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%). Suplemental 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 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 strain E. faecalis A256 (24) and E. faecium BA31, VanB phenotype strains E. faecium D366 (1) and E. faecalis 320, 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 Strept 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, Kan.). 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 x 10^8 to 2 x 10^9 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,
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 interpretive 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 an 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 7.6]–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 Smal (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-DR III; Bio-Rad, Herlev, Denmark) with ramped pulse times beginning with 0.1 s and ending with 10 s at 9 V/cm for 26 h. Lambda concatamers (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 deoxyribonucleoside 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.** Smal-digested *E. faecium* DNA fragments resolved by contour-clamped homogeneous electric field electrophoresis were transferred to positively charged nylon membranes (Boehringer Mannheim, 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 7.4])–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 susceptible (MICs, ≤500 μg/ml) to high levels of gentamicin than *E. faecalis* (83 versus 59%, respectively).
TABLE 2. Antibiotic susceptibility patterns of 157 E. faecalis and E. faecium blood isolates

<table>
<thead>
<tr>
<th>Species and antibiotic</th>
<th>MIC (μg/ml)</th>
<th>% of susceptible strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50%</td>
<td>90%</td>
</tr>
<tr>
<td>E. faecalis (n = 108)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.75</td>
<td>1</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Meropenem</td>
<td>&gt;32</td>
<td>1–&gt;32</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>&gt;1,024</td>
<td>4–1,024</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;32</td>
<td>1–&gt;32</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>&gt;256</td>
<td>0.064–&gt;256</td>
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</tbody>
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E. faecium (n = 46)

<table>
<thead>
<tr>
<th>Species and antibiotic</th>
<th>MIC (μg/ml)</th>
<th>% of susceptible strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50%</td>
<td>90%</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>125</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Imipenem</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Meropenem</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>3</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>&gt;1,024</td>
<td>6–1,024</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;32</td>
<td>1–&gt;32</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>&gt;256</td>
<td>0.032–&gt;256</td>
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</tbody>
</table>

* 50% and 90%, MICs at which 50% and 90% of strains are inhibited, respectively.
* Breakpoint criteria used to define susceptible strains were based on the NCCLS MIC interpretative standards (19) and were as follows: ampicillin, ≤8 μg/ml; ampicillin-sulbactam, ≤/8 μg/ml; imipenem, ≤/4 μg/ml; vancomycin, ≤4 μg/ml; gentamicin, ≤500 μg/ml; ciprofloxacin, ≤/1 μg/ml; and clindamycin, ≤0.5 μg/ml.
* A strain susceptible to meropenem was defined as an MIC of ≤4 μg/ml (7).

No NCCLS MIC interpretative standard for meropenem exists.

Hybridization with resistance gene probes. The SmalI-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 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 A1 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, and vanC2 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 SmalI-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
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 SmaI-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 A1a and on a 283-kb fragment of the isolate with karyotype A4 (Table 3). Weaker hybridization with a 132-kb band for the 14 isolates belonging to karyotypes A and A3b 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 SmaI-digested fragments of the two isolates belonging to karyotype A3a 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...
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<sub>10</sub> and A<sub>11</sub> (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 Smal-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.

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.
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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REFERENCES