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Antimicrobial resistance, virulence and transmission of enterococci isolated from humans and animals

사람과 동물 장구균의 항생제 내성, 병원성 및 전파에 관한 연구

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Antimicrobial resistance, virulence and transmission of enterococci isolated from humans and animals

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Antimicrobial resistance, virulence, and transmission of enterococci isolated from humans and animals

Ka Hee Kwon

(Supervised by Prof. Bong Kyun Park)

Abstract

Enterococci are commensal bacteria of humans as well as of animals and major causes of nosocomial infections. Enterococci of animals have potential risks of dissemination to human or transferring their antimicrobial resistance or virulence genes. It is concerned that companion animals can be a reservoir of infectious enterococci. Food, especially provided by food animals, has been also suspected as a carrier of enterococci of animal with antimicrobial resistance and virulence factor. Accordingly, enterococcal isolates from humans and animals were characterized and their epidemiological relation was analyzed in this study.

It was carried out to compare the phenotypes and genotypes of antimicrobial resistance and virulence genes from 184 enterococci strains isolated from chickens, pigs, companion animals (dogs and a cat), and human patients in Korea. Then, ampicillin resistant *E. faecium* (AREF) strains were selected and multilocus sequence typing was performed to investigate the dispersion of clonal complex 17 (CC17), a global epidemic strain, among animals and humans. The companion animal and human isolates showed high resistance rates against ampicillin and ciprofloxacin, whereas

food animal isolates from chickens and pigs showed high tetracycline and erythromycin resistance rates. AREF was only detected in human (21 of 21 *E. faecium*, 100%) and companion animal (3 of 5 *E. faecium,* 60%) isolates, and all human AREF strains and one of the canine AREF strains were confirmed as CC17. It was suggested that the antibiotic resistance patterns of companion animal isolates were more similar to those of humans than to those of food animals, and CC17 was also detected among only companion animal and human isolates.

Companion animal and human *E. faecium* isolates were characterized and analyzed epidemiologically. Unlike colonization isolates, companion animal infection isolates showed similar CC17 prevalence with human infection isolates. However, patterns of antimicrobial resistance, virulence and pulsed-field gel electrophoresis were different between CC17 isolates from almost all companion animals and humans.

From pork meat processing chain, slaughterhouses, processing plants and retails, 339 *E. faecalis* isolates were isolated and were compared with human infection strains for investigating the dissemination from pork meat to human via pork meat processing chain. From slaughterhouses to retails, chloramphenicol, high-level gentamicin, and erythromycin and multidrug resistance rates decreased while the penicillin resistance rate increased along the processing chain. The prevalence rate of strong or moderate biofilm forming isolates was highest at retails. Random amplified polymorphic DNA-PCR analysis was performed for genetic comparison of the isolates. No isolate seemed to be persistent through the production chain and all the isolates was not similar with human clinical isolates. Therefore, it is suggested that *E. faecalis* strains from pork meat might not be delivered to humans by consumption of pork meat. In conclusion, the results of the above studies suggested that the transmission of enterococci between animal and human is opportunistic.

Keywords: Enterococci, antimicrobial resistance, virulence, clonal complex 17, pork meat processing chain, transmission

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CONTENTS

Summary of Abbreviation

ACRONYM	FULL NAME
Ace	Adhesin of collagen from *E. faecalis*
Acm	Adhesin of collagen from *E. faecium*
AMR	Antimicrobial resistance
AREF	Ampicillin-resistant *E. faecium*
AS	Aggregation substance
BHI	Brain heart infusion
CC17	Clonal complex 17
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
$EfaA_{fs}$	*E. faecalis* endocarditis antigen
Esp	Enterococcal surface proteins
HLAR	High-level aminoglycoside resistant (or resistance)
HLGR	High-level gentamicin resistant
HLR	High-level resistance
HLSR	High-level streptomycin resistant
MDR	Multidrug-resistant (or resistance)
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
PBP	Penicillin-binding protein
PBS	Phosphate-buffered solution
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
QRDR	Quinolone resistance-determining region
RAPD-PCR	Random amplified polymorphic DNA-PCR
ST	Sequence type
UPGMA	Unweighted pair group method using arithmetic mean
UTI	Urinary tract infection
VRE	Vancomycin-resistant enterococci

Literature Review

I. The genus *Enterococcus*

The genus *Enterococcus* is Gram-positive, facultatively anaerobic, catalase-negative oval cocci in the chain form; it is hardy and has the ability to survive under harsh conditions like improper osmotic pressure or temperatures (10°C ~ > 45°C) for most bacteria (3). All the enterococci can grow in broth with 6.5% NaCl and medium with 40% bile salts. Some of them are motile (28, 29).

The genus *Enteroccocus* was quite recently valid and thoroughly separated from the genus *Streptococcus* based on comparison of 16s rRNA sequence data by Schleifer and Kilpper-Ba¨lz in 1984 (127). It belongs to the family *Enterococcaceae* with another four genera, *Melissococcus*, *Pilibacter*, *Tetragenococcus*, and *Vagococcus (41)*.

Enterococcus species are widely distributed from environmental to animal and human. Enterococci are important microbiota in humans and animals. *E. faecalis* and *E. faecium* are plentiful in the human gastrointestinal tract and some species like *E. mundtii* and *E. casseliflavus* in plants (74). The numbers of *E. faecalis* in human feces range from 10^5 to 10^7 colony-forming units (CFUs) per gram, and those of *E. faecium* from 10^4 to 10^5 CFUs per gram. The isolation rate of *E. faecium* and *E. faecalis* is higher in human feces than in livestock's (35). Also, enterococci belong to lactic acid bacteria and some of them produce enterocins, a sort of bacteriocins (31). For a long time, they have been used as probiotics or as starter cultures in fermented food industry (34).

On the other hand, enterococci are one of the most common opportunistic pathogens in hospital settings, with a high mortality rate of up to 61% (19). They frequently have virulence factors contributing infections such as bacteremia, peritonitis, endocarditis and urinary tract, wound, and device-related infections (126). Originally, *E. faecalis* was the major cause of enterococcal infection, but the ratio *E. faecalis* to *E. faecium* infections was inversed in the United States in the late 1990s (96). In addition, antibiotic resistance of enterococci dramatically increased in worldwide and, especially, vancomycin-resistant enterococci (VRE) was spotlighted because vancomycin has been used as last resort in the treatment of gram-positive bacterial infections (141). A further

important consideration is that enterococci possess the outstanding ability to acquire resistance through either chromosomal mutations or genetic exchange of mobile elements like transposons or plasmids. *E. faecium* has been suspected as a central reservoir in the acquisition, conservation and transfer of antimicrobial resistance (AMR) genes among bacteria (161).

II. Antimicrobial resistance of enterococci

Enterococci have several intrinsic resistances and easily accumulate mutations and exogenous genes leading acquired resistances. Therapeutic or subtherapeutic levels of on-going antimicrobial exposure have selected antimicrobial resistant enterococci. As a result, widespread multidrug resistant (MDR) enterococci have limited therapeutic agents and have raised the mortality caused by enterococcal infections (96).

1. Intrinsic resistance

Intrinsic resistance is due to either lack of target sites for the antimicrobial drug or insufficient penetration of the drug to the intracellular target site. Most enterococci are inherently resistant to many antimicrobials like cephalosporins, the semisynthetic penicillinase resistant penicillins (e.g., oxacillin), and therapeutic level clindamycin and aminoglycosides (98). Penicillin-binding proteins (PBPs) of enterococci possess low affinity to cephalosporins (43) (106), and aminoglycosides cannot reach their target site because of poor permeability of the enterococcal cell wall (95). *E. faecalis* is inhibited at low concentrations of trimethoprim-sulfamethoxazole readily *in vitro*, but *in vivo*, this activity is lessened by exogenous folates (166).

2. Acquired resistance

Enterococci are not only intrinsically resistant to several antibiotics but also able to readily recieve mobile genomic elements. The acquisition of resistance genes is made by conjugation using pheromone-responsive plasmids and their high-frequency transfer, broad host range conjugative plasmids, or conjugative transposons (14, 24).

(1) β-lactam resistance

Enterococcal infections have been treated by using ampicillin with an aminoglycoside for a long time. However, thesedays, most of the hospital-associated *E. faecium* isolates show ampicillin resistance (97). Originally, enterococci have intrinsic resistance to β-lactam antimicrobials because of the low affinity of their PBPs. Ampicillin is the most active agent among β-lactams and carbapenems and cephalosporins have less active than penicillin against enterococci (33).

High-level ampicillin resistance in *E. faecium* is induced by mutations in PBP5 leading even lower affinity for ampicillin or by altered PBP5 overproduction (2, 73, 82, 123, 167). Specific amino acid differences on the crystal structure of PBP5 cause resistance by changing architecture of the active site (123, 125). β-lactamase production of enterococci is rare except for a few β-lactamase-producing *E. faecalis* isolates identified in the US (99, 101, 102). Recently, a novel mechanism related with the DD-transpeptidation reaction of the final stage of peptidoglycan synthesis was elucidated (88).

(2) Aminoglycoside resistnace

Enterococci are resistant to low level aminoglycosides [minimal inhibitory concentration (MIC) 8 to 256 μg/ml] due to low cell wall penetration efficiency of these agents. However, combination with cell wall active agents such as β–lactams or glycopeptides can improve uptake of aminoglycoside and lead synergistic effect on treatment of serious enterococcal infections (99).

However, these synergistic treatments have been limited by the occurrence of

acquired high-level resistance (HLR) to all available aminoglycosides. Aminoglycoside-modifying enzymes catalyze and modify the covalent band of amino and hydroxyl groups in the aminoglycoside, thus the binding affinity between the antibiotic and the bacterial ribosome markedly decrease (94). High-level aminoglycoside resistant (HLAR) enterococci can grow on the medium with drug concentration of > 2,000 μg/ml. HLAR is caused by aminoglycoside modifying enzymes coded by transferable plasmid. The most frequently detected enzymes are bifunctional enzyme named as AAC(6′)-Ie–APH(2″)-Ia activities (HLR to all aminoglycoside except only streptomycin), ANT(3″)-Ia (HLR to kanamycin and penicillin-amikacin synergy without HLR to gentamicin), and ANT(6′)-Ia (HLR to streptomycin) (99).

(3) Glycopeptide resistance

Vancomycin was the last resort of severe enterococcal infections. However, glycopeptide resistant enterococci had emerged and rapidly spread since 1986 and become a significant clinical problem. Originally, some Gram-positive genera including *Lactobacillus*, *Leuconostoc* and *Pediococcus* have inherent resistance to glycopeptides because of their peptidoglycan precursors having low affinity to vancomycin and high-level vancomycin resistance of enterococci are supposed to be come from soil bacteria, *Paenibacillus* spp. (46, 139).

Three phenotypes of glycopeptide resistance are known well among enterococci. VanA phenotype is inducible high level resistance to both vancomycin (MIC > 64 μg/ml) and teicoplanin (MIC > 16 μg/ml) and VanB phenotype shows variable levels of inducible resistance to vancomycin (MIC 8 to 64 μg/ml) and sensitive to teicoplanin (MIC < 1 μg/ml). Enzymes encoded by VanA and VanB cluster make cell-wall precursors ending in D-Ala-D-Lac having very low affinity to vancomycin. VanA and VanB are usually associated with *E. faecalis* and *E. faecium*. VanA is the most widely distributed and predominant type. VanC phenotype have intrinsic, constitutive low level resistance to vancomycin (MIC > 8 and < 32 μg/ml) and susceptibility to teicoplanin (MIC < 1μg/ml). VanC type vancomyin resistance is induced by endogenous, species-

specific genes in *E. gallinarum* (*vanC-1*) and *E. casseliflavus*/*E. flavescens* (*vanC-2*/*vanC-3*), respectively (100). Chromosomally encoded VanC changes cell-wall precursor D-Ala-D-Ala to D-Ala-D-Ser. Except these types, there are another newly discovered vancomycin resistance types, VanD, VanE, VanG, VanL, VanM, and VanN (9, 20, 21, 30, 80, 107, 163).

(4) Macrolide resistance

Enterococccal resistance to macrolides is usually induced by the production of an enzyme that methylates an adenine residue in the 23S ribosomal RNA of the 50S ribosomal subunit (60). This mechanism influences not only macrolides (e.g., erythromycin, azithromycin and clarithromycin), but also the lincosamides (e.g., lincomycin and clindamycin) and streptogramin B antibiotics (e.g., quinupristin /dalfopristin) (157). *ErmB* is the most frequently involved gene and *ermA* gene is rare among enterococci (116). This mechanism is called "MLS_B (macrolide-lincosamice-streptograminB) system".

(5) Chloramphenicol resistance

In spite of the infrequent use of chloramphenicol in human hospitals, about a half of enterococci are resistant to chloramphenicol (112). Most of the chlroramphenicol resistances are caused by the chloramphenicol acetylase enzyme, CAT. CATs acetylate a hydroxyl group in the chloramphenicol molecule, thus the modified chloramphenicol cannot bind to the bacterial ribosome (133). The *cat* genes reside usually on the plasmids but also on the chromosome (111). Distribution of the same *cat* genes among gram-positive bacteria such as enterococci, streptococci and staphylococci implies horizontal transfer of chloramphenicol resistance genes.

(6) Tetracycline resistance

Tetracycline resistance is the most prevalent among enterococcal isolates although these antibiotics are not routinely used to treat enterococcal infections (63). Tetracyclines

block protein synthesis by interfering with the binding of aminoacyl-tRNA to the ribosome (121). There are two mechanisms of tetracycline resistance in enterococci; ribosomal protection (e.g., *tetM*, *tetO*, and *tetS*) and active efflux of the drug across the cell membrane (e.g., *tetK* and *tetL*) (6, 121). The *tetM* gene is the most common tetracycline-resistant determinant in enterococci. It is typically chromosomally located and usually carried by Tn*916* or related conjugative transposons, but it can also be found on conjugative plasmids (6, 111, 138). The *tetL* gene, located on conjugative plasmids or on the chromosome, is the more common efflux gene in enterococci (6, 113).

(7) Quinolone resistance

Ciprofloxacin was the fluoroquinolone often used for the gram-negative bacteria infection treatment. However, quinolone resistance is common among clinical enterococcal isolates. Enterococcal isolates resistant to ciprofloxacin are generally also resistant to moxifloxacin and gatifloxacin, more recently developed fluoroquinolones.

Bacteria inhibition of quinolones is performed by interacting with essential enzymes for bacterial DNA replication, type II topoisomerases, DNA gyrase, and topoisomerase IV. The primary target for quinolone activity is DNA gyrase in gram-negative bacteria, and typically the main target is topoisomerase IV in gram-positive bacteria (51). DNA gyrase is composed of two A and two B subunits, named GyrA and GyrB (152). Topoisomerase IV is composed of two subunits, named ParC and ParE, which are homologous to GyrA and GyrB, respectively (69).

Mutations in the enterococcal *parC* gene in the area corresponding to the quinolone resistance-determining region (QRDR in the *E. coli gyrA* gene may be the main event for in quinolone resistance. Additionally mutation(s) in the QRDR in the enterococcal *gyrA* gene may be followed, which makes a level of quinolone resistance higher. The quinolone MIC for the isolate having only *parC* mutations was higher than the MIC for an *E. faecalis* with no *parC* or *gyrA* mutations, but lower than the MIC for *E. faecalis* isolates with mutations in both the *parC* and *gyrA* genes (67). There has been no report about enterococcal isolates having only *gyrA* but no *parC* mutations, and most

quinolone resistant *E. faecium* and *E. faecalis* isolates have mutations in both genes (27, 67).

III. Virulence factors of enterococci

Although AMRs of enterococci play important roles in being nosocomial pathogens, virulence factors are also involved in the adaptation of enterococci in the hospital environment. Unlike streptococci and staphylococci, most enterococci do not produce a set of potent pro-inflammatory toxins, but they have many secreted factors and adhesion proteins.

1. Secreted factors

(1) Cytolysin (Hemolysin)

Over 30 % of *E. faecalis* strains produce cytolysin which is toxin encoded on pheromone-responsive plasmids or pathogenicity islands. Cytolysin is secreted extracellularly as two structural subunits (CylL-L and CylL-S) and then proteolytically activated (8, 129). The cytolysin lyses a wide range of eukaryotic and prokaryotic cells including red blood cells of some animals and some human white blood cells and enhances the enterococcal virulence in animal models (57, 61, 62).

(2) Gelatinase

Gelatinase, an extracellular zinc metalloprotease, contributes to *E. faecalis* virulence such as degradation of host tissues and modulation of the host immune response (110). It has an important role in clearing misfolded proteins and participates in the activation of autolysin, a peptidoglycan-degrading enzyme, which leads to the release of extracellular DNA and the formation of a biofilm (143, 153). *GelE*, the gene encoding gelatinase in *E. faecalis*, is regulated by the Fsr quorum sensing system (117). Recently, dissemination of gelatinase was also described in *E. faecium* (84).

(3) Hyaluronidase

Hyaluronidase, virulence factor usually detected among clinical isolates of *E. faecium*, is encoded by a gene designated *hyl* (119). The gene has a strong relation with Clonal complex 17 (CC17), a major group of *E. faecium* clinical isolates in hospitals (147). Also, megaplasmids containing the *hyl* gene cotransfer with AMR genes, thus the acquisition of these plasmids leads enhanced virulence of *E. faecium* strains (4).

(4) Extracellular superoxide

Enterococci can produce substantial amounts of superoxide. This trait is more correlated with *E. faecalis* isolates from the bloodstream. *E. faecalis* is a potent source of oxidative stress on the intestinal epithelium, which might contribute to bacterial translocation across the epithelium or chromosomal instability associated with intestinal polyps and colorectal cancer (53-55).

2. Cell surface determinants

(1) Aggregation substance

Aggregation substance (AS) is encoded by pheromone-responsive plasmids that often also harbour antibiotic resistance genes. AS makes *E. faecalis* cells clumped each other and facilitate transfer of plasmid DNA. Moreover, AS improve enterococcal abilities to bind to cultured renal epithelial cells and to survive within polymorphonuclear neutrophils and internalize into intestinal cells (108). It also affects the pathogenesis of experimental endocarditis presumably by favoring the formation of large bacterial aggregates on the cardiac valve (128, 154).

(2) Enterococcal surface proteins

Enterococcal surface protein (Esp) is a virulence factor initially derived from the original *vanB E. faecalis* clinical isolate (124). Infection-derived *E. faecalis* isolates were enriched for the *esp* gene and an *esp* homolog was recently reported in *E. faecium* isolates (26, 132, 159). The *esp* gene is contained on a pathogenicity island. The variant

esp gene was significantly enriched among epidemic vancomycin and/or ampicillin-resistant *E. faecium* (AREF) isolates (16, 159). The *esp* gene of *E. faecium* is strongly related with hospital-derived isolates belonging to the CC17 (16, 25, 26). Esp supports to form biofilm on abiotic surfaces and plays a role in colonization and persistence of *E. faecalis* in an animal model of ascending urinary tract infection (UTI) (131, 144).

(3) Collagen binding adhesin from *E. faecalis* (Ace) / *E. faecium* (Acm)

Enterococcal colonization onto human tissues is occured via interactions between specific proteins in the extracellular matrix and the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Ace is a collagen-binding MSCRAMM on enterococci (120). Ace is common among both commensal and pathogenic isolates of *E. faecalis* (23). Ace bind to collagen types I and IV, laminin and dentin (76, 103, 120). Furthermore, Ace was shown to be involved in the pathogenesis of experimental endocarditis (135).

Acm, an ace homolog, was recently identified in *E. faecium* (105). Acm binds to collagen type I and to a lesser extent to collagen type IV, which helps occurring experimental endocarditis and being antigenic in humans during endocarditis (104, 105). A functional *acm* gene is predominantly present in clinical isolates, while among non-clinical isolates, a pseudogene is mainly present (104).

(4) *E. faecalis* endocarditis antigen

E. faecalis endocarditis antigen ($EfaA_{fs}$) is a dominant 37 kDa antigen of *E. faecalis* recognized by sera from patients with infective endocarditis (1). It was different with the antigens detected from endocarditis due to other bacteria and from other *E. faecalis* infections such as UTIs (134). By a mouse model of peritoneal infection, $EfaA_{fs}$ was proved as a virulence factor (136).

IV. Transmission of enterococci among humans and animals

1. Transmission between humans and food animals

VRE strains having similar fingerprints have been isolated from humans and food animals (38, 48, 91, 149) Moreover, after prohibition of the use of avoparcin for feed additives in Europe, the decrease of VRE in food animals followed by the decrease in the prevalence of human colonization with VRE (72, 109). However, most of clinical isolates were still clustered in different groups with food animals (145, 160). VRE have been shown high recovery rates among chickens but their *Tn*1546-like elements were distinct with those of humans. Furthermore, most of the isolates showed apparent phenotypic differences in AMRs. Thus, it was suggested that there exists the possibility of VRE contamination during the processing of chicken meat or development of poultry VRE independently (64, 85, 137). As results of PhenePlate typing system based on measurements of the kinetics of 11 biochemical reactions, it also seems that VRE of humans and animals in Europe have evolved independently (77). Iversen *et al*. suggested that circulation of drug-resistant enterococci via hospital sewage and urban sewage would be a possible source of nosocomial enterococci and AMR could be a further amplified by antimicrobial usage in human medicine. Whereas it was not supposed that the transmission of eneterococci of food animals played an important role in Sweden. Overall, it was guessed that food animal VRE strains were influenced by the former use of avoparcin for growth promotors while VRE strains of humans might be developed by the result of antimicrobial use in hospitals.

Among the studies about CC17, a global epidemic hospital-adapted strain, the first study applying MLST method for typing of enterococci showed that human outbreak isolates in hospitals belonged to CC17, but most of *E. faecium* isolated from food animals clustered to other CCs (160). Some of VRE of animals belonged to CC17, which could mean a dissemination of this hospital adapted clonal lineage among food animals. However, these CC17 isolates from food animals showed ampicillin and ciprofloxacin resistance, but did not contain the *esp* virulence gene usually (85).

From the studies described above, there are possibilities that the presence of VRE among food animals affects public health, but the transmission of enterococci of food animals to humans has not been still determined.

2. Transmission between humans and companion animals

Although various surveillance studies on AMR bacteria in animals have been carried out worldwide, there are few studies include AMR bacteria of companion animals. Monitoring of AMR in dogs in Denmark, AREF CC17 isolates from dogs were reported first by Damborg *et al*. (17). Subsequently, Damborg *et al*. (18) investigated ampicillin resistant enterococci of dogs and their owners for six-months. An owner and his dog carried the same ST, ST78. However, *esp* and *hyl* were not detected. The results indicate that dogs are frequent carriers of CC17-related lineages and may play a role in the spread of this nosocomial pathogen but the difference in virulence and antimicrobial resistance profiles between humans and animals need further studies about the origin and evolution of the human and animal strains.

Fecal enterococci of dogs with administration of antimicrobial therapeutics in the veterinary intensive care unit (ICU) were investigated. Fingerprint patterns of *E. faecium* strains were not diverse suggesting their nosocomial origin. STs of some dog strains were same or closely related to STs of human clinical isolates. It was notable that enterococci of dogs with intensive antimicrobial treatment might have differed from enterococcal population of the normal healthy dogs.

Population living with companion animals has rapidly increased. Thus, there has been a concern that companion animals can transmit AMR bacteria to humans. Characterization and determination of the transmission of enterococci of companion animals would be needed.

General Introduction

Enterococcus spp. is a Gram-positive, facultative anaerobic and oval shaped organism. It is ubiquitous among humans, animals, and environment. Usually, they are commensal bacteria colonizing the skin, mucosal membranes, and the gastrointestinal tract of humans and animals. Meanwhile, enterococci are able to cause nosocomial infections including bacteremia, peritonitis, endocarditis, and device-related infections. High rates of AMRs and some virulence factors contribute enterococci to be the major opportunistic infectious agents.

A notable example is *E. faecium* CC17, one of the global epidemic clones of AREF which is receiving attention as a hospital adapted strain. Almost all CC17 has ampicillin and ciprofloxacin resistance and can acquire vancomycin resistance genes and other virulence genes.

It has been suspected that AMR and virulent enterococci can be transmitted between humans and animals via consumption of food animal products or through direct contact with companion animals. Despite their importance, few studies have been conducted to compare the enterococcal strains between animal and human. Therefore, in Chapter I, the phenotypes and genotypes of AMR as well as virulence gene profiles were investigated in enterococcal strains isolated from chickens, pigs, companion animals (dogs and a cat), and human patients in Korea. Food animals fed with antimicrobial additives and companion animals administrated similar drugs with humans might have common AMR and virulent strains with humans, such as CC17. Thus, the AREF strains were selected, and multilocus sequence typing (MLST) was performed to investigate the dispersion of CC17 among animals and humans.

In Chapter II, the further investigation about characteristic results of companion animals from Chapter I was carried out. It is possible that epidemic enterococcal strains of humans such as CC17 are distributed among companion animals associated with

antimicrobial administration or those strains are transmitted between humans and companion animals because of frequent contact. Therefore, the infectious and the colonizing *E. faecium* strains isolated respectively from dogs with or without enterococcal infections were characterized. Then, we compared them to infectious strains isolated from humans having enterococcal infections to analyze the relationship between human and canine *E. faecium* strains.

In Chapter III, the transmission of AMR and virulent *E. faecalis* of pigs such as strains appeared in food animals in Chapter I via the food processing chain was determined. It has been concerned that pathogenic enterococci can be disseminated from animals to humans through eating foods. AMR and virulent enterococci contaminating food products can be important risk factors for public health. However, there are few studies investigating whether those enterococci can be actually reach human along the processing chain from the slaughterhouse to the retail shop or not. In this study, *E. faecalis* were isolated from each steps of the pork meat processing chain and their AMR and virulence were characterized. The genetic similarity between the isolates from different steps and between isolates from the pork meat processing chain and human patients were investigated for detecting the persistent strains over processing chain and investigating the transmission between animal and human via pork meat processing chain.

Through investigation of the prevalence and characters of enterococci from human and animals, comparing companion animal and pork meat processing chain isolates with human isolates, respectively, it was determined that the transmission of enterococci with AMR and virulence factors among human and animal could occur opportunistically.

Chapter I.

Occurrence of antimicrobial resistance and virulence genes, and distribution of enterococcal clonal complex 17 from animals and humans in Korea

I. Introduction

Enterococci colonize the skin, mucosal membranes, and the gastrointestinal tract of humans and animals (140). Furthermore, these bacteria cause a wide range of nosocomial infections including bacteremia, peritonitis, endocarditis, and device-related infections in humans (126). The high rates of AMR make it difficult to treat these infections (11). Enterococci can easily acquire AMR (126). VRE have threatened human health, because vancomycin is a common therapeutic antimicrobial used for severe enterococcal infections (12). HLAR enterococci make the choice of therapeutics significantly narrower for treatment, because aminoglycosides and β-lactams are generally used for treating serious enterococcal infections (164). Several virulence factors of enterococci have been reported, including AS, gelatinase, cytolysin, Esp, Ace, and $EfaA_{fs}$ of *E. faecalis* and Esp and hyaluronidase of *E. faecium* (70, 86). Expression of these factors is related with attachment, biofilm formation, invasion into the host, evasion from killing by neutrophils, and secretion of toxins that damage host defense systems (103, 108, 118).

E. faecium CC17, one of the global epidemic clones of AREF, is receiving attention as a hospital adapted strain. CC17 has ampicillin and ciprofloxacin resistance and can acquire vancomycin resistance genes and other virulence genes such as *esp* and *hyl* genes (147, 158). Because the strong linkage between AREF and CC17 has been widely accepted (79), monitoring AREF is as important as detecting CC17.

Antimicrobial resistant and virulent enterococci can be transmitted between humans and animals via consumption of food animal products or through direct contact with companion animals (37, 59, 75). Despite their importance, few studies have been conducted to screen the distribution of CC17 in animals and compare them with human isolates. In the present study, the phenotypes and genotypes of AMR as well as virulence gene profiles were investigated in enterococcal strains isolated from chickens, pigs, companion animals, and human patients in Korea. Then, the AREF strains were

selected, and MLST was performed to investigate the dispersion of CC17 among animals and humans.

II. Materials and methods

1. Bacterial isolation and identification

In total, 184 enterococcal isolates were collected. Forty-nine isolates were collected from chicken carcasses, and 52 isolates were collected from slaughtered pigs. Companion animal-originating isolates (44 isolates) were collected from swab samples of skin and rectum of 43 dogs and one cat that visited six different veterinary clinics due to non enterococci-associated diseases. Human isolates were provided by the Asian Bacterial Bank of the Asia Pacific Foundation for Infectious Diseases. The 39 human isolates were recovered from blood, peritoneal fluid, bile, and dialysate samples of enterococci-infected patients. All samples were collected between 2008 and 2010. All isolates were confirmed as enterococci by the polymerase chain reaction (PCR) and their species were determined to *E. faecalis* or *E. faecium* by species specific-PCR or to other enterococcal species by 16s rRNA sequencing (39, 58, 71). The primers used in this study are shown in Table I-1 (7, 13, 39, 52, 58, 71, 89, 115, 151).

2. Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed for six antibiotics (vancomycin, erythromycin, tetracycline, chloramphenicol, ampicillin, and ciprofloxacin) by the disk diffusion method (15). The agar dilution method was performed according to the Clinical and Laboratory Standards Institute (CLSI) guideline to determine HLGR and HLSR (15). *Staphylococcus aureus* ATCC 25923 and *E. faecalis* ATCC 29212 were used as reference strains of disk diffusion and MIC test, respectively. A MDR isolate was defined as an isolate that was resistant to a minimum of three antibiotic categories (87).

3. Detecting AMR and virulence genes

The seven AMR associated genes and seven virulence associated genes were investigated by PCR using primers (Table I-1) from previous studies (7, 13, 52, 89, 115, 151), including vancomycin resistance (*vanA* and *vanB*), erythromycin resistance (*ermB*), tetracycline resistance (*tetM* and *tetL*), chloramphenicol resistance (*cat*), HLGR (*aac(6')-Ie-aph(2")-Ia*) and HLSR (*ant(6)-Ia*), AS (*asa1*), gelatinase (*gelE*), cytolysin (*cylA*), Esp (*esp*), hyaluronidase (*hyl*), Ace (*ace*), and $EfaA_{fs}$ ($efaA_{fs}$).

4. MLST

The twenty-four AREF isolates detected in this study (three isolates from companion animals and 21 isolates from human) were subjected to MLST as previously described (50). Briefly, seven housekeeping genes (*atpA, ddl, gdh, purK, gyd, pstS,* and *adk*) were amplified and sequenced. Allele analysis and sequence typing were performed at http://efaecium.mlst.net/.

5. Statistical analysis

Statistical analysis was conducted with the chi-square test using SPSS program (version 12; SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered significant.

TABLE I-1. Primers used in this study

Primers		Sequence	Product size (bp)	Reference
Enterococcus spp.	FW	5'-TACTGACAAACCATTCATGATG-3'	112	(71)
	RV	5'-AACTTCGTCACCAACGCGAAC-3'		
E. faecalis	FW	5'-ACTTATGTGACTAACTTAACC-3'	360	(58)
	RV	5'-TAATGGTGAATCTTGGTTTGG-3'		
E. faecium	FW	5'-GAAAAAACAATAGAAGAATTAT-3'	215	(58)
	RV	5'-TGCTTTTTTGAATTCTTCTTTA-3'		
vanA	FW	5'-CATGAATAGAATAAAAGTTGCAATA-3'	1,030	(13)
	RV	5'-CCCCTTTAACGCTAATACGATCAA-3'		
vanB	FW	5'-GTGACAAACCGGAGGCGAGGA-3'	433	(13)
	RV	5'-CCGCCATCCTCCTGCAAAAAA-3'		
ermB	FW	5'-TGGTATTCCAAATGCGTAATG-3'	745	(89)
	RV	5'-CTGTGGTATGGCGGGTAAGT-3'		
tetM	FW	5'-GTGGACAAAGGTACAACGAG-3'	406	(89)
	RV	5'-CGGTAAAGTTCGTCACACAC-3'		
tetL	FW	5'-TGGTGGAATGATAGCCCATT-3'	229	(89)
	RV	5'-CAGGAATGACAGCACGCTAA-3'		
cat	FW	5′-ATGACTTTTAATATTATTRAWTT-3′	648	(52)
	RV	5'-TCATYTACMYTATSAATTATAT-3′		
aac(6')-Ie-aph(2'')-Ia	FW	5'-CCAAGAGCAATAAGGGCATA-3'	220	(115)
	RV	5'-CACTATCATACCACTACCG-3'		
ant(6)-Ia	FW	5'-ACTGGCTTAATCAATTTGGG-3'	597	(115)
	RV	5'-GCCTTTCCGCCACCTCACCG-3'		
asa1	FW	5'-GCACGCTATTACGAACTATGA-3'	375	(151)
	RV	5'-TAAGAAAGAACATCACCACGA-3'		
gelE	FW	5'-TATGACAATGCTTTTTGGGAT-3'	213	(151)
	RV	5'-AGATGCACCCGAAATAATATA-3'		
cylA	FW	5'-ACTCGGGGATTGATAGGC-3'	688	(151)
	RV	5'-GCTGCTAAAGCTGCGCTT-3'		
esp	FW	5'-AGATTTCATCTTTGATTCTTGG-3'	510	(151)
	RV	5'-AATTGATTCTTTAGCATCTGG-3'		
hyl	FW	5'-ACAGAAGAGCTGCAGGAAATG-3'	276	(151)
	RV	5'-GACTGACGTCCAAGTTTCCAA-3'		
ace	FW	5'-GGAATGACCGAGAACGATGGC-3′	616	(7)
	RV	5'-GCTTGATGTTGGCCTGCTTCCG-3′		
efaAfs	FW	5'-CGTGAGAAAGAAATGGAGGA-3′	499	(7)
	RV	5'-CTACTAACACGTCACGAATG-3′		
16s rDNA universal primer	27F	5'- AGAGTTTGATCMTGGCTCAG-3'		(39)
	1492R	5'- TACGGYTACCTTGTTACGACTT-3'		

III. Results

1. *Enterococcus* spp. detected from animals and humans

Enterococcal species were identified, and the results are summarized in Table I-2. *E. faecalis* and *E. faecium* were detected in all samples. Other than these two major species, the other six minor species (*E. hirae, E. gilvus, E. avium, E. canintestini, E. saccharolyticus* and *E. pseudoavium*) were detected in companion animals (four species only), pigs (three species only) and humans (one species only).

TABLE I-2. *Enterococcus* spp. isolated from chickens, pigs, companion animals, and hospitalized humans

Species	No. of isolates (%) from indicated host				
	Chicken	Pig	Companion animal	Human	Total
E. faecalis	42 (85.7)	41 (78.8)	31 (70.5)	15 (38.5)	129 (70.1)
E. faecium	7 (14.3)	3 (5.8)	5 (11.4)	21 (53.8)	36 (19.6)
E. hirae	-*	1 (1.9)	5 (11.4)	-	6 (3.3)
E. gilvus	-	6 (11.5)	-	-	6 (3.3)
E. avium	-	-	1 (2.3)	3 (7.7)	4 (2.2)
E. canintestini	-	-	1 (2.3)	-	1 (0.5)
E. saccharolyticus	-	-	1 (2.3)	-	1 (0.5)
E. pseudoavium	-	1 (1.9)	-	-	1 (0.5)
Total number of isolates	49	52	44	39	184

*-; not detected

2. AMR and associated genes

The phenotypes and genotypes of the antimicrobial resistant enterococcal isolates are shown in Tables I-3 and I-4. Vancomycin resistance only occurred in six human *E. faecium* isolates (15.4% among all the human isolates), and the *vanA* genes were

detected in these isolates. No vancomycin resistant or *vanA* gene possessing strains were isolated from animals. The *vanB* gene was not detected in this study.

Erythromycin and tetracycline resistance detection rates were generally high among all sources. The chloramphenicol resistance rate was higher in companion animals than that from the other sources ($P < 0.05$). The HLGR rates of *E. faecium* from companion animal and human strains were higher than that from food animals ($P < 0.05$).

The ampicillin resistance rate was 56.4% in humans (22/39 isolates) and 11.4% in companion animals (5/44 isolates), whereas it was not detected in chicken or pig isolates. Among *E. faecium* isolates, 24 AREF isolates were detected in companion animals (3/5 isolates, 60%) and humans (21/21 isolates, 100%). Ciprofloxacin resistance was detected in human isolates (66.7%), companion animal isolates (31.8%), and chicken isolates (10.2%), but not from pig isolates. Interestingly, all of the AREF isolates from companion animals and humans were resistant to ciprofloxacin.

3. MDR enterococci

The enterococci MDR rates are summarized in Table I-3. The MDR rates for the *E. faecalis* and *E. faecium* strains in companion animals (71.0% and 80.0%) and humans (73.3% and 100%) were higher than those in chicken (38.1% and 28.6%) and pig isolates (53.7% and 0%) ($P < 0.05$). In agreement with the MDR phenotype results, the number of isolates possessing multiple AMR genes was higher in companion animals and humans than that in the chicken and pig ($P < 0.05$, data not shown). The most prevalent *E. faecalis* AMR gene patterns from companion animals and humans were *ermB/tetM/aac(6')-Ie-aph(2")-Ia/ant(6)-Ia/cat* (25.8%) and *ermB/tetM/aac(6')-Ie-aph(2")-Ia/ant(6)-Ia* (33.3%). These two patterns were similar except for the *cat* gene. The sole *E. saccharolyticus* isolate, obtained from a companion animal, was resistant to all the antibiotics tested except vancomycin and also had five AMR genes.

TABLE I-3. Antimicrobial resistance (AMR) rates of the enterococcal isolates

Origin	Species	AMR rate (%)*								Enterococcal isolates showing MDR (%)
		VA	E	TE	C	HLG	HLS	AM	CIP	
Chicken	*E. faecalis* (n=42)	0	73.8	88.1	9.5	23.8	14.3	0	9.5	38.1
	E. faecium (n=7)	0	71.4	85.7	14.3	14.3	0	0	14.3	28.6
	Total (n=49)	0	73.5	87.8	10.2	22.4	12.2	0	10.2	36.7
Pig	*E. faecalis* (n=41)	0	48.8	82.9	36.6	39	48.8	0	0	53.7
	E. faecium (n=3)	0	66.7	0	0	0	0	0	0	0
	E. hirae (n=1)	0	100	100	0	0	100	0	0	100
	E. gilvus (n=6)	0	0	66.7	0	0	0	0	0	0
	E. pseudoavium (n=1)	0	0	0	0	0	0	0	0	0
	Total (n=52)	0	44.2	75	28.8	30.8	40.4	0	0	44.2
Companion animal	*E. faecalis* (n=31)	0	71	93.5	64.5	41.9	35.5	0	25.8	71
	E. faecium (n=5)	0	80	80	0	80	40	60	80	80
	E. hirae (n=5)	0	20	80	20	0	0	0	20	20
	E. avium (n=1)	0	100	100	100	0	100	100	0	100
	E. canintestini (n=1)	0	0	0	0	0	0	0	0	0
	E. saccharolyticus (n=1)	0	100	100	100	100	100	100	100	100
	Total (n=44)	0	65.9	88.6	52.3	40.9	34.1	11.4	31.8	65.9
Human	*E. faecalis* (n=15)	0	66.7	73.3	13.3	66.7	20	0	26.7	73.3
	E. faecium (n=21)	28.6	95.2	33.3	0	81	14.3	100	100	100
	E. avium (n=3)	0	66.7	66.7	0	66.7	66.7	33.3	33.3	66.7
	Total (n=39)	15.4	82.1	51.3	5.1	74.4	20.5	56.4	66.7	87.2

* AMR, antimicrobial resistance; VA, vancomycin; E, erythromycin; TE, tetracycline; C, chloramphenicol; HLG, high-level gentamicin; HLS, high-level streptomycin; AM, ampicillin; CIP, ciprofloxacin; MDR, multiple drug resistance

TABLE I-4. Detection of antimicrobial resistance (AMR) genes among enterococcal isolates

Origin	Species	AMR gene positive isolates (%)*						
		vanA	*ermB*	*tetM*	*tetL*	*cat*	*Amg1*	*Amg2*
Chicken	*E. faecalis* (n=42)	0.0	73.8	47.6	64.3	2.4	23.8	31.0
	E. faecium (n=7)	0.0	28.6	0.0	57.1	0.0	0.0	0.0
	Total (n=49)	0.0	67.3	40.8	63.2	2.0	20.4	26.5
Pig	*E. faecalis* (n=41)	0.0	43.2	47.7	43.2	13.6	47.7	54.5
	E. faecium (n=3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	E. hirae (n=1)	0.0	100.0	100.0	0.0	0.0	0.0	0.0
	E. gilvus (n=6)	0.0	0.0	16.7	0.0	0.0	0.0	0.0
	E. pseudoavium (n=1)	0.0	0.0	100.0	0.0	0.0	0.0	0.0
	Total (n=52)	0.0	38.5	48.1	36.5	11.5	40.4	46.2
Companion animal	*E. faecalis* (n=31)	0.0	64.5	77.4	29.0	51.6	64.5	54.8
	E. faecium (n=5)	0.0	60.0	80.0	80.0	0.0	80.0	40.0
	E. hirae (n=5)	0.0	20.0	80.0	0.0	0.0	0.0	0.0
	E. avium (n=1)	0.0	100.0	100.0	0.0	0.0	0.0	100.0
	E. canintestini (n=1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	E. saccharolyticus (n=1)	0.0	100.0	100.0	100.0	0.0	100.0	100.0
	Total (n=44)	0.0	59.1	77.3	31.8	36.4	56.8	56.8
Human	*E. faecalis* (n=15)	0.0	66.7	60.0	13.3	6.7	66.7	40.0
	E. faecium (n=21)	28.6	81.0	33.3	28.6	4.8	90.5	28.6
	E. avium (n=3)	0.0	66.7	66.7	0.0	0.0	66.7	66.7
	Total (n=39)	15.4	74.4	46.2	20.5	5.1	79.5	35.9

* AMR, antimicrobial resistance; Amg1, *aac(6')-Ie-aph(2")-Ia*; Amg2, *ant(6)-Ia*

4. Detection of virulence genes

The detection rates of virulence genes are shown in Table I-5. Generally, most of the virulence genes, except the *hyl* gene, appeared in *E. faecalis.* Among *E. faecium* strains, only human isolates were associated with the *esp* (76.2%) and *hyl* (66.7%) virulence genes. In other species, *E. avium* strains isolated from companion animals (1/1 isolate) and humans (2/3 isolates) had *esp*. The *E. saccharolyticus* strain isolated from companion animals (n = 1) had *hyl*. *E. hirae*, *E. gilvus*, and *E. pseudoavium*, but *E. canintestini* did not have any virulence genes.

5. MLST

Among the three AREF isolated from companion animals (dogs), sequence type (ST) 202 (n = 1), ST590 (n = 1), and ST591 (n = 1) were detected, with ST202 being part of CC17. Twenty-one human AREF isolates represented six STs, ST78 (n = 9, 42.9%), ST192 (n = 7, 33.3%), ST17 (n = 2, 9.5%), ST18 (n = 1, 4.8%), ST323 (n = 1, 4.8%), and ST631 (n = 1, 4.8%). All the STs identified in human-sourced AREF belonged to CC17.

TABLE I-5. Incidence of virulence genes among enterococcal isolates

Origin	Species	Virulence gene positive isolates %						
		asa1	*gelE*	*cylA*	*esp*	*hyl*	*ace*	$efaA_{fs}$
Chicken	*E. faecalis* (n=42)	78.6	100.0	11.9	0.0	0.0	71.4	100.0
	Total (n=49)	67.3	85.7	10.2	0.0	0.0	61.2	85.7
Pig	*E. faecalis* (n=41)	63.6	75.0	50.0	40.9	0.0	81.8	93.2
	Total (n=52)	53.8	63.5	42.3	34.6	0.0	69.2	78.8
Companion animal	*E. faecalis* (n=31)	74.2	51.6	58.1	61.3	0.0	77.4	96.8
	E. avium (n=1)	0.0	0.0	0.0	100.0	0.0	0.0	0.0
	E. saccharolyticus (n=1)	0.0	0.0	0.0	0.0	100.0	0.0	0.0
	Total (n=44)	52.3	36.4	40.9	45.5	2.3	54.5	68.2
Human	*E. faecalis* (n=15)	66.7	93.3	53.3	66.7	0.0	86.7	100.0
	E. faecium (n=21)	0.0	0.0	0.0	76.2	66.7	0.0	0.0
	E. avium (n=3)	0.0	0.0	0.0	66.7	0.0	0.0	0.0
	Total (n=39)	25.6	35.9	20.5	71.8	35.9	33.3	38.5

* Some enterococcal species isolated from each origin did not have any virulence gene (*E. faecium* isolates from chicken; *E. faecium*, *E. hirae*, *E. gilvus*, *E. psuedoavium* isolates from pig; *E. faecium*, *E. hirae*, *E. canintestini* isolates from companion animals).

IV. Discussion

E. faecalis and *E. faecium* were detected more than other species, except in pigs in which *E. faecium* was the third most detected species after *E. gilvus*. *E. faecalis* is the major cause of enterococcal infection in humans, and *E. faecium,* such as CC17, has also emerged as an epidemic pathogen (146, 148). *E. faecalis* is the most frequently isolated species in animals as well (56, 59, 75). In the current study, the human isolates were collected from enterococcal-associated infections while the animal isolates might include normal flora isolates. Indeed, diverse species, an expected finding with normal flora, were identified from pigs and companion animals, while only *E. faecalis* and *E. faecium* were detected in chickens. This difference in the species diversity may have been influenced by the isolation and identification methodology, the age of the animals, feed, and geographical differences (56, 75).

VRE harboring *vanA* were only detected in human isolates (15.4%), which was consistent with studies in other countries, including Japan, Italy, and Portugal where no VRE has been reported among animal enterococcal isolates (75, 90, 114). VRE detection rates have gradually decreased among food animal isolates after a ban on avoparcin as a feed additive In Korea, the use of avoparcin in feed was banned in 1998 (83). In 2002, the incidence of VRE was reported as 16.7% in chickens and 1.9% in pigs (83). In 2003, 7.7% of enterococci from Korean poultry were VRE (65). As twelve years after banning avoparcin, VRE were not detected among animal isolates, it appears that the glycopeptide prohibition in food animals might be effective in Korea. The absence of VRE detection among companion animal isolates could be related to the rare use of glycopeptides in companion animal medicine (156). In contrast, VRE have been steadily isolated from hospitalized human patients at rates of 5.7–32% (11, 81, 165), and were also detected in this study. It appears that a decrease in VRE among animal enterococci might not immediately contribute to a decrease in humans.

Erythromycin and tetracycline resistance rates were generally high in all sources, presumably reflecting the use of erythromycin and tetracycline in human and animal

therapy. The incidence of chloramphenicol resistance was high only among companion animals. This may have been caused by the chloramphenicol ban in food animals in 1991 in Korea and the observation that it is used only for limited purposes in human medicine (78) and for treating companion animals and horses. The HLGR rates of *E. faecium* were high in companion animals and humans but not in food animals ($P < 0.05$). This similarity between isolates from companion animals and humans might reflect the similar therapeutic antimicrobial use in human and companion animal medicine. Taken together, these results suggest that antimicrobials should be used more prudentially in companion animals as well as humans.

Penicillin and ampicillin are important antimicrobials for enterococcal infections. In particular, the combination of β-lactams and aminoglycosides leads to synergistic bactericidal effects (164). We did not detect any ampicillin-resistant isolates in food animals. However, companion animal and human isolates showed 11.4% and 56.4% ampicillin resistance rates, respectively. Ciprofloxacin resistance rates among companion animals and humans were also higher than those of food animals ($P < 0.05$). Although there was no detection of ampicillin-resistant isolates from food animals in this study, there has been a report of CC17 of food animals elsewhere. Continuous screening of ampicillin-resistant enterococci and major human adapted strains, such as CC17, is also important in Korea.

MDR rates of companion animal and human isolates were higher than those of food animals ($P < 0.05$). Moreover, the most prevalent AMR gene patterns of the isolates were similar between companion animals and humans except the *cat* gene, suggesting that MDR is associated with intensive antimicrobial therapy in human and companion animal medicine. MDR enterococci can be a serious problem not only in human hospitals but also in veterinary clinics, causing an increasing therapy failure rate.

An *E. saccharolyticus* isolate from a companion animal sample was resistant to most of the antibiotics tested except vancomycin, and it harbored five resistance genes. We presume that minor enterococcal species such as this *E. saccharolyticus* isolate

might be active reservoirs for AMR genes. More screening and investigations should be performed targeting minor enterococcal species as a pool of AMR genes.

Among virulence factors, almost all genes except the *hyl* gene were detected from *E. faecalis* from all four sources. Interestingly, *E. faecalis* isolates with more than four virulence genes were detected in humans and animals at similar rates, although the animal samples probably included normal flora. The abundant number of virulence genes in *E. faecalis* might be needed for colonization and infection of the species in animal and human. In contrast, the *esp* and *hyl* genes among the *E. faecium*, *E. avium*, and *E. saccharolyticus* strains were only detected from companion animals and human isolates. The *esp* and *hyl* genes are linked with ampicillin and ciprofloxacin resistant enterococci, particularly in CC17 (147). Indeed, 12 of 21 human CC17 *E. faecium* isolates in this study had the *esp* and *hyl* genes (data not shown). Those two virulence factors seem to be related with the adaptation of *E. faecium* and other species to human and companion animal hospital settings.

MLST was performed with 21 human and 3 companion animal AREF strains to detect the presence of isolates belonging to CC17. All human isolates and one companion animal isolate were classified as CC17. The CC17 isolate from a canine patient may have been transmitted from a human, reflecting frequent contact between animal and human. Alternatively, it is also possible that CC17 is adapting to veterinary hospital settings independently. Larger scale surveillance to search for CC17 among companion animals and studies comparing human isolates are required.

Fortunately, no VRE was detected in food and companion animal isolates in this study. *E. faecium* isolate belonging to CC17 was detected in one companion animal and 21 humans. As companion animals come in frequent contact with humans and usually receive intensive drug therapy individually for disease treatment like humans, more attention is required on the use of antibiotics in companion animal clinics as well as human hospitals. The epidemiological relationships among enterococcal isolates from food animals, companion animals, and human patients should be further studied in-depth.

Chapter II.

Detection of CC17 *E. faecium* in dogs and a comparison with human isolates

I. Introduction

Enterococci are one of the most prevalent zoonotic pathogens and cause opportunistic bacteremia, endocarditis, UTIs, and surgical wound infections (126). In particular, the *E. faecium* CC17 strain is an epidemic clone associated with human enterococcal infections. The AMRs and virulence factors of CC17 enable this microorganism to adapt to the hospital environment. In most cases, CC17 is resistant to ampicillin and ciprofloxacin. It also possesses virulence genes encoding Esp (*esp*), hyaluronidase (*hyl*), and collagen binding protein (*acm*) which are markers for CC17 (147, 158).

This globally epidemic clone has been extensively studied in human medicine, but only a few case reports are available in dogs (17, 18, 44). Dogs often come in contact not only with their owners but also with other people in the community. Moreover, *E. faecium* isolates from dogs having enterococcal infections could possess common AMR with humans due to the similar drug prescriptions in veterinary clinics, except the few drugs forbidden for use in animals such as vancomycin. In this study, we characterized the infectious and the colonizing *E. faecium* strains isolated respectively from dogs with or without enterococcal infections. Then, we compared them to infectious strains isolated from humans having enterococcal infections to analyze the relationship between human and canine *E. faecium* strains.

II. Materials and methods

1. Sampling

Thirty-eight *E. faecium* strains were isolated from domestic dogs who visited two veterinary teaching hospitals and three local veterinary hospitals in Seoul, Republic of Korea from January 2010 to May 2011. The strains (28 isolates) isolated from enterococcal UTI diagnosed dogs were defined as infectious strains. The other ten isolates were defined as colonizing strains that originated from rectum of dogs having

fractures due to a car accident or the dislocation of patella without an infection. Urine samples were aseptically collected by bladder puncture and fecal samples were swabbed from rectum of dogs.

2. Isolation and identification of *E. faecium*

Urine and fecal samples of dogs were cultured on blood agar (KOMED, Korea) at 37°C for 24 h. Colonies showing enterococci-specific morphology were identified as *E. faecium* by PCR. Genomic DNA was extracted by a DNeasy Blood & Tissue Kit (Qiagen Inc., Mississauga, ON, Canada) according to the manufacturer's instructions. PCR for identification of *E. faecium* were carried according to previous study (58).

Twenty-one human isolates were provided by the Asian Bacterial Bank of the Asia Pacific Foundation for Infectious Diseases. These isolates were recovered from blood, peritoneal fluid, bile, and dialysate samples of enterococci-infected patients who visited a tertiary hospital in the same area as the veterinary hospitals, Seoul, from May 2010 to May 2011.

3. Antimicrobial susceptibility test

Antimicrobial susceptibility tests were carried out for eight antibiotics [vancomycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg), high-level gentamicin (HLG, 120 μg), high-level streptomycin (HLS, 300 μg), ampicillin (10 μg), and ciprofloxacin (5 μg)] using the disk diffusion method according to the CLSI guidelines (15). *S. aureus* ATCC 25923 was used as a reference strain.

4. PCR for detecting virulence genes

Three virulence genes, *esp*, *hyl*, and *acm*, were also identified by PCR amplification according to previous studies (10, 151). The amplified PCR products were resolved by electrophoresis in a 1% agarose gel at 100 V for 30 min.

5. MLST

STs and CCs of all *E. faecium* isolates were determined by MLST as previously described (50). The allele type of *atpA, ddl, gdh, purK, gyd, pstS,* and *adk* genes of each isolate was analyzed and designation of ST and CC was performed at http://efaecium.mlst.net/.

6. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as described previously with some modification (66). Sample plugs were digested with 20 U of *Sma*I (New England Biolabs, Beverly, Mass.), and electrophoresed on a CHEF-Mapper electrophoresis systems (Bio-Rad, USA). Electrophoresis was performed according to following conditions: voltage, 6 V/cm; temperature, 14°C; initial switch, 1 s; final switch, 30 s; run time, 19 h; angle, 120°; molecular weight, 10-500 kb. A Lambda ladder (New England Biolabs) was used as the PFGE size marker. After ethidium bromide staining, the gel was visualized under UV. PFGE patterns were analyzed with BioNumerics software package v.6.0 (Applied Maths, Sint-Martens-Latem, Belgium) using Dice coefficient (optimization, 0.5%; band matching tolerance, 1%) and the unweighted pair group method using arithmetic mean (UPGMA).

7. Statistical analysis

All statistical comparisons were performed using the chi-square test and SPSS program (version 12; SPSS Inc).

III. Results

1. AMR of *E. faecium* isolated from dogs and humans

The rate of AMR of dog and human isolates are shown in Table II-1. The majority of human and domestic dog strains were resistant to ampicillin and ciprofloxacin. All

human isolates (100%) were resistant to ampicillin and ciprofloxacin, and had high HLG (81%) and erythromycin resistant levels (95.2%). Ampicillin and ciprofloxacin resistant levels were also high in domestic dog strains regardless of the infectious (AM, 82.1%; CIP, 92.9%) or colonizing (AM, 70%; CIP, 80%) strains. Chloramphenicol resistance was not shown in any three groups. Human and domestic dog isolates showed different resistance levels to other antimicrobials. For example, vancomycin-resistant *E. faecium* strains were detected only among human isolates (6/21, 28.6%). The tetracycline resistant level in animal strains was 82.1% in infectious strains and 90% in colonizing strains and both were significantly higher than the level in human isolates (33.3%).

Table II-1. Antimicrobial resistance rate of colonizing and infectious *E. faecium* isolates from dogs and human infectious *E. faecium* isolates

	Antimicrobial resistance rate (%)							
	VA	E	TE	C	HLG	HLS	AM	CIP
Dog (col) (n=10)	0.0	80.0	90.0	0.0	80.0	60.0	70.0	80.0
Dog (inf) (n=28)	0.0	64.3	82.1	0.0	35.7	50.0	82.1	92.9
Human (inf) (n=21)	28.6	95.2	33.3	0.0	81.0	14.3	100.0	100.0

* VA, vancomycin; E, erythromycin; TE, tetracycline; C, chloramphenicol; HLG, high-level gentamicin; HLS, high-level streptomycin; AM, ampicillin; CIP, ciprofloxacin; col, colonizing isolates; inf, infectious isolates

2. Virulence gene profiling

Acm genes were detected in all human and canine isolates. *Esp* (76.2%) and *hyl* (66.7%) were prevalent among human infectious isolates. However, among the dog strains, only one infectious and one colonizing isolate carried *esp-hyl-acm* and *hyl-acm* respectively (Figure II-1).

3. MLST

Most of the infectious isolates from humans (20/21) and domestic dogs (24/28) belonged to CC17, and the prevalences were significantly higher ($P < 0.05$) than those of colonizing isolates from domestic dogs (2/10). Moreover, any of the STs of colonizing isolates of dogs did not coincided with those of human isolates. However, infectious isolates of dogs and humans shared some STs (ST323, ST17, ST78) (Table II-2).

4. PFGE

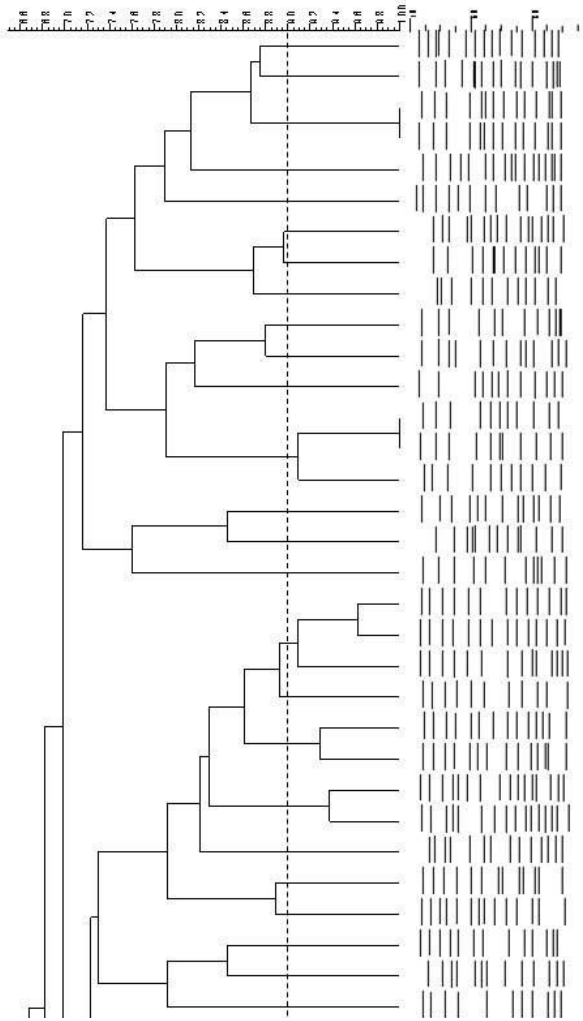
The PFGE results revealed that two clusters of infectious strains, which were isolated from humans and dogs respectively, had over 90% similarity; cluster 1 included the no. 34 (canine) and no. 58 (human) strains and cluster 2 included no. 32 (canine), no. 41 (human), and no. 45 (human) strains (Figure II-1).

Table II-2. The number of *E. faecium* isolates belonged to each sequence type (ST)

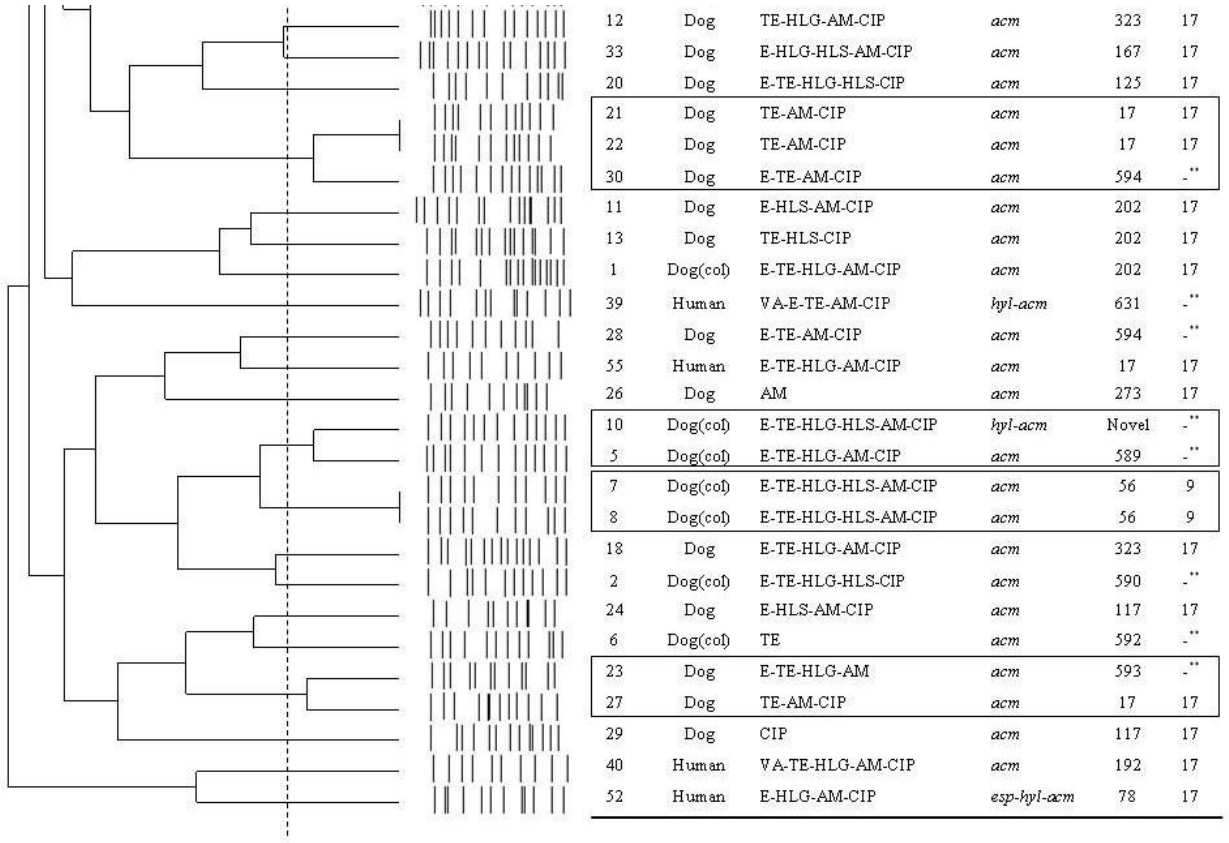
ST	Dog (col)	Dog (inf)	Human (inf)
590	1		
591	1		
588	1		
589	1		
592	1		
56	2		
202*	2	7	
323*		2	1
262*		4	
125*		1	
17*		4	2
117*		3	
78*		1	9
273*		1	
167*		1	
593		1	
594		2	
595		1	
631			1
192*			7
18*			1
Novel	1		
Total	10	28	21

* These STs are included into CC17.

† col, colonizing isolates; inf, infectious isolates



No.	Origins	AMR	Virulence genes	ST	CC
42	Human	VA-E-HLG-AM-CIP	*esp-hyl-acm*	78	17
54	Human	E-HLG-AM-CIP	*esp-hyl-acm*	78	17
48	Human	E-HLG-AM-CIP	*esp-acm*	192	17
49	Human	E-HLG-HLS-AM-CIP	*esp-acm*	192	17
50	Human	E-TE-HLG-HLS-AM-CIP	*esp-acm*	323	17
15	Dog	TE-HLG-AM-CIP	*acm*	262	17
47	Human	E-HLG-HLS-AM-CIP	*esp-acm*	192	17
51	Human	E-AM-CIP	*esp-hyl-acm*	192	17
25	Dog	E-TE-HLS-AM-CIP	*esp-hyl-acm*	78	17
56	Human	E-HLS-AM-CIP	*esp-hyl-acm*	78	17
57	Human	E-TE-HLG-AM-CIP	*esp-hyl-acm*	78	17
44	Human	VA-E-HLG-AM-CIP	*esp-hyl-acm*	78	17
41	**Human**	**VA-E-HLG-AM-CIP**	***esp-hyl-acm***	**192**	**17**
45	**Human**	**E-HLG-AM-CIP**	***esp-hyl-acm***	**78**	**17**
32	**Dog**	**E-HLG-HLS-AM-CIP**	***acm***	**117**	**17**
43	Human	VA-E-AM-CIP	*esp-hyl-acm*	78	17
46	Human	E-HLG-AM-CIP	*esp-hyl-acm*	192	17
59	Human	E-AM-CIP	*esp-hyl-acm*	78	17
37	Dog	E-TE-HLS-AM-CIP	*acm*	262	17
38	Dog	E-TE-HLS-AM-CIP	*acm*	262	17
16	Dog	E-TE-HLG-HLS-CIP	*acm*	202	17
36	Dog	E-TE-HLG-HLS-AM-CIP	*acm*	262	17
34	**Dog**	**E-TE-HLS-AM-CIP**	***acm***	**17**	**17**
58	**Human**	**E-TE-HLG-AM-CIP**	***acm***	**17**	**17**
14	Dog	E-TE-HLG-HLS-AM-CIP	*acm*	202	17
17	Dog	E-TE-AM-CIP	*acm*	202	17
4	Dog(col)	E-TE-HLG-HLS-AM-CIP	*acm*	588	-**
19	Dog	TE-CIP	*acm*	202	17
35	Dog	E-TE-AM-CIP	*acm*	595	-**
53	Human	E-TE-HLG-AM-CIP	*hyl-acm*	78	17
9	Dog(col)	E-TE-HLG-HLS-AM-CIP	*acm*	202	17
31	Dog	E-TE-HLG-HLS-AM-CIP	*acm*	202	17



12	Dog	TE-HLG-AM-CIP	*acm*	323	17
33	Dog	E-HLG-HLS-AM-CIP	*acm*	167	17
20	Dog	E-TE-HLG-HLS-CIP	*acm*	125	17
21	Dog	TE-AM-CIP	*acm*	17	17
22	Dog	TE-AM-CIP	*acm*	17	17
30	Dog	E-TE-AM-CIP	*acm*	594	-**
11	Dog	E-HLS-AM-CIP	*acm*	202	17
13	Dog	TE-HLS-CIP	*acm*	202	17
1	Dog(col)	E-TE-HLG-AM-CIP	*acm*	202	17
39	Human	VA-E-TE-AM-CIP	*hyl-acm*	631	-**
28	Dog	E-TE-AM-CIP	*acm*	594	-**
55	Human	E-TE-HLG-AM-CIP	*acm*	17	17
26	Dog	AM	*acm*	273	17
10	Dog(col)	E-TE-HLG-HLS-AM-CIP	*hyl-acm*	Novel	-**
5	Dog(col)	E-TE-HLG-AM-CIP	*acm*	589	-**
7	Dog(col)	E-TE-HLG-HLS-AM-CIP	*acm*	56	9
8	Dog(col)	E-TE-HLG-HLS-AM-CIP	*acm*	56	9
18	Dog	E-TE-HLG-AM-CIP	*acm*	323	17
2	Dog(col)	E-TE-HLG-HLS-CIP	*acm*	590	-**
24	Dog	E-HLS-AM-CIP	*acm*	117	17
6	Dog(col)	TE	*acm*	592	-**
23	Dog	E-TE-HLG-AM	*acm*	593	-**
27	Dog	TE-AM-CIP	*acm*	17	17
29	Dog	CIP	*acm*	117	17
40	Human	VA-TE-HLG-AM-CIP	*acm*	192	17
52	Human	E-HLG-AM-CIP	*esp-hyl-acm*	78	17

Figure II-1. The results from antimicrobial resistance (AMR) and virulence analysis, MLST, and PFGE of *E. faecium* isolates from humans and dogs. Isolate numbers 1–10, colonizing isolates from dogs (col); 11–38, infectious isolates from

dogs; 39–59, infectious isolates from humans. The PFGE result of No.3 isolate is not shown, because it was not digested by *Sma*I. No.3 isolate had *acm* gene and no AMR, and it belonged to ST591, not to CC17. The 90% similarity is indicated by the dotted line and clusters having over 90% similarity were indicated by boxes.

[*] Abbreviations. Vancomycin, VA; erythromycin, E; tetracycline, TE; chloramphenicol, C; high-level gentamicin, HLG; high-level streptomycin, HLS; ampicillin, AM; ciprofloxacin, CIP; *aac(6')-Ie-aph(2")-Ia, Amg1;ant(6)-Ia, Amg2*

[†] Newly assigned STs. The CC number has not been designated.

IV. Discussion

In the present study, most of the isolates from dogs as well as humans had ampicillin and ciprofloxacin resistances in common. Wide spreading of these resistant isolates among dogs seems to resemble that of the human hospital setting. However, except for those, their resistance patterns were not similar. The resistance to vancomycin which is usually used for treatment of human's infections was not detected among isolates from dogs. On the other hand, the resistance to tetracycline which is widely used in veterinary clinics was highly prevalent among dog isolates. These patterns might reflect different antimicrobial use in human and veterinary medicine.

Acm, a gene encoding a collagen binding protein that plays an important role in colonization, was detected in all human and canine isolates (105). The expression of *acm* in those isolates will be analyzed further because this gene is usually a non-expressed pseudogene among non-clinical isolates (105). Many human infectious strains had *esp* (76.2%) and *hyl* (66.7%) genes and a few dog strains had *esp* and *hyl* genes. These genes are rare in canine strains also in other countries (18, 44). The difference in virulence might be due to host specificity or a reflection of an intermediate evolutionary step of the canine strains adapting to hospital settings by acquiring virulence genes, such as *esp* and *hyl*.

Both infectious isolates from humans and dogs showed the higher prevalence of CC17 than dog's colonizing isolates. Another study also reported isolating CC17 from dogs in the intensive care unit (44). This could indicate that CC17 can adapt well to the hospital environment either for humans or for dogs. ST56, a strain first found in the feces of a non-hospitalized subject, was also detected among the dog colonizing strains (155). Moreover, there were same STs between infectious isolates of dogs and humans while all the STs of colonizing isolates of dogs were different with human's infectious isolates. Association with hospital setting seemed to influence of distribution of *E. faecium* STs.

There were two clusters including both of infectious isolates from humans and dogs based on the PFGE results. Among these isolates, particularly the cluster 1 (no. 34 and 58) strains belonged to ST17, the founder of CC17, and had only one virulence factor, *acm*. They differed only in aminoglycoside resistance; resistance to HLS in the dog strain and resistance to HLG in the human strain. They might start from the similar phylogenetic lilneage, but it is suggested that they acquired different characters suited for their own environment.

In conclusion, we determined that CC17 was the major infectious lineage in dogs as well as in humans and that the ampicillin and ciprofloxacin resistant levels were commonly high. However, some differences were observed in the AMR phenotype, such as resistance to high-level aminoglycosides, and in the distribution of virulence genes, as the *esp* and *hyl* genes were more prevalent in human strains. Our results indicate that CC17 members might possess advantages for infecting both humans and animals, although their antimicrobial resistant phenotypes and virulence might have independently evolved due to different uses of various antimicrobials in human and veterinary medicine or due to host specificity.

Chapter III.

Characterization of *E. faecalis* isolates from the pork meat processing chain and comparison with human clinical isolates

I. Introduction

Enterococci are widely distributed over nature and in the intestinal tract of animals and humans (49, 140) so that enterococci are used as indicators of fecal contamination through food processing chain (34, 40, 45, 142). Meanwhile, enterococci are the leading causes of opportunistic infections including bacteremia, peritonitis, endocarditis and device-related infections. It has been concerned that pathogenic enterococci can be disseminated from animals to humans via foods. Therefore, the AMR and virulence factors of enterococci contaminated food products are important risk factors for public health (11).

Especially, VRE was regarded as a disaster, thus use of antimicrobials including glycopeptides as feed additives was restricted in food animals (5, 56, 150). In Korea, high resistance rates against feed additive antimicrobials were observed in enterococci originated from fecal samples of chickens and pigs at the slaughterhouse (56, 68). However, there are few studies screened enterococci along the processing chain from the slaughterhouse to the retail shop that would be essential information for risk assessment of pork meat processing. In this study, *E. faecalis* were isolated along the pork meat processing chain and their AMR and virulence were characterized. The genetic similarity between the isolates from different steps was investigated for detecting the persistent strains on the meat product and the point of contamination step. Then the genetic comparison of pork meat isolates to human clinical strains was performed for investigating the transmission between animals and human via food processing chain in Korea.

II. Materials and methods

1. Sampling and bacterial isolation

Samples including porcine carcasses, pork meat, and devices (knives, cutting boards, rasps, and gloves) were collected from the five pork meat processing chains (K, B, H, P, and D) including slaughterhouses, processing plants and retail shops near Seoul, the capital of Korea. Each processing chain was visited three times for sampling from March to September, 2010. Twenty-five grams of carcass or meat samples were homogenized with 225 ml buffered-peptone water [Beckton Dickson (BD), Sparks, MD, USA] and then one milliliter of them were inoculated into 9 ml of Enterococcosel broth (BD). After incubation at 37°C overnight, they were streaked onto Enterococcosel agar (BD). Colonies with bile-esculin activity were inoculated to tryptic soy broth (BD) and subcultured on blood agar. Colonies showing enterococci-specific morphology on blood agar were confirmed as *E. faecalis* by PCR (71).

Fifteen human clinical strains isolated from blood and dialysate samples in 2008 were kindly provided by the Asian Bacterial Bank of Asia Pacific Foundation for Infectious Diseases.

2. Antimicrobial susceptibility tests

The disk diffusion susceptibility tests were carried out for determining antimicrobial susceptibility for 11 antimicrobials including chloramphenicol, tetracycline, HLG, HLS, erythromycin, ciprofloxacin, penicillin, ampicillin, vancomycin, teicoplanin, and linezolid according to the CLSI guideline (15). The criterion of MDR was the resistances to minimum 3 antimicrobial classes.

3. Hemolysin and gelatinase activity and biofilm formation assay

Activity of hemolysin was analyzed by culture on 5% fresh horse blood agar for 2 days at 37°C (25). Clear zones around colonies indicated hemolysis. Gelatinase activity was determined by cultured on Todd-Hewitt agar (Oxoid, UK) containing 30 g of gelatin/L

(Duksan, Korea) for 24 h at 37°C. From 4 h before the observation of clear halo around colonies, the plates were cooled at 4°C (25). The biofilm formation ability was evaluated as the method described by Toledo-Arana *et al*. (144). *E. faecalis* isolates were cultured in brain heart infusion (BHI) broth (BD) containing 0.25% glucose (Sigma-Aldrich, USA) at 37°C overnight. One microliter of the culture suspension was inoculated into 200 μl BHI containing 0.25% glucose in sterile 96-well polystyrene microplates (Nalge Nunc Int., Naperville, IL, USA) and incubated at 37°C for 24 h. The plates were washed using phosphate-buffered solution (PBS) and dried inverted. Dried plates were stained with 200 μl 1% crystal violet for 15 min and washed by PBS again. Crystal violet absorbed by enterococcal cell wall was solubilized by adding 200 μl ethanol-acetone (80:20, *v*/*v*). Optical density values (A_{550}) were measured at 550 nm by the microplate reader. Mean A_{550} of blank wells was 0.14 which was used as the background value. Degrees of biofilm formation were divided into four groups: negative, $A_{550} \leq 0.14$; weak, $0.14 < A_{550} \leq 0.3$; moderate, $0.3 < A_{550} \leq 0.45$; strong, $A_{550} > 0.45$.

4. DNA extraction and PCR

For DNA extraction, DNeasy Blood & Tissue Kit (Qiagen Inc.) was used following the manufacturer's instructions. PCR procedures were performed for detecting the eight genes encoding AMR (*vanA*, *vanB, ermB*, *tetM*, *tetL*, *cat*, *aac(6')-Ie-aph(2")-Ia*, and *ant(6)-Ia*) and three virulence associated genes (*gelE*, *cylA*, and *esp*) in accordance with previous studies (7, 13, 52, 89, 115, 151) .

5. Random amplified polymorphic DNA (RAPD)-PCR

RAPD-PCR procedures were performed to investigate the genetic similarities between *E. faecalis* strains from the each step of pork meat processing chain and between pork meat *E. faecalis* strains and human clinical strains. M13 universal primer was used for RAPD-PCR procedures as previously described (122). The fingerprints were analyzed by GelComparII software (version 6.5; Applied Maths BVBA, Belgium) using the UPGMA.

6. Statistical analysis

Statistical significance was determined by a chi-square test using SPSS program (version 12; SPSS Inc). *P*-value of < 0.05 was accepted as statistically significance.

III. Results

1. Isolation of *E. faecalis* from pork meat processing chain

Six-hundreds nineteen samples were gathered from carcasses (n=326) at the slaughterhouses, middle products (n=178) at the processing plants, and final products (n=115) at the retail shop. A total of 339 *E. faecalis* isolates (54.8%) were isolated and the final products at retail shops showed lowest detection rate (32/115, 27.8%) followed by middle products (87/178, 48.9%) and carcasses (220/326, 67.5%).

2. AMR of *E. faecalis* isolates

Tetracycline resistance rate (60.5%) was the highest among all AMR rates regardless of the processing steps (Table III-1). Among the pork meat isolates, slaughterhouses step showed the highest chloramphenicol (21.4%), HLG (17.3%), and erythromycin (28.6%) resistance rates which were significantly higher than those of other steps ($P < 0.05$). On the other hand, penicillin resistance was significantly higher in retails (6.3%) than other steps ($P < 0.05$). MDR was most frequently detected in isolates from slaughterhouse (27.7%) than in those from the processing plants and retail shops (14.9% and 15.6%, respectively, $P < 0.05$). The resistance against vancomycin, teicoplanin, and linezolid were not appeared in all origins.

In human clinical isolates, the tetracycline resistance rate (73.3%) was the highest among all AMR rates as in the pork meat isolates. HLG (66.7%), erythromycin (66.7%), ciprofloxacin (26.7%), penicillin (26.7%) resistance and MDR (66.7%) rates in human

clinical isolates were significantly higher than those in isolates from all pork meat processing steps ($P < 0.05$, respectively).

Among the AMR associated genes (Table III-1), *tet(M)* (50.7%) was the most prevalent in the pork meat isolates. Genes of *ermB*, *tet(L)*, *aac(6')-Ie-aph(2")-Ia* and *ant(6)-Ia* were more frequently detected in slaughterhouse originated isolates than in processing plant and retail ones ($P < 0.05$). Among human clinical isolates, *ermB* (66.7%) and *aac(6')-Ie-aph(2")-Ia* (66.7%) were the most prevalent and the detection rates of these genes were significantly higher than those from all pork meat processing steps ($P < 0.05$). The genes encoding vancomycin resistance were not detected in this study.

3. Virulence of *E. faecalis* isolates

The prevalence of virulences and the related genes are shown in the Table III-2. Hemolysin activities were more detected in isolates from slaughterhouses than in those from other steps ($P < 0.05$) and the highest hemolysin activity was observed from human clinical isolates ($P < 0.05$). The gelatinase activity was not different between the processing steps and between the human and pork meat isolates. The rate of strong or moderate biofilm forming isolates was increased along the processing from slaughterhouse (32.2%), processing plant (49.4%) to retail shop (59.4%) and the most highest in the human isolates (86.7%).

The genes of *gelE*, *cylA*, and *esp* were more frequent among isolates from slaughterhouses (71.8%, 24.5%, and 20.9%, respectively) than from processing plants (49.4%, 4.6%, and 3.4%, respectively, $P < 0.05$) and retail shops (65.6%, 9.4%, and 9.4%, respectively, $P < 0.05$). *CylA* and *esp* were significantly more detected in the human clinical isolates (53.3% and 66.7%, respectively) than in the pork meat isolates (18.0% and 15.3%, respectively, $P < 0.05$).

4. RAPD-PCR fingerprint patterns

In this study, a putative persistent strain was defined when different isolates originated from different processing step and showed > 90% of genetic similarity in the RAPD-PCR result. There were 14 groups consisted of 44 putative persistent strains analyzed (Figure III-1). Thirteen groups included isolates from slaughterhouse and processing plant. Only one group contained isolates from slaughterhouse and retail shop suggested that the clone could be delivered from slaughterhouse to retail. Comparing with human strains, the genetic similarity was not high because no strains originated from human and pork meat processing showed > 90% of similarity.

Table III-1. Antimicrobial resistance (AMR) of *E. faecalis* isolates from the processing chain of pork meat and human patients

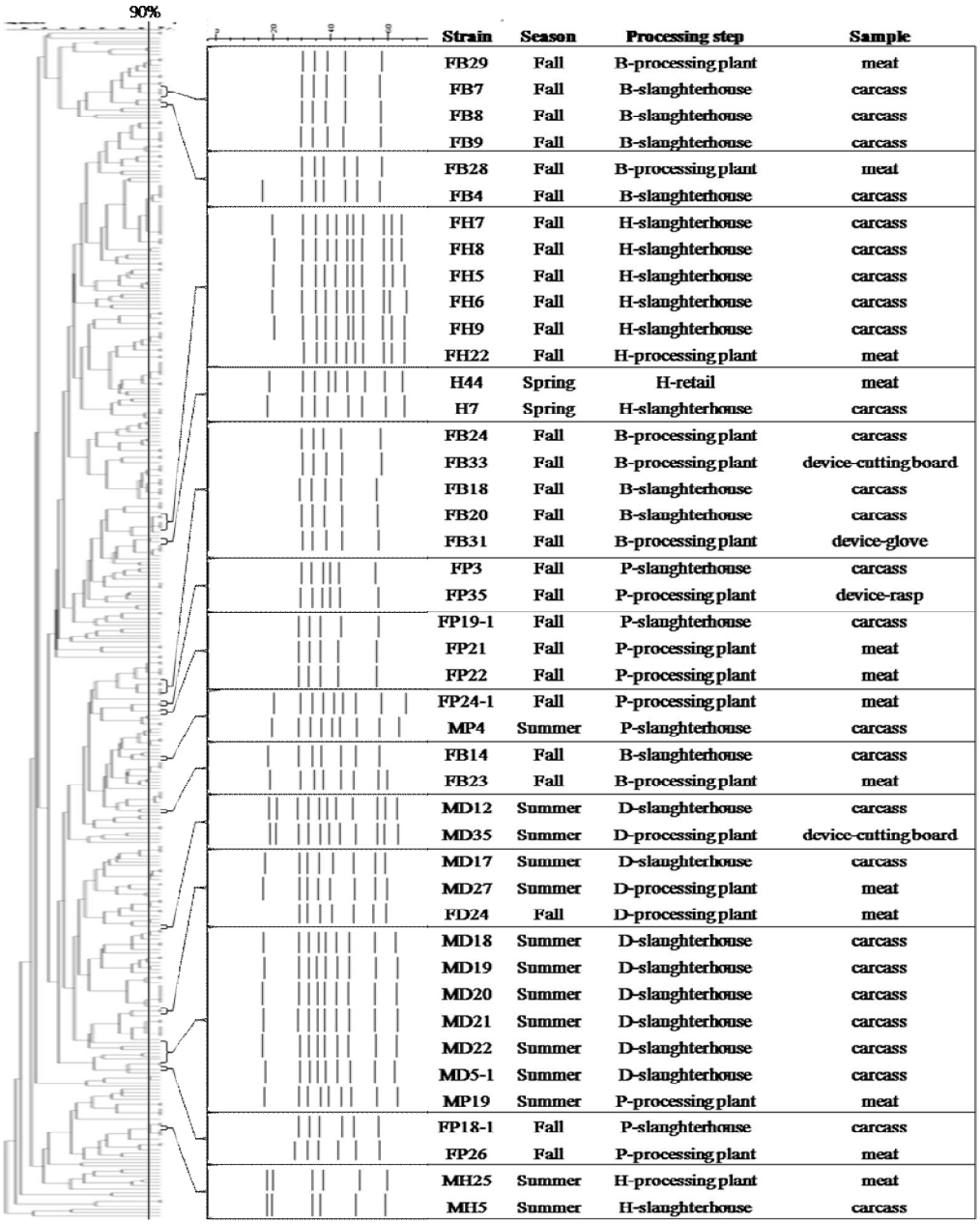
Sources	AMR rate* (%)											
	C	TE	HLG	HLS	E	CIP	P	AM	VA	TEC	LZD	MDR
Slaughter house (n=220)	21.4	61.8	17.3	32.3	28.6	4.1	0.5	0.5	0.0	0.0	0.0	27.7
Processing plant (n=87)	4.6	52.9	3.4	25.3	13.8	4.6	0.0	0.0	0.0	0.0	0.0	14.9
Retail (n=32)	6.3	71.9	9.4	12.5	15.6	6.3	6.3	0.0	0.0	0.0	0.0	15.6
Whole processing chain of pork meat (n=339)	15.6	60.5	13.0	28.6	23.6	4.4	0.9	0.3	0.0	0.0	0.0	23.3
Human patients (n=15)	13.3	73.3	66.7	20.0	66.7	26.7	26.7	0.0	0.0	0.0	0.0	66.7

Sources	Gene detection rate (%)							
	vanA	*vanB*	*ermB*	*tet(M)*	*tet(L)*	*cat*	*aac(6')-Ie-aph(2")-Ia*	*ant(6)-Ia*
Slaughter house (n=220)	0.0	0.0	22.7	50.0	34.1	11.4	22.7	26.8
Processing plant (n=87)	0.0	0.0	5.7	44.8	17.2	4.6	3.4	6.9
Retail (n=32)	0.0	0.0	15.6	71.9	15.6	6.3	9.4	9.4
Whole processing chain of pork meat (n=339)	0.0	0.0	17.7	50.7	28.0	9.1	16.5	20.1
Human patients (n=15)	0.0	0.0	66.7	60.0	13.3	6.7	66.7	40.0

* C, chloramphenicol; TE, tetracycline; HLG, high-level gentamicin; HLS, high-level streptomycin; E, erythromycin; CIP, ciprofloxacin; P, penicillin; AM, ampicillin; VA, vancomycin; TEC, teicoplanin; LZD, linezolid; MDR, multidrug resistance

Table III-2. Virulence of *E. faecalis* isolates from the processing chain of pork meat and human patients

Sources	Virulence phenotype (%)						Gene detection rate (%)		
	Hemolysis	Gelatinase	Degree of biofilm formation				*gelE*	*cylA*	*esp*
			Negative	Weak	Moderate	Strong			
Slaughterhouse (n=220)	10	65.9	12.3	55.5	17.7	14.5	71.8	24.5	20.9
Processing plant (n=87)	2.3	35.6	11.5	39.1	37.9	11.5	49.4	4.6	3.4
Retail (n=32)	6.3	59.4	9.4	31.3	34.4	25.0	65.6	9.4	9.4
Whole processing chain of pork meat (n=339)	7.7	57.5	11.8	49.0	24.5	14.7	65.5	18.0	15.3
Human patients (n=15)	33.3	46.7	0.0	13.3	40.0	46.7	93.3	53.3	66.7



Strain	Season	Processing step	Sample
FB29	Fall	B-processing plant	meat
FB7	Fall	B-slaughterhouse	carcass
FB8	Fall	B-slaughterhouse	carcass
FB9	Fall	B-slaughterhouse	carcass
FB28	Fall	B-processing plant	meat
FB4	Fall	B-slaughterhouse	carcass
FH7	Fall	H-slaughterhouse	carcass
FH8	Fall	H-slaughterhouse	carcass
FH5	Fall	H-slaughterhouse	carcass
FH6	Fall	H-slaughterhouse	carcass
FH9	Fall	H-slaughterhouse	carcass
FH22	Fall	H-processing plant	meat
H44	Spring	H-retail	meat
H7	Spring	H-slaughterhouse	carcass
FB24	Fall	B-processing plant	carcass
FB33	Fall	B-processing plant	device-cutting board
FB18	Fall	B-slaughterhouse	carcass
FB20	Fall	B-slaughterhouse	carcass
FB31	Fall	B-processing plant	device-glove
FP3	Fall	P-slaughterhouse	carcass
FP35	Fall	P-processing plant	device-rasp
FP19-1	Fall	P-slaughterhouse	carcass
FP21	Fall	P-processing plant	meat
FP22	Fall	P-processing plant	meat
FP24-1	Fall	P-processing plant	meat
MP4	Summer	P-slaughterhouse	carcass
FB14	Fall	B-slaughterhouse	carcass
FB23	Fall	B-processing plant	meat
MD12	Summer	D-slaughterhouse	carcass
MD35	Summer	D-processing plant	device-cutting board
MD17	Summer	D-slaughterhouse	carcass
MD27	Summer	D-processing plant	meat
FD24	Fall	D-processing plant	meat
MD18	Summer	D-slaughterhouse	carcass
MD19	Summer	D-slaughterhouse	carcass
MD20	Summer	D-slaughterhouse	carcass
MD21	Summer	D-slaughterhouse	carcass
MD22	Summer	D-slaughterhouse	carcass
MD5-1	Summer	D-slaughterhouse	carcass
MP19	Summer	P-processing plant	meat
FP18-1	Fall	P-slaughterhouse	carcass
FP26	Fall	P-processing plant	meat
MH25	Summer	H-processing plant	meat
MH5	Summer	H-slaughterhouse	carcass

Figure III-1. Isolates showing over 90% similarities on the PCR-RAPD patterns. Persistent strain groups were defined as *E. faecalis* strains collected from the different processing place at the same sampling time. Boxes shown in the figure indicate groups.

IV. Discussion

In this study, the isolation rates of *E. faecalis* decreased along the processing chain from slaughterhouses, processing plants to retail shops. This suggested that contaminated enterococci seemed to decrease as the meat products was undergone the processing by washing and chilling.

Resistances to chloramphenicol, HLG, and erythromycin and MDR rates decreased along the chain. Other resistances did not significantly increase and showed stable rates along the chain. Penicillin and ciprofloxacin resistance were rarely detected in the pork meat isolates while those were frequent among human clinical isolates. These suggested that AMR of enterococci might not be beneficial to come up with meat processing conditions. Vancomycin, teicoplanin, and linezolid resistances and associated genes were not observed in both isolates from the whole pork meat processing chain and human patients, which was consistent with previous data in Korea (81, 130).

Different to other antimicrobials, tetracycline resistance did not decrease along the chain. This was opposite to our expectation because Wu S *et al.*(162) observed that tetracycline resistant *Escherichia coli* isolates survived less than susceptible isolates during carcass processing. The high rate of tetracycline resistance in human strains suggested that the tetracycline resistance and associated genes might be ubiquitous in animal and human *E. faecalis* strains.

Virulences like hemolysin, gelatinase, and biofilm formation are contributors to the nosocomial infection of *E. faecalis* (22, 47). In this study, hemolysin activity and the related gene, *cylA*, in isolates from retail shops were lower than those from slaughterhouse. On the other hand, gelatinase activity and the associated gene, *gelE*, were prevalent among isolates from all the three steps of the pork meat processing chain. It has been known that gelatinase is prevalent among enterococci originated from protein-rich environment such as pork meat (36). Biofilm formation ability was tending to increase although the enterococci-isolation rate decreased along the processing. It is suggested that the moderate and strong biofilm forming strains are advantageous to

persist along the chain. Indeed, biofilm helps enterococci colonize the abiotic surfaces (42).

RAPD-PCR was used for evaluation of intraspecies diversity of enterococci isolates. This typing method has been chosen to characterize enterococci isolated from meat in many studies (32, 92, 93). In this study, there were 14 groups of isolates having 90% similarity and being originated from different processing steps from the results of RAPD-PDR. Particularly, one group of isolates originated from the slaughterhouse and the retail shop showed the possibility that *E. faecalis* strains can reach consumers through the meat processing chain. However, they were not identical strains because they showed very different gene profiles of AMRs and virulences (data not shown). Accordingly, most putative persistent strains were seemed to be maintained instantly along the pork meat processing chain. Comparing with human strains, the genetic similarity was not high because no strains originated from human and pork meat processing showed > 90% of similarity. Consequently, it would occur opportunistically that porcine *E. faecalis* isolates are conveyed to humans and cause infections.

In this study, we compared AMRs, virulences, and fingerprint patterns of *E. faecalis* strains from each step of the processing chain of pork meat and human patients. *E. faecalis* strains from the pork meat processing chain and human patients showed dissimilar appearances in AMRs and virulences generally and no isolate from the processing chain similar to the human isolates. Moreover, only few isolates seemed to be persistent through the processing chain. It is suggested that *E. faecalis* strains from pork meat could be opportunistically delivered to humans by consumption of pork meat. Consistent monitoring of *E. faecalis* isolates of the pork meat processing chain encompassing from farms to retails and seeking for the way of reducing the transmission of *E. faecalis* to consumers through the processing chain of pork meat would be necessary.

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General Conclusion

Enterococci are one of the major commensal microorganisms of humans and animals. Meanwhile, these bacteria cause a wide range of nosocomial infections in humans and some of them possess serious AMR such as VRE. It has been concerned that pathogenic enterococci can be transmitted between animals and humans through diverse ways. It is needed to characterize enterococci disseminated over animals, food, and environment and to estimate the transmission of pathogenic enterococci from various sources to humans.

1. Enterococcal isolates from chickens, pigs, companion animals and human patients were analyzed and compared in aspects of phenotype and genotype of antimicrobial resistance and virulence genes. Enterococci harboring *vanA* were detected only in human isolates (15.4%) and chicken and pig isolates showed high tetracycline and erythromycin resistance rates. Most of the companion animal and human isolates showed similar resistance rates against ampicillin and ciprofloxacin and some of companion animal isolates possessed *esp* and *hyl* genes like human's. Furthermore, CC17 *E. faecium* were detected in companion animal and human isolates while was not in food animal isolates. It seemed that the antibiotic resistance and virulence gene patterns of companion animal isolates were more similar to those of humans than those of food animals. CC17 *E. faecium* from companion animals was reported for the first time in Korea.

2. Characterization and epidemiological analysis were performed over *E. faecium* isolates from healthy dogs and dogs and humans with enterococcal infections. The prevalences of ampicillin (humans with infections, 100%; dogs with infections, 82.1%; dogs with no infection, 70%) and ciprofloxacin resistance (100%, 92.9%, 80%) were similar level regardless of origins. *Esp* (76.2%) and *hyl* (66.7%) were

prevalent among human infection isolates, but among companion animal, only one infection isolate had *esp-hyl-acm* and one colonized isolate carried *hyl-acm*. Unlike colonization isolates (2/10), dog infectious isolates (24/28) showed similar CC17 prevalence with human infectious isolates (20/21). However, patterns of antimicrobial resistance, virulence and PFGE were different between CC17 from companion animals and humans. Only one pair of CC17 isolates from a dog and a human patient coincide in the PFGE profile. However, they differed in aminoglycoside resistance. CC17 members might possess advantages for infecting both humans and animals, although their antimicrobial resistant phenotypes and virulence might have independently evolved due to different uses of various antimicrobials in human and veterinary medicine or due to host specificity.

3. *E. faecalis* isolates from the each step of pork meat processing chain and human isolates were compared for investigating the possibility of dissemination from animal to human via food processing chain. AMRs commonly found among pigs were decrease along with the processing steps while penicillin resistance, frequently detected from human isolates (26.7%), was higher in retails (6.3%) than in other steps of processing chains (0.5% and 0% in slaughterhouses and processing plants, $P < 0.05$, respectively). The detection rates of hemolysin (human, 33.3%; pork meat processing chain, 7.7%) and biofilm formation were higher among human isolates (100%, 88.2%) than among isolates from the pork meat processing chain while that of gelatinase was not significantly different between two origins (46.7%, 57.5%). C*ylA* and *esp* were significantly more detected in human clinical isolates (53.3% and 66.7%) than in the pork meat isolates (18.0% and 15.3%, $P < 0.05$). As a result of RAPD-PCR, most of the strains but one pair were not identical at the 90% similarity even in the same facilities in the same season. There was no isolate from pork meat processing chain similar with humans'. It is suggested that *E. faecalis* strains did not persist through the pork meat processing chain and they were not delivered to humans by pork meat consumption.

So long as humans get along with animals, there are still possibilities for pathogenic bacteria to be transmitted from animals to humans. In this study, however, we found that the characteristics such as AMR, virulence and epidemiological profiles of major enterococcal strains of humans and those of animals were different. It is suggested that the transmission of enterococci between animal and human occur opportunistically according to this study. Enterococci have to overcome host specificity and competition among microorganism to be transmitted to other host and colonize. Transmission of enterococci via food is also interrupted by food processing procedures such as washing and chilling. This study will help to be able to relieve some anxiety about animals as reservoirs of pathogens.

사람과 동물 장구균의 항생제 내성, 병원성, 및 전파에 관한 연구

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장구균은 동물과 사람의 장내세균총인 동시에 대표적인 병원감염의 원인체이다. 동물은 장구균과 장구균의 항생제 내성 및 병원성 유전자를 사람으로 전파하는 역할을 할 것으로 추측되어 왔다. 특히, 사람과 접촉이 많은 반려동물과 사람이 섭취하게 되는 축산 유래 식품이 전달체로 빈번히 지목되어 사람과 동물의 장구균 분리주의 특성 규명과 역학적 분석을 실시하였다.

국내의 닭, 돼지와 같은 산업동물, 개와 고양이를 포함하는 반려동물, 사람 환자로부터 분리된 184개 장구균의 항생제 내성과 병원성 표현형과 유전형을 비교하고 사람과 반려동물로부터 분리된 ampicillin 내성 장구균에 대해서는 multilocus sequence typing를 실시하였다. 반려동물과 사람의 분리주는 ampicillin과 ciprofloxacin에 대해, 산업동물 분리주는 tetracycline과 erythromycin에 대해 높은 내성율을 나타냈다. Ampicillin 내성 장구균 중 모든 사람균주와 3주의 반려동물 분리주 (3/5) 가 사람의 주요 병원감염 균주 계통인 clonal complex 17 (CC17) 에 속하였다. 반려동물의 항생제 내성 패턴 및 CC17 분포에 있어서 산업동물보다는 사람 균주와 유사한 것으로 여겨진다.

반려견의 *E. faecium* 균주를 사람 감염 균주와 비교한 결과 CC17 빈도는 반려견 colonization 균주 중에서는 사람 감염균주보다 유의적으로 낮은 반면 반려견 감염균주에서는 사람균주와 유사하게 높았다. 그러나 반려견 감염균주 또한 항생제 내성, 병원성 유전자 패턴과 pulsed-field gel electrophoresis 결과에 있어서는 사람 감염균주와 다른 양상을 나타내었다. 반려견과 사람간의 균주 전달보다는 반려견의 CC17이 사람 CC17의 경우와 마찬가지로 반려견 감염에 유리한 특징을 나타내는 방향으로 진화한 것으로 추측된다.

도축장으로부터 가공장, 소매점에 이르는 돈육가공체인으로부터 분리된 339개 *E. faecalis* 균주의 특성 규명 및 사람 감염 균주와의 역학적 비교를 실시하였다. 도축장에서 소매점으로 갈수록 chloramphenicol, high-level gentamicin, erythromycin 등 대부분의 내성과 다제내성 비율이 감소하였다. 병원성 중 hemolysin 생성능은 도축장에서, biofilm 형성능은 소매점에서 가장 높게 나타났다. Random amplified polymorphic DNA-PCR 분석 결과, 동일 *E. faecalis* 균주가 돈육가공체인에 지속적으로 존재하여 소매점까지 전달되는 경우는 없었으며 돈육가공체인 유래 균주가 사람 균주와 같은 그룹에 속하는 경우도 없었다. 이와 같이 균주의 특성과 fingerprint 결과로 보아 돈육가공체인을 통해 돼지의 *E. faecalis* 균주가 사람으로 전파될 가능성은 매우 낮을 것으로 여겨진다. 결론적으로 종 특이성이나 식품가공공정 등의 다양한 원인으로 인해 사람과 동물 간 장구균의 전파는 매우 드물 것으로 판단된다.

주요어: 장구균, 항생제 내성, 병원성, clonal complex 17, 돈육가공체인, 균주 전파

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