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Prevalence and Characterization of Vancomycin-Resistant Enterococci in Chicken Intestines and Humans of Korea

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The prevalence, genotype for antibiotic resistance and antibiotic susceptibility of vancomycin resistant enterococci (VRE) were determined. And molecular typings of the *Enterococcus faecium* isolates were analyzed. Prevalence of VRE in chickens, healthy children and intensive care unit (ICU) patients was 41.6%, 7.9%, and 20.4%, respectively. Forty out of 54 isolates from chicken intestines, and 9 out of 11 from ICU patients were identified as *Enterococcus faecium.* Eleven out of 13 isolates from non-hospitalized young children were *E. gallinarium.* Twelve strains of *E. faecalis* were isolated from chicken intestines. The gene for the antibiotic resistance in *E. faecium,* and *E. faecalis* was *vanA,* while that in *E. gallinarium* was *vanC1. E. faecium* isolates were resistant to most of antibiotics except ampicillin and gentamicin. Molecular typing of the *E. faecium* strains obtained by pulse field gel electrophoresis and repetitive sequence-based PCR suggest that VRE transmit horizontally from poultry to humans, especially young children, *via* the food chains in Korea.

Key words: Vancomycin resistant enterococci (VRE), Prevalence, Molecular typing, Chicken intestine, *Enterococcus faecium*, *E. faecalis*

INTRODUCTION

Vancomycin resistant enterococci (VRE) cause a health risk, especially in patients with severe underlying disease or immunosuppression (Chadwick *et al.*, 1996; Van den Braak *et al.*, 1998). Over the last few years, intrinsic or acquired resistance of enterococci to many antibiotics, in particular, to glycopeptides, has become a major problem in clinical medicine. Since the first report on VRE in 1986 in Europe, their presence has increasingly been detected throughout the world (Leclertcq *et al.*, 1988; Van Horn *et al.*, 1996). The increase of VRE in nosocomial infection is due to oral use of vancomycin or other antibiotics (Gold, H. S. 2001; Husni *et al.*, 2002; Rice, L. B. 2001). In Korea, VRE strain *E. durans* was first isolated in 1992 (Park *et al.*, 1992). Since then, the prevalence of VRE in hospitalized patients has been rising significantly (Kim *et al.*, 1997; Kim *et al.*, 1995; Shin *et al.*, 2003). Also, there are several cases on occurrence of VRE from animals and environmental sources. The increasing use of antimicrobial agents as animal growth promoters has been related to the emergence of VRE (Devriese *et al.*, 1996; Leclercq *et al.*, 1989). In Europe, antimicrobial agents are widely used as feed additives for promotion of growth in animal husbandry (Woodford *et al.*, 1995). Avopracine is a glycopeptide antibiotic used for this purpose in poultry, and it appears to be associated with the emergence of resistance to glycopeptides in general (Klare *et al.*, 1995; Woodford *et al.*, 1995).

Enterococci is known to belong to the natural intestinal flora of poultry. It is, thus, likely that transmission of VRE occurs through human contact with poultry meat contaminated with resistant bacteria. However, such a route of transmission of VRE from poultry to humans has not been unequivocally documented so far (Gambartto *et al.*, 2000; Van den Braak *et al.*, 1998). It is presumed that the introduction and spread of the resistant VRE into humans results from transmission of resistance genes (Arthur *et al.*, 1997; Salyers and Amabile-Cuevas, 1997). Several studies suggest that high-level of resistance to glycopeptides in enterococci is mediated by transposons similar to Tn*1546* (Van den Braak *et al.*, 1998).

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The development of strategies to control the spread of glycopeptide resistance among bacterial species pathogenic to humans are of considerable concern. To approach this goal, the clarification and quantification of antibiotic resistance gene transfer from animals to humans are needed. We therefore undertook an investigation of VRE in intestinal colonization among chickens, patients, and non-hospitalized children. Then we determined the resistance genotype of the isolates and analyzed the molecular types of VREs isolated in Korea.

MATERIALS AND METHODS

Isolation and identification of VRE

Chickens, patients, and healthy young children were screened for gastrointestinal carriage of VRE. For a period of 8 months, from January to August, 1998, a total of 130 chicken intestinal gut samples were collected from retail dealer in Iksan, Jeonbuk, Korea. From February to March 1999, rectal swabs were taken from the 166 healthy young children (age range 3 to 5 years) in Suncheon, Jeonnam (102) and Iksan (64), and 54 patients in the intensive care unit (ICU) of Wonkang University Hospital, Iksan, Korea.

Intestinal swabs of chickens and rectal swabs of humans were inoculated onto bile esculin azide agar (Oxoid) supplemented with 6 mg of vancomycin per liter, which was then incubated at 37°C for 24 h (Gambartto *et al.*, 2000). Colonies grown on agar with a dark brown halo and morphologically resembling enterococci were primarily identified by Gram staining, and the presence or absence of catalase and pyrase (Dryslide Pyrkit; Difco). Species identification was performed with the API 20 STREP system (Biomerieux) and physiological tests (Carvalho *et al.*, 1998). Motility, yellow pigmentation, and acidification of methyl-α-D-glucopyranoside (MGP), arabinose, lactose, raffinose, and mannose of the VRE isolates were determined as recommended by Carvalho *et al.* (1998). *E. casseliflavus* ATCC25788 (VANC2), *E. faecium* ATCC19434 (VANA), *E. faecalis* CDC-286 (VANA), and *E. gallinarium* CDC-42 (VANC1) were used as reference strains. The confirmation of the identification was done by detection of vancomycin resistance gene (*vre*) with PCR analysis.

Antimicrobial susceptibility test

VRE strains were tested for antibiotic resistance using agar dilution methods. Bacterial suspension in tryptic soy broth (Difco) with a turbidity equivalent to that of a 0.5 McFarland standard was diluted 1:10 with saline. The cell suspension was inoculated onto Mueller-Hinton agar (Difco) supplemented with antibiotics. Inoculated plates were read after incubation at 37°C for 24 h. The results were interpreted according to the guidelines set forth by NCCLS (National Committee for clinical Laboratory Standards).

DNA isolation

VRE strains were grown overnight at 37°C on brain heart infusion (BHI) agar plates. Ten colonies were mixed with 30 μL of lysis buffer (Genotek, Korea) and overlaid with one drop of mineral oil. This mixture was heated for 5 min in a microwave oven (750w), then centrifuged for 10 min at 12,000 rpm. The supernatant containing DNA was stored at -20°C until further use.

PCR

Confirmation of species identification and determination of glycopeptide resistant genotypes were performed by PCR. The DNAs encoding *vanA, vanB, vanC1,* and *vanC2* were amplified with the primers described by Dukta-Malen *et al.* (1995). The PCR amplification mixture consisted of PCR buffer (Genotek, Korea), 0.2 mM (each) dATP, dCTP, dGTP, and dTTP (Genotek), 50 pmol of each primer, 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, and about 3 uL of DNA sample in a total volume of 25 μL. DNA amplification was carried out with a Turbo thermal cycler (Perkin-Elmer, USA). The reaction mixture was heated to 94°C for 5 min, followed by 30 cycles each consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. A final elongation step was carried out at 72°C for 5 min. A reagent blank (containing all the components of the reaction mixture except DNA) and positive control for each *van* genotypes were run in PCR procedures as controls.

PFGE

Pulse field gel electrophoresis (PFGE) was performed with the slight modification of Cameron *et al.* (1994). Four hundred μL of cell suspension, grown on a brain heart infusion broth, were harvested and washed with SE buffer (75 mM NaCl, 25 mM EDTA [pH 8.0]). Cell pellet was suspended in 300 μL of Pett IV sol. (1 M NaCl, 10 mM EDTA [pH 8.0]). An amount of this suspension (130 μL) was transferred and mixed with 1 μL of lysostaphin (5 mg/ml) and 130 μL of 1% agarose (InCert agarose, FMC) in SE buffer. Then 200 μL of this solution was pipetted into small plug molds.

The cells suspended in the agarose plugs were lysed by incubation for 1 h at 37°C in 1 mL of lysis solution (1 M NaCl, 6 mM Tris, 10 mM EDTA, 0.5% Sarkosyl [pH 8.0]) containing 0.5 mg of lysozyme, and 5 mg lysostaphin. Next, lysis solution was replaced by a 1 mL ES solution (0.25 M EDTA, 1% Sarkosyl [pH 8.0]) containing 10 μL proteinase K (10 mg/mL), which was then incubated at 50°C for 18 h. The plugs were then washed four times (10 min each time at room temperature) with TE buffer (10 mM Tris-HCl, 0.1 mM EDTA [pH 8.0]) containing 1 mM

phenylmethylsulfonyl fluoride (PMSF). Plugs were then stabilized for 30 min in 1.5 mL restriction buffer solution (10 mM Tris-HCl, 7 mM $MgCl_2$, 20 mM KCl, 3.5 mM 2-mercaptoethanol), and approximately 40 U of the restriction enzyme *Sma* I was added, and incubated at 30°C for 16 h. Electrophoresis (1% SeaKem agarose in 0.5×Tris-borat-EDTA [pH 8.0]) was performed with a contour-clampled homogeneous electric field apparatus (CHEF-DR II system, Bio-Red, USA), programmed in the auto-algorithm mode (block 1 run time, 8 h; switch time, 0.5 to 15 s; block 2 run time, 10 h; switch time, 15 to 30 s). The gels were stained with ethidium bromide for 15 min and then destained in distilled water for 1 h before being photographed under UV irradiation.

REP-PCR

Repetitive sequence based PCR was performed using a thermal cycler. Each 25 μL PCR reaction contains 50 pmol each of two opposing oligonucleotide primer, BOXA1R (Versalovic *et al.*, 1994), 100 ng of chromosomal DNA, 1.25 mM of each of 4 dNTPs, 2 units of *Taq* DNA ploymerase (Bioneer, Korea) in a reaction buffer with 10% DMSO (v/v). The following conditions were used for amplification: denaturation at 95°C for 30 s, annealing at 52°C for 1 min, and elongation at 65 for 8 min for each cycle. A total of 30 cycles were performed followed by a final elongation step at 65°C for 16 min. For the comparative analysis of DNA fingerprint patterns, 5 μL of each PCR reaction was electrophoresed at 5 V/cm directly on 1% agarose gels containing 1×Tris acetate-EDTA (TAE) and 0.5 μg/mL ethidium bromide.

Data analysis

The gels were visualized and the data obtained from two DNA fingerprints were analyzed with Gelcompar software (Applied Maths. Gent, Belgium). Dice analysis of peak positions was executed, and unweighted pair group method using arithmetic averages (UPGMA) was applied to get dendrograms.

RESULTS

Prevalence of VRE and antibiotic susceptibility

Seventy-eight VRE strains were isolated: 54 from chicken intestines, 11 from ICU patients, and 13 from healthy young children. It is observed that the VRE carriage rate was 41.5%, 20.4%, and 7.8%, respectively (Table I). Forty of 54 (74.1%) and 7 of 11 (81.8%) VRE strains isolated from chickens and patients, respectively, were identified as *E. faecium*. Twelve of 54 (22.2%) VRE strains from chickens were *E. faecalis*. In contrast, 11 of 13 VRE strains (84.6%) from healthy young children were identified as *E. gallinarium*.

Sixty-three strains showed VANA phenotype VRE: 52 from chickens, 9 from patients and 2 from healthy young children. Their vancomycin MIC ranged from 256 to ≥1,024 mg/L, and teicoplanin MIC ranged from 16 to ≥256 mg/L (Table II). Among them, 61 strains belonged to *E. faecium* and 2 strains, *E. faecalis*, which carried the *vanA* gene, as identified by PCR.

Fifteen VANC phenotype strains were isolated. For vancomycin resistant *E. gallinarum*, their vancomycin MIC was 8 mg/L, and teicoplanin MIC was ≤0.5 mg/L. PCR analysis confirmed that all *E. gallinarum* strains had the *vanC1* gene. While none had *vanB*.

Most of the VANA phenotype VRE strains, *E. faecium* and *E. faecalis* showed multi-drug resistance to erythromycin, kanamycin, tetracycline, and tobramycin. In contrast, most of the VANC phenotype VRE strains were susceptible to all the antibiotics tested (Table II).

Table I. Numbers and percentages of VRE isolated from chickens, non-hospitalized children and ICU patients

Genotype	Species	No. (%) of strains isolated from:		
		Chicken (n=130)	Healthy young children (n=166)	ICU patients (n=54)
vanA	*E. faecalis*	12(9.2)	–	–
	E. faecium	40(30.8)	2(1.2)	9(16.7)
vanC1	*E. gallinarum*	2(1.5)	11(6.6)	2 (3.7)
Total (%)		54(41.5)	13(7.8)	11(20.4)

Table II. Antibiotic MIC range of VRE isolates

Antibiotics	MIC (mg/L, range)	
	VANA VRE (63)	VANC VRE (15)
Vancomycin	512 - 1,024	8
Teicoplanin	16 - ≥256	≤0.5
Ampicillin	4	0.5 - 2
Ciprofloxacin	1 - 4	0.5 - 8
Erythromycin	≥256	≤1 - 256
Gentamicin	≤1	≤1 - 16
Kanamycin	≥128	16 - 32
Tetracycline	≥128	≤0.1 - 128
Tobramycin	≥128	4 - 16

Molecular typing of VRE

Twenty-one VANA *E. faecium* isolates were analyzed by PFGE using *Sma* I (Fig. 1). Most of the banding patterns comprised 9 to 16 DNA fragments ranged from 24.5 kb to 194 kb. Five genotypes of VANA *E. faecium* were identified at the similarity (S) level of 75%. The biggest cluster contained all the 3 isolates originated from healthy young children and 5 from chicken intestines. Single member cluster (strain 28, from chicken intestine) and two-mem-

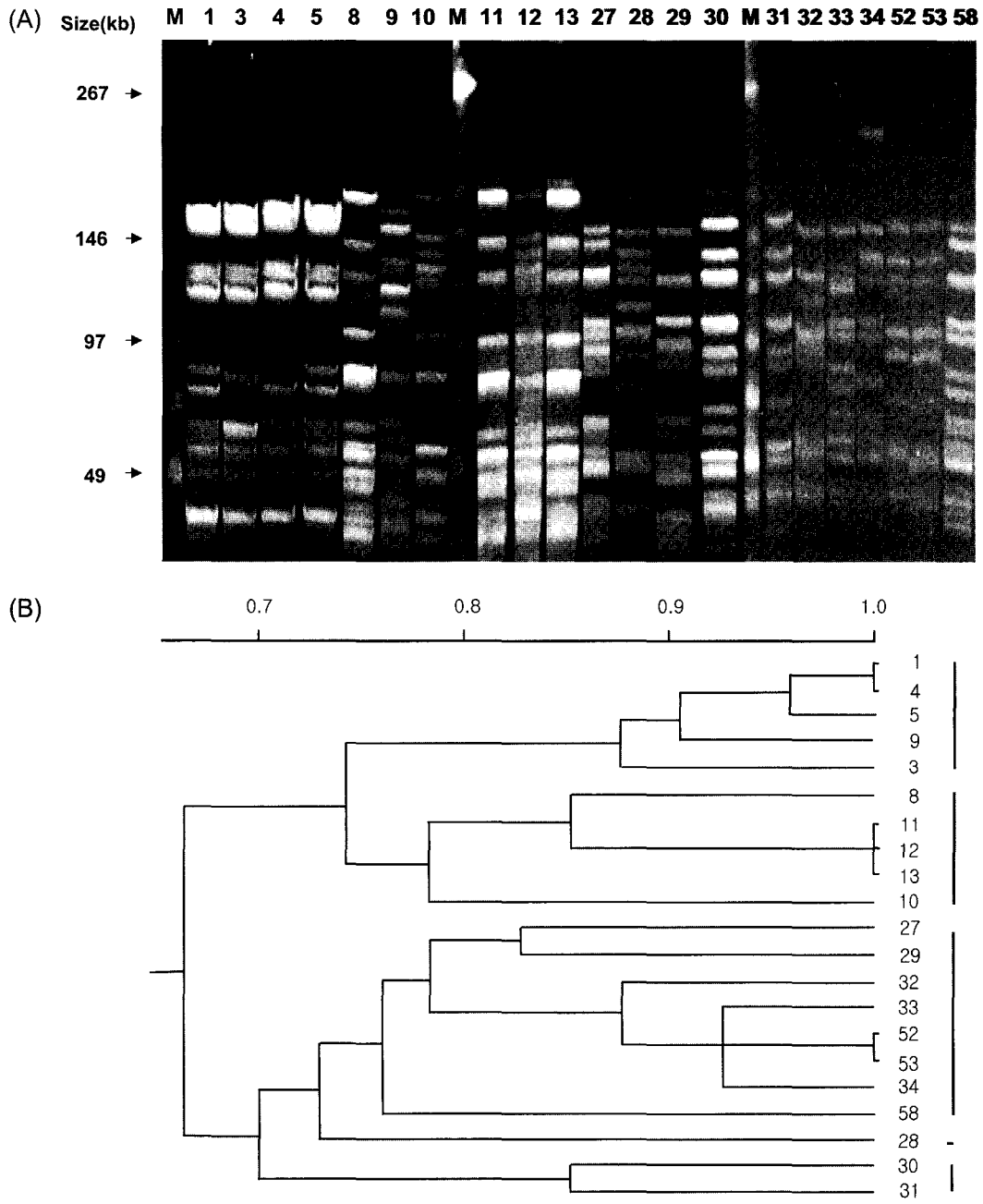

Fig. 1. *Sma*I restriction endonuclease pattern obtained by PFGE (A) and dendrogram (B) for *vanA E. faecium* isolates. Strains were described in Table III.

bered cluster (strain 30 and strain 31, from chicken intestine) located outside the biggest cluster. But these clusters joined as one group at 70% *S*-level. All the isolates originated from ICU patients clustered as one group at the 74% *S*-level. This cluster was distinct from the group composed by childrens and chicken isolates (Table III).

Twenty-six VANA *E. faecium* isolates containing 21 strains analyzed by PFGE, were again analyzed by Rep-PCR using BOXA1R primer. Most of the banding patterns comprised 7 to 15 different kinds of DNA fragments, and three bands were found in all isolates (Fig. 2). Five genotypes of VANA *E. faecium* were identified at the 60% *S*-level. Ten strains originated from chicken intestines formed the biggest cluster. Each 3 strains from healthy young children and chicken intestines clustered at the 65% *S*-level. This cluster was more similar to the biggest cluster than to other clusters. Other three clusters contained the isolates originated from ICU patients and joined as one group at the 50% *S*-

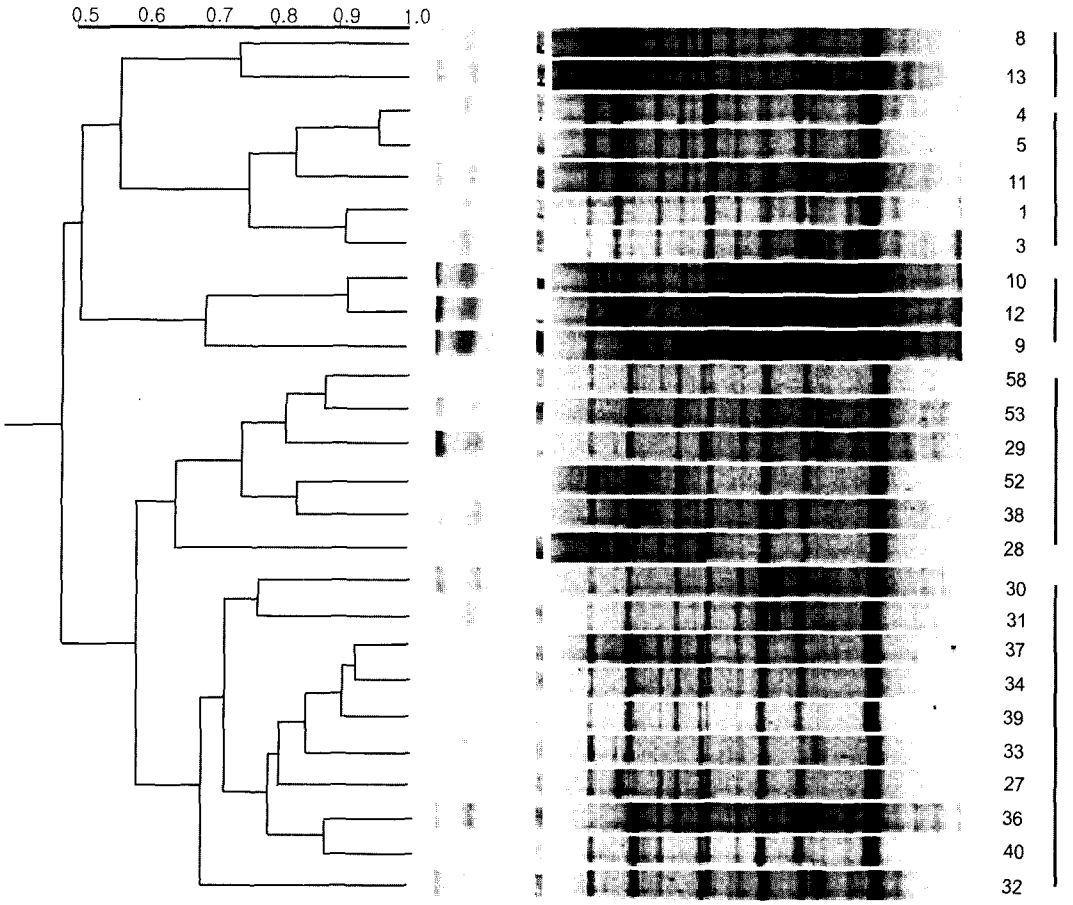

Fig. 2. Repetitive sequence based PCR fragment pattern obtained by electrophoresis and dendrogram for vancomycin-resistant *E. faecium*. strains were described in Table III.

Table III. Origin and antibiotic MICs of VANA *E. faecium* isolates

Strain No.	Vancomycin MIC (mg/L)	Teicoplanin MIC (mg/L)	Origin
1	1,024	128	Urine, ICU patient, Wonkang hospital, Iksan, Jeonguk, Korea
3	512	128	Wound, ICU patient, Wonkang hospital
4	1,024	128	Catheter, ICU patient, Wonkang hospital
5, 10	1,024	>256	Urine, ICU patient, Wonkang hospital
8, 9, 12	1,024	256	Urine, ICU patient, Wonkang hospital
11	512	64	Urine, ICU patient, Wonkang hospital
13	512	64	Wound, ICU patient, Wonkang hospital
27, 38	512	128	Chicken intestine, Iksan
28, 29, 32, 33, 37, 39, 40	512	64	Chicken intestine, Iksan
30	512	32	Chicken intestine, Iksan
31	512	128	Chicken intestine, Iksan
34	1,024	64	Chicken intestine, Iksan
36	512	16	Chicken intestine, Iksan
52, 53	512	64	Children, Play group, Suncheon, Jeonnam
58	512	64	Child, Play group, Iksan

level (Table III).

Two molecular types obtained by different methods showed that all 3 isolates from children, strain 52, strain 53, and strain 58 had higher similarity with the isolates

from chicken intestines. And the strains from ICU patients showed higher intra-cluster similarity, and this cluster was distinct from other clusters.

DISCUSSION

Glycopeptide resistance in enterococci isolated from living poultry has been associated with the use of oral glycopeptide antibiotics in animal feed (Gambartto *et al.*, 2000; Woodford *et al.*, 1995). High-level of resistance to glycopeptides has been shown to be mediated by transferable plasmids that may harbor resistant determinants to other drugs as well (Leclercq *et al.*, 1989). Therefore, other antimicrobial agents used as feed additives in veterinary medicine may also select for vancomycin resistance (Noble *et al.*, 1992). Comparison of resistant microorganisms derived from poultry with those derived from humans may shed light on the role of poultry as a possible reservoir of VRE.

Our study reports a high prevalence of VRE in chicken intestines and fecal samples from hospitalized patients and nonhospitalized young children. Because the subjects, children and patients, were collected from a place near an agricultural area where avopracin has been used in animal husbandry, VRE would be detected highly in non-hospitalized children as well as patients and chickens.

We found that 41.6% of chickens at the retail level were contaminated with VRE. The majority of these were *E. faecium* containing *vanA* gene. A study from the United Kingdom documented that 22 of 52 farm animals studied were colonized with vancomycin resistant *E. faecium*. Another study from Manchester, U.K. revealed that 90% of all uncooked chicken specimens contained VRE that were genetically distinguishable (Chadwick *et al.*, 1996). In the Netherlands 70% of the poultry products were contaminated with VRE containing *vanA* gene. Interestingly, VRE have so far not been recovered from animal sources in the United States, which may be related to the fact that glycopeptides are not licensed for use as feed additives in animal husbandry (Van den Braak *et al.*, 1998). There were few reports on the isolation of VRE from poultry products in Korea.

VRE were found in 20.4% of ICU patients. The prevalence found in this study seems close to the levels observed in the United States: 16% in Texas (Coque *et al.*, 1996) and 28% in New York (Montecalvo *et al.*, 1995). In contrast, in Europe, the prevalence of VRE in patients ranged from 2% in the Netherlands (Endtz *et al.*, 1997) to 37% of hematology patients in France (Gambartto *et al.*, 2000). Compared with the prevalence of VRE in patients (Kim *et al.*, 1997), we found that the carriage rate is increasing in Korea. However, comparison of the data is very difficult and should be done cautiously since the populations studied differ in size, age, sex ratio, method of principal diagnosis, etc.

The majority of the clinical isolates in France were *E. gallinarium* (Gambartto *et al.*, 2000). However, *E. faecium* was the prevalent isolates in patients in this study, but *E. faecalis* was not. This species distribution is similar to those frequently reported in previous studies. For example, majority (58.7%) of the VRE isolated from poultry products in the Netherlands were *E. faecium* (Coque *et al.*, 1996; Endtz *et al.*, 1997). A high prevalence of VRE in patients, in particular of *E. faecium,* in our study would pose a problem on the source and transmission of VRE in hospitals in Korea.

To the best of our knowledge this is the first study reporting the prevalence of VRE in non-hospitalized healthy young children whose ages range from 3 to 5 years. VRE were found in 13 out of 166 young children. Although their clinical history was not analyzed in detail, a high carriage rate of VRE in outpatient young children was intriguing. This rate seems closest to the levels of 11.8% of the control group whose average age is 36.2 years in France (Gambartto *et al.*, 2000). Fortunately, the majority of the VRE strains from children were *E. gallinarium*. Presence of these species is not always taken into account because their resistance to glycopeptides is intrinsic and their pathogenicities are very low. In our study, these species were susceptible to most of the antibiotics tested. However, additional research on the relevance of *E. gallinarium* as a potential pathogen in humans is needed.

Two main routes of dissemination of vancomycin resistance genes can be envisaged. First resistant strains may spread in a clonal fashion from host to host. Second, the resistance determinant may be passed on to other bacterial strains through conjugation (Salyers and Amabile-Cuevas, 1997). Molecular typing of the VRE strains isolated from different patients or poultry products in our study exhibited different patterns with those in France and the Netherlands (Gambartto *et al.*, 2000; Van den Braak *et al.*, 1998). This genetic unrelatedness of the VRE strains between the isolates from different sources such as patients or chickens possibly excludes transmission from host to host. On the other hand, most outbreaks in the United States are caused by the intra- or inter-hospital spread of clonal strains (Handwerger *et al.*, 1993; Perlada *et al.*, 1997) which suggests that host-to-host transmission is the major factor responsible for dissemination of VRE.

In this study, DNA fingerprinting patterns using Rep-PCR and PFGE pose the possibility of host-to-host transmission of VRE. VREs originated from young children and chicken intestines were identical or similar. While, strains from ICU patients were different from those from healthy

young children and chicken intestines. Thus our study suggests that the horizontal transmission of VRE occurred from poultry to humans, especially young children, *via* the food chains in Korea.

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