

Molecular Epidemiology and Antibiotic Susceptibility of Enterococci in Cincinnati, Ohio: a Prospective Citywide Survey

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To determine patterns of antimicrobial susceptibility among enterococci and to assess molecular characteristics of vancomycin-resistant enterococci, 157 clinical blood isolates of enterococci from 10 hospitals in Cincinnati, Ohio, were prospectively collected during a 6-month period from February to July 1995. The isolates included 108 (69%) *E. faecalis* isolates, 46 (29%) *E. faecium* isolates, and 1 isolate each of *E. avium*, *E. durans*, and *E. gallinarum*. The *E. faecalis* and *E. faecium* isolates differed in their susceptibilities to ampicillin (100 versus 20%), ampicillin-sulbactam (100 versus 13%), vancomycin (100 versus 57%), imipenem (94 versus 2%), and high levels of gentamicin (59 versus 83%). Supplemental susceptibility testing of the 21 vancomycin-resistant isolates showed that 21 (100%) were susceptible to chloramphenicol and that only 7 (33%) were susceptible to doxycycline. Nineteen (90%) of the vancomycin-resistant *E. faecium* isolates were of the VanB phenotype, with *vanB* resistance genes detected by PCR and hybridization with gene-specific probes; and the *E. gallinarum* isolates demonstrated the VanC phenotype with the *vanC1* gene. One vancomycin-resistant *E. faecium* isolate was highly resistant to both teicoplanin and vancomycin, corresponding to the VanA phenotype; however, it was found to have the *vanB* gene. Pulsed-field gel electrophoresis (PFGE) revealed that all of the 19 *E. faecium* isolates with the VanB phenotype had identical to closely related banding patterns. Hybridization of restriction enzyme-digested DNA separated by PFGE with a *vanB* gene probe demonstrated differences in the locations of *vanB* genes that corresponded closely to the PFGE banding patterns. Our study has documented that the emerging vancomycin resistance in our city was mainly due to the clonal dissemination of a single strain of *E. faecium* VanB.

Enterococci are the second most common cause of nosocomial infections and the third most common cause of hospital-acquired bacteremia in the United States (23). Nosocomial vancomycin-resistant enterococci (VRE) in the United States increased from 0.3% of enterococcal isolates in 1989 to 7.9% in 1993 and account for 14% of enterococcal isolates causing infection in critical-care units (5). VRE was first detected in Cincinnati, Ohio, in August 1993 from one area hospital (hospital IV). After that single blood isolate, no further isolate of VRE was reported in that facility up to the end of our study. However, in November of that year, an isolate of VRE from an urethral discharge was detected at another local hospital (hospital X). In early 1994, a prospective screening of enterococcal isolates from routine cultures (e.g., urine and wounds) in hospital X showed an increasing prevalence of vancomycin resistance over a 3-month period, from 4 to 16%. Subsequently, 36% of patients at hospital X screened by obtaining rectal swabs were found to be colonized with VRE (13). All the isolates from these two local hospitals were *E. faecium* of the VanB phenotype, and upon analysis of their genomic DNA by pulsed-field gel electrophoresis (PFGE) they were found to be similar, suggesting possible clonal dissemination. By late 1994, VRE had been detected in a third area hospital (hospital IX). The appearance of these vancomycin-resistant strains prompted us to do a prospective citywide survey to (i) determine the patterns of antimicrobial susceptibility among enterococci causing bacteremia in our area, (ii) determine the hos-

pital and species prevalence of the vancomycin-resistant isolates, and (iii) assess their molecular characteristics in the hope of elucidating the epidemiology of the emergence of vancomycin-resistant enterococci in Cincinnati. The ability of specific vancomycin resistance gene probing was explored as a means of differentiating isolates that appeared to be clonally related by their PFGE patterns.

MATERIALS AND METHODS

Bacterial isolates. From February to July 1995, all clinical blood isolates of enterococci regardless of species and antimicrobial susceptibility were collected from 10 hospitals in Cincinnati. The 10 hospitals, all within an 8-mile radius, included primary-care and tertiary-care community hospitals and university teaching facilities (230 to 550 beds). The isolates were inoculated and transported on Trypticase soy agar slants (Difco, Detroit, Mich.). The control strains used in the study were obtained from the American Type Culture Collection (ATCC) and other investigators and included vancomycin-sensitive strain *E. faecalis* ATCC 29212, VanA phenotype strains *E. faecalis* A256 (24) and *E. faecium* BA31, VanB phenotype strains *E. faecium* D366 (1) and *E. faecalis* J20, VanC phenotype strain *E. gallinarum* ATCC 49573, and *E. casseliflavus* ATCC 49604.

Identification of *Enterococcus* species. All isolates were identified to the genus and species levels with the API 20 Strep system (bioMérieux Vitek, Inc., Hazelwood, Mo.) (3, 18, 21, 22). The Vitek GPI system (bioMérieux Vitek, Inc.) was used as an alternative method of identification. All isolates identified as *E. faecium* were tested for motility by using Motility B medium (Remel, Lenexa, Kans.). Identification of *E. gallinarum* was confirmed by the presence of motility, the absence of yellow pigmentation (4), and amplification of *vanC1* genes (6, 14) by PCR.

Antibiotic susceptibility testing. The isolates were tested by the E test (AB Biodisk, Piscataway, N.J.) (12, 20). Isolates were grown overnight on 5% sheep blood agar plates at 37°C. Inocula were then prepared by suspending the freshly grown organisms in sterile normal saline adjusted to a 0.5 McFarland standard (approximately 1×10^8 to 2×10^8 CFU/ml) and were directly inoculated onto unsupplemented Mueller-Hinton agar (Difco) plates. The inoculated plates were allowed to dry for 15 to 20 min before one to six E test strips were applied to each plate in an equidistant radial fashion (12). After incubation at 35°C for 24 h (19), the MIC was read at the intersection of growth and the MIC scale of the strip.

All strains were tested for susceptibility to ampicillin, ampicillin-sulbactam,

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TABLE 1. Oligonucleotide primers

Amplified gene	Size of PCR product (bp)	Oligodeoxynucleotide		Annealing temp (°C)	Position
		Pair	Sequence		
<i>vanA</i>	1,029	A ₁	5'-ATGAATAGAATAAAAGTTGCAATAC	54	1-25 1029-1009
		A ₂	5'-CCCCTTTAACGCTAATACGAT		
<i>vanB</i>	457	B ₁	5'-CCCGAATTTCAAATGATTGAAAA	54	440-462 896-879
		B ₂	5'-CGCCATCCTCCTGCAAAA		
<i>vanC1</i>	811	C ₁	5'-GCTGAAATATGAAGTAATGACCA	56	93-115 904-924
		C ₂	5'-CGGCATGGTGTGTGATTTCTGTT		
<i>vanC2</i>	439	D ₁	5'-CTCCTACGATTCTCTTG	56	455-486 885-869
		D ₂	5'-CGAGCAAGACCTTTAAG		

ciprofloxacin, clindamycin, high levels of gentamicin, imipenem, meropenem, and vancomycin. In addition, all isolates for which vancomycin MICs were >4.0 µg/ml were tested for their susceptibilities to teicoplanin, doxycycline, and chloramphenicol. Interpretations of MICs and breakpoint criteria were according to the interpretative standards set by the National Committee for Clinical Laboratory Standards (NCCLS) (19) except for the following: (i) meropenem (7), for which there is no NCCLS MIC interpretative standard, and (ii) vancomycin, of which a MIC of >4 µg/ml was considered resistant in this study (this category included isolates with low-level resistance such as isolates with the VanC phenotype).

PFGE. Genomic DNA from all vancomycin-resistant *E. faecium* isolates and from control isolates were prepared by previously described methods, with slight modifications (15). Briefly, pure enterococcal isolates were grown overnight on a blood agar plate, harvested, and suspended in 1.0 ml of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) to an optical density at 540 nm of 1.2 to 1.5. After embedment in 1% (final concentration) SeaPlaque GTG low-melting-point agarose, the agarose plugs were lysed overnight at 37°C in 6 mM Tris-HCl (pH 7.6)-1 M NaCl-100 mM EDTA (pH 7.5)-0.5% Brij 58-0.2% deoxycholate-0.5% Sarkosyl-20 µg of RNase per ml-1 mg of lysozyme per ml, followed by a second lysis in 0.5 M EDTA (pH 9 to 9.5)-1% Sarkosyl-50 µg of proteinase K per ml for an additional 24 h. The plugs were washed three times with TE and stored at 4°C.

The agarose-embedded chromosomal DNAs were digested with 20 U of *Sma*I (Promega Corp., Madison, Wis.) for 6 to 24 h at 25°C after a 30- to 60-min dialysis in 1× restriction buffer. Digested plugs were electrophoresed through a 1.2% SeaKem GTG agarose gel (FMC, Rockland, Maine) in 0.5× TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA [pH 8.3]) by using a contour-clamped homogeneous electric field apparatus (CHEF-DRIII; Bio-Rad, Hercules, Calif.) with ramped pulse times beginning with 0.1 s and ending with 10 s at 9 V/cm for 26 h. Lambda concatemers (Bio-Rad) were used as size standards in all gels. Interpretation of the chromosomal DNA restriction patterns generated by PFGE was based on a set of guidelines proposed by Tenover et al. (25). Isolates were considered (i) indistinguishable if there were no differences in the banding pattern, (ii) closely related if there were two to three band differences, (iii) possibly related if there were four to six band differences, and (iv) different if there were seven or more band differences.

Amplification of vancomycin resistance genes by PCR. The oligonucleotide primers used for amplification of the *vanA*, *vanB*, *vanC1* (14), and *vanC2* (6) genes were selected from previously published primer sequences (Table 1). A piece (1 by 1 mm) of agarose embedded with enterococcal DNA was dissolved in 50 µl of TE and was used as the DNA template. PCR amplifications were carried out on a Gene Amp PCR System 2400 instrument (Perkin-Elmer, Foster City, Calif.). The final reaction volume of 25 µl contained 2.5 mM MgCl₂, 200 µM deoxynucleoside triphosphates, 20 nM primers, and 5 µl of enterococcal DNA. The PCR program consisted of an initial denaturation step at 94°C for 5 min; this was followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at the appropriate temperature for each set of primers for 30 s, and DNA extension at 72°C for 30 s. After the last cycle, the reaction was terminated by incubation at 72°C for 7 min, and the products were stored at 4°C. The amplicons were resolved by electrophoresis on a 0.7% agarose gel in 1× TBE buffer for 1 h at 70 V, stained with ethidium bromide, and visualized under UV light.

Resistance gene probes. Probes for *vanA* and *vanB* genes were generated by PCR amplification of DNA from the reference strains by use of the appropriate primers (Table 1). The amplicons were resolved by electrophoresis on a 0.7% agarose gel at 70 V for 1 h, stained with ethidium bromide, and excised from the gel, and the DNA was extracted with a GeneClean II kit (Bio 101, Inc., La Jolla, Calif.). Randomly primed [α -³²P]dCTP-labeled probes were generated with the Klenow fragment of DNA polymerase.

Southern blotting and hybridization. *Sma*I-digested *E. faecium* DNA fragments resolved by contour-clamped homogeneous electric field electrophoresis were transferred to positively charged nylon membranes (Boehringer Mann-

heim, Mannheim, Germany) by capillary action. Blots were prehybridized for 2 h at 60°C in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7])-5× Denhardt's solution-0.5% sodium dodecyl sulfate (SDS)-100 µg of salmon sperm DNA per ml and hybridized overnight under the same conditions. The membranes were then washed twice for 15 min at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.2% SDS and twice for 15 min at 60°C with 0.2% SSC-0.2% SDS and exposed to Fuji X-ray film with an intensifying screen for 4 to 7 days at -70°C.

RESULTS

A total of 157 isolates were prospectively collected from the blood of 135 patients during the 6-month study period (Fig. 1). The majority of the isolates were from 4 of the 10 hospitals; 17 (11%) were from hospital IV, 23 (15%) were from hospital VI, 57 (36%) were from hospital IX, and 24 (15%) were from hospital X. Of the 157 isolates, 108 (69%) were *E. faecalis*, 46 (29%) were *E. faecium*, and 3 (2%) were other *Enterococcus* spp. (1 each of *E. avium*, *E. durans*, and *E. gallinarum*).

Antibiotic susceptibility testing. The results of the antimicrobial susceptibility tests are summarized in Table 2. All 108 *E. faecalis* isolates were susceptible to both ampicillin (MICs, ≤8 µg/ml) and vancomycin (MICs, ≤4 µg/ml). Only 9 (20%) and 26 (57%) of the *E. faecium* isolates were susceptible to ampicillin and vancomycin, respectively. *E. faecium* isolates were more frequently sensitive (MICs, ≤500 µg/ml) to high levels of gentamicin than *E. faecalis* (83 versus 59%, respec-

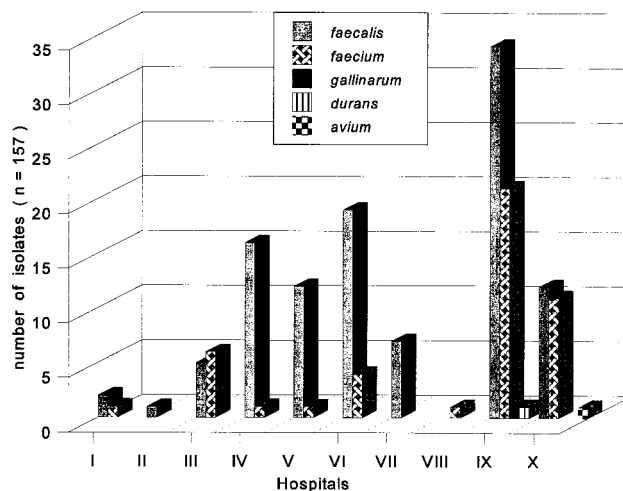


FIG. 1. Distribution of enterococcal bacteremia in the participating hospitals by enterococcal species, February through July 1995.

TABLE 2. Antibiotic susceptibility patterns of 157 *E. faecalis* and *E. faecium* blood isolates

Species and antibiotic	MIC ($\mu\text{g/ml}$) ^a			% of susceptible strains ^b
	50%	90%	Range	
<i>E. faecalis</i> (n = 108)				
Ampicillin	0.75	1	0.125–6	100
Ampicillin-sulbactam	1	1.5	0.25–8	100
Imipenem	2	4	0.38–16	94
Meropenem ^c	12	>32	1–>32	19
Vancomycin	2	4	1–4	100
Gentamicin	64	>1,024	4–>1,024	59
Ciprofloxacin	3	>32	0.5–>32	18
Clindamycin	64	>256	0.064–>256	3
<i>E. faecium</i> (n = 46)				
Ampicillin	128	>256	0.5–>256	20
Ampicillin-sulbactam	>256	>256	0.5–>256	13
Imipenem	>32	>32	4–>32	2
Meropenem ^c	>32	>32	>32	0
Vancomycin	3	>256	1–>256	57
Gentamicin	24	>1,024	6–>1,024	83
Ciprofloxacin	>32	>32	1–>32	2
Clindamycin	>256	>256	0.032–>256	4

^a 50% and 90%, MICs at which 50 and 90% of strains are inhibited, respectively.

^b Breakpoint criteria used to define susceptible strains were based on the NCCLS MIC interpretative standards (19) and were as follows: ampicillin, ≤ 8 $\mu\text{g/ml}$; ampicillin-sulbactam, $\leq 8/4$ $\mu\text{g/ml}$; imipenem, ≤ 4 $\mu\text{g/ml}$; vancomycin, ≤ 4 $\mu\text{g/ml}$; gentamicin, ≤ 500 $\mu\text{g/ml}$; ciprofloxacin, ≤ 1 $\mu\text{g/ml}$; and clindamycin, ≤ 0.5 $\mu\text{g/ml}$.

^c A strain susceptible to meropenem was defined as an MIC of ≤ 4 $\mu\text{g/ml}$ (7). No NCCLS MIC interpretative standard for meropenem exists.

tively). Among the carbapenems, *E. faecalis* isolates were more susceptible (MICs, ≤ 4 $\mu\text{g/ml}$) to imipenem than to meropenem (102 [94%] and 20 [19%] isolates, respectively). In contrast, *E. faecium* isolates were only 0 to 2% susceptible to both carbapenems. The other enterococcal species in the study (*E. avium*, *E. durans*, and *E. gallinarum*) were all susceptible to ampicillin, ampicillin-sulbactam (MICs, $\leq 8/4$ $\mu\text{g/ml}$), and high levels of gentamicin. Of the 157 total isolates, 20 (13%) and 5 (3%) were susceptible to ciprofloxacin (MICs, ≤ 1 $\mu\text{g/ml}$) and clindamycin (MICs, ≤ 0.5 $\mu\text{g/ml}$), respectively.

Twenty-one vancomycin-resistant isolates (MICs, 8 to ≥ 256 $\mu\text{g/ml}$) from 15 patients were identified. These isolates came from 3 of the 10 hospitals; 1 was from hospital I, 11 were from hospital IX, and 9 were from hospital X. With the single exception of an *E. gallinarum* isolate from hospital IX, all VRE were *E. faecium* isolates (n = 20). The prevalence of vancomycin resistance among the *E. faecium* strains from hospitals IX and X were 48% (10 of 21) and 82% (9 of 11), respectively. Supplemental susceptibility testing with all 21 isolates of VRE showed that 21 (100%) were susceptible to chloramphenicol and that 7 (33%) were susceptible to doxycycline. Among the 20 vancomycin-resistant *E. faecium* isolates, 19 were of the VanB phenotype (resistant to vancomycin but susceptible to teicoplanin) and 1 was found to be constitutively resistant to teicoplanin and vancomycin, a VanA phenotype (Table 3). The *E. gallinarum* isolate had a VanC phenotype (low-level resistance to vancomycin and susceptible to teicoplanin).

PFGE. Analysis of the *Sma*I restriction digests of genomic DNAs of the 20 vancomycin-resistant *E. faecium* isolates by PFGE resulted in two main banding patterns or karyotypes, designated karyotypes A and B (Table 3). Karyotype A, the predominant karyotype, has four subtypes (subtypes A_{1a}, A_{3a}, A_{3b}, and A₄) that differed by one to four bands, as indicated by

TABLE 3. Molecular and phenotypic characteristics of vancomycin-resistant *E. faecium* blood isolates

Karyotype by PFGE	No. of isolates	Phenotype ^a		van genes		Hospital prevalence ^b
		Vancomycin	Teicoplanin	Type	Location (kb) ^c	
A	13	R	S	vanB	132 ^d	IX, X
A _{1a}	2	R	S	vanB	291	IX, X
A _{3a}	2	R	S	vanB	— ^e	X
A _{3b}	1	R	S	vanB	132 ^d	X
A ₄	1	R	S	vanB	283	X
B	1	R	R	vanB	388/340	I

^a R, resistant; S, susceptible.

^b Hospital where isolates were identified.

^c Hybridization of *vanB*-specific gene probes to *Sma*I-digested DNA fragments.

^d Weak hybridization to 132-kb fragment.

^e No hybridization to *Sma*I-digested fragments noted with *vanB*-specific gene probe.

their numbered subscripts (Fig. 2A). Nineteen of the 20 isolates (95%) belonged to karyotype A; 13 belonged to the main karyotype A and 6 belonged to its subtypes. The one remaining isolate demonstrated a karyotype B pattern. Karyotypes A and A_{1a} were seen in hospitals IX and X, whereas karyotype B was detected in hospital I.

The vancomycin-resistant *E. faecium* isolates from Chicago, Ill. (BA31), and Cleveland, Ohio (D366), used as geographic controls, showed different banding patterns compared to those of the Cincinnati isolates (Fig. 2A). Analysis of 26 vancomycin-sensitive *E. faecium* isolates from the study collection by PFGE revealed 15 unique karyotype patterns. Four isolates from hospital IX were observed to be of the karyotype A pattern, whereas the remaining 22 isolates were distributed among 14 karyotype patterns, designated by letters D through Q (data not shown).

Vancomycin resistance genotyping. To identify the vancomycin resistance genes, the 21 vancomycin-resistant isolates (20 *E. faecium* isolates and 1 *E. gallinarum* isolate) were subjected to PCR analysis with primers specific for *vanA*, *vanB*, *vanC1* (14), and *vanC2* (6) genes. Twenty *E. faecium* isolates generated amplification products only with *vanB* primers, producing the expected 457-kb product (Fig. 3). These included 19 isolates that were phenotypically VanB and, unexpectedly, also 1 isolate that was phenotypically VanA. This latter isolate repeatedly failed to produce products on amplification with *vanA* primers.

The *E. gallinarum* isolate with the VanC phenotype produced an 811-bp product with the *vanC1* primers but no products with the other primer pairs tested, including *vanC2* primers. The *vanC1* primers are specific for *E. gallinarum* (14), confirming the species identification of this isolate. Neither *vanC1* nor *vanC2* primers generated PCR products with any of the 20 *E. faecium* isolates.

Control isolates produced appropriate PCR products only with the expected primers: BA31 from Chicago with the *vanA* primer, D366 from Cleveland with the *vanB* primer, ATCC 49573 (*E. gallinarum*) with the *vanC1* primer, and ATCC 49604 (*E. casseliflavus*) with the *vanC2* primer.

Hybridization with resistance gene probes. The *Sma*I-digested DNA fragments of the vancomycin-resistant *E. faecium* isolates separated by PFGE were transferred to nylon membranes for hybridization with radiolabeled *vanA* and *vanB* gene probes. All 20 isolates that produced a *vanB* PCR product, including the 1 isolate with the VanA phenotype, hybridized

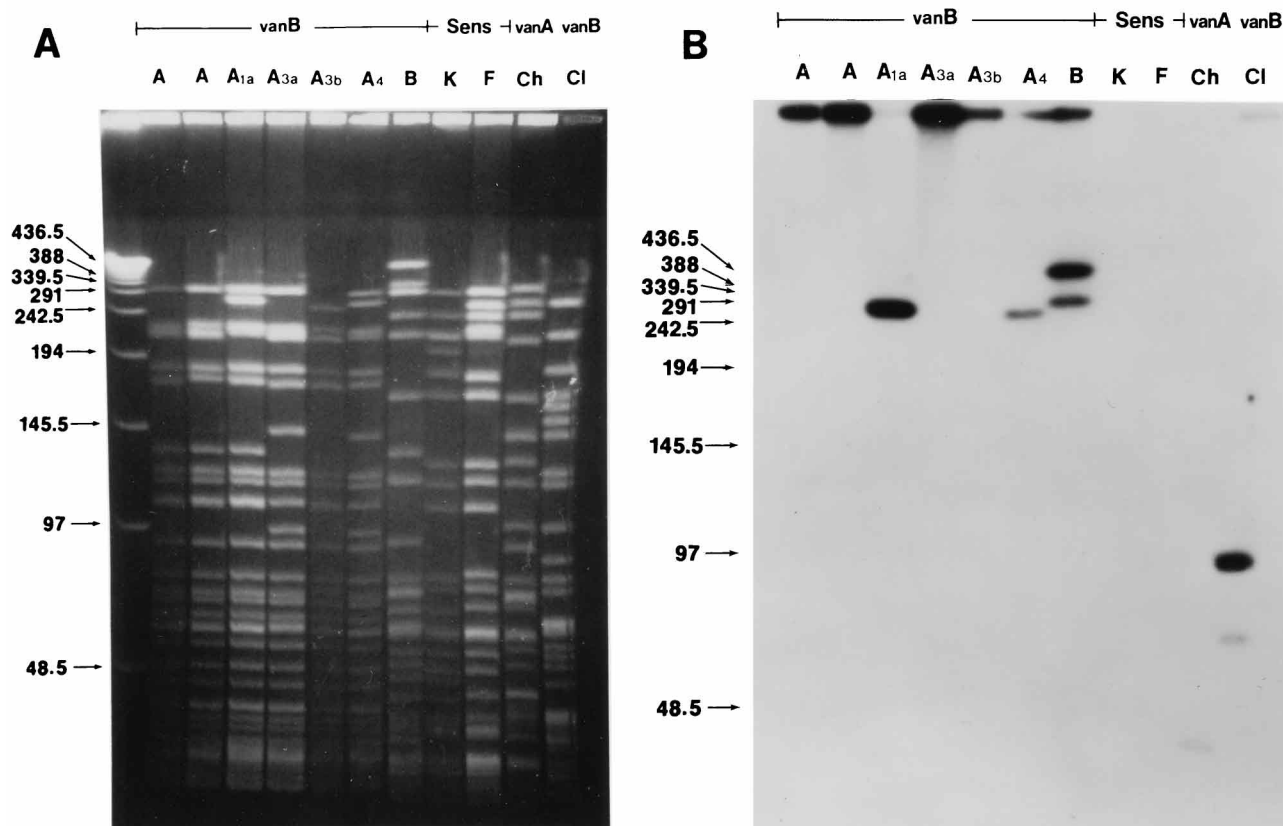


FIG. 2. Analysis of chromosomal DNA of isolates of VRE with representative karyotypes compared with those of isolates of VRE from Chicago and Cleveland and with vancomycin-sensitive isolates in the study. (A) Contour-clamped homogeneous electric field banding patterns (karyotypes) of *Sma*I-digested genomic DNA showing representative karyotypes. Lanes 1 to 12 are the unnumbered lanes in the figure, from left to right, respectively. Lane 1, lambda concatemer; lanes 2 to 8, isolates of VRE from the study showing the different karyotypes; lanes 2 and 3, isolates IX-5 and X-18 (karyotype A), respectively; lane 4, isolate X-10 (A_{1a}); lane 5, isolate X-29 (A_{3a}); lane 6, isolate X-1 (A_{3b}); lane 7, isolate X-20 (A_4); lane 8, isolate IX-7 (karyotype B); lanes 9 and 10, contain vancomycin-sensitive isolates; lane 9, isolate IX-9 (karyotype K); lane 10, isolate IX-27 (karyotype F); lanes 11 and 12, control isolates BA31 from Chicago and D366 from Cleveland, respectively. (B) Result of hybridizing the gel in panel A with a *vanB*-specific gene probe, showing the differences in location of the gene depending on the karyotype (Table 3). Numbers to the left of each panel are in kilobase pairs.

with the *vanB* gene probe (Fig. 2B). None of these 20 isolates hybridized with a *vanA* gene probe, but the VanA *E. faecium* control isolate from Chicago (BA31) showed strong hybridization to a 46-kb band (data not shown). Interestingly, the location of the *vanB* hybridization to the *Sma*I-digested genomic DNAs of these isolates differed according to the PFGE karyotype. Strong hybridization with the *vanB* probe revealed that the *vanB* genes resided on a 291-kb fragment of the two isolates with karyotype A_{1a} and on a 283-kb fragment of the isolate with karyotype A_4 (Table 3). Weaker hybridization with a 132-kb band for the 14 isolates belonging to karyotypes A and A_{3b} suggested that this was the location of their *vanB* genes (Table 3). Strong hybridization to the DNA remaining in the wells with these isolates was noted. None of the *Sma*I-digested fragments of the two isolates belonging to karyotype A_{3a} hybridized with the *vanB* probe; however, the probe hybridized strongly to the DNA remaining in the well of the electrophoresis gel. The *vanB* genes localized to a 95-kb fragment for the VanB *E. faecium* control isolate from Cleveland (isolate D366).

In contrast, the *vanB* probe hybridized with two fragments, a 340-kb and a 388-kb fragment, for the one isolate with karyotype B pattern. The hybridization of this isolate with a *vanB* probe was consistent with the finding of *vanB* gene amplification by PCR. Despite the VanA phenotype pattern of this

isolate with the karyotype B pattern, a hybridization and PCR analysis demonstrated the presence of a *vanB* gene.

DISCUSSION

Enterococci have been identified in the recent report from the National Nosocomial Infections Surveillance in U.S. hospitals as the second most frequent nosocomial pathogen that plays a causal role in 12% of all hospital-acquired infections (23). This problem is magnified by the intrinsic resistance of enterococci to many antibiotics and the emergence of resistance to glycopeptides. Since first described clinically in 1988, the literature has recorded the rapid emergence of vancomycin resistance in enterococci in different cities throughout the United States (8, 16, 17). In this study, we determined the antibiotic susceptibilities and species prevalence of enterococci and the emergence of vancomycin resistance in the city of Cincinnati.

The majority of the clinical isolates (98%) were *E. faecalis* or *E. faecium*, while other *Enterococcus* spp. accounted for only 2% of isolates, comparable to the distribution of species in previous studies (3, 9, 18). However, unlike those studies, our proportion of *E. faecium* strains was higher than those reported previously (29 versus 8 to 20%). This finding is of clinical importance since *E. faecium* is often more resistant

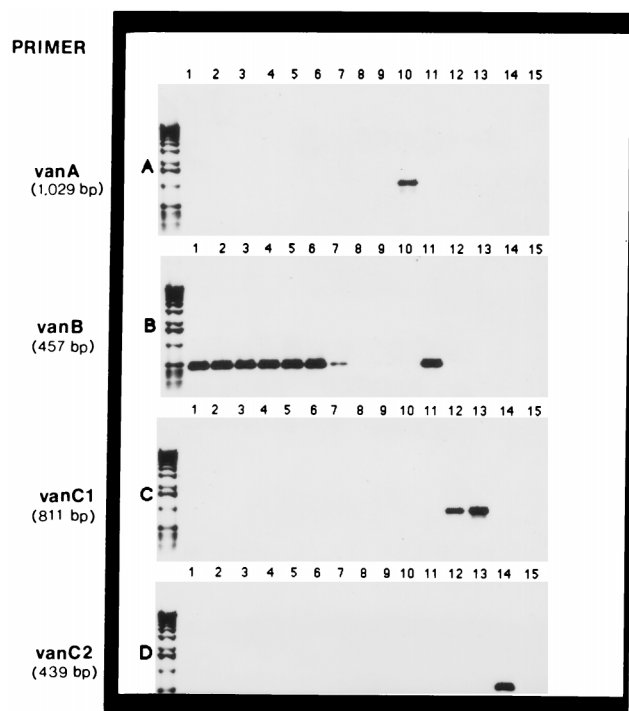


FIG. 3. Amplification of vancomycin resistance genes of representative isolates of VRE and control strains by PCR. The DNA templates for lanes 1 to 11 are the same as those depicted in lanes 1 to 11 of Fig. 2, respectively. Lanes: 12, study isolate *E. gallinarum* IX-10; 13, *E. gallinarum* ATCC 49573 (*vanC1* control); 14, *E. casseliflavus* ATCC 49604 (*vanC2* control); and 15, negative control. (A) *vanA* primer. Lane 10, VanA *E. faecium* reference strain from Chicago (strain BA31), which has the expected *vanA* gene product of 1,029 bp; lane 7, phenotypically VanA study isolate that failed to produce *vanA* gene products. (B) *vanB* primer. Lanes 1 to 6, VanB study isolates of VRE; lane 11, VanB *E. faecium* reference strain from Cleveland (strain D366). All of these isolates produced the expected *vanB* gene product of 457 bp. The isolate in lane 7, however, is phenotypically VanA but instead produced a *vanB* gene product, in keeping with its gene probe hybridization result. (C) *vanC1* primer. Lanes 12 and 13, *E. gallinarum*. This *vanC1* primer is specific for *E. gallinarum* and generated the expected 811-bp *vanC1* gene products with both isolates. (D) *vanC2* primer. This primer is specific for *E. casseliflavus*. Lane 14, *E. casseliflavus* ATCC reference strain and the only isolate that produced the expected *vanC2* gene products of 439 bp.

than *E. faecalis*, thus limiting the therapeutic options. The only *E. gallinarum* isolate in the study was initially identified as *E. faecium* but was correctly identified after being found to be motile, and its identity was subsequently confirmed by the lack of yellow pigment production (4), low-level vancomycin resistance, and PCR amplification of the *vanC1* gene (6, 14). The inability of the commercially available test systems to identify this intrinsically vancomycin-resistant organism may partly explain the rarity of finding this organism in clinical specimens.

Species identification of isolates enabled us to assess species-specific antibiotic susceptibility patterns in our area. It was reassuring that *E. faecalis* isolates remained 100% susceptible to the usual first-line and alternative treatments, ampicillin and vancomycin, respectively. Imipenem could also be considered a good alternative therapy for *E. faecalis* infections because 94% of the isolates remained susceptible. Only 18% of *E. faecalis* isolates were susceptible to ciprofloxacin, which differs markedly from the 97% reported by Gordon et al. (9) in 1992. *E. faecium* strains, on the other hand, were less sensitive to most antibiotics tested, including ampicillin (20%), imipenem (2%), and vancomycin (57%). Since 83% of *E. faecium* isolates demonstrated susceptibility to high levels of gentamicin, it should

remain a useful agent that is synergistic when used in combination with cell wall-active agents for clinical infections caused by this strain. A multicenter study examining the susceptibilities of 42 multidrug-resistant enterococci found that all were susceptible to chloramphenicol and 93% were susceptible to doxycycline (22). Similarly, all 21 isolates of VRE in our study were susceptible to chloramphenicol; however, none were susceptible to doxycycline. The overall prevalence of vancomycin resistance among enterococcal isolates in our study was 13% (21 of 157). They were mostly limited to hospitals IX and X. The first known isolate of VRE in Cincinnati, from August 1993 (hospital IV), and the subsequent clinical and stool surveillance isolates of VRE detected from late 1993 to early 1994 (hospital X) were *E. faecium* of the VanB phenotype. By use of the technique of PFGE typing, these isolates belonged to karyotypes A_{1b} and A_{3a} (data not shown). Similarly, 20 of the 21 blood isolates of VRE obtained during our study period were *E. faecium* of the VanB phenotype, and 19 of these 20 were genetically related, belonging to karyotype A and its subtypes by PFGE typing. When 24 vancomycin-resistant isolates from hospital IX from sources other than blood (urine, drainage, catheter tip, etc.) were similarly analyzed by PFGE, 14 belonged to karyotype A and 8 belonged to subtypes of karyotype A (data not shown). Taken together, this information points to the persistence of a single strain of VanB *E. faecium* in hospital X from 1993 and the eventual clonal dissemination to hospital IX. Extensive staff sharing between these two hospitals might be partly responsible for this clonal dissemination. Moreno et al. (16) reported similar findings in southern Texas, where the clonal dissemination of a single strain of VanB *E. faecium* among six hospitals was documented.

When analyzed by hybridization and PCR gene amplification, all 19 phenotypically VanB *E. faecium* isolates produced the expected 457-kb product with *vanB* primers and also hybridized to the *vanB* probe. However, we identified a discrepancy between the phenotype and the genotype in the remaining 1 of the 20 vancomycin-resistant *E. faecium* isolates in our study. This isolate was resistant to both vancomycin and teicoplanin, corresponding to a VanA phenotype; however, it hybridized with a *vanB* probe and produced a 457-kb product when amplified by PCR with *vanB* primers. This same isolate failed to produce a product when amplified with both *vanA* and *vanC* primers and did not hybridize to a *vanA* or a *vanC* probe. It is postulated that this isolate is a variant of the VanB phenotype, with possible differences in the regulation of the resistance genes (2). Similar derivatives of a VanB strain have been reported to appear in vivo under conditions of vancomycin therapy (11) and under laboratory conditions by selection on teicoplanin (10). Further characterization of the genetics of this variant are under way.

Southern blot analysis of *Sma*I-digested genomic DNAs of the isolates of VRE confirmed the PCR amplification results and determined the location of the resistance genes within the genome. Since the vancomycin resistance genes can be acquired through mobile elements (e.g., plasmids and transposons) (2), they could theoretically integrate in different locations in the genome. This might serve to further differentiate otherwise similar isolates. In our study, we found the location of the *vanB* genes to be similar among the strains that were indistinguishable by PFGE banding pattern or karyotype but different between strains that were either closely related or possibly related (e.g., one to two band differences) by PFGE karyotype. Hybridization analysis may therefore provide a useful additional epidemiologic tool for differentiating isolates that otherwise seem clonally related by PFGE.

In summary, our study has documented that the emerging vancomycin resistance in Cincinnati was mainly due to the clonal dissemination of a single strain of VanB *E. faecium*. This has led to the reevaluation of infection control policy regarding VRE in both hospitals IX and X and has raised the awareness of the medical community in our area.

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