Prevalence and Detection of Mixed-Population Enterococcal Bacteremia

Ana María Cárdenas, Kathleen A. Andreacchio, Paul H. Edelstein

Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Mixed-population (heterogeneous) enterococcal bacteremia (MEB) is rarely reported. Based on one occasion in which Vitek2 missed a vancomycin-resistant subpopulation isolated from a patient, we developed a simple method to detect this subpopulation and determined MEB frequency. The four patients presented here had either Enterococcus faecium or Enterococcus faecalis bacteremia caused by both vancomycin-resistant enterococci (VRE) and vancomycin-susceptible enterococci (VSE). No prior common antibiotic therapy was observed, and bacteremia resolved with daptomycin, gentamicin, and/or linezolid treatment. In two cases, VRE presence was missed by Vitek2. To detect the VRE subpopulation, tryptic soy broth was inoculated from positive blood cultures and a saline suspension was inoculated to a vancomycin (6-μg/ml) (V6) plate. Two isolates from each patient were studied further. Relatedness was assessed by multilocus sequence typing, fitness was evaluated by growth curve and competition assays, and vanA presence was determined by PCR. MEB represented ~9% of all enterococcal bacteremias. All VRE subpopulations grew on V6 plates but were missed in two instances by Vitek2. VRE and VSE isolates from each patient were closely related and did not differ in overall fitness. All four VRE isolates and 2/4 VSE isolates were vanA positive. MEBs occur regardless of prior antimicrobial therapy, are relatively common in our hospital, and are important to detect. As far as we know, this study is the first to report heterogeneous E. faecalis bacteremia. There is a simple method to detect VRE subpopulations that may be missed by Vitek2.

Enterococci can cause serious infections, including bacteremia, endocarditis, and urinary tract and wound infections (1). Treatment of infections caused by vancomycin-resistant enterococci (VRE) poses a challenge because of multidrug resistance. Therefore, prompt and accurate detection of VRE and determination of antimicrobial susceptibilities are necessary for appropriate patient care. Various types of vancomycin resistance have been described that are distinguished by location (plasmid or chromosomal) as well as mode of gene expression regulation (2). The most frequent mechanism of glycopeptide resistance in enterococci is VanA presence. Dissemination of resistance by the vanA gene cluster can occur through both clonal expansion and horizontal transfer of genes either on self-transferable plasmids or by conjugation from chromosome to chromosome.

Variation in the ability of different antimicrobial susceptibility testing methods to detect vancomycin resistance in enterococci has been previously described (3, 4). In examples of heteroresistance, homogenous bacterial populations show different susceptibility results depending on the method used. In cases of population heterogeneity (mixed populations of susceptible and resistant bacteria), i.e., the presence of a subpopulation of resistant organisms within a larger population of fully susceptible microorganisms, some methods may not detect the resistant subpopulation altogether. Therefore, depending on the methods used for susceptibility testing, the resistant subpopulation may be missed by the clinical laboratory. Reasons for the differences in susceptibility profiles among the various methods are not well understood, and the differences have been thought to be due to either fitness variability between susceptible and resistant subpopulations or inadequate inoculum size used for susceptibility testing.

Mixed-population (heterogeneous) enterococcal bacteremias (MEBs) have been rarely reported for Enterococcus faecium (5–7) and never for Enterococcus faecalis, possibly because the prevalence of glycopeptide resistance in E. faecium is higher than that in E. faecalis. Based on an occasion in which our automated antimicrobial susceptibility system failed to identify a subpopulation of vancomycin-resistant enterococci (VRE) in the blood culture of a patient who had both VRE and vancomycin-susceptible enterococcus (VSE) bacteremia, a method to detect mixed-population enterococcal bacteremia was devised and implemented in our laboratory. Of the four cases of mixed-population enterococcal bacteremia discussed here, three were caused by E. faecium and one was caused by E. faecalis. These four patients had no prior common antibiotic therapy; bacteremia lasted on average 34 days and resolved with combination treatment using daptomycin, gentamicin, and/or linezolid.

The present study describes a simple and inexpensive method to detect VRE subpopulations in heterogeneous enterococcal bacteremias that may be missed by commercially available automated systems, as well as determination of the frequency of such mixed infections in our hospital. It is also apparently the first to report this phenomenon in E. faecalis bacteremia.

(A portion of this work was presented at the 53rd Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Denver, CO, 2013.)
TABLE 1  Patient 1’s antibiotic course and organism isolated (from blood)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Day from 1st admission</th>
<th>Day or wk from discharge</th>
<th>Day from 2nd admission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16 17 18 19 20 21 22 23 24 25 26 27 28 29</td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15</td>
<td></td>
</tr>
<tr>
<td>Cefepime</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daptomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linezolid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vancomycin</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Antibiotic administration is highlighted by shading, and susceptibility interpretations for each drug tested are shown. R, resistant; S, susceptible; \( + \), same organism isolated, no further workup.

\textsuperscript{b} S/R, VSE recovered on Vitek2 and VRE detected by V6 plate (retrospectively, VRE was present on day 1). V6 screening was not implemented until a month later.

\textsuperscript{c} Resolution of bacteremia.

**CASE REPORTS**

**Case 1.** A 52-year-old neutropenic man with refractory acute myeloid leukemia was admitted to the hospital for fever of unknown origin several weeks after a stem cell bone marrow transplant. The patient was empirically treated with cefepime, metronidazole, and vancomycin. Sixteen days after admission, multiple blood cultures grew VRE; vancomycin was discontinued, and daptomycin and gentamicin were given (Table 1). Consecutive blood cultures yielded VRE for the following 12 days. A transthoracic echocardiogram performed on hospital day 25 showed no evidence of endocarditis, and the patient was discharged 4 days later. Three days after discharge, blood cultures grew VSE. Two weeks later, the patient was readmitted and VSE was recovered repeatedly over the first 5 days of hospitalization (Table 1). Because the patient had been recently reported to have VRE, the lab was asked to double-check the susceptibility profile of the recent blood isolate. Retrospective testing was done by inoculating both an automated system (Vitek2) card and a plate containing vancomycin (6 \( \mu \)g/ml) (V6) using the same inocula from a positive blood culture, and growth on the V6 plate showed that the VRE subpopulation was present on readmission. On day 6 of the patient’s second hospitalization, both VSE (from Vitek2) and VRE (from V6) isolates were reported. The patient was successfully treated when linezolid was added to his vancomycin therapy and was discharged 5 days later (Table 1). Had susceptibility testing been performed using only the Vitek2, vancomycin resistance would have been detected on hospital day 11 as opposed to day 6 (Table 1).

**Case 2.** A 64-year-old morbidly obese male with widely metastatic prostate cancer refractory to multiple chemotherapeutic agents was hospitalized because of altered mental status, hypoxia, and hypotension. Urine culture from the day of hospitalization grew *Klebsiella pneumoniae* and *Enterococcus* spp.; the patient was given vancomycin, cefepime, ceftriaxone, and azithromycin for urosepsis and multilobar pneumonia. Blood cultures grew a mixed population of vancomycin-resistant (by V6 plate) and -susceptible (by Vitek2) *E. faecalis* the next day. During an attempted arterial line placement 1 day later, the patient had an acute brady-cardiac episode. Resuscitation efforts were initiated, but the patient expired.

**Case 3.** A 73-year-old male with a medical history of acute myeloid leukemia and multiple recent admissions for an *Escherichia coli* liver abscess and diverticulitis was admitted to the hospital for treatment of a worsening intrahepatic fluid collection, leukemic bowel infiltration, and continued diverticulitis. Blood cultures collected 11 days after admission grew *E. coli* and VSE, and the patient was placed on vancomycin. Subsequent blood cultures on hospital day 21 grew VRE. Vancomycin was stopped, and the patient was given daptomycin and imipenem. Subsequent blood cultures on hospital days 35 and 37 yielded VRE and VSE, respectively, both detected by Vitek2. Blood cultures remained positive for two more days. An echocardiogram performed 39 days after hospitalization showed no sign of endocarditis. Daptomycin therapy was halted and replaced with gentamicin and linezolid; the bacteremia resolved 2 days after the antibiotic switch.

**Case 4.** A 65-year-old man with a history of heavy alcohol use and aortic valve replacement 6 years previously was admitted to the hospital for the treatment of infection-related alcoholic pancreatitis complicated by VRE bacteremia. The infection was treated successfully with linezolid for 2 weeks. The patient returned 5 months later with joint, abdominal, and lower back pain and 1 month of increasing weakness and weight loss. Blood cultures grew VRE, and the patient was treated with daptomycin and gentamicin. Transesophageal echocardiogram revealed a mitral valve vegetation and severe mitral regurgitation. The patient remained bacteremic for 9 days after admission with blood cultures yielding both VRE (hospital days 1 and 9) and VSE (hospital day 6). Both the VRE and VSE were detected by Vitek2. Of note, vancomycin had not been previously administered. Twelve days after admission, the patient underwent mitral valve replacement while being treated with daptomycin and gentamicin; all subsequent blood cultures were negative.

**MATERIALS AND METHODS**

**Antibiotic susceptibility testing.** At our institution, organism identification and susceptibility testing are usually performed with the Vitek2 system (software version 05.04 at the time of this study, and currently 06.01) (bioMérieux, Inc., Hazelwood, MO). When a blood culture is flagged as positive by the Bactec system (BD, Sparks, MD), it is subcultured onto a blood agar plate (BD, Sparks, MD). The next day, a bacterial suspension (McFarland density of 0.5 to 0.63) is made in a 0.45% sodium chloride solution from the subculture plate and a Vitek2 GP67 card is set up according to the manufacturer’s instructions. A month after the clinical laboratory became aware that the Vitek2 system missed detection of the VRE subpopulation (case 1), a new protocol was implemented. In addition to what has been performed, the new protocol includes the inoculation of tryptic soy broth (TSB) (BD, Sparks, MD) with 2 to 3 drops of...
blood, when a positive blood culture bottle shows Gram-positive cocci in pairs or short chains. The broth is incubated at 35°C until turbid, and a 0.5 McFarland density suspension in saline is made. The suspension is then spot inoculated onto a V6 plate (BD, Sparks, MD) and incubated overnight. If there is growth on the V6 plate and the isolate is reported as vancomycin resistant on Vitek2, it is reported as vancomycin resistant. If the isolate is vancomycin susceptible by Vitek2 and no growth is observed on the V6 plate, it is reported as vancomycin susceptible. Any discrepant results are checked by Etest (bioMérieux, Inc., Hazelwood, MO) on both isolates, and the MICs are interpreted according to the Clinical and Laboratory Standards Institute guidelines (8). Two specimens, one vancomycin susceptible and one resistant, from each of the four patients presented here were isolated separately and saved for further study. The Etest method was used to determine the MICs of these individual subpopulations and to confirm that they were a homogeneous subpopulation. Of note, no resistant subpopulations were observed when testing the VRE isolates by Etest.

Molecular methods. Multilocus sequence typing (MLST) was performed on all eight isolates according to the previously described protocol (9) in order to determine that these were indeed part of a heterogeneous population and not a co- or reinfection. Internal fragments of 7 housekeeping genes were amplified by PCR and directly sequenced using Sanger automated cycle sequencing and an ABI 3730 sequencer (University of Pennsylvania Genomics Analysis Core). The allele number for each gene was assigned according to the respective MLST database (http://www.mlst.net). The combination of the allelic sequences for the 7 genes yielded the sequence type (ST) for each isolate.

To determine the mechanism of vancomycin resistance in the VRE subpopulation, primers A1 (5′-GGGAAAACGACAATTTGCTATTCAGC-3′) and A2 (5′-CAGTACATTGGGGCGGTATTC-3′) were used for PCR amplification of the vanA gene. These were modified from those previously described by Dutka-Malen et al. (10). Primers P1 (5′-GAAATTC TCAAAGGTATCTTTACCTTTTTATG-3′) and P2 (5′-TAAAGACCA ACCCTTTCTGTGAAGGCAC-3′) were used for PCR amplification of the promoter region. The thermal cycler conditions were 95°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s; and extension at 72°C for 2 min.

Growth curves and competition experiments. It has been previously reported that some vancomycin-resistant isolates of Staphylococcus aureus may grow more slowly than susceptible isolates (11). Since no differences in colony morphology or growth rates on blood agar plates were noted (not shown), enterococcal growth rates were determined by spectrophotometric analysis of inoculated broth cultures. Bacterial isolates were grown overnight in brain heart infusion (BHI) (BD, Sparks, MD) broth at 37°C. A 1:500 dilution from the overnight cultures was inoculated into BHI broth in a 96-well plate. The plate was incubated at 37°C with shaking, and optical density was measured at 595 nm every hour for 10 h. Each isolate was tested on three different days; growth curves were plotted and analyzed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA).

Fitness cost associated with antimicrobial resistance may also account for differences in laboratory detection. To address this point, competition experiments were performed between paired isolates from patients 1 and 2. Isolates from these two patients were selected for study because the paired isolates had nearly identical doubleting times and because the VRE isolate from each pair was missed by Vitek2 susceptibility testing. Overnight bacterial cultures of individual isolates were incubated in BHI at 37°C. The following day, a 1:200 dilution was started with a 1:1 VRE-to-VSE ratio with isolates from the same patient. Serial dilutions were spotted on Mueller-Hinton (MH) plates with and without 10 µg/ml of vancomycin at 0, 2, 4, 5, 6, and 9 h. To measure plating efficiency, the VRE isolates were incubated individually and serial dilutions were spotted at 0, 5, and 9 h. The densities of the competing isolates were estimated by the number of colonies obtained on MH plates with and without vancomycin. From these densities, the net population growth was expressed as the Malthusian parameter. Relative fitness was obtained using the ratio of their Malthusian parameters (see Fig. 3) (12). By definition, a relative fitness of 1 indicates no fitness effect or growth rate difference between the competing isolates.

RESULTS AND DISCUSSION

The prevalence of heterogeneous enterococcal populations in patients with bacteremia has not been previously reported, and the clinical impact of these infections is not clear. In our hospital setting, MEB accounted for approximately 5% of all patients having enterococcal bacteremia over a 9-month period (7 cases out of 152 total). The majority of cases (6/7) were caused by E. faecium, and one was caused by E. faecalis. Out of the four cases described here, three patients had a history of cancer (two with acute myeloid leukemia and one with metastatic prostate cancer). Of note, only one patient had endocarditis. Importantly, the emergence/detection of mixed-population bacteremia had no correlation with antibiotic therapy given. None of the patients received glycopeptide therapy in the month before the detection of the bacteremia. One patient had received cefepime, metronidazole, linezolid, and piperacillin-tazobactam; one had received piperacillin-tazobactam; and one had received meropenem. They all received at least one dose of vancomycin while being bacteremic.

In our clinical microbiology laboratory, repeat susceptibility testing is not performed on multiple blood cultures with the same organism unless more than 4 days have elapsed. Therefore, we are unable to accurately determine the ability of the Vitek2 system to detect the vancomycin-resistant subpopulations. In our experience, however, there were two instances in which the Vitek2 failed to detect the VRE subpopulation. These instances were on two separate occasions in two different patients. In patient 1, it occurred after a month of being bacteremic. The patient had initially been placed on vancomycin therapy, but after the VRE subpopulation was reported, linezolid was administered and bacteremia resolved 5 days later. This episode prompted us to implement the enhanced vancomycin resistance detection method described here.

The other instance of VRE bacteremia missed by the Vitek2 occurred early during patient 2’s bacteremic course. The initial blood cultures sent to the laboratory were positive for bacterial growth, and since we had implemented our new method, both VSE and VRE subpopulations were detected. The vancomycin MICs determined by Etest were >256 µg/ml for the VRE detected on the V6 plate and 2 µg/ml for the VSE detected by the Vitek2. The patient had been placed on vancomycin therapy before susceptibilities were reported, but since the patient expired the following day, we were not able to assess the effect of our new VRE detection method on this patient’s care.

Once the laboratory detected mixed-population bacteremia in our four patients, we immediately isolated the two subpopulations (one VRE and one VSE) for further study. Vitek2 was able to detect the VRE subpopulations once these had been subcultured. Isolate pairs from the same patient were closely related based on MLST pattern analysis, with a total of four different sequence types among all eight isolates (Fig. 1). These results are indicative of a mixed-population bacteremia in the same individual rather than coinfections or reinfections. Vancomycin MICs for all eight isolates were determined by Etest. The four vancomycin-resistant isolates had vancomycin MICs of >256 µg/ml, while the susceptible isolates had MICs in the 1- to 2-µg/ml range without observation of heteroresistance (Table 2). All four VRE isolates were
also positive for the vanA gene, as were 2/4 VSE isolates (Table 2).

Because mixed populations of E. faecium have been reported previously, due to either vanA acquisition or differential expression of vanA, we sequenced both the promoter and vanA regions of the paired isolates; no mutations were found in either the promoter or the vanA regions. The mechanism for suppression of vancomycin resistance was undetermined. We did not identify different phenotypic characteristics on the subcultured blood plates between the VRE and VSE subpopulations that could account for differential detection of VRE by the Vitek2 and screening agar methods.

In order to determine if bacterial growth rates had an effect on VRE detection, we compared individual growth curves for each isolate and calculated doubling times. There was no significant (P < 0.05, unpaired t test) doubling time difference between paired isolates from the same patient (Fig. 2). Therefore, growth rate differences between VRE and VSE pairs cannot explain the lack of Vitek2 detection of some VRE isolates. Competition experiments were also performed between isolates from patients 1 and 2 over 9 h. The magnitude of the fitness difference, either greater or less than 1, corresponded to how rapidly or slowly one isolate grew relative to the other. The relative fitness for the VRE recovered from patient 1 was 1.11 (Fig. 3). That is, the VRE subpopulation increased at a rate about 11% higher than did the VSE subpopulation. The VRE from patient 2 had a relative fitness of 0.98, showing no significant difference in growth rate from the VSE subpopulation (Fig. 3). Therefore, we conclude that differences in fitness do not correlate with preferential VSE detection by the Vitek2 method.

In conclusion, MEBs occur more often than initially thought, with a prevalence of 5% in our patient population regardless of prior antimicrobial therapy. These MEBs occur with both E. faecalis and E. faecium species. Because not all VRE subpopulations are detected by Vitek2, it is possible that some patients may receive suboptimal antimicrobial therapy for these infections, stressing the importance of using an alternative method to detect MEB. Here, we describe a method implemented in our laboratory for vancomycin susceptibility testing of enterococcal isolates recovered from blood. By inoculating a V6 plate as well as using Vitek2, we were able to detect and recover the vancomycin-resistant organisms. This method would be useful in clinical microbiology laboratories that use Vitek2, and presumably other automated systems, for the detection of VRE. We realize that our screening strategy may not detect VSE subpopulations, but this is not clinically relevant because antibiotic therapy is directed against the more resistant strain and not both the VSE and the VRE.

It is not clear if this phenomenon would be detected using automated systems other than the Vitek2 or if direct molecular testing of positive blood cultures would detect the mixed populations. Additional studies with other automated systems, and molecular resistance detection methods, are required before this is

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**TABLE 2 Summary of isolate characteristics and susceptibility profiles**

<table>
<thead>
<tr>
<th>Patient sample/strain</th>
<th>Date isolated (mo/day/yr)</th>
<th>Organism</th>
<th>Sequence type</th>
<th>V MIC (µg/ml)</th>
<th>vanA presence</th>
<th>Antibiotic susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A/VSE</td>
<td>1/2/12</td>
<td>E. faecium</td>
<td>412</td>
<td>1</td>
<td>–</td>
<td>G, L, V</td>
</tr>
<tr>
<td>1B/VRE</td>
<td>1/11/12</td>
<td>E. faecium</td>
<td>412</td>
<td>&gt;256</td>
<td>+</td>
<td>G, D, L</td>
</tr>
<tr>
<td>2A/VRE</td>
<td>2/1/13</td>
<td>E. faecalis</td>
<td>525</td>
<td>&gt;256</td>
<td>+</td>
<td>A, G, L</td>
</tr>
<tr>
<td>2B/VSE</td>
<td>2/1/13</td>
<td>E. faecalis</td>
<td>525</td>
<td>2</td>
<td>+</td>
<td>A, G, L, V</td>
</tr>
<tr>
<td>3A/VRE</td>
<td>2/26/13</td>
<td>E. faecalis</td>
<td>736</td>
<td>&gt;256</td>
<td>+</td>
<td>G, L</td>
</tr>
<tr>
<td>3B/VSE</td>
<td>2/28/13</td>
<td>E. faecium</td>
<td>736</td>
<td>1.5</td>
<td>+</td>
<td>G, V</td>
</tr>
<tr>
<td>4A/VRE</td>
<td>3/3/13</td>
<td>E. faecium</td>
<td>750</td>
<td>&gt;256</td>
<td>+</td>
<td>G, D, L</td>
</tr>
<tr>
<td>4B/VSE</td>
<td>3/9/13</td>
<td>E. faecium</td>
<td>750</td>
<td>1</td>
<td>–</td>
<td>G, V</td>
</tr>
</tbody>
</table>

**Notes:**

- **Abbreviations:** A, ampicillin; D, daptomycin; G, gentamicin; L, linezolid; V, vancomycin; S, susceptible; I, intermediate; R, resistant.
- **Nonsusceptibility** to daptomycin was reported.

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**FIG 1** Dendrogram based on unweighted pair group method using average linkages and built on the pairwise differences in the MLST allelic profiles. Numbers above the lines indicate genetic distances between the strains. Sequence type is on the right, and numbers in parentheses refer to the patient and isolate.

**FIG 2** Doubling times for each isolate are shown. White bars, VSE; gray bars, VRE. Error bars indicate ± standard errors.
known. We suggest that our mixed-population enterococcal bacteremia method be used by all Vitek2 users where VRE is endemic and by users of other resistance detection methods until these other systems show adequate performance.

ACKNOWLEDGMENTS

We thank Todd Barton for initially alerting us to the apparently fluctuating vancomycin susceptibility in one patient’s isolate and both he and Marissa Wilck for allowing us to report patient clinical details. We thank Prashanth Vallabhajosyula and Meeta Kerlin for allowing us to report clinical details of their patients. We also thank Diana Panesso for her critical review of the manuscript.

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