

## Vancomycin-Resistant Enterococci from Nosocomial, Community, and Animal Sources in the United States

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**The presence of vancomycin-resistant enterococci (VRE) was looked for in fecal samples from 104 healthy volunteers (3 with hospital exposure), 100 selected hospitalized patients, and various environmental sources (44 commercial chickens, 5 farm-raised chickens, 3 turkeys, and 2 chicken farm lagoon slurries). Five probiotic preparations were also studied. No VRE with *vanA* or *vanB* genes were isolated from the healthy volunteers without hospital exposure, environmental sources, or probiotic preparations. VRE with *vanB* were found in the stools of 16% of the high-risk hospitalized patients and in one volunteer with hospital contact. All VRE examined could be classified into one of two clones by pulsed-field gel electrophoresis. VRE from 11 of the colonized patients were quantified and ranged from  $10^3$  to  $10^6$  CFU/g of stool. This study, in contrast to findings in Europe, failed to find evidence of VanA- or VanB-type VRE in the community or environmental sources in Houston, Texas, and suggests that these settings are not a likely source of VRE in hospitals in this geographic area.**

Vancomycin resistant enterococci (VRE) were isolated in Europe in 1986 (28, 46) and in 1987 in the central United States (37). In recent years, the nosocomial prevalence of VRE in the United States has increased from 0.3% in 1989 to 7.9% in 1993 (7), and clonally related isolates have often been observed in different patients, including some hospitalized in different cities (9, 31). In contrast to findings in the United States, the presence of clinical isolates of VRE in European hospitals is still rather infrequent (51), and great heterogeneity has been reported among typed isolates from the nosocomial setting (5, 8, 22, 36).

Three phenotypic classes of glycopeptide resistance have been described: VanA, VanB, and VanC (3). *Enterococcus gallinarum* and *Enterococcus casseliflavus* contain the species-specific genes *vanC1* and *vanC2*, respectively, which can confer low to moderate levels of resistance (VanC phenotype) (29, 35). Acquired resistance to glycopeptides (VanA and VanB) has been reported for *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus raffinosus*, *Enterococcus avium*, *Enterococcus durans*, *Enterococcus hirae*, *Enterococcus mundtii*, *E. gallinarum*, and *E. casseliflavus* (10, 15, 20, 26, 45, 47). *E. faecium* has been the most often isolated species and VanA has been the most often reported phenotype (7). Nonetheless, because of difficulties in detecting some VanB-type VRE (41, 42) and because some studies have been designed to detect the high levels of resistance more characteristic of the VanA phenotype (24, 25), VanB-type VRE could be more widespread than previously thought.

Although major strides have been made toward understanding the mechanism of vancomycin resistance (3) and the spread of VRE within the nosocomial environment (31), the epidemiology of VRE outside the hospital setting remains less well defined. In Europe, investigators have found VRE in healthy

volunteers (22, 24, 48), sewage (23, 44), and animal sources, including uncooked chickens purchased from retail outlets (1, 4, 25). However, the presence of VRE in community and environmental settings in the United States has not been defined.

Following the first isolation of VRE in our hospital in June 1994, we initiated a study to determine if spread of this organism to other patients could be documented and to determine if the outpatient community was a likely source. In this study, we surveyed fecal samples of hospitalized patients, healthy volunteers, and different environmental sources including commercial chickens, farm-raised chicken and turkeys, chicken farm lagoon slurries, and probiotic preparations from the Houston, Texas, metropolitan area, for the presence of VRE and classified these VRE with respect to the type of vancomycin resistance gene and clonal relatedness.

### MATERIALS AND METHODS

**Sources of bacterial isolates.** Between June 1994 and July 1996, single-stool specimens were obtained from the following sources: (i) healthy volunteers, 18 to 45 years of age, without prior medical illness or recent antimicrobial exposure ( $n = 104$ ; 101 of 104 had no hospital exposure); (ii) inpatients at a tertiary care university-affiliated hospital who had been hospitalized for at least 8 days ( $n = 100$ ); samples included fecal specimens submitted to the microbiology laboratory for other purposes ( $n = 47$ ), stool samples from patients in the same units in which clinical isolates of VRE had been recovered ( $n = 14$ ), and stool samples from patients treated with vancomycin ( $n = 39$ ); and (iii) freshly slaughtered farm-raised chickens ( $n = 5$ ) and turkeys ( $n = 3$ ). Cultures were also obtained from 44 fresh chickens purchased from six different local grocery stores (Houston, Texas), two samples from chicken farm lagoon slurries in Texas, and five probiotic preparations processed in geographically diverse locations (Super Carrot Acidophilus, Source Naturals, Inc., Scotts Valley, Calif.; Power Dophilus, Country Life, Hauppauge, N.Y.; Protec Acidophilus; Natural Factors, Santa Barbara, Calif.; Children's PB8, Nutrition Now, Portland, Oreg.; and PrimeLife, Klair Laboratories, Inc., San Marcos, Calif.).

Informed consent was obtained from all participating volunteers. This study was approved by the University of Texas Committee for the Protection of Human Subjects.

**Sample processing.** For human fecal samples from 104 healthy volunteers and 61 hospitalized patients, 0.5 g of feces was suspended in 4.5 ml of normal saline and serially 10-fold diluted. A 100- $\mu$ l aliquot of the dilutions was used to inoculate *Streptococcus faecalis* medium (SF agar; Difco Laboratories, Detroit,

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Mich.) containing 6 µg of vancomycin (Van) per ml; CNA agar (Difco Laboratories) containing 6 µg of vancomycin per ml plus 10 µg of amphotericin per ml; and SF agar and CNA plates without antibiotics. The latter two were used as controls for detection of any enterococci. Thirty-nine fecal specimens from hospitalized patients were plated directly on the above media. The plates were incubated at 37°C and examined at 24, 48, and 72 h. For 40 of the samples isolated from healthy volunteers, a 100-µl aliquot of the above suspension was also inoculated into 4.5 ml of SF broth plus 6 µg of vancomycin per ml. For 10 of the samples from normal volunteers, 0.5 g was inoculated into 4.5 ml of brain heart infusion (BHI) (Difco Laboratories) broth, SF broth, and SF broth plus 6 µg of vancomycin per ml. Tubes were incubated at 37°C for 48 h with shaking. Aliquots of 100 µl from the tubes showing growth were then plated on the above media. Five colonies of each morphology seen on plates containing antibiotics were subcultured onto BHI agar plus 6 µg of vancomycin for identification and further confirmation of vancomycin resistance. Only one stool specimen per person and only one isolate of a given species per specimen were evaluated.

For animal sources, the whole eviscerated cavity was swabbed and plated on the above media, and some samples were also plated on SF containing kanamycin (2,000 µg/ml), streptomycin (1,000 µg/ml), or gentamicin (500 µg/ml). Aliquots of 200 µl from chicken farm lagoons were plated on the same media. Probiotics were pulverized, and 0.5 g was inoculated into 5 ml of BHI broth. Subcultures from tubes showing growth were plated on the above media.

Vancomycin was kindly provided by Eli Lilly & Co. (Indianapolis, Ind.); kanamycin, streptomycin, and gentamicin were purchased from Sigma Chemical Co. (St. Louis, Mo.).

**Susceptibility testing.** MICs of vancomycin, teicoplanin, ampicillin, and clindamycin were determined by following the National Committee for Clinical Laboratory Guidelines (33). Susceptibility to tetracycline, chloramphenicol, and ciprofloxacin was determined by disk diffusion (34). High-level resistance to aminoglycosides was determined by plating onto BHI agar containing 2,000 µg of streptomycin per ml and BHI agar containing 500 µg of gentamicin per ml (33). Teicoplanin and ciprofloxacin were kindly provided by Marion Merrell Dow (Cincinnati, Ohio) and Miles Laboratories Inc. (West Haven, Conn.), respectively. Ampicillin, tetracycline, and chloramphenicol were purchased from Sigma Chemical Co., and clindamycin was purchased from Boehringer Mannheim Inc. (Indianapolis, Ind.).

The control strains used were *Staphylococcus aureus* ATCC 29213, *E. faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *E. faecalis* A256 (40), *E. faecium* D366 (50), *E. faecium* D399 (39), *E. gallinarum* UCLA11 (49), and *E. casseliflavus* C11 (kindly provided by David M. Shlaes).

**Genetic characterization of vancomycin resistance.** The presence of DNA homologous to *vanA*, *vanB*, *vanC1*, and *vanC2* genes was tested by colony lysis hybridization under high-stringency conditions (38). Intragenic probes for *vanA* and *vanB* were generated by PCR from *E. faecalis* A256 and *E. faecium* D366 (40, 50), respectively, by using two primers selected from previously published sequences (16, 18): (*vanA*, F/5'-GCAAGTCAGGTGAAGATGG-3' and R/5'-ACCTGCCAACAACTAACGC-3'; *vanB*, F/5'-ACCCTGTCTTTGTGAAGCCGGCAC-3' and R/5'-CAAAAAAGATCAACACGAGCAAGCCC-3'). Oligonucleotides chosen from published sequences were used as *vanC1* and *vanC2* probes (17, 35) (*vanC1*: +ATGGGAATCGCTAGTGC; *vanC2*: +CATTGGCGTACAAA GTGC).

Radiolabeled PCR fragments representing *vanA* and *vanB* were prepared by incorporation of  $\alpha$ -<sup>32</sup>P-dCTP-labeled deoxyribonucleotides with a random primed DNA labeling kit in accordance with the manufacturer's instructions (Boehringer Mannheim). Oligonucleotide probes for *vanC1* and *vanC2* were end labeled with [ $\gamma$ -<sup>32</sup>P]dATP (38).

**PFGE.** Genomic DNA preparation and conditions for pulsed-field gel electrophoresis (PFGE) were performed as described by Murray et al. (32), and preparations were digested with *Sma*I (New England Biolabs, Beverly, Mass.).

**Identification.** Strains were presumptively identified as enterococci by colony morphology and the results of testing for catalase production, hydrolysis of esculin, growth in 6.5% NaCl, and hydrolysis of L-pyrrolydonyl-β-naphthylamide (Difco Laboratories) (19). Further identification was performed by standard biochemical tests (19) and API 20STREP (BioMerieux, Plainview, N.Y.).

To corroborate the identification to species level, the isolates were also tested for the presence of genes coding for GyrA and AAC(6')-II, derived from *E. faecalis* and *E. faecium* (13, 27), respectively, by using colony lysis hybridization and high-stringency conditions (38). Intragenic probes for *gyrA* and for *aac(6')-II* were generated by PCR from *E. faecalis* OG1RF (ATCC 47077) and from *E. faecium* GE1 (ATCC 51558), respectively, by using the following primers: for *aac(6')-II*, F/5'-GCGGTAGCAGCGGTAGACCAAG-3' and R/5'-GCATTGTGAAGACACTACG-3'; for *gyrA*, F/5'-CGGGATGAACGAATTGGGTGTGA-3' and R/5'-AATTTTACTCATACGTGCTTCGG-3'. Radiolabeled probes for *gyrA* and *aac(6')-II* were prepared as described above. The use of these probes for identification purposes has been previously reported (12, 13).

## RESULTS

Enterococci were isolated from the feces of 75% of healthy volunteers and from 80% of hospitalized patients. Vancomycin-resistant gram-positive organisms other than enterococci

were recovered from 42 and 39% of the healthy volunteers and the inpatient groups, respectively; further identification was not performed. VRE were isolated from 16 of the 100 (16%) selected inpatients studied (6 of whom had prior known infections by VRE). Twelve of these patients (75%) had severe underlying diseases, and 10 (63%) had been treated with intravenous vancomycin in the previous 2 months. In 11 colonized patients, VRE were quantified and ranged from 10<sup>3</sup> to 10<sup>6</sup> CFU/g of stool, constituting less than 1% of the enterococcal flora. VRE could not be quantified in five samples because they had been submitted on a swab. VRE were found in two healthy volunteers without hospital exposure and in one with hospital exposure. VRE were usually recovered on SF agar containing 6 µg of vancomycin per ml after 48 to 72 h of incubation and on CNA containing 6 µg of vancomycin per ml at 24 h.

VRE were identified as *E. faecalis* (three isolates) and *E. faecium* (nine isolates) in the hospitalized group; four VRE were not saved for further characterization. All of these VRE were susceptible to teicoplanin and hybridized to the *vanB* probe but not to the *vanA*, *vanC1*, or *vanC2* intragenic probe (Table 1). The *E. faecalis* isolates (vancomycin MICs, 512 µg/ml; teicoplanin MICs, ≤0.25 µg/ml) were susceptible to ampicillin (MICs, 1 to 2 µg/ml) and resistant to clindamycin (MICs, >16 µg/ml) and ciprofloxacin (by disk) and highly resistant to gentamicin and streptomycin. The *E. faecium* strains (vancomycin MICs, 32 to 256 µg/ml; teicoplanin MICs, 0.5 µg/ml) were resistant to ampicillin (MICs, 64 to 128 µg/ml), clindamycin (MICs, >16 µg/ml), ciprofloxacin (by disk), and tetracycline (by disk) and highly resistant to streptomycin. The two VRE isolated from stools of the healthy volunteers with no hospital exposure were identified as *E. gallinarum* and hybridized to the *vanC1* probe but not to the *vanA*, *vanB*, or *vanC2* probe. The VRE isolate recovered from a healthy volunteer with hospital exposure hybridized to the *vanB* probe and was identified as *E. faecalis*.

All *E. faecalis* isolates, including the isolate recovered from a healthy volunteer with hospital exposure, and all *E. faecium* isolates were classified as belonging to a single clone per species by PFGE (pattern 1 for *E. faecalis* and pattern 2 for *E. faecium*) (Fig. 1). *E. faecalis* isolates showed a pattern of *Sma*I-digested genomic DNA more similar to that typical of *E. faecium* than *E. faecalis* (all fragments less than 350 kb) as previously reported (12) (Fig. 1). These strains hybridized to the *E. faecalis gyrA* probe but not to the *E. faecium aac(6')-II* probe, corroborating their classification as *E. faecalis* (12).

None of the VRE recovered from animal sources and chicken farm lagoons hybridized to the *vanA* or *vanB* probe, although *E. gallinarum* isolates which hybridized to the *vanC1* probe were isolated. Also, a number of *E. faecalis* and *E. faecium* highly resistant to aminoglycosides (kanamycin, streptomycin, and/or gentamicin) were detected.

Enterococci were recovered from all probiotic preparations studied; however, no VRE were found.

## DISCUSSION

VRE have been increasingly reported in recent years, although the percent varies widely depending on the geographic location and the setting (31, 51). In Europe, the isolation of VRE from healthy volunteers, animals, and environmental sources indicates that these organisms are part of the normal human flora and suggests that the food chain may be the origin of VRE in these countries (1, 4, 22, 23, 25, 44, 48). The presence of VRE in environmental and community settings in

TABLE 1. Fecal carriage of enterococci and VRE among persons and environmental sources examined

Population (no. of isolates)	Enterococci (%)	No. of VRE	Species	PFGE result	No. of strains hybridizing to the following probes:					
					<i>vanA</i>	<i>vanB</i>	<i>vanC1</i>	<i>vanC2</i>	<i>E. faecalis</i> <i>gyrA</i> <sup>a</sup>	<i>E. faecium</i> <i>aac(6')-II</i> <sup>a</sup>
Hospitalized (100)	75	3	<i>E. faecalis</i>	Pattern 1	0	3	0	0	3	0
			<i>E. faecium</i>	Pattern 2	0	9	0	0	0	9
			Not saved							
Community (104)	80	2	<i>E. gallinarum</i>	NT <sup>b</sup>	0	0	2	0	NT	NT
			<i>E. faecalis</i>	Pattern 1	0	1	0	0	1	0
Animal (52)	100	16	<i>E. gallinarum</i>	NT	0	0	16	0	NT	NT
Farm lagoon (2)	100	2	<i>E. gallinarum</i>	NT	0	0	2	0	NT	NT
Probiotic (5)	100	0								

<sup>a</sup> Different enterococcal species (32 *E. faecium*, 19 *E. faecalis*, 2 *E. casseliflavus*, 2 *E. gallinarum*, 2 *E. solitarius*, 2 *E. hirae*, 1 *E. mundtii*, and 1 *E. raffinosus* isolates) were also tested with *aac(6')-II* and *gyrA* probes. All *E. faecalis* strains hybridized to the *gyrA* probe but not to the *aac(6')-II* probe. All *E. faecium* strains hybridized to the *aac(6')-II* probe but not to the *gyrA* probe. None of the non-*E. faecalis* strains hybridized to the *gyrA* probe, and none of the non-*E. faecium* strains hybridized to the *aac(6')-II* probe.

<sup>b</sup> NT, not tested.

<sup>c</sup> From a healthy volunteer with hospital exposure.

the United States remains largely unknown, although nosocomial outbreaks are commonly reported (6, 9, 21, 31).

Although we did not determine the overall incidence of VRE in our hospital, their presence in 16% of high-risk patients and the demonstration that all isolates tested belonged to one of only two clones is similar to results of other reports in this country (6, 9, 21, 31). Moreover, in the same period as this study, other clinical isolates of *E. faecalis* (six isolates) and *E. faecium* (three isolates) with the same *Sma*I-digested genomic DNA patterns described here were recovered at different hospitals in Houston, and the one VRE isolate from the healthy volunteer with hospital exposure was found to be an *E. faecalis* isolate of pattern 1, indicating intra- and interhospital transmission of these two strains.

Despite a number of studies of VRE in the nosocomial setting, including those implicating fecal contamination of environmental objects (6, 30), little information on the concentration of VRE in feces is known. We found that the counts of VRE in 11 colonized patients (6 of whom had been treated with vancomycin in the previous 2 months) ranged from 10<sup>3</sup> to 10<sup>6</sup> CFU/g of fecal sample. Up to 10<sup>6</sup> to 10<sup>8</sup> CFU of VRE per g were found in healthy Belgian volunteers after oral glycopeptide administration, while very low concentrations were found in stool specimens of community-based Belgian volun-

teers without hospital or glycopeptide exposure (48). The large numbers of VRE observed in our hospitalized patients and in the volunteers after glycopeptide administration (48) might result in an increased risk of nosocomial transmission of these VRE as well as spread of this resistance to other species in the intestinal flora.

The absence of *vanA* or *vanB* VRE in the healthy volunteers in Houston who lacked hospital contact contrasts with results of several studies performed in Europe in which 2 to 28% of community-based persons without previous hospital or glycopeptide exposure have been reported to be colonized by VRE (22, 24, 48). Comparison of the data should be done cautiously, however, since different media (Enterococcosel, kanamycin-aesculin-azide, CNA, and BHI), different concentrations of antibiotics, and different plating or enrichment methods were used in each of these studies, and this might result in the differences in the recovery rates (22, 24, 48). In the study by van der Auwera et al., VanA-type VRE were isolated from the stools of 28% of healthy volunteers by directly plating on CNA containing 16 µg of vancomycin per ml, and the authors were able to detect low numbers of organisms (48). Although SF has not previously been reported for detection of VRE, all our isolates were detected on both CNA and SF supplemented with vancomycin, suggesting that either medium can be used for this purpose. CNA detected VRE earlier than did SF, but SF has the advantage in that overgrowth of yeast- and vancomycin-resistant organisms other than enterococci is unusual and enterococcal colonies can be easily differentiated.

In agreement with other studies performed with animals in the United States (26, 43), we did not recover VRE from animal sources. This might be due to the fact that in the United States, in contrast to Europe, glycopeptides are not used in animal feeds. In some European countries, the glycopeptide avoparcin is used for this purpose, and it has been demonstrated as a risk factor for VRE (24, 25). Although the size of our animal sample ( $n = 52$ ) might represent a limitation of this study, Bates et al. isolated VRE (4) in a large percentage of samples studied (22 of 52 farm-raised animals and from 5 of 5 uncooked chickens). In other studies, the number of total samples evaluated was not specified (24, 25). The food chain has not clearly been demonstrated to be a source of VRE acquisition in the United States, but this possibility cannot be

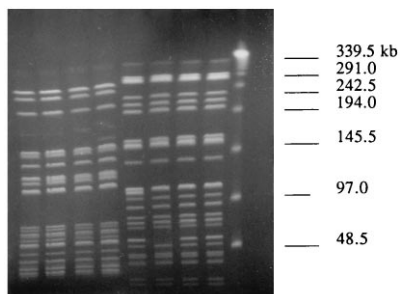


FIG. 1. PFGE of *Sma*I-digested genomic DNA from *vanB*-containing *E. faecium* (pattern 2) (lanes 1 to 4 from left) and from *vanB*-containing *E. faecalis* (pattern 1) (lanes 5 to 8). Lane 9 shows successively larger concatemers of a lambda phage DNA ladder standard (48.5 to 1,000 kb). Each lane represents an independent isolate corresponding to a different patient.



eliminated. Recently, a vancomycin-resistant *E. hirae* strain has been isolated from creek water, but whether this creek was close to an animal source or to a hospital setting is unknown (26). Also, a VanA *E. faecium* strain has been recovered from dry dog food (14).

Together with our failure to recover enterococci from non-hospitalized patients or environmental sources, we did not find VRE in different brands of probiotics. However, this is another possible route for the introduction of VRE to the community, since an isolate containing the *vanB* gene recovered from a bacterial preparation has been described previously (2).

VRE have been recovered from a large number of samples by a direct plating method or broth enrichment in a highly selective medium in some studies (4, 25, 48). Despite the use of these methods in our study, we failed to find evidence of *vanA* or *vanB* VRE in community or environmental sources. Although the use of a direct plating method on antibiotic-containing medium has been shown to increase the recovery rate of resistant enterococci (11), Klare et al. have recently reported isolation of VRE only after broth enrichment in antibiotic-free medium in some of the samples they studied, suggesting that with the use of selective media, low numbers of organisms might be missed (24). Further studies to establish an optimal medium and method for the recovery and detection of VRE are necessary.

The results of this study indicate that in our geographic area, enterococci with acquired vancomycin resistance are rare to nonexistent in community sources and thus these are unlikely sources of VRE now found in the hospital setting. However, our findings represent sampling of sources restricted to the greater Houston metropolitan area and may not be representative of other areas, in particular locations where VRE have been long established in the hospital environment or where introduction via the food chain may be more likely.

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