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Antimicrobial Resistance and Virulence Profiles of *Enterococcus* spp. isolated from Horses and Horse-associated Environments in Korea

국내 말 및 말 사육환경에서 분리된 장구균 분리주의 항생제 내성 및 병원성 특징

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Abstract

Antimicrobial resistant (AR) Enterococci have emerged as leading nosocomial pathogens. Transmission of AR Enterococci from animals to humans was demonstrated. However, limited information is available for Enterococci from horses. The goal of this study was to characterize Enterococci isolated from horse-associated-samples for their AR profiles and virulence traits. From 3,078 horsed-associated-samples, 264 Enterococci were isolated in Korea during 2013. AR profiling revealed that the average ratio of AR Enterococci was 54.2 %. Seven isolates (2.7 %) were defined as multi-drug resistant *E. faecalis*. Most of tetracycline resistant Enterococci harbored either of *tetM* or *tetL*, or both. The detection rates of other AR genes in Enterococci resistant to other kinds of antimicrobials were relatively low. Biofilm formation and gelatinase activities

were observed in 50.8 % and 47.3 % of Enterococci, respectively. Most of them were *E. faecalis* carrying *gelE* gene. Pulsed filed gel electrophoresis analysis indicated transmission of AR Enterococci between horses and their environments. These results might suggest a possibility of transmission of horse AR Enterococci to horse-riders and horse-care-workers by close contacts.

Keywords: Enterococci, Antimicrobial resistance, Virulence, Horses, Cross-transmission

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INTRODUCTION

Enterococci are Gram-positive, facultative anaerobic bacteria found in animal and human intestines. They were formerly considered as non-pathogenic commensals with little clinical problem. However, Enterococci have emerged as leading nosocomial pathogens causing infections of surgical wounds, blood stream, urinary tracts, meninges, and inner layers of heart (endocarditis) [1]. A recent surveillance indicated Enterococci are the second most frequent pathogens causing nosocomial infections in Gram-positive bacteria [2]. Enterococci are instinctively resistant to several antimicrobials [3]. Enterococci can acquire mobile genetic elements for the antimicrobial resistance either by mutation of existing genes or by horizontal gene transfer via conjugation, transduction and transformation [4, 5]. The increased nosocomial infections with Enterococci are partly related with the intensive use of antimicrobials in hospitals and intrinsic or acquired antimicrobial resistances of Enterococci [6]. In Enterococci, relatively fewer virulence or putative virulence genes are present such as genes for aggregation substance (*asa1*), cytolysin (*cylA*), enterococcal surface protein (*esp*), and gelatinase (*gelE*) [7-9]. However, with the increasing antimicrobial resistance, those virulence factors attributed to establish nosocomial infections [7].

Several studies have shown the potential transmission of antimicrobial resistant (AR) *Enterococcus* spp. between companion or food animals and their surrounding environments [10, 11]. A study showed that ingested AR Enterococci originated from animals can establish in the human GI tract and transfer AR genes to other human commensals [12]. As *Enterococcus* spp. can serve as a bio-indicator to monitor antimicrobial resistance, analysis of AR profile of *Enterococcus* spp. isolated from various animals and their environment should be informative [11]. Compared to other pet or industry animals, very limited studies were conducted to characterize the virulence factors and AR profile of *Enterococcus* spp. isolated from horses [13-15]. According to Korea Racing Authority, there are 132 horse riding clubs in Korea and the total number of horse riders are estimated to be 420,000 [16]. Since horse riders have consistently increased in recent years, the risk of transmission of horse-originated zoonotic pathogens including AR Enterococci to humans might increase. Nevertheless, no study was conducted in Korea to characterize Enterococci from horses. In the present study, we investigated the prevalence of *Enterococcus* spp. isolated from samples collected from horses and their surrounding environments in Korea. The isolates were then characterized for AR patterns in phenotypes and genotypes. Furthermore, clonal relatedness was

analyzed among the isolates from different locations, using pulsed filed gel electrophoresis (PFGE).

MATERIALS AND METHODS

1. Sampling

A total of 3,078 swab and specimen cup samples were collected from three national race parks and fourteen private riding clubs during 2013 in Korea. The samples included 1,926 horse samples taken from 649 horses (637 from feces, 645 from skin, and 644 from nasal cavity) and 1,152 samples taken from the environment around the horses (495 from drinking water, 646 from feedboxes, and 11 from beddings). Swab method was used to collect skin, feedbox (from surface of feedbox after removing residual feeds), and nasal cavity samples. The swab samples were kept in sterile tubes containing Amies transport medium (Yu-Han Lab Tech, Seoul, Korea). The other kinds of samples (feces, drinking water, and bedding) were collected in sterile specimen cups (Mediland, Seoul, Korea). All the samples were transported to the laboratory on ice within 6 hrs.

2. Isolation and identification of *Enterococcus* spp.

Isolation of *Enterococcus* spp. from collected samples was performed using Enterococci selective media according to the laboratory protocol [17]. The putative enterococcal colonies were selected and confirmed the genus using genus-specific polymerase chain reaction (PCR) [18, 19]. Determination of the

species was performed by species-specific PCR and the VITEK II bacterial identification system (BioMerieux, Craponne, France). The PCR primers used in this study are listed in Table 1.

3. Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed by disc diffusion method according to Clinical and Laboratory Standard Institute guideline [10]. The following antimicrobial disks (Beton Dickinson, Sparks, MD, USA) were used to determine the susceptibility of the enterococcal isolates to ampicillin (AMP, 10μg), chloramphenicol (CAM, 30μg), ciprofloxacin (CIP, 5μg), erythromycin (ERM, 15μg), high-level gentamicin (HLG, 120μg), high-level streptomycin (HLS, 300μg), linezolid (LZD, 30μg), quinupristin-dalfopristin (SYN, 15μg), teicoplanin (TEC, 30μg), tetracycline (TET, 30μg) and vancomycin (VAN, 30μg). The enterococcal cultures were diluted with 0.85 % saline to a 0.5 McFarland standard and applied onto Mueller-Hinton agar plates and then the discs were placed on the plates. After incubation at 37 ℃ for 24 hrs the diameter of growth inhibition around each disk was measured. *E. faecalis* ATCC (American Type Culture Collection) 29212 was used as a reference strain. An enterococcal isolate resistant to at least three different antimicrobial categories was defined as multi-drug resistant (MDR) strain.

4. Identification of AR and virulence associated genes

The presence of the seven AR genes, one integrase gene, and four virulence genes were determined by PCR using sequence specific primer sets as described below. All the isolates resistant to ERM, TET, CAM, HLG, and HLS were PCR screened for the presence of corresponding AR genes as following; ERM (*ermB*), TET (*tetM* and *tetL*), CAM (*cat*), HLG (*aac(6')-Ie-aph(2'')-Ia*), and HLS (*ant(6)-Ia and ant(3'')-Ia*) [20-23]. The integrase gene (*int*) was also included to detect the Tn*916*/Tn*1545* conjugative transposon family in all the isolates with TET or ERM resistant phenotype [24]. The integrase (*int*) gene associated with the Tn*916*/Tn*1545* transposon family was amplified by specific primers [25]. To detect virulence associated genes, all the isolates were subjected to the PCR analyses for detection of the following genes: *asa1*, *gelE*, *cylA* and *esp* [26, 27]. Primer pairs specific to the respective target genes were listed in Table 1.

5. Gelatinase activity assays

Gelatinase activity was measured on tryptic soy agar supplemented with 1.5 % skim milk. After overnight incubation at 37 °C the plates were cooled for 5 hrs at 4 °C. Hydrolysis of gelatin was considered as positive by screening the plates for the observation of clear halo around the colonies. *E. faecalis* OG1RF [28] was used as a positive control. The experiments were repeated at least two times.

6. Biofilm formation assay

Biofilm formation assay was performed under static culture conditions as described previously [29]. Briefly, a 1:100 dilution of overnight bacterial cultures in tryptic soy broth (TSB) containing 0.25 % glucose was dispensed into sterile 96-well microtitre polystyrene plate. TSB alone was inoculated into three wells per tray as negative control. After overnight growth at 37 °C the plates were carefully washed three times with phosphate-buffered saline. The plates were inverted and dried at 60 °C for 1 hr, stained with 200 µl of 1 % crystal violet for 30 min, and rinsed with distilled water. Two hundred µl of ethanol-acetone solution (80:20) was added, and absorbance of each well was read at 570 nm wave length. All the OD_{570} values were adjusted by the sample OD_{570} minus the negative control OD, then samples with the adjusted $OD_{570}>0.5$ were defined as positive for biofilm formation. The ability of biofilm formation was further categorized as follows: strong, $OD_{570}>2$; moderate, $1< OD_{570}<2$; weak, $0.5< OD_{570}<1$; negative, $OD_{570}<0.5$ [29]. All biofilm formation assays were performed in triplicate.

7. Pulsed-field gel electrophoresis

PFGE was performed to analyze clonal relatedness among the isolates as previously described with minor modifications [10]. Briefly, chromosomal DNA

sample plugs were digested with 20 U of *Sma*I (Takara Bio Inc., Shiga, Japan). Digested plugs were electrophoresed on a CHEF-Mapper (Bio-Rad, Hercules, CA, USA) through 1.2 % SeaKem Gold agarose (Lonza, Allendale, NJ, USA) in 0.5X Tris-Borate-EDTA buffer at 14 °C for 19 hrs. The electrophoretic parameters used were as follows: initial switch time, 1.0 s; final switch time, 20.0 s; angle, 120 °; gradient, 6.0 V/cm; ramping factor, linear. Analysis of PFGE banding patterns was performed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Un-weighted pair group method with arithmetic mean (UPGMA) dendrograms were represented using Dice coefficient and optimization set at 0.5 %, with tolerance of 1.0 % for band comparison [10]. *E. faecalis* OG1RF was used as a reference strain.

RESULTS

1. Isolation and identification of *Enterococcus* spp. from the horse associated samples

Overall, 264 *Enterococcus* spp. were isolated from the 3,078 horse associated samples (8.6 %) and 6 groups of enterococcal species were identified as shown in Table 2. *E. faecalis* were the most frequently detected (50.0 %) followed by *E. faecium* (22.3 %). The two species, *E. faecalis* and *E. faecium,* constituted 72.3 % of the overall enterococcal isolates. The other 4 species identified were *E. hirae* (14.8 %), *E. gallinarum* (7.2 %), *E. durans* (3.8 %), and *E. casseliflavus* (1.9 %). Generally, the isolation rates of enterococcal isolates from horses were higher than those from environment samples (Table 2).

2. Antimicrobial resistance profiles of the *Enterococcus* spp.

All the enterococcal isolates were tested for antimicrobial susceptibility against 11 different antimicrobials representing 9 classes. The 9 classes tested in this study included antimicrobial drugs most frequently prescribed to treat human/animal enterococcal infections, such as vancomycin and aminoglycosides [30]. Among the 11 antimicrobial agents tested, no AR isolates were detected against VAN, TEC, and LZD (data not shown). The highest resistance rate was shown against SYN (43.6 %). However, most of the SYN resistant Enterococci

were *E. faecalis* isolates. If SYN excluded, the highest AR pattern was found against TET (49 out of 264 isolates, 18.6 %). Overall, *E. faecalis* showed higher AR rate than other Enterococci in this study. The frequency of resistance to TET, ERM, CAM, SYN, HLS, and HLG was higher in *E. faecalis* than those were observed in other species of Enterococci (Table 3). Antimicrobial resistance against to CAM, HLS, and HLG were only found in *E. faecalis* isolates (3, 5, and 2 isolates, respectively). In addition, MDR Enterococci were also detected only in *E. faecalis* isolates (7 isolates, Table 3). When compared AR rates between isolates from horses and environments, there was no significant difference.

3. Genetic profiles of Antimicrobial resistant enterococcal isolates

As shown in Table 4, the AR isolates were screened for the presence of antimicrobial resistance genes acquired by horizontal gene transfer. Of the 6 ERM resistant Enterococci isolates, 2 isolates harbored *ermB* gene. Of 49 TET resistant isolates, 45 harbored either of *tetM* gene, encoding ribosomal protection protein mechanism, or *tetL* gene, encoding proteins involved in the efflux mechanism [21], or both. The *tetM* gene was detected in 43 (87.8 %) TET resistant isolates. Also 14 (28.6 %) of TET resistant isolates contained *tetL*. Twelve TET resistant isolates harbored both *tetM* and *tetL* genes. The *int* was

detected from all of enterococcal isolates carrying *tetM* gene and from two *ermB* positive isolates (data not shown). The *aac(6')-Ie-aph(2'')-Ia* gene was detected in the one HLG resistant *E. faecalis* isolated from drinking water. HLS (*n*=5) and CAM (*n*=3) resistant isolates did not harbor the AR genes screened in this study (*ant(6)-Ia, ant(3'')-Ia* and *cat*, Table 4).

4. Characterization of virulence factors among enterococcal isolates

Gelatinase activity was found in most of *E. faecalis* (94.7 %), but not in other enterococcal isolates (Table 5). In consistent with the phenotypes, most *E. faecalis* (98.5 %) harbored *gelE* gene which has been known to be related to the gelatinase activity [27]. Some of *E. gallinarum* isolates (5.3 %) carried *gelE* gene, but they were negative for gelatinase activity (Table 5).

For biofilm formation, 50.8 % of isolates were biofilm positive (weak, 37.1 %; moderate, 26.5 %; strong, 0.4 %, data not shown). Most of *E. faecalis* (95.5 %) were biofilm positive, but only small portions of *E. casseliflavus* (20 %), *E. hirae* (15.4 %), and *E. gallinarum* (5.3 %) isolates showed the ability to form biofilm. Among the positive isolates, moderate and strong biofilm formation abilities were observed only in *E. faecalis* isolates (25.5 % and 0.8 %, respectively, data not shown). However, all of *E. faecium* and *E. durans* isolates were negative for biofilm formation (Table 5). The average OD value of *E. faecalis* was 0.89,

whereas the OD values of other species were less than 0.5, the cutoff value for biofilm formation [29] (data not shown). The ability of biofilm formation was often related to the presence of *gelE*, *asa1* or *esp* gene [31, 32]. The *asa1* and *esp* genes were only found in *E. faecalis* (17.4 %, and 0.8 %, respectively). However, the biofilm formation of isolates was more correlated with the presence of the *gelE* gene in this study (Table 5). The gene encoding bacteriocin, *cylA* [33], was only found 1 isolate of *E. faecalis* (Table 5).

5. PFGE analysis

Biofilm producing AR Enterococci have a high risk of antimicrobial resistance transfer to public health [34]. Therefore, all 34 biofilm-producing AR *E. faecalis* identified in this study were selected for the analysis of genetic relatedness by PFGE. Since *E. faecalis* has inherited resistance to SYN intrinsically [30], *E. faecalis* isolates resistant to only SYN were excluded from this test. PFGE analyses produced 19 distinct PFGE types, and identified 5 clonal sets among the 34 *E. faecalis* isolates tested (Fig. 2). The results revealed that there were horizontal spreads of AR *E. faecalis* among horses and horse-associated environment within the same locations, but no cross-transmission among the different locations.

DISCUSSION

The increasing AR bacteria in human and animal medicine are a global problem. Enterococci are considered as opportunistic pathogens in humans and animals. However, they are now leading nosocomial pathogens which pose a serious threat to the public health [1, 2]. Ingested Enterococci can establish infection in human and animal intestine. The established Enterococci can easily acquire and transfer AR genes among other commensals in the intestine [12, 35]. A recent study also showed that *E. faecium* from animal origin can easily transfer vancomycin-resistant-gene to the same species of human origin in the GI tract of co-infected mice [36]. Those studies indicate that the transmission AR Enterococci from animals to humans should be intensively managed for human health. However, compared to other animals, the risk of AR Enterococci transmission from horses to humans was not well evaluated. In this study, we investigated the prevalence of AR Enterococci and their clonal expansion in horses and their environments as the initial step to analyze the risk factors.

From a total of 3,078 horse and environment samples, 264 *Enterococcus* spp. were isolated. Among the enterococcal isolates, the most frequently isolated species is *E. faecalis* (50 %) followed by *E. faecium* (22.3 %). These two species were reported to be the causative pathogens of more than 95 % of human enterococcal infections [9]. Hence, our results might suggest that the high

isolation rates of these two species from horses could be potentially hazard to human health in Korea. Compared to other countries, the predominant species of Enterococci isolated from horses are different. In the United States, *E. casseliflavus* is the predominant species, whereas *E. faecium* in Portugal [37, 38].

Compared to other domestic animals in Korea, the prevalence of AR Enterococci isolated from horses was lower than other industrial animals (Table 3) [17, 39, 40]. The apparent difference might be partly due to the use of antimicrobials as feed additives in industrial animals [40]. In comparison, the use of antimicrobials in the horses was well controlled in Korea and antimicrobials were rarely prescribed to horses only when infected (personal communications). The AR ratios of the enterococcal isolates from horses against most tested antimicrobials in this study were also much lower than those reported in north India [14]. Interestingly, although the isolation of vancomycin-resistance Enterococci (VRE), one of the most serious AR bacteria threatening the public health, from healthy horses were reported in several countries including India (70.6 %) and Portugal (8.8 %) [14, 15], no VRE was detected in this study. The results might have reflected the well-controlled use of antimicrobial drugs for horse care in Korea.

To most of the antimicrobials tested in this study, *E. faecalis* showed higher AR rate compared to those of other enterococcal species. Consistent with this

result, a previous study reported that *E. faecalis* can easily acquire AR genes by pheromone-mediated, conjugative plasmids [4, 5]. The highest AR rate of *E. faecalis* to SYN (81.1 %, Table 3) might be due to the intrinsic resistance mechanism by the presence of *lsa* gene [41]. In contrast, other Enterococci do not harbor the *lsa* gene. So they are susceptible to SYN. Therefore, SYN can be used to treat infections caused by vancomycin-resistant *E. faecium* (VREF) [42]. In the current study, four *E. faecium* isolates (6.8 %) showed resistance to SYN. Considered with the usefulness of SYN to VREF treatment, the results indicate that there will need to monitor the spread of SYN resistant *E. faecium* form horses to humans. If SYN excluded, the enterococcal isolates in this study showed the highest resistant rate to TET (49 isolates, 18.6 %, Table 3). TET has been widely used in veterinary medicine in Korea [40], and this might be correlated to the high AR rate of Enterococci against TET shown in this study.

The screening of AR genes in the AR isolates revealed a high correlation between the presence of TET resistant genes and TET resistant phenotypes. Of 49 TET resistant enterococcal isolates, 45 isolates (91.8 %) harbored *tetM* or *tetL* gene, or both (Table 4). Consistent with a previous study, most TET resistant Enterococci had *tetM* gene (n=43, 87.8 %), which indicates the *tetM* gene plays the major role of TET resistance phenotype in Enterococci [21]. Fourteen TET resistant Enterococci carried *tetL* gene (n=14, 28.6 %), and all of them also

carried *tetM* gene, except two isolates. A previous study suggested that the presence of both *tetM* and *tetL* genes resulted in higher MIC values than presence of only one AR gene in AR bacteria [43]. The AR genes responsible for the resistance to other kinds of antimicrobials screened in this study were detected at low frequencies in each AR phenotype isolated. The resistance genes screened in this study responsible for the AR phenotype (*ermB* for ERM resistance, *cat* for CAM, *aac(6')-Ie-aph(2'')-Ia* for HLG, *ant(6)-Ia* and *ant(3'')-Ia* for HLS, respectively, Table 4) have been known as the most frequently detected AR genes in AR Enterococci isolated from other animals in Korea and other countries [10, 21, 30, 39]. However, the detection rates of those resistance genes in each type of AR Enterococci isolated from horses were relatively low in this study, which indicates most of the AR Enterococci isolated from horses acquired other resistance genes or resistant mechanism responsible for the AR phenotypes.

It has been known that the presence of *int* gene, a gene related to mobile conjugative transposon Tn*916*/Tn*1545* family, in Enterococci is highly related to the transfer of *tetM* or *ermB* gene [24]. In consistent with the study [24], all of the AR isolates harboring *tetM* or *ermB* gene carried the *int* gene (data not shown). The high level prevalence of TET resistant Enterococci harboring both *tetM* and *int* genes might be a high risk factor for spreading of the AR gene to

same or different bacterial species infecting other animals because the Tn*916*/Tn*1545* family had a wide range of bacterial hosts [24].

Gelatinase activity is one of the most important virulence factors of *E. faecalis*. Thurlow et al. reported that gelatinase contributed to development of endocarditis and reduced neutrophil recruitment to the site of infection by cleavage of anaphylatoxin complement C5a [44]. In the current study, gelatinase activity was observed in most of *E. faecalis* (*n*=125, 94.7 %) but none of other species, which consists with a previous finding [8]. The gelatinase activity is mainly mediated by the function of *gelE* gene [7]. In this study, all of the gelatinase positive *E. faecalis* harbored *gelE* gene (Table 5), indicating the gelatinase activity are mainly mediated through the function of *gelE* gene.

The ability of biofilm formation is also considered as an important virulence factor regarding the spread of AR gene and AR bacteria. Of 264 enterococcal isolates, 134 isolates (50.8 %) showed biofilm formation ability. Most of them were identified as *E. faecalis* (*n*=126, 94.0 %, Table 5). In previous studies, *gelE*, *esp*, and *asa1* genes were proposed to be correlated to the ability of biofilm formation of Enterococci [45, 46]. In the present study, the results revealed that 94.8 % (127/134) of biofilm forming Enterococci isolates had *gelE* gene. In contrast, only 15.7 % (21/134) and none of biofilm forming Enterococci isolates carried *asa1* and *esp*, respectively. All the *asa1* gene carrying Enterococci with

biofilm forming ability also had *gelE* gene. These results indicate the *gelE* gene is more related to the ability of biofilm formation in Enterococci.

The PFGE results demonstrated that Enterococci could spread among different horses and horse associated environments although the transmission was limited within the same facilities (Figure 1). No clonal expansion of AR Enterococci among different facilities was detected. The result might be due to the limited horse moves between different facilities. Nevertheless, Enterococci can survive under the harsh condition in environment. Therefore, our results indicate a possible transmission of AR Enterococci to humans by contacts with horses or the contaminated materials.

In conclusion, this is the first study to analyze the frequency and clonal distribution of AR Enterococci in healthy horses in Korea. Our study revealed the lower prevalence of AR Enterococci in horse-associated samples in Korea compared to other countries. The most predominantly isolated enterococcal species was *E. faecalis*. Among the enterococcal species tested in this study, *E. faecalis* showed more AR and virulent phenotypes and harbored more virulence and antimicrobial resistance genes. The transmission of AR Enterococci between horses or horses and environment was demonstrated in this study. This result might suggest a possible transmission of AR Enterococci from horses to human communities. Due to the close contact with horses, the horse-riders and horse-

care-workers might be in a high risk of exposure to AR bacteria originated from horses and horse-associated materials. Therefore, they can serve as the primary carrier of the AR bacteria to introduce into human communities. A further study might need to evaluate the risk of transmission of AR Enterococci to humans by analyzing the clonal distribution of AR Enterococci in horses, horse-riders, and horse-care-workers.

Table 1. PCR primers used in this study

Targets[a]	Nucleotide sequences (5′ to 3′)	Product size (bp)	Reference
Enterococcus spp.	FW: TACTGACAAACCATTCATGATG RV: AACTTCGTCACCAACGCGAAC	112	[18]
E. faecalis	FW: ACTTATGTGACTAACTTAACC RV: TAATGGTGAATCTTGGTTTGG	360	[19]
E. faecium	FW: GAAAAAACAATAGAAGAATTAT RV: TGCTTTTTTGAATTCTTCTTTA	215	[19]
E. gallinarum	FW: TTACTTGCTGATTTTGATTCG RV: TGAATTCTTCTTTGAAATCAG	173	[19]
E. casseliflavus	FW: TCCTGAATTAGGTGAAAAAAC RV: GCTAGTTTACCGTCTTTAACG	288	[19]
ermB	FW: TGGTATTCCAAATGCGTAATG RV: CTGTGGTATGGCGGGTAAGT	745	[20]
tetM	FW: GTGGACAAAGGTACAACGAG RV: CGGTAAAGTTCGTCACACAC	406	[20]
tetL	FW: TGGTGGAATGATAGCCCATT RV: CAGGAATGACAGCACGCTAA	229	[20]
cat	FW: ATGACTTTTAATATTATTRAWTT RV: TCATYTACMYTATSAATTATAT	648	[21]
aac(6')-Ie-aph(2'')-Ia	FW: CCAAGAGCAATAAGGGCATA RV: CACTATCATACCACTACCG	220	[22]
ant(6)-Ia	FW: ACTGGCTTAATCAATTTGGG RV: GCCTTTCCGCCACCTCACCG	597	[22]
ant(3'')-Ia	FW: TGATTTGCTGGTTACGGTGAC RV: CGCTATGTTCTCTTGCTTTTG	284	[23]
asa1	FW: GCACGCTATTACGAACTATGA RV: TAAGAAAGAACATCACCACGA	375	[27]
gelE	FW: TATGACAATGCTTTTTGGGAT RV: AGATGCACCCGAAATAATATA	213	[27]
cylA	FW: ACTCGGGGATTGATAGGC RV: GCTGCTAAAGCTGCGCTT	688	[27]
esp	FW: TTGCTAATGCTAGTCCACGACC RV: GCGTCAACACTTGCATTGCCGAA	933	[26]
int	FW: GCGTGATTGTATCTCACT RV: GACGCTCCTGTTGCTTCT	1,046	[25]

[a] Targets indicate the name of bacterial species or target genes for PCR identifications conducted.

Table 2. Prevalence of *Enterococcus* spp. isolated from horse riding clubs in Korea

Group of samples		No. of samples	No. of the *Enterococcus* species / No. of the samples tested (%)						
			Total	***E. faecalis***	***E. faecium***	***E. hirae***	***E. gallinarum***	***E. durans***	***E. casseliflavus***
Horse	**Feces**	637	111[a] (17.4)	63 (9.9)	14 (2.2)	24 (3.8)	4 (0.6)	5 (0.8)	1 (0.2)
	Nasal cavities	644	34 (5.3)	12 (1.9)	1 (2.3)	3 (0.5)	2 (0.3)	1 (0.2)	1 (0.2)
	Skins	645	45 (7.0)	15 (2.3)	19 (2.9)	4 (0.6)	6 (1.0)	1 (0.2)	0
	Subtotal	1926	190 (9.9)	90 (4.7)	48 (2.5)	31 (1.6)	12 (0.6)	7 (0.1)	2 (0.1)
Environ-ment	**Drinking water**	495	32 (6.5)	20 (4.0)	4 (0.8)	6 (1.2)	1 (0.2)	0	1 (0.2)
	Feed boxes	646	38 (5.9)	21 (3.3)	5 (0.8)	2 (0.3)	6 (0.9)	3 (0.5)	1 (0.2)
	Beddings	11	4 (36.4)	1 (9.1)	2 (18.2)	2 (18.2)	0	0	1 (9.1)
	Subtotal	1152	74 (6.4)	42 (3.6)	11 (1.0)	10 (0.9)	7 (0.6)	3 (0.3)	3 (0.3)
Total		**3,078**	**264 (8.6)**	**132 (4.3)**	**59 (1.9)**	**39 (1.3)**	**19 (0.6)**	**10 (0.3)**	**5 (0.2)**

[a] The number of AR isolates to each antimicrobial is presented with percentage (in parenthesis).

Table 3. Antimicrobial resistance rates of Enterococci isolates from horses and horse associated environments

Origin	Species	ERM[a]	TET	CAM	AMP	CIP	SYN	HLS	HLG	MDR	AR
Horse	*E. faecalis* (*n*=90)	4[b] (4.4)	23 (25.6)	1 (1.1)	1 (1.1)	6 (6.7)	73 (81.1)	3 (3.3)	1 (1.1)	5 (5.6)	78 (86.7)
	E. faecium (*n*=48)	1 (2.1)	4 (8.3)	0	1 (2.1)	2 (4.2)	4 (8.3)	0	0	0	11 (22.9)
	E. hirae (*n*=31)	0	6 (19.4)	0	0	1 (3.2)	1 (3.2)	0	0	0	8 (25.8)
	E. gallinarum (*n*=12)	0	3 (25.0)	0	0	0	1 (8.3)	0	0	0	3 (25.0)
	E. durans (*n*=7)	0	0	0	0	0	1 (14.3)	0	0	0	1 (14.3)
	E. casseliflavus (*n*=2)	0	0	0	0	0	0	0	0	0	0
	Subtotal (*n*=190)	5 (2.6)	36 (18.9)	1 (0.5)	2 (1.1)	9 (4.7)	80 (42.1)	3 (1.6)	1 (0.5)	5 (2.6)	101 (53.2)
Environ -ment	*E. faecalis* (*n*=42)	1 (2.4)	10 (23.8)	2 (4.8)	1 (2.4)	0	34 (80.9)	2 (4.8)	1 (2.4)	2 (4.8)	36 (85.7)
	E. faecium (*n*=11)	0	0	0	0	2 (18.2)	0	0	0	0	2 (18.2)
	E. hirae (*n*=8)	0	2 (25.0)	0	0	0	0	0	0	0	2 (25.0)
	E. gallinarum (*n*=7)	0	1 (14.3)	0	0	0	0	0	0	0	1 (14.3)
	E. durans (*n*=3)	0	0	0	0	0	1 (33.3)	0	0	0	1 (33.3)
	E. casseliflavus (*n*=3)	0	0	0	0	0	0	0	0	0	0
	Subtotal (*n*=74)	1 (1.4)	13 (17.6)	2 (2.7)	1 (1.4)	2 (2.7)	35 (47.3)	2 (2.7)	1 (1.4)	2 (2.7)	42 (56.8)
Total (*n*=264)		6 (2.3)	49 (18.6)	3 (1.1)	3 (1.1)	11 (4.2)	115 (43.6)	5 (1.9)	2 (0.8)	7 (2.7)	143 (54.2)

[a] Abbreviations: ERM (Erythromycin), TET (Tetracycline), CAM (Chloramphenicol), AMP (Ampicillin), CIP (Ciprofloxacin), SYN (quinupristin-dalfopristin), LZD (Linezolid), HLS (High-level streptomycin), HLG (High-level gentamicin), MDR (Multi-drug resistance) and AR (Antimicrobial resistance to at least one category of antimicrobials)

[b] The number of each *Enterococcus* spp. presented with percentage (in parenthesis).

Table 4. Detection of the antimicrobial resistance genes in AR *Enterococcus* spp.

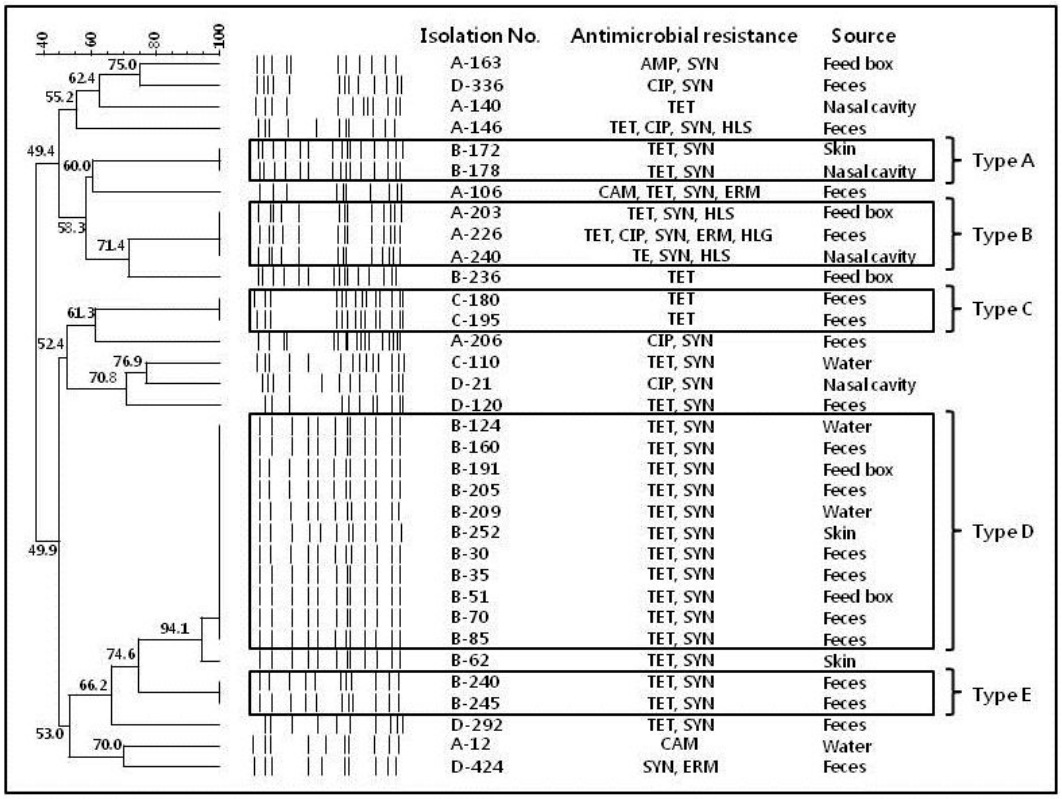
Origin	Species	No. of isolates carrying the gene[a] / No. of resistant isolates (%)						
		ERM[b]	TET		CAM	HLG	HLS	
		ermB	*tetM*	*tetL*	*cat*	*aac(6')-Ie-aph(2'')-Ia*	*ant(6)-Ia*	*ant(3'')-Ia*
Horse	*E. faecalis*	1 / 4 (25.0)	20 / 23 (87.0)	5 / 23 (21.7)	0 / 1 (0)	0 / 1 (0)	0 / 3 (0)	0 / 3 (0)
	E. faecium	0 / 1 (0)	4 / 4 (100)	1/ 4 (25.0)	-	-	-	-
	E. hirae	-	4/ 6 (66.7)	5/ 6 (83.3)	-	-	-	-
	E. gallinarum	-	2 / 3 (66.7)	0 / 3 (0)	-	-	-	-
	Subtotal	1 / 5 (20.0)	30 / 36 (83.3)	11 / 36 (30.6)	0 / 1 (0)	0 / 1 (0)	0 / 3 (0)	0 / 3 (0)
Environ-ment	*E. faecalis*	1 / 1 (100)	10 / 10 (100)	2 / 10 (20.0)	0 / 2 (0)	1 / 1	0 / 2 (0)	0 / 2 (0)
	E. hirae	-	2/ 2 (100)	1/ 2 (50.0)	-	-	-	-
	E. gallinarum	-	1 / 1 (100)	0 / 1 (0)	-	-	-	-
	Subtotal	1 / 1 (100)	13 / 13 (100)	3 / 13 (23.1)	0 / 2 (0)	1 / 1 (100)	0 / 2 (0)	0 / 2 (0)
Total		2 / 6 (33.3)	43 / 49 (87.8)	14 / 49 (28.6)	0 / 3 (0)	1 / 2 (50)	0 / 5 (0)	0 / 5 (0)

[a] *ermB,* erythromycin resistance gene; *tetM* & *tetL*, tetracycline resistance gene; *cat*, chloramphenicol resistance gene; *aac(6')-Ie-aph(2'')-Ia*, high-level gentamicin resistance gene; *ant(6)-Ia* & *ant(3'')-Ia*; high-level streptomycin resistance gene

[b] Abbreviations: ERM (Erythromycin), TET (Tetracycline), CAM (Chloramphenicol), HLS (High-level streptomycin) and HLG (High-level gentamicin)

Table 5. Phenotypic and genetic characterization of virulence in *Enterococcus* spp.

Species	Virulence phenotype (%)			Gene detection rates (%)			
	Gelatinase Activity	Ability of biofilm formation		*gelE*	*asa1*	*esp*	*cylA*
		Negative	Positive				
***E. faecalis* (*n*=132)**	94.7	4.5	95.5	98.5	17.4	0.8	0.8
***E. faecium* (*n*=59)**	0.0	100	0.0	0.0	0.0	0.0	0.0
***E. hirae* (*n*=39)**	0.0	84.6	15.4	0.0	0.0	0.0	0.0
***E. gallinarum* (*n*=19)**	0.0	94.7	5.3	5.3	0.0	0.0	0.0
***E. durans* (*n*=10)**	0.0	100.0	0.0	0.0	0.0	0.0	0.0
***E. casseliflavus* (*n*=5)**	0.0	80.0	20.0	0.0	0.0	0.0	0.0
Total (*n*=264)	47.3	49.2	50.8	49.6	8.7	0.4	0.4



Figiure 1. PFGE analysis of the 34 AR biofilm forming *E. faecalis*.

The PFGE dendrogram of *Sma*I-digested genomic DNA of 34 AR biofilm forming *E. faecalis* isolated in this study is presented. PFGE types were defined based on a 100 % similarity cut-off.

PFGE patterns are summarized with isolation number (indicating the location of equine facilities and sample number), antimicrobial resistance profiles and sample sources. Abbreviations: AMP, ampicillin; TET, tetracycline; SYN, quinupristin-dalfopristin; CIP, ciprofloxacin; ERM, erythromycin; CAM,

chloramphenicol; HLS, high-level streptomycin; HLG, high-level gentamicin; H, horse samples; E, environmental samples.

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국문초록

국내 말 및 말 사육환경에서 분리된 장구균 분리주의 항생제 내성 및 병원성 특징

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장구균에 의한 질병은 전 세계적으로 증가하는 추세이고, 그 심각성 또한 대두되고 있다. 산업동물, 반려동물 그리고 사람에서 분리된 장구균에 대한 연구는 많이 이루어져 있다. 하지만 이에 비하여 말에서 분리된 장구균의 특징과 교차전파 가능성에 대한 연구는 미비한 수준이다. 따라서 이 논문에서는 한국 말과 그 환경에서 분리된 장구균의 항생제 내성 및 병원성 특징 그리고 교차전파 가능성을 조사하였다. 2013 년에 한국의 3 개의 국립경마장 및 14 개의 승마장을 대상으로부터 채취한 3,078 개의 시료들 중에 총 264 개의 장구균이 분리되었고, 6 종의 서로 다른 장구균이 동정되었다. 그 중 사람에서 특히 문제를 일으키는 2 종인 *E. faecalis* 와 *E. faecium* 이 많은 비율을 차지하고 있었다 (각각 50 %, 22 %).

한 개 이상의 항생제에 내성을 가지는 장구균은 143 주 (54.2 %)였으며, 3 개 이상의 서로 다른 항생제에 내성을 가지는 Multi-drug resistant (MDR) 장구균은 단 7 주 (2.7 %)에 불과하였다. Tetracycline 내성을 가지는 균주의 경우 항생제 내성 유전자인 *tetM*, *tetL* 유전자를 많이 보유하는 것으로 관찰되었으나 (각각 87.8 %, 28.6 %), 다른 항생제들의 경우 항생제 내성 표현형과 유전형간의 차이가 관찰되었다. 병원성을 확인하기 위한 biofilm formation assay 와 gelatinase activity assay 에서 *E. faecalis* 가 다른 종에 비하여 높은 양성률을 나타내었고 (각각 95. 5%, 94.7 %), 병원성 유전자의 경우 역시 주로 *E. faecalis* 에서 검출되었다. 항생제 내성과 병원성 모두 *E. faecalis* 에서 높게 나타났고, Pulsed-field gel electrophoresis 를 통해 이 장구균을 대상으로 유전적 상동성을 비교 분석한 결과 서로 다른 말 그리고 그 환경 사이에 교차전파가 관찰되었다. 이러한 결과는 공중보건학적으로 승마자 또는 말 관리사 같은 말산업 종사자에게 전파될 수 있다는 것을 시사하기 때문에, 지속적인 관찰이 필요할 것이다.

주요어: Enterococci, Antimicrobial resistance, Virulence, Horses, Cross-transmission

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