In Vitro Activity of Daptomycin against Vancomycin-Resistant Enterococci of Various Van Types and Comparison of Susceptibility Testing Methods

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Received 27 March 2003/Returned for modification 19 August 2003/Accepted 9 September 2003

The increasing prevalence of vancomycin-resistant enterococcal (VRE) infections and the limited number of antimicrobial agents for their treatment emphasize a need for new, more effective agents. In this study, the in vitro activity of daptomycin was determined against a collection of 156 VRE from seven different institutions. Van types were characterized by PCR, and pulsed-field gel electrophoresis was performed to exclude isolates with >85% relatedness by dendrogram. Included were 126 Enterococcus faecium (vanA), 17 Enterococcus faecalis (vanA), 5 Enterococcus faecalis (vanA,2,2 vanB) isolates, 2 Enterococcus avium (vanA) isolates, 1 Enterococcus durans (vanA) isolate, 10 Enterococcus gallinarum (vanC1) isolates, and 12 Enterococcus casseliflavus (vanC2) isolates. MICs of daptomycin and five additional agents were determined by the NCCLS broth microdilution method with Mueller-Hinton (MH) broth containing supplemental calcium. MICs were also determined using two investigational E-test strip formulations, and disk diffusion testing was performed by the standard NCCLS method. The MIC of daptomycin at which 50% of the isolates tested were inhibited for this isolate collection was 4 μg/ml, and the MIC at which 90% of the isolates tested were inhibited was 8 μg/ml. Two isolates of vanA E. faecium were resistant to linezolid, and one isolate was resistant to quinupristin-dalfopristin. MICs of daptomycin were determined by the E test with and without added calcium varied by 8- to 16-fold, and disk diffusion zones varied by 3 to 6 mm according to the calcium content of the commercial MH agar lots used in the study. This study has shown daptomycin to have good activity against a diverse collection of contemporary VRE isolates. However, improved standardization of the calcium content of MH agar will be important for reliable testing of daptomycin by clinical laboratories using either the E test or disk diffusion methods.

Vancomycin-resistant enterococci (VRE) have emerged as one of the most common antibiotic-resistant nosocomial pathogens encountered in American health care institutions (6, 12, 17, 22). Few options are currently available for effective chemotherapy of serious VRE infections, especially if a bactericidal effect is deemed to be necessary. A bactericidal effect is not achieved against VRE by current therapeutic options, including linezolid, quinupristin-dalfopristin, chloramphenicol, or tetracyclines (9, 13, 17). Daptomycin is an investigational lipopeptide antibiotic that can be administered once daily by the intravenous route. It has previously been demonstrated to have good inhibitory and bactericidal effects on Enterococcus spp. (1, 3, 4, 14, 16, 21, 23, 24) as well as other resistant gram-positive pathogens (13, 14, 21, 23, 24) due to its novel mechanism of action at the cytoplasmic membrane level (2).

The present in vitro study has assessed the activities of daptomycin against a selected group of vancomycin-resistant isolates of Enterococcus species from several medical institutions. The collection included 126 unique strains of Enterococcus faecium (both Van A and Van B types) in addition to smaller numbers of Enterococcus faecalis, Enterococcus avium, Enterococcus durans, Enterococcus casseliflavus, and Enterococcus gallinarum isolates. The susceptibilities of a total of 156 enterococcal isolates were determined using the NCCLS-recommended broth microdilution MIC procedure incorporating calcium-supplemented Mueller-Hinton broth (18). Calcium supplementation of test media has been shown in prior studies to be necessary for the mode of action of daptomycin (15). MICs of daptomycin were also determined using specially prepared E-test strips, one version of which contained calcium supplementation. Finally, the NCCLS disk diffusion procedure was performed on each isolate.

MATERIALS AND METHODS

Test isolates. A collection of 156 VRE from seven different institutions was examined in this study. Each isolate was identified to the species level by biochemical substrate tests, including use of Vitek GPI and Vitek 2 GPC cards (bioMerieux, Hazelwood, Mo.). Van types were characterized phenotypically by susceptibility to teicoplanin (i.e., teicoplanin MIC of ≥32 μg/ml, probable Van A; MIC of ≤2 μg/ml, probable Van B) and by the magnitude of the vancomycin MIC (i.e., vancomycin MIC of 4 to 16 μg/ml, probable Van C) (12, 17). Isolates were definitively classified by PCR amplification of chromosomal DNA regions that encoded vanA, vanB, vanC1, and vanC2 resistance types (6, 7, 8, 10). Pulsed-field gel electrophoresis was performed on SmaI digests of isolates of the same Van genotypes to exclude isolates with >85% relatedness by dendrogram (Fingerprinting version 1.12, Molecular Analyst software). This resulted in 126 E. faecium (109 vanA and 17 vanB) isolates, 5 E. faecalis (3 vanA and 2 vanB) isolates, 2 E. avium (vanA) isolates, 1 E. durans (vanA) isolate, 10 E. gallinarum (vanC1) isolates, and 12 E. casseliflavus (vanC2) isolates.

Antibiotics tested. MICs of daptomycin and five additional agents, including ampicillin, vancomycin, doxycycline, linezolid, and quinupristin-dalfopristin, were determined for each isolate. Daptomycin reagent powder was kindly provided by Cubist Pharmaceuticals (Lexington, Mass.). Reagent powders of the comparator agents were either provided by their respective manufacturers or obtained from Sigma Chemical Company (St. Louis, Mo.).

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NCCLS broth microdilution susceptibility tests. Each isolate was tested by the broth microdilution procedure recommended by the NCCLS (18). The test medium was cation-adjusted Mueller-Hinton broth (Difco formulation; Becton Dickinson, Cockeysville, Md.) for the comparative agents. Daptomycin was tested by the same medium supplemented with calcium to a target level of 50 mg/liter, as suggested by the NCCLS (18, 20). The calcium was added to the medium as recommended by the NCCLS and based upon the initial calcium content provided by the medium’s manufacturer. The total calcium concentration of the final supplemented Mueller-Hinton broth was measured initially using a COBAS INTEGRA 700 chemistry analyzer. Inocula of the test organisms were prepared from colonies grown on sheep blood agar plates incubated for 20 to 24 h. Colonies were suspended in 0.9% saline to obtain a suspension equivalent to the turbidity of a 0.5 McFarland standard and further diluted 1:20 in 0.9% saline within 15 min. This provided a final inoculum density of approximately 5 x 10⁶ CFU/ml in the wells of the microdilution panels following transfer with disposable inoculators. Colony counts of positive control wells were performed to verify that the desired inoculum concentration was obtained. The microdilution panels were incubated at 35°C in ambient air for 16 to 20 h prior to visual determination of MICs.

E tests. MICs were also determined for each isolate using specially prepared daptomycin E-test strips (prepared by AB Biodisk, Solna, Sweden, and provided by Cubist) with a concentration range of 0.016 to 256 μg/ml. One lot of E-test strips contained only a daptomycin concentration range; a second lot contained a COBAS INTEGRA 700 chemistry analyzer. Inocula of the test organisms were prepared from colonies grown on sheep blood agar plates incubated for 20 to 24 h. Colonies were suspended in 0.9% saline to obtain a suspension equivalent to the turbidity of a 0.5 McFarland standard and further diluted 1:20 in 0.9% saline within 15 min. This provided a final inoculum density of approximately 5 x 10⁶ CFU/ml in the wells of the microdilution panels following transfer with disposable inoculators. Colony counts of positive control wells were performed to verify that the desired inoculum concentration was obtained. The microdilution panels were incubated at 35°C in ambient air for 16 to 20 h prior to visual determination of MICs.

NCCLS disk diffusion tests. Disk diffusion tests with 30-μg daptomycin disks (prepared by Becton Dickinson and kindly provided by Cubist) were performed on all isolates according to the methods recommended by the NCCLS (19) with 150-mm Mueller-Hinton agar plates (Becton-Dickinson). In addition, a subset of strains was examined using Remel Mueller-Hinton agar plates (Becton-Dickinson; and with a subset of strains with Remel [Lenexa, Kans.] prepared plates). Linezolid E strips were included on each plate as a control drug. Plates were inoculated using a 0.5 McFarland density organism suspension prepared in 0.9% saline as described above. Plates were incubated at 35°C in ambient air for 16 to 20 h prior to determination of MICs. The MIC was defined by the intersection of the organism growth ellipse margin with the E-test strip using reflected light. Because the E-test strips were marked in one-half-log2 concentration increments, it was possible to record MICs in smaller than the usual twofold concentrations. In such instances in this study, E-test MICs were rounded to the next-higher log2 MICs for purposes of comparison with the reference MICs.

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Quality control organisms. *E. faecalis* ATCC 29212 was tested initially and with each day’s tests by both MIC test methods. Since the *E. faecalis* strain is not recommended for the NCCLS disk diffusion procedure, the *Staphylococcus aureus* ATCC 25923 strain was used for quality control testing of daptomycin disk tests (20).

RESULTS

Daptomycin showed very good activity against this diverse collection of VRE species and Van types. The MIC at which 50% of the isolates were inhibited (MIC₅₀) was 4 μg/ml and the MIC at which 90% of the isolates were inhibited (MIC₉₀) was 8 μg/ml for the entire collection based upon MICs determined using the NCCLS broth microdilution test method and calcium-supplemented Mueller-Hinton medium. The collection included some strains with resistance to linezolid, quinupristin-dalfopristin, ampicillin, or doxycycline (Table 1). There was no difference in susceptibility to daptomycin based upon the species of *Enterococcus* with Van A or Van B resistance or between those two acquired Van resistance types (data not depicted further). The Van C1 and Van C2 strains (*E. gallinarum* and *E. casseliflavus*) were generally more susceptible to daptomycin and the comparative agents than were the Van A and Van B strains of the other species.

The measured calcium concentration of the Mueller-Hinton broth prepared for the reference microdilution panels was 42 μg/ml, somewhat lower than the intended concentration of 50 μg/ml for unclear reasons. The calcium content of the Mueller-Hinton test agars affected the daptomycin MIC when determined using the E-test strips (Tables 2 and 3). Table 2 lists the agreement between the broth microdilution and E-test methods when a select group of strains was examined using two different manufacturers’ Mueller-Hinton agars and both varieties of daptomycin E strips. Table 3 lists similar comparisons with a larger number of strains with both E-test formulations tested on a single lot of Mueller-Hinton agar. The daptomycin E strips without added calcium provided the best correlation with MICs determined by the NCCLS broth microdilution method when applied to the Mueller-Hinton agar that contained the highest calcium content (24 μg/ml), i.e., 93.5 to 240 μg/ml, depending upon the agar lot used.

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (μg/ml)</th>
<th>50%</th>
<th>90%</th>
<th>Range</th>
<th>% Resistant*</th>
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<tr>
<td>Van A and Van B strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Daptomycin</td>
<td>4</td>
<td>8</td>
<td>0.5–8</td>
<td>NA</td>
<td></td>
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<tr>
<td>Linezolid</td>
<td>2</td>
<td>2</td>
<td>1–32</td>
<td>1.5</td>
<td></td>
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<tr>
<td>Quinupristin-dalfopristin</td>
<td>0.5</td>
<td>1</td>
<td>0.25–16</td>
<td>6.0</td>
<td></td>
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<tr>
<td>Ampicillin</td>
<td>64</td>
<td>128</td>
<td>≤0.5–128</td>
<td>93.2</td>
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<tr>
<td>Doxycycline</td>
<td>4</td>
<td>16</td>
<td>≤0.25–32</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>32–&gt;128</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1.** Overall susceptibilities of the vancomycin-resistant *Enterococcus* species strain collection to daptomycin and the comparative agents by the NCCLS broth microdilution method using calcium-supplemented Mueller-Hinton broth test medium

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (μg/ml)</th>
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<th>Range</th>
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<td>Van C1 and Van C2 strains</td>
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<tr>
<td>Daptomycin</td>
<td>1</td>
<td>2</td>
<td>0.5–8</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Linezolid</td>
<td>2</td>
<td>2</td>
<td>2–4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Quinupristin-dalfopristin</td>
<td>0.5</td>
<td>1</td>
<td>0.5–2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>2</td>
<td>2</td>
<td>1–2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≤0.25–16</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>4</td>
<td>4</td>
<td>4–8</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* MIC interpretive criteria from NCCLS M100-S13 (20). NA, not applicable.

**TABLE 2.** Comparison of daptomycin E test and NCCLS broth microdilution methods according to the Mueller-Hinton agar test medium and E-test formulation tested
The acceptable limit with the agar lots that contained only 8 to 9 μg of calcium/ml (data not depicted further).

**DISCUSSION**

Daptomycin showed very good activity against a diverse collection of VRE species and Van types included in this study, including strains resistant to quinupristin-dalfopristin or linezolid. The MIC	extsubscript{50} was 4 μg/ml and the MIC	extsubscript{90} was 8 μg/ml for the entire strain collection based upon MICs determined using the NCCLS broth microdilution test method and calcium-supplemented medium. Based upon the results of this study and those previously published (3, 11, 14, 21, 23, 24), daptomycin seems to have the advantage of uniform, predictable activity against *Enterococcus* species, regardless of susceptibility to other agents. As noted previously, quinupristin-dalfopristin has significant activity only against *E. faecium* isolates, and resistance has emerged during clinical use with both quinupristin-dalfopristin and linezolid (5; R. D. Gonzales, P. C. Schreckenberger, M. B. Graham, S. Kelkar, K. DenBesten, and J. P. Quinn, Letter, Lancet 357:1179, 2001). Furthermore, daptomycin appears to represent the only antimicrobial agent that provides bactericidal activity as a single drug (1, 4, 14, 16). In the era of ampicillin and penicillin resistance among most *E. faecium* isolates (12, 17), vancomycin resistance in a substantial percentage of *E. faecium* isolates (17, 22), and a substantial prevalence of high-level aminoglycoside resistance among current enterococcal isolates (12, 17), the ability to provide bactericidal therapy for very serious infections (e.g., endocarditis and meningitis) has become very problematic. Thus, daptomycin could provide an advantage over linezolid or quinupristin-dalfopristin, which are only bacteriostatic against enterococci (17).

The calcium content of the susceptibility test agar lots markedly influenced the daptomycin susceptibility of test strains in this study by the E-test method. This finding is very consistent with the findings of prior studies that have documented the critical influence of calcium content of the test medium on generation of accurate and reproducible MICs of daptomycin (3, 11, 24). Indeed, in earlier years when the activity of daptomycin was first described (9, 14), the NCCLS recommended 50 μg of calcium/ml (in addition to 25 μg of magnesium/ml) for testing of all drugs and aerobic bacteria in the form of cation-supplemented Mueller-Hinton broth (3). The calcium content was reduced to the present level of 25 μg/ml and the magnesium level also was reduced by 50% (i.e., cation-adjusted Mueller-Hinton broth) in recent years to facilitate more uniform testing of aminoglycosides (3). However, appropriate levels of calcium in Mueller-Hinton agar have never been specified. The agar calcium content also affected daptomycin zones of the NCCLS disk diffusion method, although to a somewhat lesser degree. In both cases, the quality control tests recommended by the NCCLS (20) served to identify agar formulations that were suboptimal for testing of daptomycin. The NCCLS does not recommend testing the *E. faecalis* control strain by the disk method but instead recommends testing with an *S. aureus* control strain (20). While the *S. aureus* test did detect agar lots with low calcium content, it might be advisable for the future to develop quality control ranges for *E. faecalis* ATCC 29212 as a possibly more sensitive indicator of low calcium content.
Currently, reliable determination of susceptibility of enterococci to daptomycin by clinical laboratories using E strips or disks would require knowledge of the calcium content of Mueller-Hinton test media, and in the case of the E strips, selecting the E test formulation to specifically complement the test medium lot. Manufacturers of prepared Mueller-Hinton agar plates should either increase the concentration of calcium in their medium or routinely provide an analysis of their medium’s calcium content if daptomycin testing is contemplated by the users of the medium. An NCCLS subcommittee is currently performing studies to select a new reference lot of Mueller-Hinton agar to be used as a reference standard by media manufacturers (R. Rennie, personal communication). The calcium content and proper performance with daptomycin will be considered in selection of the new reference agar lot. It will also be important to determine whether increasing the calcium content would result in alterations in susceptibility to certain other drug classes, e.g., aminoglycosides and tetracyclines.

In summary, this study has demonstrated the very good in vitro activity of daptomycin against a diverse collection of vancomycin-nonsusceptible Enterococcus species. Susceptibility to daptomycin was not different in VRE isolates with resistance to linezolid or quinupristin-dalfopristin compared to results with isolates susceptible to those agents. However, this very encouraging activity of daptomycin against highly resistant enterococci can only be documented using susceptibility testing reagents that contain appropriate amounts of calcium. Efforts are under way to standardize the calcium content of standard Mueller-Hinton medium to allow for reliable testing of daptomycin in a clinical laboratory setting.

ACKNOWLEDGMENT

This study was supported in part by a grant from Cubist Pharmaceuticals, Lexington, Mass.

REFERENCES