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# Prevalence and Antibiotic Susceptibility of Vancomycin-Resistant Enterococci in Chicken Intestines and Fecal Samples from Healthy Young Children and Intensive Care Unit Patients

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**The prevalence, resistance genotype and antibiotic susceptibility of vancomycin-resistant enterococci (VRE) were determined. Prevalence of VRE in chickens, healthy children and intensive care unit (ICU) patients was 43.0%, 12.7% and 24.1%, respectively. Forty out of 56 isolates from chicken intestines were identified as *Enterococcus faecium*, and 12 were *E. faecalis*. All the isolates contained the *vanA* gene. Nine out of 13 VRE isolates from patients and two out of 21 from healthy young children were identified as *E. faecium*. The resistance types of *E. faecium*, *E. gallinarium* and *E. casseliflavus* were VanA, VanC1, and VanC2, respectively. The mimimum inhibitory concentrations (MICs) of *E. faecium*, *E. gallinarium*, and *E. casseliflavus* to vancomycin were 512, 8 and 4 g/ml, respectively. Specifically, *E. faecium* isolates were resistant to most of antibiotics except ampicillin and gentamicin. This is the first report of high VanA type VRE prevalence in nonhospitalized young children in Korea.**

***Key words***: prevalence, antibiotic susceptibility, vancomycin-resistant Enterococci (VRE), healthy young children

Vancomycin-resistant enterococci (VRE) cause a health risk, especially in patients with severe underlying disease or immunosuppression (4, 24). High degree of heterogeneity is observed among the VRE strains (2, 6, 24). According to the vancomycin MIC and teicoplanin MIC, several phenotypes of VRE exist, and are determined by the determinant gene of *vanA, vanB, vanC1,* and *vanC2*. Because of the high level of vancomycin MIC and teicoplanin MIC, the VanA phenotype is distinct from other phenotypes. In contrast, low vancomycin MIC and teicoplanin MIC of other phenotypes make the identification of resistance phenotype difficult (3, 9, 11, 24).

Over the last few years, intrinsic or acquired resistance of enterococci to many antibiotics, in particular, to glycopeptides, has become a major cause of concern. Since the first report on VRE in 1986 in Europe, their presence has increasingly been detected throughout the world (15, 25). In Korea, VRE strain *Enterococcus durans* was first isolated in 1992 (20). Since then, the prevalence of VRE in hospitalized patients has been rising significantly (11, 12).

Furthermore, there are cases on record of the isolation of VRE from animals and from environmental sources. Nobel *et al.* (19) reported the *in vitro* conjugative transfer of high level vancomycin resistance from *E. faecalis* to *Staphylococcus aureus*. The increasing use of antimicrobial agents in human medicine and as animal growth promoters has been related to the emergence of VRE (17). In Europe, antimicrobial agents are widely used as feed additives for growth promotion in animal husbandry (23). 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 (13, 23). 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 (9, 24). Rather, the introduction and spread of the resistant VRE into humans may be by transmission of resistance genes (1, 22). Several studies suggest that high-level resistance to glycopeptides in enterococci is mediated by transposons similar to Tn*1546* (24).

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 (9, 24). We, therefore undertook an investigation of VRE intestinal colonization among chickens, patients, and healthy children. Then we determined the resistance

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**Table 1.** Nucleotide sequences of PCR primers

| Gene | Primer pair | Nucleotide sequence (5'-3') | Size of PCR product (bp) |
|---|---|---|---|
| *vanA* | vanA-1 | GCGGTATTGGAAACAGTGCC | 356 |
| | vanA-2 | GCGGRCAATCAGTTCGGGAAGTGC | |
| *vanB* | vanB-1 | GGAATGGGAAGCCGATAGTCTCC | 741 |
| | vanB-2 | GTTTAGAACGATGCCGCCATCC | |
| *vanC1* | vanC1-1 | CCCACTTTCCTTTTATCCCGC | 429 |
| | vanC1-2 | ACCCGTCAATCCCAAGTTTCG | |
| *vanC2* | vanC2-1 | CCTCTCTTTGATCGCC | 322 |
| | vanC2-2 | TGCAGCTTGATGCAGCC | |

genotype and antibiotic susceptibility of the isolates.

## Materials and Methods

***Chickens, patients, and healthy children***

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. From February to March, 1999, rectal swabs were taken from the 166 healthy young children (age range 3 to 5 years) in Sunchon (102) and Iksan (64) and 54 patients in the intensive care unit(ICU) of Won-kang University Hospital, Iksan, Korea.

***Isolation and identification of VRE***

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 (9). 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 (3). Motility, yellow pigmentation, and acidification of methyl-α-D-glucopyranoside (MGP), arabinose, lactose, raffinose, and mannitol of the VRE isolates were determined as recommended by Carvalho *et al.* (3). *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 PCR analysis. The motile isolates were stained with uranyl acetate (4%, w/v) and observed with transmission electron microscope (Hitachi 7100, Japan).

***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 no. 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 (18).

***DNA isolation***

VRE strains were grown overnight at 37°C on 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 genes *vanA, vanB, vanC1,* and *vanC2* were amplified with the primers (Table 1) described by Dukta-Malen *et al.* (7). The PCR amplification mixture consisted of PCR buffer (Genotek, Korea), 0.2 mM (each) dATP, dCTP, dGTP, and dTTP (Genotek, Korea), 50 pmol of each primer, 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase, and about 3 μl of DNA sample in a total volume of 25 μl. DNA amplification was carried out with a Turbo thermal cycler (Bioneer, Korea). 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 controls for each *van* genotype were run in PCR procedures as controls.

## Results and Discussion

Ninety VRE strains were isolated: 56 from chicken intestines, 13 from ICU patients, and 21 from healthy young children, thus the VRE carriage rate was 43.1%, 24.1%, and 12.7%, respectively (Table 2). Forty of 56 (71.4%) and 9 of 13 (69.2%) VRE strains isolated from chickens and patients, respectively, were identified as *E. faecium.*

**Table 2.** Numbers and percentages of VRE isolated from chickens, healthy young children and ICU patients

| Genotype | Species | No. (%) of strains isolated from: | | |
|---|---|---|---|---|
| | | Chickens (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) |
| *vanC2* | *E. casseliflavus* | 2(1.5) | 8(4.8) | 2(3.7) |
| Total(%) | | 56(43.1) | 21(12.7) | 13(24.1) |

**Table 3.** Properties for differentiating between *E. faecium, E. faecalis, E. gallinarum,* and *E. casseliflavus* isolates

| Tests[a] | Species | | | |
|---|---|---|---|---|
| | *E. faecium* (n=11)[b] | *E. faecalis* (n=12) | *E. gallinarum* (n=13) | *E. casseliflavus* (n=10) |
| TEL | 0[c] | 100 | 0 | 0 |
| PIG | 0 | 0 | 0 | 100 |
| MOT | 0 | 0 | 54 | 100 |
| ADH | 100 | 100 | 100 | 50 |
| PYR | 100 | 100 | 100 | 100 |
| MGP | 0 | 0 | 100 | 100 |
| ARA | 0 | 0 | 100 | 100 |
| LAC | 100 | 100 | 100 | 100 |
| RAF | 0 | 0 | 62 | 100 |
| MAN | 100 | 100 | 92 | 100 |

[a]TEL, growth at 0.04% tellurite; PIG, production of yellow pigment; MOT: motility; ADH, arginine dihydrolase; PYR, pyrase; ARA, acid production from arabinose; LAC, acid production from lactose; RAF, acid production from raffinose; MAN, acid production from mannitol.
[b]Number of strains tested.
[c]Percent positive.

In contrast, 11 of 21 VRE strains (52.4%) from healthy young children were identified as *E. gallinarium. E. faecalis* was isolated only from chickens.

The physiological properties of the isolates are summarized in Table 3. *E. faecium* and *E. faecalis* had the same properties except resistance to 0.04% tellurite. The two species were distinguished from *E. gallinarium* and *E. casseliflavus* in the acidification of MGP and arabinose. Differentiation of *E. gallinarium* from *E. casseliflavus* was done by yellow pigmentation. Fifty-four percent of *E. gallinarium* had a single polar flagellum and were motile (Fig. 1).

Sixty-three strains of the VanA phenotype were isolated: 52 from chickens, nine from patients and two from healthy young children. Vancomycin MICs ranged from 256 to ≥ 1,024 mg/l, and teicoplanin MICs ranged from 16 to ≥ 256 mg/l (Table 4). All these strains belonged to either *E. faecium* or *E. faecalis,* and carried the *vanA* gene, as shown by PCR (Fig. 2). Twenty-seven VanC phenotype strains were isolated. For vancomycin resistant *E.*

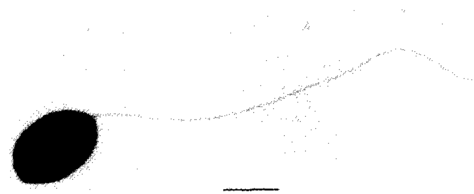

**Fig. 1.** Transmission electron microscopy of *E. gallinarum* W243 (VanC1) isolated from healthy child (bar=0.5 μm).

**Table 4.** *In vitro* activities of nine antimicrobial agents against VRE isolates

| Organism and antimicrobial agents | MIC (μg/ml)[a] | | | % resistant |
|---|---|---|---|---|
| | Range | 50% | 90% | |
| *E. faecium* (n=11)[b] | | | | |
| Vancomycin | 512 | 512 | 512 | 100 |
| Teicoplanin | 64-128 | 64 | 128 | 100 |
| Ampicillin | 4 | 4 | 4 | 0 |
| Ciprofloxacin | 1-4 | 1 | 4 | 27 |
| Erythromycin | ≥ 256 | ≥ 256 | ≥ 256 | 100 |
| Gentamicin | ≤ 1 | ≤ 1 | ≤ 1 | 0 |
| Kanamycin | ≥ 128 | 128 | ≥ 128 | 100 |
| Tetracycline | ≥ 128 | ≥ 128 | ≥ 128 | 100 |
| Tobramycin | ≥ 128 | ≥ 128 | ≥ 128 | 100 |
| *E. gallinarum* (n=13) | | | | |
| Vancomycin | 8 | 8 | 8 | 0 |
| Teicoplanin | ≤ 0.5 | ≤ 0.5 | ≤ 0.5 | 0 |
| Ampicillin | 0.5-2 | 1 | 2 | 0 |
| Ciprofloxacin | 0.5-8 | 1 | 4 | 8 |
| Erythromycin | ≤ 1-256 | ≤ 1 | ≤ 1 | 46 |
| Gentamicin | ≤ 1-16 | ≤ 1 | ≤ 1 | 46 |
| Kanamycin | 16-32 | 32 | 32 | 0 |
| Tetracycline | ≤ 0.1-128 | 64 | 128 | 62 |
| Tobramycin | 4-16 | 4 | 8 | 0 |
| *E. casseliflavus* (n=10) | | | | |
| Vancomycin | 4 | 4 | 4 | 0 |
| Teicoplanin | ≤ 0.5 | ≤ 0.5 | ≤ 0.5 | 0 |
| Ampicillin | 1 | 1 | 1 | 0 |
| Ciprofloxacin | 2-4 | 2 | 4 | 50 |
| Erythromycin | 8-16 | 8 | 16 | 100 |
| Gentamicin | ≤ 1 | ≤ 1 | ≤ 1 | 0 |
| Kanamycin | 32 | 32 | 32 | 0 |
| Tetracycline | ≤ 1 | ≤ 1 | ≤ 1 | 0 |
| Tobramycin | 4 | 4 | 4 | 0 |

[a]50% and 90%, MICs at which 50% and 90% of strains are inhibited, respectively.
[b]Number of strains tested.

*gallinarum*, vancomycin MICs were 8 mg/l, and teicoplanin MICs were ≤ 0.5 mg/l. For vancomycin resistant *E. casseliflavus*, vancomycin MICs were 4 mg/l, and teicoplanin MICs were ≤ 0.5 mg/l. PCRs confirmed that all *E. gallinarum* strains had the *vanC1* gene and that all *E. casseliflavus* strains had the *vanC2* gene. No *vanB* strains were detected. The problem of the lower van-

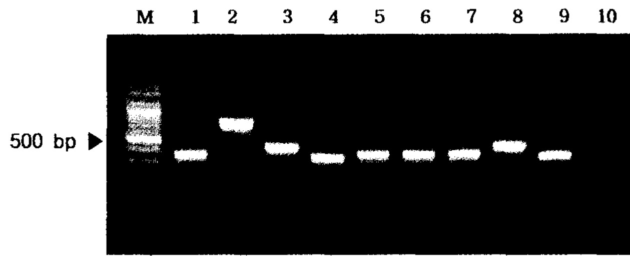


**Fig. 2.** Agarose gel electrophoresis of *vanA*, *vanB*, *vanC1* and *vanC2* enterococci genes by PCR. Lane M, Marker; lane, 1 *E. faecalis* CDC-286 (*vanA* positive control); lane 2, *E. faecalis* CDC-583 (*vanB* positive control); lane, 3 *E. gallinarum* CDC-42 (*vanC1* positive control); lane 4, *E. casseliflavus* ATCC25788 (*vanC2* positive control); lane 5, *E. faecium* H232; lane 6, *E. faecium* H233; lane 7, *E. faecium* H238, lane 8, *E. gallinarum* H243; lane 9, *E. casseliflavus* H228; lane 10, negative control (without DNA).

comycin MIC for *E. casseliflavus* compared to the vancomycin concentration in isolation procedure was a task to solve. One of the possible reason is the use of different medium in each step.

Most of the VanA phenotype VRE strains, *E. faecium* and *E. faecalis* showed multi-drug resistance to erythromycin, kanamycin, tetracycline, and tobramycin. Most of the VanC phenotype VRE strains were susceptible to all the antibiotics tested except *E. casseliflavus* to erythromycin (Table 4).

Glycopeptide resistance in enterococci isolated from living poultry has been associated with the use of oral glycopeptide antibiotics in animal feed (9, 23). High-level resistance to glycopeptides has been shown to be mediated by transferable plasmids that may harbor resistant determinants to other drugs as well (14). Therefore, other antimicrobial agents used as feed additives in veterinary medicine may also select for vancomycin resistance (19).

We found that 43% 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 (4). 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 there (24). There were few reports on the isolation of VRE from poultry products in Korea.

VRE were found in 24% of ICU patients. The prevalence found in this study seems closest to the levels observed in the United States: 16% in Texas (5) and 28% in New York (16). In contrast, in Europe, the prevalence of VRE in patients ranged from 2% in the Netherlands (8) to 37% of hematology patients in France (9). When compared with the 1997 prevalence of VRE in patients (11), 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, principal diagnosis, etc.

The majority of the clinical isolates were *E. faecium*, and no *E. faecalis* strains were detected, while, the majority in France was *E. gallinarium* (9). 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* (5, 8). 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 from Korea reporting the prevalence of VRE in healthy young children whose ages range from 3 to 5 years. VRE were found in 21 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 interesting. This rate seems closest to the levels of 11.8% of the control group whose average age is 36.2 years in France (9). Fortunately, the majority of the VRE strains from children were *E. gallinarium*. These species are 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 (22). Molecular typing of the VRE strains isolated from different patients or poultry products showed different patterns in France and the Netherlands (9, 24). 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 (9, 24). On the other hand, most outbreaks in the United States are caused by the intra- or interhospital spread of clonal strains (10, 21), which suggests that host-to-host transmission is the major factor responsible for dissemination of VRE.

In conclusion, our study reports a high prevalence of VRE in chicken intestines and fecal samples from hospitalized patients and nonhospitalized young children living in the same local community. The fact that the subjects, children and patients were collected from a place near an agricultural area where avopracin has been used in animal husbandry may contribute to the high levels of VRE detected in healthy children as well as patients and chick-

ens. Further studies, such as molecular typing of the isolates, are needed to clarify the route of VRE transmission.

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