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Development of multiplex PCR for detection of vancomycin resistant enterococci(VRE) and epidemiological application in Korea

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Abstract : Vancomycin resistant enterococci (VRE) have emerged as an important nosocomial pathogen. Since 1989 the Center for Disease Control, United States, has reported a rapid increase in the incidence of enterococcal bacteremia and endocarditis infection by VRE. It was suggested that the use of avoparcin was associated with the appearance of VRE in animal husbandry. To date, several detection methods have been used based on conventional methods of culture and gene detection. However, these methods have some limitations such as time-consuming, laborious and additional differential needs. Therefore, In this study a multiplex PCR method was established to detect and differentiate resistance types of enterococci which specifically amplify the four *van* genes encoding vancomycin resistance elements.

Using the method, we investigated the incidence rates and types of VRE from farms using or not using avoparcin. A total of 1091 animal fecal samples were collected from 70 pig and 32 poultry farms. A total of 425 of enterococci were isolated from samples. Of the 425 isolates, 11 of the them showed a pattern of high-level vancomycin resistance (MIC : 64~256μg/ml) which was associated with the presence of the *vanA* or *vanB* gene. Fifty-seven isolates showed a pattern of low-level vancomycin resistance (MIC : 3~8μg/ml) associated with the *vanC-1* or *vanC-2* gene. Interestingly, all isolates with high-level vancomycin resistance were from farms that have never used avoparcin. Moreover, the high-level VRE isolation rate in Korea (2.58%) was much lower than that of other countries (50% in England, 7% in Belgium) where avoparcin have been used.

In conclusion, the multiplex PCR method established in this study could be applied for detection of VRE.

Key words : vancomycin resistant enterococci (VRE), avoparcin, multiplex-PCR.

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Introduction

The genus *Enterococcus* is gram-positive, facultatively anaerobic organisms that are catalase negative and reacts with group D antisera[1,2]. Hydrolysis of L-purrolidonyl-β-naphthylamide (PYR) and the ability to grow in 6.5% NaCl is a characteristic feature[3]. Enterococci cause an estimated 5 to 15% bacterial endocarditis infections. *E. faecalis* is the most frequent isolate[4~7]. Enterococcal endocarditis occasionally occurs in children and rarely in infants. Enterococcal bacteremia is much more common than enterococcal endocarditis and mortality of enterococcal bacteremia has generally been high due to the acquisition of resistance to antimicrobial agents[8].

Glycopeptide antibiotics, vancomycin and teicoplanin, inhibit cell wall synthesis by formation of a complex with peptidyl-D-Ala-D-Ala termini of peptidoglycan precursors in the outer surface of the bacterial membrane[9].

VanA and VanB type resistance evade inhibition of cell wall assembly by synthesizing the peptidoglycan precursor, UDP-N-acetylmuralmyl-L-Ala-γ-Glu-L-Lys-D-Ala-D-lactate. Replacement of D-Ala by D-lactate (D-Lac) in the C-terminal portion of the precursor results in 1,000 fold decreased affinity of glycopeptides antibiotics for the precursor which allows bacterial survival[10,11].

Vancomycin resistant enterococci (VRE) first reported in Europe in 1988, are emerging as an important nosocomial infections[12]. In Korea, since the first isolation of VRE from hospital infection in 1992, the isolation rate has increased from 2% to 11%[13].

Among enterococci, five phenotypic classes of vancomycin resistance have been described associated with resistance genes : *vanA* , *vanB* , *vanC-1* , *vanC-2* , *vanC-3* and *vanD* [14]. The VanA type showed a high-level of resistance to both vancomycin (〉 256μg/ml) and teicoplanin, whereas the VanB type showed various levels of vancomycin resistance (256~64μg/ml) and remained susceptible to teicoplanin. The *vanB* gene has been found only *E. faecium* and *E. faecalis*, whereas *vanA* gene has been found in various enterococcal species. The *vanC-1* , *vanC-2* and *vanC-3* genes have been found only in *E. gallinarum* , *E. casseliflavus* and *E. flavescens* , respectively[15~17]. VanA and VanB type resistance are encoded by gene clusters that are acquired and are often transferable but VanC-1, VanC-2 and VanC-3 type resistance are not normally transferable. The *vanA* gene cluster has been found in a small transposon, *Tn* 1546, which has been found on both transferable and nontransferable plasmids, as well as on the chromosome of the host strain[18,19]. The *vanB* gene cluster has been found on a large (90kb to 250kb) chromosomally located transferable element. Recently, it has been found as part of transferable plasmid[18,19]. The more serious problem is with the *vanA* and *vanB* gene clusters that have been found in a number of different bacterial species[20]. The *vanA* gene has been found in lactococci, *Orskovia* and *Arcanobacteria*. The *vanB* gene has also been found in *Streptococcus bovis* [20~22]. These findings indicate that it may be possible for the vancomycin resistance genes to be transferred to streptococci and staphylococci[20].

The origin of VREs and the transmission route have not been fully understood. Klare *et al* [23] has found VRE in manure samples from pig and poultry farms in Germany[21]. They have suggested there is an association between the recovery of VRE from animal husbandry and the use of avoparcin which is a glycopeptide antimicrobial drug used as a livestock feed additive in many European countries[23]. Denmark, Norway and the Netherlands have suggested there is an association between the recovery of VRE from food animals and the use of avoparcin at subtherapeutic doses for growth promotion[24,25]. VREs were also isolated from poultry and pork meat products, which were collected from slaughterhouses and retail markets[24,25]. If VREs from poultry, swine and other food animals play an important role in human colonization and infection, identical genotypes should be found in isolates from animals and human. However, a relatively low incidence of human VRE infection has been found in European countries where avoparcin has been used for many years. In Europe, VREs have been detected among persons outside the health-care setting in several studies[26~29]. VREs were isolated from the stool of 3 (2%) of 184 persons in Oxford, England (Jordens *et al* [26]), 7 (7%) of 40 persons living in Charleroi, Belgium, without recent ex-

posure to a health-care setting[28]. 22 (3.5%) of 636 patients cultured within 2 days of entering a hospital in Belgium[27]. Moreover, pulse field gel electrophoresis analysis between human isolates and animal isolates has not shown a clear relatedness. In contrast, in the United States, where avoparcin has not been used, a higher incidence rate of human VRE infection has been noted that observed in European Countries. Although VRE infection is endemic in the United States, there has been no report of human VRE colonization outside hospital infections or associated with the presence of VRE from animals or human foods of animal origin[30].

In this study, we compared the occurrence of VRE on animal farms which were using avoparcin with farms not-using avoparcin. We developed a multiplex PCR method to differentiate the vancomycin resistance genotypes.

Materials and Methods

Isolation and identification of enterococci : A total of 1091 fecal samples were collected from pig and poultry farms. To isolate enterococci spp., fecal swab samples were inoculated on *Streptococcus* selective agar (Merck 5468, Darmstadt, Germany) and incubated for 24hrs at 37℃. Enterococci-like colonies were subcultured on 5% sheep blood agar (KOMED, Korea) and subsequently identified as follows; Catalase negative, esculin positive, growth on MacConkey agar, 6.5% salt tolerance positive, L-pyrrolidonyl-naphtylamide (PYR) test positive and gram positive cocci in short chains. Sixty-three human clinical isolates of *Enterococcus* spp., identified by Vitek system (bioMerieux Inc., Hazalwood, Mo. USA), were collected from hospitalized patient's blood, urine, bile and abscesses.

Reference strains : Reference strains used in this study were shown in Table 1. The following strains were used as positive control for multiplex PCR which were kindly gifted from Dr. Robin Patel (Mayo Clinic and Foundation, USA); *E. faecium* BM7641, *E. faecalis* V583, *E. gallinarum* GS, *E. casseliflavus* ATCC25788. In addition, 13 other pathogenic strains were obtained from the National Veterinary Research and Quarantine Service in Korea, International *Escherichia* and *Klebsiella* Centre (Statens Serum Institute, Copenhagen, Denmark) and *Escherichia coli* reference center (Pennsylvania State Univ., USA).

Table 1. Reference strains used in this study

Strain	Gene	Remark (Vancomycin MIC)
Enterococcus faecium BM7641	*vanA*	〉256μg/ml
Enterococcus faecalis V583	*vanB*	64μg/ml
Enterococcus gallinarum GS	*vanC-1*	4μg/ml
Enterococcus casseliflavus ATCC 25788	*vanC-2*	4μg/ml
Enterococcus faecium ATCC 21912		〈 4μg/ml
Staphylococcus aureus FRI 913		
Escherichiae coli (LT positive)		
Escherichiae coli (ST positive)		
Escherichiae coli O157 : H7		
Salmonella dubulin		
Yersinia enterocolitica		
Staphylococcus epidermidis ATCC 12228		
Streptococcus uberis ATCC 27958		
Streptococcus agalactiae ATCC 13813		
Streptococcus dysagalactiae ATCC 27957		
Streptococcus pyogenes ATCC 21059		
Clostridium perfringens ATCC 3628		
Clostridium perfringen ATCC 3629		

Antimicrobial susceptibility test of *Enterococcus* isolates : Vancomycin and teicoplanin susceptibility tests were performed by E-test (AB BioDisk, Solna, Sweden) on BHI agar (Difco 0418, Detroit, USA) as described by the manufacturer. The Minimal Inhibitory Concentration (MIC) values of vancomycin were detected at the range from 0.023 to 256μg/ml.

DNA preparation : To prepare total DNA, enterococci were cultured in 3ml of BHI broth and harvested after overnight incubation at 37℃. Total DNA was prepared by the guanidinium thiocyanate method of Pitcher *et al*[31]. Briefly, bacterial pellets were harvested by centrifugation at 12,000×

g for 3 min, washed twice in 700μl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and suspended in lysis buffer (0.1M Tris-HCl, 0.2M EDTA, GuSCN, pH 8.0). Diatomaceous earth (Sigma, USA) suspension was added immediately after mixings and the tubes were then centrifuged at 12,000rpm briefly. After standing 10 min at room temperature, the tubes were vortexed again (5 sec) and the supernatant was decanted. The silica-nucleic acid pellet was subsequently washed twice with washing buffer (0.1M Tris-HCl, GuSCN pH 6.4), twice with ethanol 70% (vol/vol), and once with acetone. After removal of the acetone, the tubes were dried at 56℃ with open lids in a heating block for 10 min. After addition of elution buffer (10mM Tris-HCl, 1mM EDTA pH 8.0), vortexed briefly, and incubated for 10 min at 56℃. The tubes were then briefly vortexed again and centrifuged for 2 min at 12,000×g. The supernatants containing the DNA were collected. The concentration of the DNA was measured at 260nm with a spectrophotometer (Pharmacia Biotech, Piscataway, N.J.) and kept at -20℃ unitl use.

Oligonucleotide primers for multiplex PCR : Eight oligonucleotide primers (Table 2) were synthesized by a DNA synthesizer (Expedite Nucleic Acid System, Perseptive Biosystem, USA) and purified by SEP-PAK and used to amplify the *vanA*, *vanB*, *vanC-1*, *vanC-2* gene, base on the published DNA sequences for theses genes[16,17,32,33].

Multiplex PCR : PCR amplifications were performed by using a Gene Amp PCR system 2400 thermocycler (Perkin-Elmer). Amplifications were performed in a total volume of 25μl in mixtures containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.0mM $MgCl_2$, 1mM deoxynucleoside triphosphate, 50 pmol of each primer, 2U of *Taq* polymerase, and 50pg of DNA template. Thermocycler conditions were as follow ; PCR amplicons of *vanA*, *vanB*, *vanC-1* and *vanC-2* were produced by an initial cycle of 95℃ for 5 min, followed by 30 cycles of 95℃ for 1 min, 58℃ for 1 min, 72℃ for 30 sec. An extension step of 72℃ for 5 min was added after the final cycle. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide and photographed on a UV transilluminator.

Table 2. Sequence of oligonucleotide primers used for detection of *van* genes

Gene	Oligonucleotide sequence (5'to 3')	PCR products size (bp)
vanA	ATTGCTATTCAGCTGTACTC GGCTCGACTTCCTGATGAAT	559
vanB	AACGGCGTATGGAAGCTATG CCATCATATTGTCCTGCTGC	467
vanC-1	GGCATCGCACCAACAATGGA TCCTCTGCCAGTGCAATCAA	902
vanC-2	TTCAGCAACTAGCGCAATCG TCACAAGCACCGACAGTCAA	663

Specificity and sensitivity of the multiplex PCR : The specificity of the PCR was confirmed using thirteen bacterial strains which were pathogenically related or other enteric bacteria. To examine the sensitivity of the PCR, template DNA of reference strains were extracted as described above. Ten-fold serially diluted template DNA was used for PCR after measuring the concentration of extracted DNA by GeneQuant at 260nm (Pharmacia, USA).

Sequencing of PCR products : Each amplified PCR product was eluted from low melting point agarose gel (Promadisa, Hispanlab, USA), using Geneclen kit II and then cloned into pGEM T vector system (Promega Corp. Madison, WI, USA). The cloned PCR products were sequenced by the dye termination method using an automated ABI 377 DNA sequencer (The Perkin Elmer Corp. Norwalk, CT, USA) after purification with Quiagen column (Promega). Cloning and sequencing were performed as described by the manufacturer. The identities of the products were confirmed by comparison with the Genebank sequences (National Institute of Health, USA).

Result

Amplification of *vanA*, *vanB*, *vanC-1* and *vanC-2* gene by multiplex PCR : As shown in Fig 1, the method established in this study showed that each of *van* gene was specifically amplified (*vanA* : 559bp, *vanB* : 467bp, *vanC-1* : 902bp, *vanC-2* : 663bp). To examine the detection ability of

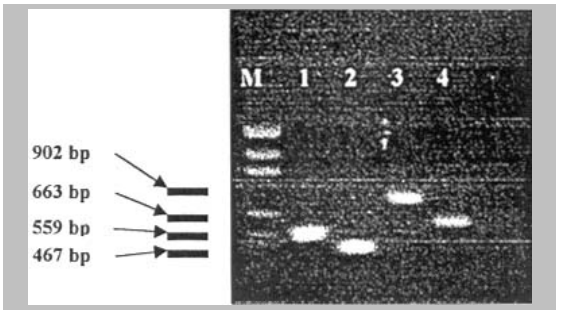

Fig 1. Detection of vancomycin resistant genes from reference strains.
M : pGEM DNA markers (36-2645bp) : lane 1. *E faecium* BM 7641(*vanA*) ; lane 2. *E faecalis* V583 (*vanB*) ; lane 3. *E gallinarum* (*vanC-1*) ; lane 4. *E casseliflavus* (*vanC-2*).

the established multiplex PCR for multiple *van* genes, each reference strains' DNA was mixed and then amplified. Four different *van* genes were successfully amplified(Fig 2).

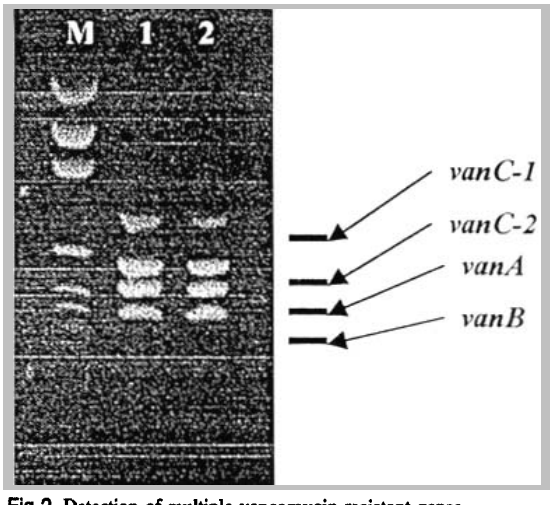

Fig 2. Detection of multiple vancomycin resistant genes.
M : pGEM DNA markers (36~2645bp) ; lane 1. Four reference strains' DNA mixture was used as template DNA (using 3 Unit *Taq* polymerase) ; lane 2. Four reference strains' DNA mixture was used as template DNA (using 2 Unit *Taq* polymerase).

The specificity of established multiplex PCR method :

The specificity of the PCR assay for detection of the *vanA*, *vanB*, *vanC-1* and *vanC-2* gene was compared with the other 13 pathogenic bacteria. The strains possessing *vanA*, *vanB*, *vanC-1* or *vanC-2* genes were specifically amplified, otherwise other strains which did not possess *van* gene were not amplified(Fig 3).

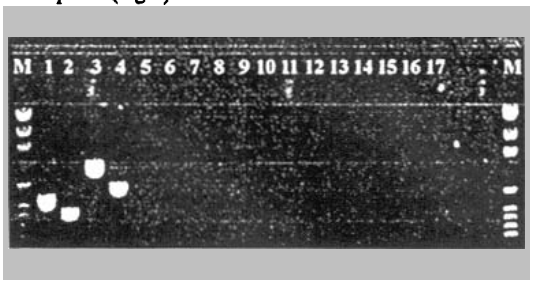

Fig 3. Specificity of a multiplex PCR method established in this study.
The method established in this study could specifically amplify the *van* genes of the references.
Lane 1 to 4, each of reference VRE strain was used as template.
Lane 5 to 17, pathogenic enteric bacteria or pathogenically related bacteria were used as template. M : pGEM DNA markers (36~2645bp). Lane 1. *E faecium* (*vanA*) ; Lane 2. *E faecalis* (*vanB*) ; Lane 3. *E gallinarum* (*vanC-1*), Lane 4. *E casseliflavus* (*vanC-2*), Lane 5. *E faecium*, Lane 6. *Staphy. aureus* ; Lane 7. *E coli* ; Lane 8. *E coli* ; Lane 9. *E coli* O157 : H7 ; Lane 10. *Sal. dubulin* ; Lane 11. *Yer. enterocolitica* ; Lane 12. *Staphy. epidermidis*, Lane 13. *Strepto. uberis* ; Lane 14. *Strepto. agalactiae* : Lane 15. *Strepto. dysagalactiae* ; Lane 16. *Strepto. pyogenes* ; Lane 17. *Cl. perfringens*.

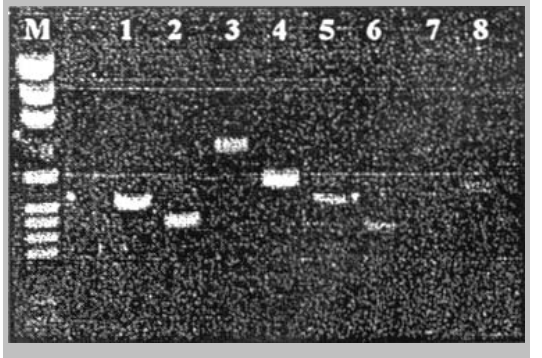

Fig 4. Sensitivity of a multiplex PCR method established in this study for detecting the *vanA, B, C-1, C-2* genes of the reference strains.
Each of template DNA was used as low as 8ng/ml concentration.
Lane 1 to 4, 80ng/ml template DNA was used.
Lane 5 to 8, 8ng/ml template DNA was used.
M : pGEM DNA markers (36~2645bp) : Lane 1. *E faecium* (*vanA*) ; Lane 2. *E faecalis* (*vanB*), Lane 3. *E gallinarum* (*vanC-1*), Lane 4. *E casseliflavus* (*vanC-2*)-template DNA conc. : 80ng/ml, Lane 5. *E faecium* (*vanA*)) ; Lane 6. *E faecalis* (*vanB*). Lane 7. *E gallinarum* (*vanC-1*), Lane 8. *E casseliflavus* (*vanC-2*)-template DNA conc. : 8ng/ml.

Table 3. Phenotypic ad genotypic characteristics of human isolates

	vanA	*vanB*	*vanC-1*	*vanC-2*	Vancomycin MIC (μg/ml)		
					〉256	64	4
E. faecium	15*	10	0	0	14	11	0
E. faecalis	5	16	0	0	5	16	0
E. gallinarum	0	0	8	0	0	0	8
E. casseliflavus	0	0	0	9	0	0	9

*No. of isolates.

The sensitivity of established multiplex PCR method : As shown in Fig 4, the method established in this study could detect all of *van* genes as low as 8pg/μl concentration. *VanA* and *vanB* gene could be detected as low as 0.8pg/μl, however *vanC-1* and *vanC-2* could not be detected at 0.8pg/μl concentration (Data not shown).

Amplification of *vanA*, *vanB*, *vanC-1* and *vanC-2* gene from animal origin VRE : We applied the method established in this study to detect *van* genes from isolates from animal source. Of the 425 isolates, ten isolates were associated with *vanA* gene. One isolate was associated with *vanB* gene. Fifty-seven isolates were associated with *vanC-1* and *vanC-2* genes (Fig 5).

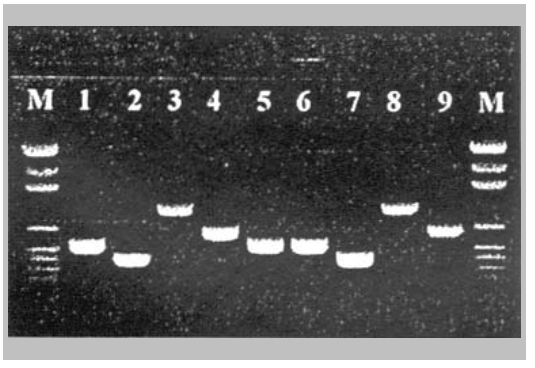

Fig 5. Detection of vancomycin resistant genes from animal isolates by multiplex PCR.
Lane 1 to 4, reference VRE strains.
Lane 5 to 9, VREs isolated from animal fecal samples.
M : pGEM DNA markers (36~2645bp). Lane 1. *E faecium* (*vanA*), Lane 2. *E faecalis* (*vanB*), Lane 3. *E gallinarum* (*vanC-1*), Lane 4. *E casseliflavus* (*vanC-2*), Lane 5. 100-1 (*vanA*), Lane 6. 100-2 (*vanB*), Lane 7. 89-4 (*vanB*), Lane 8. 100-3(*vanC-1*), Lane 9. 100-4 (*vanC-2*).

Isolation and identification of enterococci and antibiotic susceptibility test : A total of 425 enterococci spp. were isolated from 1091 animal fecal samples and 63 VRE were collected from human specimens. Of the 425 animal isolates. 11 isolates showed high-level vancomycin resistance (MIC : 〉256μg/ml) and 57 isolates showed low level vancomycin resistance (MIC : 4~8μg/ml). Phenotypic and genotypic characteristics of the human isolates are shown in Table 3. The characteristics of VRE isolated from animal fecal samples are shown in Table 4.

Table 4. Characteristics of vancomycin resistant animal isolates

No. of isolate	Source	MIC(μg/ml)		Genotype Results
		Vancomycin	Teicoplanin	
*89-4	Poultry farm	〉256	0.38	*vanB*
*100-1	Poultry farm	〉256	〉256	*vanA*
*100-2	Poultry farm	〉256	〉256	*vanA*
119-1	Poultry farm	〉256	〉256	*vanA*
119-2	Poultry farm	〉256	〉256	*vanA*
128-1	Poultry farm	〉256	〉256	*vanA*
154-1	Poultry farm	〉256	〉256	*vanA*
191-1	Poultry farm	〉256	〉256	*vanA*
191-3	Poultry farm	〉256	〉256	*vanA*
192-1	Poultry farm	〉256	〉256	*vanA*
357-4	Poultry farm	〉256	〉256	*vanA*
*57 isolates	Pig and poultry farm	8-4	〈0.38	*vanC-1* or *vanC-2*

*Fifty-seven isolates showed low level vancomycin resistance.

Table 5. Comparison of VRE occurrence and their MIC values to vancomycin between avoparcin-using farms and non-using farms

	No. of farms	No. of fecal samples	No. of isolated enterococci	Vancomycin MIC (μg/ml)				
				≥256	64	8	4	⟨4
Avoparcin using pig farm	23	215	81		0	6	27	48
Avoparcin non-using pig farm	47	524	193	0	0	4	19	170
Avoparcin using poultry farm	8	105	47	3	0	0	5	39
Avoparcin non-using poultry farm	24	247	104	8	0	0	6	90
Total	102	1091	425	11	0	10	57	347

Discussion

Increased number of colonizied and infected patients by vancomycin resistant enterococci (VRE) have been reported from many European countries, Korea and United States[34-36]. Enterococci have been known as a cause of infective endocarditis, more recently have been recognized as a cause of nosocomial infection in patients receiving antimicrobial agents. Enterococci have been focused because of theirs resistance to multiple antimicrobial drugs. The hospital outbreaks of clonally related VRE have been reported[35,37]. The rapid appearance of VRE in humans in recent years was attributable to an increased selective pressure due to increasing use of vancomycin in man and the spread of in hospital settings of a highly mobile genetic determinant of resistance[34,38]. High-level vancomycin resistance was mediated by transferable plasmids that may harbor resistance determinants to other drug as well[10]. It was suggested that glycopeptide resistant enterococci isolated from animal fecal sample had been associated with the use of glycopeptide antibiotics as feed additives[39]. Bate *et al*[40], Klare *et al*[23] and Aarestrup[25] isolated VRE from domestic animals and from food of animal origin, and Klare *et al*[23] and Aarestrup[25] suggested that this finding could be explained by the use of the glycopeptide antibiotics, avoparcin in food animals[23,25,40]. The Swedish National Veterinary Institute investigated the occurrence of VRE in six poultry flocks and five pig herds in Sweden. Enterococci were recovered from most of samples and all were fully susceptible to vancomycin though avoparcin has not been used in Sweden since 1986[41]. In Norway, avoparcin was used in poultry between 1986 and 1995. Kruse (1995) found VRE in 81 of 89 Norwegian broiler flocks that had used avoparcin[42]. Bates *et al*[40] reported 22 of 52 farm animals were colonized with VRE in England[40]. Therefore, European Countries and other countries including Japan prohibited the use of avoparcin. The results shown in this study was different to many other reports[26-28]. In Korea, we had also used avoparcin between 1983 and 1997 and the use was temporarily stopped for further investigation. However, the occurrence rate of high-level VRE was significantly lower (2.58%) than that of European Countries. We selected the avoparcin-using farms which were supplied avoparcin as feed additives from an animal feed company until 1997. The results of vancomycin susceptibility test in this study showed that most of isolates from animals had a high susceptibility to vancomycin. Interestingly, all of high-level vancomycin resistance isolates were only obtained from poultry farms.

In this study, we established a multiplex PCR to detect the 4 genotypes of VRE. The PCR has the potential ability in reducing time and cost detecting VREs[43,44]. As illustrated in Fig 1 and 2, the genes encoding the vancomycin resistant element could be specifically amplified. Eleven high-level VRE isolated from animal produced the *vanA* and *vanB* gene products by the method established in this study. The results showed that the established method might be applicable to detect and differentiate types of *van* gene of the

field isolates. All isolates' phenotypes were consistent with genotype confirmed by the established method. The sequence similarity of each of PCR gene products compared with reference GenBank sequence was shown follow ; 100% in *vanA*, 100% in *vanB*, 99.9% in *vanC-1*, 99.8% in *vanC-2* .

Although low-level vancomycin resistance (MIC ; 3~8μg/ml) enterococci were less important than high level resistant enterococci, a recent study reported an increased number of *E. gallinarum* and *E. caselliflavus* caused hospital infection[45]. We could also isolate fifty-seven strains possessing *vanC-1* or *vanC-2* genes from animal source. We should pay more attention to the antimicrobial reactivity of these strains and need to differentiate enterococci at the subspecies level. However, most of laboratories have been concentrated on *E. faecium* and *E. faecalis* .

In conclusion, the study had revealed that the use of avoparcin in animal farms did not affect to induce vancomycin resistance of *Enterococcus* in Korea. Also, we could find only eleven high-level VRE from animal farms. The occurrence rate of VRE in animal farms in the country was much lower than that of other countries.

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