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# Vancomycin-Resistant Enterococci in Humans and Imported Chickens in Japan

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**The phenotypes and genotypes of 22 VanA-type vancomycin-resistant enterococci that had been isolated in Japan were examined. The VanA resistance determinant was plasmid mediated in each of the 22 strains. Of the 22 strains, 8 were isolated from different patients and 11 and 3 were obtained from different samples of chickens imported from Thailand and France, respectively. Three of the strains that were isolated from patients and the 11 strains isolated from the Thai chickens showed high-level vancomycin resistance (MICs, 512 to 1,024 μg/ml) and low-level teicoplanin resistance (MICs, 0.5 to 4 μg/ml). Each of these strains had three amino acid substitutions in the N-terminal region of the deduced VanS sequence. L50 was converted to V, E54 was converted to Q, and Q69 was converted to H compared to the *vanS* gene sequence of Tn*1546*.**

Since the first reports of infections and colonizations by vancomycin-resistant enterococci (VRE) in France (10) and the United Kingdom (15), there have been reports of VRE in many locations in the United States and the European Union. The major factors contributing to the dissemination of VRE in the United States and Europe are now evident. In the United States, the major factor is likely the excessive use of glycopeptide antibiotics in the health care environment, resulting in a selective increase in VRE in human intestines (8, 11). In Europe, it is strongly suggested that the use of avoparcin as a growth promoter in animal feed has resulted in the selective increase in VRE in animal intestines, and the VRE subsequently appear in the human community (9, 13, 16, 17). In Japan, vancomycin injections have been used for the treatment of methicillin-resistant *Staphylococcus aureus* infections since November 1991 and infection or colonization due to VRE is rare (6, 7, 9). Until 2000, a total of eight patients with VRE were detected in four hospitals. We have conducted an investigation of VRE in imported and domestic chickens every year since 1998 with the support of the Japanese Ministry of Health, Labor, and Welfare (9). VRE have never been isolated from domestic chickens. However, VRE are isolated at a high frequency in samples from imported chickens. VRE isolation frequencies of about 20% and 30 to 50% have been observed in Thailand and France, respectively, where avoparcin has been used in animal feed (9). We compared the phenotypes and genotypes of VRE isolates from humans with those of isolates from imported chickens in Japan.

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**Glycopeptide resistance and pulsed-field gel electrophoresis (PFGE) analysis of VRE strains.** Twenty-two VRE strains were examined in this study. Of these strains, eight strains were isolated from eight different patients at four hospitals in Japan up until 2000 and 11 and 3 strains were obtained from chickens imported from Thailand and France, respectively, in investigations carried out in 1998 and 1999 (Table 1).

The levels of glycopeptide resistance were examined in the VRE strains (Table 1). Of the eight strains isolated from the patients, five strains from patients at two hospitals showed high-level vancomycin and teicoplanin resistance, with MICs of 256 to 512 μg/ml and 64 to 128 μg/ml, respectively, and the other three strains from the patients at two hospitals showed high-level vancomycin resistance, with MICs of 512 to 1,024 μg/ml, and low-level teicoplanin resistance, with MICs of 0.5 to 4 μg/ml. Of the strains isolated from the imported chickens, all four strains isolated from French chicken samples showed high-level vancomycin and teicoplanin resistance, with MICs of 512 μg/ml and 64 to 128 μg/ml, respectively. All 11 strains isolated from Thai chicken samples showed high-level vancomycin resistance, with MICs of 256 to 1,024 μg/ml, and relatively low-level teicoplanin resistance, with MICs of 2 to 8 μg/ml.

The DNA from each of the VRE strains was analyzed by PCR for the presence of vancomycin resistance gene(s) with *vanA*-, *vanB*-, and *vanC*-specific primers (4, 5, 12). All strains gave rise to the expected 732-bp product with the primer specific for the *vanA* gene, indicating that the strains were VanA-type VRE (data not shown).

Banding patterns obtained by PFGE of *Sma*I-digested genomic DNA were used to compare the VRE strains. Figure 1 shows the PFGE patterns of VRE strains isolated from Thai chickens and VRE strains KV12, KV21, and CV1 isolated from patients, which showed high-level vancomycin resistance and low-level teicoplanin resistance, and also VRE strain FN1

TABLE 1. Characteristics of VRE isolated from humans and imported chickens in Japan

| Class and species | Strain[a] | Origin | Source (specimen and/or reference) | Transfer frequency of vancomycin[b] resistance from wild-type strain to *E. faecalis* FA2-2 (no. of transconjugants/ donor cell) | MIC (μg/ml)[c] | | Deduced amino acid sequence or amino acid substitution(s) of VanS of VanA-type determinant |
|---|---|---|---|---|---|---|---|
| | | | | | Vancomycin | Teicoplanin | |
| High teicoplanin resistance | | | | | | | |
| *E. faecium* | BM4147 | France | Human (1) | $<10^{-8}$ | 1,024 | 256 | Prototype |
| *E. faecium* | FN1 | Japan | Human (urine; 6) | $<10^{-8}$ | 256 | 128 | Prototype |
| *E. faecium* | EF1 | Japan | Human (feces) | $<10^{-8}$ | 512 | 128 | Prototype |
| *E. faecium* | EF2 | Japan | Human (feces) | $<10^{-8}$ | 512 | 64 | Prototype |
| *E. faecium* | EF3 | Japan | Human (drainage) | $<10^{-8}$ | 512 | 64 | Prototype |
| *E. faecium* | EF4 | Japan | Human (feces) | $<10^{-8}$ | 512 | 64 | Prototype |
| *E. faecium* | FV1 | France | Chicken | $<10^{-8}$ | 512 | 64 | Prototype |
| *E. faecium* | FV2 | France | Chicken | $10^{-5}$ | 512 | 64 | Prototype |
| *E. faecalis* | FV3 | France | Chicken | $<10^{-8}$ | 512 | 128 | D58L |
| Low teicoplanin resistance | | | | | | | |
| *E. faecium* | KV12 | Japan | Human (feces) | $10^{-4}$ | 1,024 | 4 | L50V, E54Q, Q69H |
| *E. faecium* | KV21 | Japan | Human (feces) | $10^{-4}$ | 512 | 2 | L50V, E54Q, Q69H |
| *E. faecium* | CV1 | Japan | Human (feces) | $<10^{-8}$ | 512 | 0.5 | L50V, E54Q, Q69H |
| *E. faecalis* | TV1 | Thailand | Chicken | $10^{-4}$ | 512 | 4 | L50V, E54Q, Q69H |
| *E. faecalis* | TV2 | Thailand | Chicken | $<10^{-8}$ | 512 | 4 | L50V, E54Q, Q69H |
| *E. faecalis* | TV3 | Thailand | Chicken | $<10^{-8}$ | 512 | 16 | L50V, E54Q, Q69H |
| *E. faecalis* | TV4 | Thailand | Chicken | $<10^{-8}$ | 1,024 | 16 | L50V, E54Q, Q69H |
| *E. faecalis* | TV5 | Thailand | Chicken | $10^{-5}$ | 1,024 | 2 | L50V, E54Q, Q69H |
| *E. faecalis* | TV6 | Thailand | Chicken | $<10^{-8}$ | 256 | 4 | L50V, E54Q, Q69H |
| *E. durans* | TV7 | Thailand | Chicken | $<10^{-8}$ | 512 | 2 | L50V, E54Q, Q69H |
| *E. durans* | TV8 | Thailand | Chicken | $<10^{-8}$ | 512 | 2 | L50V, E54Q, Q69H |
| *E. durans* | TV9 | Thailand | Chicken | $10^{-5}$ | 512 | 4 | L50V, E54Q, Q69H |
| *E. durans* | TV10 | Thailand | Chicken | $10^{-4}$ | 512 | 16 | L50V, E54Q, Q69H |
| *E. durans* | TV11 | Thailand | Chicken | $10^{-5}$ | 1,024 | 16 | L50V, E54Q, Q69H |
| *E. faecalis* | GV2 | Japan | Broiler farm droppings (18) | $10^{-4}$ | 512 | 2 | L50V, E54Q, Q69H |

[a] *E. faecium* BM4147 is a representative VanA-type strain, and the glycopeptide resistance determinant is encoded on transposon Tn*1546*. Strains TV1, TV2, TV3, TV7, and FV3 and strains TV4, TV5, TV6, TV8, TV9, TV10, TV11, FV1, and FV2 were isolated in investigations done in March 1998 and March 1999, respectively. Strains KV12 and KV21 and strain CV1 were isolated in November 1997 and July 1998, respectively.

[b] Filter matings were performed with a donor/recipient ratio of 1:4. The recipient strain was *E. faecalis* FA2-2 (Rif$^r$ Fus$^r$). The donor and recipient cells were trapped on a membrane filter (Millipore Corporation, Bedford, Mass.), incubated on a Todd-Hewitt broth (THB) agar plate at 37°C for 18 h, and suspended in 1 ml of THB. Appropriate dilutions of the mating mixture were plated onto THB agar plates containing vancomycin at 12.5 μg/ml plus rifampin at 25 μg/ml and fusidic acid at 25 μg/ml for counterselection of the donor strain, and the plates were incubated for 48 h at 37°C.

[c] Glycopeptide resistance levels were determined by the agar dilution method with Mueller-Hinton agar in accordance with NCCLS criteria.

(6) (Fig. 1; Table 1). Of these strains, two (KV12 and KV21) isolated from patients at a hospital and three (TV9, TV10, and TV11) isolated from Thai chickens, respectively, differed by one or two bands, indicating that strains of each group were related. Other strains showed different patterns, indicating that these VRE strains are different (Fig. 1)

**Conjugative transfer of vancomycin resistance.** Each of the 22 VRE strains harbored several plasmids. The *Eco*RI restriction profiles of total plasmid DNAs isolated from the VRE strains were different (data not shown). The v*anA* probe hybridized to a specific *Eco*RI fragment (4.1 kb) of plasmid DNA in each of the strains that is specific to the prototype VanA-type gene encoded on Tn*1546* (5), with the exception of the plasmid DNAs of one strain (FV2), where the probe hybridized to a 5-kbp *Eco*RI fragment (data not shown). The transferability of the vancomycin resistance of the VRE strains was examined between each of the strains and recipient strain *Enterococcus faecalis* FA2-2 (Rif$^r$ Fus$^r$) (3) by filter mating. The vancomycin resistances of 5 of the 11 strains from Thai chickens and 2 of the 3 strains from the patients, which were high-level vancomycin resistant and low-level teicoplanin resistant, and the vancomycin resistance of one of the three strains from French chickens, which were high-level vancomycin and teicoplanin resistant, were transferred to the recipient *E. faecalis* FA2-2 (Rif$^r$ Fus$^r$) strain at about $10^{-4}$ to $10^{-5}$ cells per donor cell by filter mating (Table 1). After repeated transfer experiments done by short mating between *E. faecalis* FA2-2 (Rif$^r$ Fus$^r$) and *E. faecalis* JH2SS (Str$^r$ Spc$^r$) (14), the transferable plasmid from each of the strains was identified from the transconjugant (Fig. 2). The plasmid DNA of the transconjugants of two strains (KV12, and KV21) isolated from patients at a hospital and three strains (TV9, TV10, and TV11) isolated from Thai chickens, respectively, exhibited the same *Eco*RI restriction profiles (Fig. 2). The *vanA* probe hybridized to a specific *Eco*RI fragment (4.1 kb) (5) of conjugative plasmid DNA of each the transconjugants, with the exception of the plasmid DNA isolated from strain FV2. These results indicated that the VanA determinants, which are encoded on

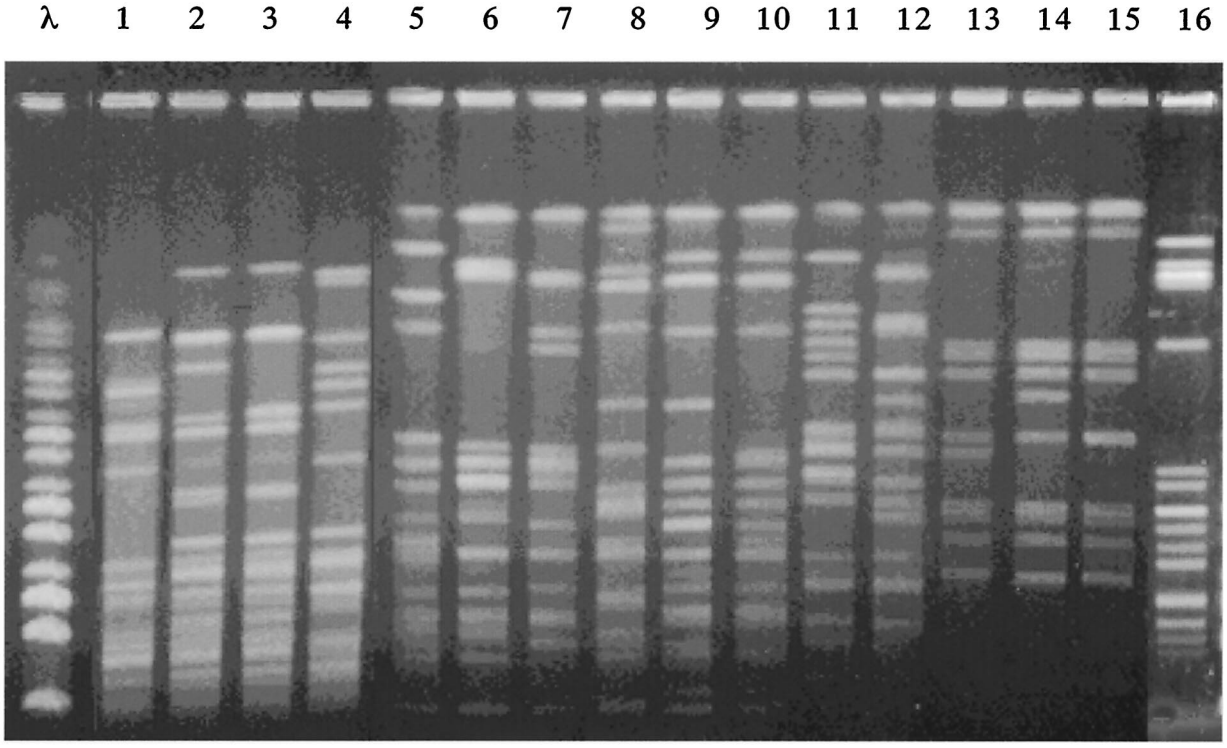


FIG. 1. PFGE of *Sma*I-digested chromosomal DNAs isolated from VRE strains. λ, a bacteriophage λ DNA ladder used as a molecular size marker. Lanes 1 to 16, VRE strains CV1, KV12, KV21, FN1, TV1, TV2, TV3, TV4, TV5, TV6, TV7, TV8, TV9, TV10, TV11, and GV2, respectively.

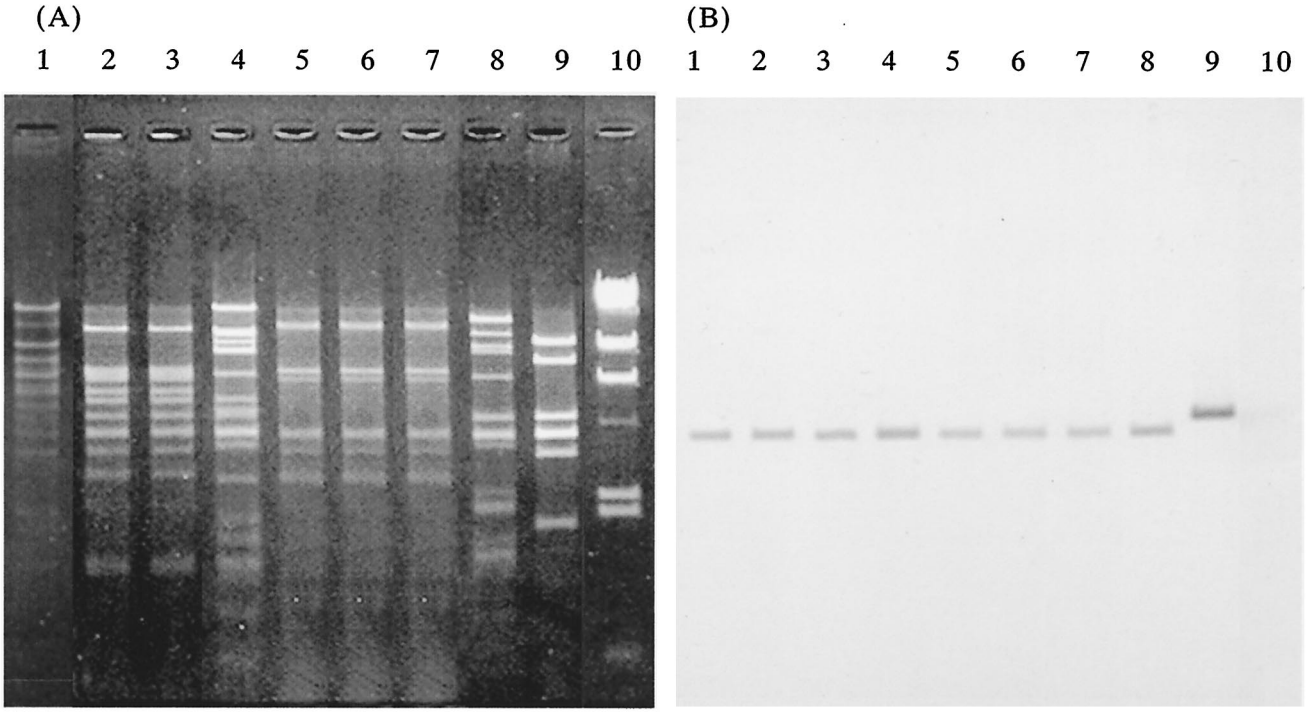


FIG. 2. Agarose gel electrophoresis of restriction endonuclease-digested conjugative plasmid DNA and hybridization with a *vanA*-specific probe. (A) Agarose gel electrophoresis of *Eco*RI-digested plasmid DNA isolated from each the transconjugants. (B) The gel was Southern blotted and hybridized to a *vanA*-specific probe. Lanes 1 to 9, plasmid pMG2 and plasmid DNAs isolated from transconjugants of VRE strains KV12, KV21, TV1, TV9, TV10, TV11, TV5, and FV2, respectively. Lanes 10, *Hin*dIII-digested lambda DNA.

TABLE 2. Nucleotide sequences of PCR primers for *vanS* of the VanA-type determinant encoded on Tn*1546*

| Primer no., name[a] | Target gene | Nucleotide sequence | Position[b] | Size of PCR product (bp) | Reference |
|---|---|---|---|---|---|
| 1, VanS | *vanS* | 5′-AACGACTATTCCAAACTAGAAC | 4676–4697 | 1,094 | 12 |
| 2, VanS1 | *vanS* | 5′-GCTGGAAGCTCTACCCTAAA | 5769–5749 | | 12 |
| 3, VanS2 | *vanS* | 5′-CTTAAATCACCTGGACGCGATG | 4822–4843 | | 7 |
| 4, VanS3 | *vanS* | 5′-GCAGGATGCAAAGCTGGCCG | 5083–5102 | | 7 |
| 5, VanS4 | *vanS* | 5′-GAATACTAATGACAATCGCC | 4913–4894 | | 7 |

[a] Primers 1 and 2 are a primer pair for amplification of *vanS*. Primers 3 to 5 were used to determine the nucleotide sequence of *vanS*.
[b] The positions given are from the first base of the coding sequence of the left inverted repeat of Tn*1546*. The nucleotide sequence of Tn*1546* was obtained from the GenBank nucleotide sequence database (accession no. M97297).

transposons such as Tn*1546*, are carried on a conjugative plasmid and can transfer between enterococci and also to different replicons by transposition.

**DNA sequence analysis of the *vanS* gene of the vancomycin determinants encoded on the plasmids.** A nationwide survey was conducted to investigate VRE in broiler farms in Japan in December 1996 (18). Three VanA-type VRE strains were isolated from droppings obtained from 3 of 35 broiler farms in 24 prefectures in Japan that used avoparcin (18). The three VanA-type VRE isolates showed high-level vancomycin resistance and relatively low-level teicoplanin resistance, and the VanA determinant of *E. faecalis* GV2, which is one of the three isolates, was analyzed in detail (7). The glycopeptide resistance of *E. faecalis* GV2 is mediated by conjugative plasmid pMG2 (85 kb). The nucleotide sequences of the genes for the VanA determinant encoded on pMG2 are completely identical to those of the prototype VanA-type gene encoded on Tn*1546*, with the exception of the gene for glycopeptide sensor protein VanS (1). There are three amino acid substitutions in the N-terminal region of the deduced sequence encoded by *vanS* (Table 1).

Nucleotide sequence analysis of the *vanS* gene of the VRE strains was performed by sequencing PCR products with *vanS*-specific primers (Table 2). To analyze the *van* determinant corresponding to *vanS* of the VanA-type determinant encoded on Tn*1546* (2), one or two pairs of PCR primers specific for *vanS* were used for PCR amplifications. The pairs of PCR primers were also used to sequence the amplified genes. The nucleotide sequence and the deduced amino acid residues of the *vanS* gene of the VRE strains isolated from Thai chickens and from three patients at two hospitals revealed three amino acid substitutions in the N-terminal region of the deduced VanS sequence. L50 was converted to V, E54 was converted to Q, and Q69 was converted to H compared to the *vanS* gene sequence of Tn*1546* (Table 1).

The VRE strains gave rise to the expected PCR products of 1,744, 1,856, and 926 bp with the primers specific for open reading frame (ORF) 1, both ORFs 1 and 2, and ORF2, respectively, which are located on Tn*1546* and encode a transposase (ORF1) and a resolvase (ORF2) (data not shown) (12). These results suggested that the VanA-type determinants of VRE strains were encoded on transposon Tn*1546* or a closely related transposon.

**Concluding comments.** The VRE strains isolated from French chickens exhibited high-level vancomycin and teicoplanin resistance and could not be distinguished from the representative VanA-type VRE (2, 5, 12). The VRE strains isolated from Thai chickens exhibited high-level vancomycin resistance and relatively low-level teicoplanin resistance, had three amino acid substitutions in the *vanS* gene of the VanA-type determinant, and were distinguished by these characters from the representative VanA-type VRE. The VRE strains isolated from three patients at the two hospitals showed the same characteristics as the VRE strains from the Thai chickens. It is very rare that identical substitutions would occur independently in each of the strains. Japan imports about 600,000 tons of chicken a year, while the nation's domestic chicken production is about 1.2 million tons a year. About 100,000 tons (16.6%) of the imported chicken come from Thailand. Although isolation of VRE is rare in enterococci from inside or outside the health care environment, these results imply that the VanA-type vancomycin resistance determinant, which resides on transposons such as Tn*1546* and can be transferred between enterococci by a conjugative plasmid, can transfer from chickens to humans.

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