1.0% tolerance) and the unweighted pair group method. *E. coli* ATCC 25922 was used as a reference strain.

III. Results

1. Isolation of *E. coli* from swab samples

A total of 31 *E. coli* isolates were obtained from 48 swab samples (64.6%), with 9/14 (64.3%) and 22/34 (64.7%) collected from owners and non-owners, respectively (Table 1).

2. Antibiogram of 31 *E. coli* isolates

The number of *E. coli* isolates showing resistance to each antimicrobial is shown in Table 1. A total of 20/31 isolates (64.5%) from non-owners (n=15) and owners (n=5) were resistant to at least one antimicrobial (Table 1). All isolates were susceptible to CAZ, CTT, IMP and ATM. The antibiogram analysis revealed frequencies of AR *E. coli* isolates of 51.6% (n=16) for AM followed by 48.4% (n=15) for NA, 41.9% (n=13 each) for AMC and TE, 29.0% (n=9) for SXT and 16.1% (n=5) for CIP. In addition, nine isolates showed resistance to each of GM, CRO and CTX. A total of 16 isolates (51.6%) were identified as harboring MDR *E. coli*.

3. Detection of integrase genes in *E. coli* isolates

Six of the 31 *E. coli* isolates (19.4%) harbored integrase genes (Table 2). Of these, four originated from non-owners, and two were from owners. The four isolates from non-owners harbored only the class 1 integrase gene *intI1*, whereas both *intI1* and the class 2 integrase gene *intI2* were detected in the two isolates from owners. All six isolates were defined as having MDR, since they harbored at least three different antimicrobial resistance genes.

4. Genetic relatedness of *E. coli* isolates from owners and non-owners

To determine the risk of cross-transmission between owners and non-owners, we analyzed the genetic relatedness of the 31 *E. coli* isolates by PFGE. Three clonal sets

(PFGE types 5, 6 and 24) were identified (Fig. 1A and 1B). For type 5, two *E. coli* isolates (nos. P106-1 and P124) were obtained from two students (one non-owner and one owner) who shared a classroom, whereas the other isolate (no. P119) was from a family member of an owner student. For type 6, the three isolates (nos. P102, P103 and P99) were obtained from three non-owner students, but they had slightly different antibiogram profiles. For type 24, the two isolates (nos. P108 and P109) were from two non-owners living in the same household who showed identical antibiogram profiles.

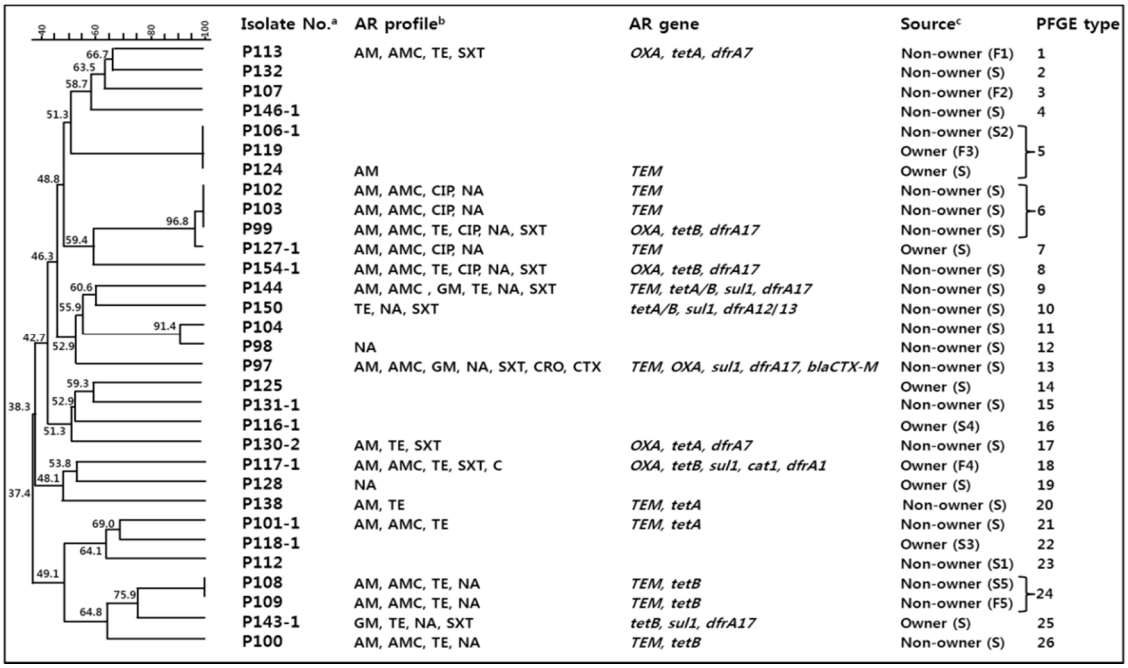
IV. Discussion

AR bacteria can be cross-transmitted between humans and animals [57, 105]. Transmission is usually determined by detecting the same clonal isolates from different hosts [25]. Livestock (e.g., horse, goat and cattle) and wild animals (e.g., free-roaming elk) are sources of enteric pathogens and AR bacteria that can be transmitted to humans via direct or indirect contact [62]. Companion animals are also considered as sources of AR bacteria and infectious human pathogens [35, 57]. However, there is no direct evidence that AR bacteria originating from companion animals are cross-transmitted between owners and non-owners living in a confined community. Common enteric microorganisms, such as *E. coli*, are easily and inadvertently transferred between individuals via hand-to-hand contact [103]. In addition, *E. coli* isolated from feces is considered as a good indicator for antimicrobial resistance in a population [8, 41]. The current study was carried out in order to establish the risk of cross-transmission of AR

bacteria originating from companion animals within a confined human community. Of 31 *E. coli* isolates, 64.5% were identified as AR bacteria showing resistance to at least one antimicrobial. This rate is similar to that in healthy humans (67.1%), but much lower than that in human clinical specimens (98.5%) reported by a previous study from Korea [82]. Integrons are known to play an important role in the horizontal transfer of antimicrobial resistance genes by conjugative plasmids and transposons, and closely associated with the development of MDR in enterobacteria [124, 156]. Class I is the predominant class of integron detected in many countries including Korea [82, 111, 171]. Likewise, all integron positive *E. coli* isolates in this study were found to be multi-drug resistant, and the prevalent type was class I. PFGE analysis revealed that all isolates carrying integrons had distinct PFGE types (Fig. 1A). This suggests that the dissemination of integrons was not due to clonal spread, but to horizontal gene transfer of plasmids or transposons, emphasizing the important role of integrons in the spread of antimicrobial resistance genes. The prevalence of integrons (19.4%) in *E. coli* isolates from healthy humans was higher than that reported in an earlier study from Korea [82], but lower than that in other countries [111, 194]. Three clonal sets were identified among 31 *E. coli* isolates, providing evidence of clonal expansion of resistant strains within the study population (Fig. 1A and 1B). PFGE types 6 and 24 indicated the spreading of AR *E. coli* within a classroom and family, respectively, whereas PFGE type 5 included three isolates from an owner student, a non-owner student and an owner family member. Although we did not analyze *E. coli* isolates from the dogs in this study, our results indirectly demonstrate the possibility of cross-transmission of AR bacteria

from companion animals to non-owners. In most countries, the overall amount of antimicrobials used for companion animals is not reliably measured. However, antimicrobials used in human and veterinary hospitals are almost identical [57]; as such, resistance patterns in bacteria originating from animals and humans are very similar [179, 204]. There is an increasing concern that AR bacteria from companion animals can spread among humans. Although this study only investigated the clonal expansion of AR *E. coli* in a confined human community, the results indicate that once these bacteria are transmitted from companion animals to their owners, they can spread to other humans through social activities. Further investigations are required to provide more direct evidence and identify the risk factors of secondary transmission by studying larger numbers of bacterial isolates from companion animals, their owners and non-owners in a community.

(A)



Isolate No.[a]	AR profile[b]	AR gene	Source[c]	PFGE type
P113	AM, AMC, TE, SXT	*OXA, tetA, dfrA7*	Non-owner (F1)	1
P132			Non-owner (S)	2
P107			Non-owner (F2)	3
P146-1			Non-owner (S)	4
P106-1			Non-owner (S2)	5
P119			Owner (F3)	
P124	AM	*TEM*	Owner (S)	
P102	AM, AMC, CIP, NA	*TEM*	Non-owner (S)	6
P103	AM, AMC, CIP, NA	*TEM*	Non-owner (S)	
P99	AM, AMC, TE, CIP, NA, SXT	*OXA, tetB, dfrA17*	Non-owner (S)	
P127-1	AM, AMC, CIP, NA	*TEM*	Owner (S)	7
P154-1	AM, AMC, TE, CIP, NA, SXT	*OXA, tetB, dfrA17*	Non-owner (S)	8
P144	AM, AMC , GM, TE, NA, SXT	*TEM, tetA/B, sul1, dfrA17*	Non-owner (S)	9
P150	TE, NA, SXT	*tetA/B, sul1, dfrA12/13*	Non-owner (S)	10
P104			Non-owner (S)	11
P98	NA		Non-owner (S)	12
P97	AM, AMC, GM, NA, SXT, CRO, CTX	*TEM, OXA, sul1, dfrA17, blaCTX-M*	Non-owner (S)	13
P125			Owner (S)	14
P131-1			Non-owner (S)	15
P116-1			Owner (S4)	16
P130-2	AM, TE, SXT	*OXA, tetA, dfrA7*	Non-owner (S)	17
P117-1	AM, AMC, TE, SXT, C	*OXA, tetB, sul1, cat1, dfrA1*	Owner (F4)	18
P128	NA		Owner (S)	19
P138	AM, TE	*TEM, tetA*	Non-owner (S)	20
P101-1	AM, AMC, TE	*TEM, tetA*	Non-owner (S)	21
P118-1			Owner (S3)	22
P112			Non-owner (S1)	23
P108	AM, AMC, TE, NA	*TEM, tetB*	Non-owner (S5)	24
P109	AM, AMC, TE, NA	*TEM, tetB*	Non-owner (F5)	
P143-1	GM, TE, NA, SXT	*tetB, sul1, dfrA17*	Owner (S)	25
P100	AM, AMC, TE, NA	*TEM, tetB*	Non-owner (S)	26

(B)

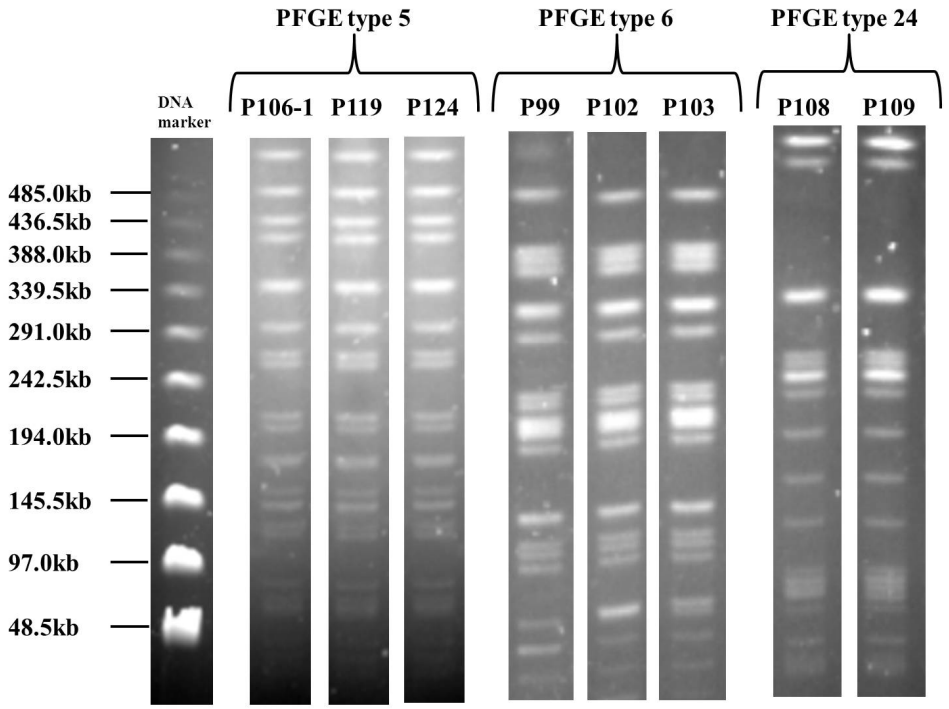


Fig. 1. PFGE analysis of 31 *E. coli* isolates. (A), Dendrogram of all PFGE patterns; (B), PFGE results of types 5, 6, and 24. Levels of similarity were determined using the Dice coefficient (0.5% optimization, 1.0% tolerance) and the unweighted pair-group method. Individual PFGE patterns are summarized with their antimicrobial resistance profiles and genes and sample sources (A). PFGE results of each isolate belonging to types 5, 6, and 24 are presented (B). AM, ampicillin; AMC, amoxicillin/clavulanic acid; C, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; GM, gentamicin; NA, nalidixic acid; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline.

[a]Identification number of each *E. coli* isolate from owners and non-owners.

[b]Antimicrobial resistance profiles.

[c]Sample sources: college students (S) and family members (F). Students and family members living in the same house are represented by a combination of a letter and the same number, as follows: S1 (college student) and F1 (family member who lives in the same household as S1).

Table 1. Antibiogram of 31 *E. coli* isolates from owners and non-owners

Antimicrobial	Non-owner (n = 22)	Owner (n = 9)	Total (n = 31)
AM	13 (59.1)[a]	3 (33.3)	16 (51.6)
AMC	11 (50.0)	2 (22.2)	13 (41.9)
GM	2 (9.1)	1 (11.1)	3 (9.7)
TE	11 (50.0)	2 (22.2)	13 (41.9)
CIP	4 (18.2)	1 (11.1)	5 (16.1)
NA	11 (50.0)	4 (44.4)	15 (48.4)
SXT	7 (31.8)	2 (22.2)	9 (29.0)
C	0	1 (11.1)	1 (3.2)
CRO	1 (4.5)	0	1 (3.2)
CTX	1 (4.5)	0	1 (3.2)
CAZ	0	0	0
CTT	0	0	0
IMP	0	0	0
ATM	0	0	0
MDR	13 (59.1)	3 (33.3)	16 (51.6)
AR[b]	15 (68.2)	5 (55.6)	20 (64.5)

[a]Number of *E. coli* isolates showing resistance to each indicated antimicrobial is presented with the antimicrobial resistant rate in parenthesis.

[b]Antimicrobial resistant. This row shows the number of *E. coli* isolates showing resistance to at least one antimicrobial tested.

AM, ampicillin; AMC, amoxicillin/clavulanic acid; ATM, aztreonam; C, chloramphenicol; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTT, cefotetan; CTX, cefotaxime; GM, gentamicin; IMP, imipenem; MDR, multidrug resistance; NA, nalidixic acid; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline.

Table 2. Characterization of six AR *E. coli* isolates harboring integrase genes

Isolate no.	Integron class	Sample group	Antibiogram	Integron-associated genes[a]
P99	Class 1/*intI1*	Non-owner (S)[b]	AM, AMC, TE, CIP, NA, SXT	*OXA*, *tetB*, *dfrA17*
P130-2	Class 1/*intI1*	Non-owner (S)	AM, TE, SXT	*OXA*, *tetA*, *dfrA7*
P154-1	Class 1/*intI1*	Non-owner (S)	AM, AMC, TE, CIP, NA, SXT	*OXA*, *tetB*, *dfrA17*
P150	Class 1/*intI1*	Non-owner (S)	TE, NA, SXT	*tetA*, *tetB*, *sul1*, *dfrA12/13*
P143-1	Class 1/*intI1*	Owner (S)	GM, TE, NA, SXT	*tetB*, *sul1*, *dfrA17*
P117-1	Class 2/*intI2*	Owner (F)	AM, AMC, TE, SXT, C	*OXA*, *tetB*, *sul1*, *dfrA1*, *cat1*

[a]*cat1*, chloramphenicol resistance gene; *dfrA1*/*A7*/*A17*/*A12*/*A13*, trimethoprim resistance genes; *OXA*, ampicillin resistance gene; *sul1*, sulfamethoxazole resistance gene; *tetA*/*B*, tetracycline resistance genes.

[b]S and F indicate college student and family member, respectively.

AM, ampicillin; AMC, amoxicillin/clavulanic acid; C, chloramphenicol; CIP, ciprofloxacin; GM, gentamicin; NA, nalidixic acid; SXT sulfamethoxazole/trimethoprim; TE, tetracycline.

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General Conclusion

In this study, we demonstrated the potential risk of infection with AR *E. coli* originating from companion animals, such as horses, in the related people. We further examined the (F)Q resistance mechanisms in *E. coli* isolated from dogs and their owners to compare the resistance mechanisms acquired by AR bacteria between companion animals and humans. The results demonstrated that the (F)Q resistance mechanisms in the isolates from companion animals are very similar to those found in the human isolates. The findings emphasized the risk of transmission of AR bacteria from companion animals to humans. Therefore, we evaluated the possibility of cross-transmission of AR bacteria from companion animals to non-owners, mediated by their owners. Although our study investigated the clonal expansion of AR *E. coli* only in a confined human community, the results indicated that once AR bacteria are transmitted from companion animals to their owners, they can spread to other humans through social activities.

In conclusion, we demonstrated that AR bacteria from companion animals have similar antimicrobial resistance mechanisms found in the human isolates, and they can be transmitted to surrounding environments and humans by direct or indirect contacts. Antimicrobial resistance is an urgent global problem. There are increasing concerns about the emergence of multi-drug resistant bacteria in humans, animals and environments. Like our study, the antimicrobial resistance is a complex phenomenon driven by many factors such as the interaction of humans, animals and environmental

sources. Therefore, the aims of combating antimicrobial-resistant bacteria and preserving the efficacy of the currently available antimicrobials in human and veterinary medicine as well as in ecological systems should be addressed in an interdisciplinary effort within a “One Health” approaches. Further studies are clearly warranted to identify the exact risk and mechanism of transmission of AR bacteria from animals and humans.

반려동물과 관련·종사자에서 분리된 항생제 내성 대장균의 상관성 분석

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오늘날, 우리는 소, 돼지와 같은 가축보다 말, 개 그리고 고양이와 같은 반려동물에 더 큰 가치를 두고 있으며, 반려동물의 수의학적 치료에 대한 관심 또한 더 높다. 한국의 경우, 반려동물 시장의 규모가 매년 급격히 커지고 있으며 2020 년에는 그 규모가 5 조 4000 억원에 달할 것으로 예상된다. 또한, 매년 더 많은 한국 사람들이 말 산업의 중요성을 인지하고

있다. 하지만, 현재 국내의 말과 연관된 항생제 내성균에 대한 연구는 많지 않다.

첫 번째 연구는 건강한 말에서 채취한 시료들로부터 분리된 항생제 내성 대장균의 특성을 파악하는데 그 목적이 있다. 세 곳의 국립 마사회와 14 곳의 개인 마장에서 채취한 총 3,078 개의 샘플로부터 143 주의 대장균(4.6%)이 분리되었으며 이 중, 30 주 (21%)는 적어도 1 종류의 항생제에 내성을 나타내었다. 또한, 30 주의 항생제 내성 대장균 분리주 중에서 4 주(13.3%)는 3 종류 이상의 항생제에 내성을 나타내는 다제내성균으로 확인되었다. Pulsed-field gel electrophoresis (PFGE) 분석을 통하여 분리된 항생제 내성 대장균들의 유전적 상동성을 비교해 본 결과, 대부분의 마장으로부터 각기 다른 PFGE 타입의 항생제 내성 대장균이 동정 되었다. 하지만, 두 곳의 마장에서 분리된 항생제 내성 대장균들의 유전적 상동성을 확인한 결과, 말과 말이 거주하는 환경간에는 내성균이 교차 전파 되었음을 확인할 수 있었다. 이는 비록, 말과 환경으로부터 분리된 내성균이 교차 감염되는 가능성은 낮을지라도 말과 접촉하는 사람에게까지 말 유래의 내성균이 전파될 수 있음을 보여주고 있다.

퀴놀론과 플루오르퀴놀론 계열의 항생제는 사람과 동물에서 널리 사용되고 있는 항생제이다. 특히, 이 항생제들은 대장균과 같은 그람음성균에 효과적이기 때문에 인의와 수의학에서 균 감염을 치료하는데 주로 사용된다. 하지만, 이는 전세계적으로 퀴놀론 내성균의 출현을 조장하였으며 사람과

동물의 건강을 크게 위협하는 수준에 이르렀다. 따라서, 두 번째 연구를 통하여 반려동물과 그들의 주인, 그리고 반려동물을 키우지 않는 일반인들로부터 분리된 대장균의 퀴놀론 내성 기전을 확인함으로써 내성이 나타나는 원인을 규명하고자 하였다. 총 104 개의 항문 시료로부터 63 주의 대장균이 분리되었으며 이중, 27 주는 nalidixic acid 에 내성을 보였다. 또한, 27 주의 nalidixic acid 내성 분리주 중에 10 주는 ciprofloxacin 에 내성을 나타내었다. 내성 기전을 구체적으로 살펴보면, 1 주는 plasmid 상에 존재하는 퀴놀론 내성 유전자를 가지고 있었으며 18 주 (66.7%)는 efflux pump 의 활성을 가지고 있었다. 하지만, 무엇보다도 quinolone resistance-determining regions (QRDRs)라고 불리는 특정 유전자 염기서열에 발생하는 돌연변이가 퀴놀론 내성 기전의 가장 주요한 원인이었다. QRDRs 상에서의 점돌연변이는 모든 nalidixic acid 내성 분리주로부터 관찰되었으며 이와 같은 점돌연변이의 갯수는 최소성장억제농도 (MICs)와 연관성이 다소 낮았다. 흥미롭게도, 반려동물로부터 분리된 퀴놀론 내성균의 내성기전은 사람으로부터 분리된 내성 분리주의 내성기전과 동일하였으며 이는 반려동물로부터 사람으로의 퀴놀론 내성균의 상호 전파를 방지하기 위하여 인의와 수의에서 모두 신중한 항생제의 처방이 필요함을 보여준다.

반려동물은 흔히 사람에게 항생제 내성균을 전파하는 보균체로 여겨진다. 또한, 동물과 사람간에 직접적이거나 간접적인 접촉으로 인하여 발생하는 항생제 내성의 전파는 공중보건학적으로 큰 문제를 야기하고 있다. 하지만,

반려동물 유래의 항생제 내성균이 그들의 주인을 통하여 주인들과 접촉하는 사람들에게까지 2 차적인 경로로 전파될 수 있는지에 대한 연구는 아직까지 이루어지지 않았다. 따라서, 마지막 연구 내용으로 반려동물의 주인들과 이들과 같은 환경을 공유하고 있는 반려동물을 키우지 않는 사람들로부터 분리된 항생제 내성균의 유전적 상동성을 확인함으로써 이들간에 항생제 내성균의 상호 전파 가능성을 확인하고자 하였다. 14 명의 반려동물의 주인들과 34 명의 반려동물을 키우지 않는 사람들로부터 총 48 개의 항문 시료를 채취하였으며 이로부터 31 주의 대장균 (반려동물의 주인으로부터 9 주의 대장균이 분리되었으며, 반려동물을 키우지 않는 사람으로부터 22 주가 분리되었다.)을 분리하였다. 또한, 31 주의 대장균 분리주중에서 20 주 (64.5%)는 적어도 1 종류의 항생제에 내성을 나타내고 있음을 확인하였으며, 3 종류 이상의 항생제에 내성을 나타내는 다제내성균은 16 주 (51.6%)가 확인되었다. 유전적 상동성을 비교한 결과, 유전적으로 100% 일치하는 3 쌍의 클론이 확인되었으며 이는 같은 환경을 오랜 시간 동안 공유하는 반려동물의 주인들과 반려동물을 키우지 않는 사람들간에는 항생제 내성균의 상호 전파가 가능함을 보여주었다. 더 나아가, 이는 반려동물 유래의 항생제 내성균이 그들의 주인을 통하여 지역사회로도 쉽게 전파될 수 있음을 나타낸다.

항생제 내성은 전 세계적으로 큰 문제이며, 사람, 동물 그리고 환경으로부터 유래된 다제내성균의 출현은 거대한 위협이 되고 있다. 항생제

내성은 사람, 동물 그리고 환경간의 밀접한 접촉에 의하여 발생하는 복합적인 현상이다. 본 연구의 결과를 통하여 사람, 동물 그리고 환경은 항생제 내성균의 보균체일 뿐만이 아니라 항생제 내성균의 전파체임을 확인하였다. 따라서, 향후에 항생제를 사용함에 있어서는 사람, 동물, 환경의 경계를 가릴 것 없이 “One Health” 개념으로 이해하여야만 효율적인 항생제의 사용을 유지할 수 있을 것이다.

항생제 내성 문제를 해결하는 대표적인 방안에는 사람과 동물에 사용하는 항생제의 지속적인 모니터링 및 관리, 새로운 항생제의 개발, 항생제 내성 기전의 연구 등이 있다. 본 연구를 통하여 반려동물 유래 균주의 퀴놀론계 항생제 내성 기전과 사람 유래 균주의 내성 기전이 동일하다는 것을 확인하였다. 따라서, 동물과 사람에서의 항생제 내성균의 발생을 방지하기 위해서는 수의사와 의사의 신중한 항생제의 처방이 뒤따라야 할 것이다.

주요어: 항생제 내성, 대장균, one health, 말, 반려동물의 주인, 퀴놀론계 항생제

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