Problems with the Disk Diffusion Test for Detection of Vancomycin Resistance in Enterococci

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A total of 53 strains of enterococci, including recently isolated strains with high-level resistance to vancomycin, were tested for vancomycin susceptibility by broth microdilution and disk diffusion using Mueller-Hinton media with and without supplementation with 5% blood. By using currently published parameters of the National Committee for Clinical Laboratory Standards for the disk diffusion test, we found that strains for which MICs were 8 to 32 µg/ml were incorrectly placed in the susceptible or intermediate category, which caused both very major (1.9%) and minor (11.5%) errors. When we used newer, recently proposed breakpoints for vancomycin, we found 13.5% minor errors but no very major errors. Changing disk diffusion breakpoints to ≤14 mm for susceptible and ≥15 mm for resistant would eliminate the problem for the strains with MICs of 32 µg/ml but not for those with MICs of 8 µg/ml. For those strains, it is necessary to perform an MIC test to differentiate them from strains with MICs of ≥4 µg/ml.

Beginning as early as 1957, the existence of enterococci with decreased susceptibility to vancomycin (MICs, >4 µg/ml) has been reported, usually in general surveys of the susceptibility of gram-positive cocci (1, 2, 5–11, 14–21). However, not until 1988 were isolates with indisputable, clinically significant vancomycin resistance (MICs, ≥32 µg/ml) reported, including those isolated from an outbreak among 22 patients in England (21) in which eight strains were isolated from blood; highly resistant strains have also recently been described in France (10) and the Federal Republic of Germany (11).

Several years ago, during a larger study on susceptibility of streptococci, we noticed that for several strains of the enterococci we had tested, MICs of vancomycin were 8 to 16 µg/ml, yet the strains looked susceptible with the disk diffusion test, with zones of 17 to 18 mm (unpublished data). Because of this and because of the apparent current increase in vancomycin resistance, we initiated studies to determine whether the standard disk diffusion test yielded accurate results for vancomycin and enterococci.

Initially, 20 strains, including as many as we could find with elevated MICs, were tested by broth microdilution, agar dilution, and disk diffusion using Mueller-Hinton medium (Difco Laboratories, Detroit, Mich.; and BBL Microbiology Systems, Cockeysville, Md.) (data not shown). Because all MICs obtained with the agar dilution test were within ±1 dilution of the values obtained with the broth microdilution test (MICs, ≤0.5 to 8 µg/ml), we concluded that the two methods gave comparable results. Zone diameters were in the range of 15 to 29 mm. Since we had more recently received several strains with high-level resistance to vancomycin, we repeated the disk diffusion and MIC (using broth microdilution) tests and we included some of the more recently described species of enterococci (3).

Organisms. The microorganisms used in this study were selected from our culture collection and included strains with all levels of vancomycin susceptibility and representatives from as many species as possible.

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strains were received from Missouri (*E. faecalis*; MICs, 32 and >128 μg/ml), Minnesota (*E. faecium*; MIC, 32 μg/ml), and Spain (*E. faecium*; MIC, >128 μg/ml). When subcultured on receipt, both the Missouri strain and the Minnesota strain appeared to have two distinct colony types, each with different levels of susceptibility. The second colony type of the Minnesota strain was susceptible to vancomycin, with an MIC of 1.0 μg/ml.

The most vancomycin-susceptible species were *E. avium*, *E. durans*, *E. mundii*, *E. pseudoavium*, and *E. raffinosus*, which had MICs of 0.25 to 1.0 μg/ml. For the remaining species, MICs of vancomycin ranged from 0.5 to >128 μg/ml; for the susceptible strains, the order of activity of vancomycin, from most active to least active, was *E. faecium > E. faecalis > E. casseliflavus > E. gallinarum*. All of the strains for which the MICs of vancomycin were 8 μg/ml were *E. gallinarum*. We do not mean to infer, however, that a vancomycin MIC of 8 μg/ml indicates *E. gallinarum*.

**Disk diffusion.** The addition of 5% sheep blood did not appear to affect the zone diameters when compared to zone diameters in medium without blood. Of the 52 strains tested (1 isolate of *E. avium* did not grow on agar with or without blood and could not be tested by disk diffusion), 47 (90.4%) gave zone diameters within ±1 mm on the two media; 98% of the values were within 2 mm; and 100% were within 3 mm (data not shown).

Scatterplots for MICs (Mueller-Hinton broth without blood) and zone diameters of vancomycin (30-μg disks) are shown in Fig. 1. By using the current NCCLS breakpoints (Fig. 1), 1 of 52 (1.9%) strains was categorized as susceptible by disk diffusion and resistant by MIC (very major error); 5 strains (9.6%) were categorized as susceptible by disk diffusion and intermediate by MIC, and 1 strain (1.9%) was categorized as intermediate by disk diffusion and resistant by MIC (minor errors). In 1986, Barry et al. (1) suggested modifying the vancomycin breakpoints for the disk diffusion test from ≤9 to ≤10 mm for resistant and from ≥12 to ≥15 mm for susceptible. When the breakpoints proposed by Barry et al. (Fig. 2) were used, only minor errors occurred (7 of 52 strains, 13.5%).

These data suggest that with the current NCCLS guidelines for vancomycin, clinical laboratories which use the disk diffusion test may fail to categorize as resistant those strains of enterococci that have decreased susceptibility to vancomycin, i.e., those that require MICs of 8 to 32 μg/ml. If the proposed breakpoints of Barry et al. (1) are used, only minor errors are seen; however, all strains for which MICs of vancomycin are 8 μg/ml were incorrectly categorized as susceptible by the disk diffusion test and could not be distinguished from the more susceptible strains (MICs of 0.5 to 4 μg/ml).

All strains that had MICs of 8 μg/ml were identified as *E. gallinarum*, which suggests that this species is inherently more resistant to vancomycin than are the other species of enterococci. Kaplan et al. reported a case of bacteremia caused by this species in a 67-year-old man who had been undergoing vancomycin prophylaxis during hemodialysis (8). When we originally confirmed the vancomycin resistance of this strain, we found the MIC to be 16 μg/ml; on several repeat testings, the MIC was 8 μg/ml. Of the six strains of *E. gallinarum* included in this study, all were originally identified as *E. faecium* or as a variant of *E. faecium*. Four of the strains were isolated from blood; one was isolated from cerebrospinal fluid; and one was isolated from peritoneal fluid. Although this species of enterococcus has rarely been implicated in clinical disease, it may cause disease more frequently than previous investigators have shown, given that it has only recently been described as a species (3).
Although Thornberry and Facklam (C. Thornberry and R. R. Facklam, Antimicrob. Newsl. 8:63-64, 1984) suggest that the vancomycin resistance previously reported in some strains of enterococci may have been in error in some cases, given these data as well as those of recent investigators, the increased resistance in *E. gallinarum* and in some strains of the more commonly isolated species is now undeniable. This may not be surprising because vancomycin is being used much more frequently to treat infections caused by multiresistant gram-positive organisms, especially methicillin-resistant staphylococci.

If breakpoints were changed to eliminate the moderately susceptible category (for example, if resistance were indicated by ≤14 mm for disk diffusion and an MIC of >8 μg/ml), the strains that require an MIC of 32 μg/ml would then be correctly categorized as resistant, but the strains with an MIC of 8 μg/ml, i.e., in this study, *E. gallinarum*, would be considered susceptible. However, the *E. gallinarum* strains with MICs of 8 μg/ml should not be considered susceptible, since Kaplan et al. (8) reported them to be clinically resistant (the fact that they had obtained an MIC of 16 μg/ml for their strain is irrelevant in this regard).

Although moving the resistant breakpoint to ≤14 mm eliminates the problem with the strains that have an MIC of 32 μg/ml, it appears that there is little that can be done to separate the strains with MICs of 8 μg/ml by the disk test, since 14 other strains with MICs ranging from 0.5 to 4 μg/ml had the same zone diameters (16 or 17 mm). The only way to circumvent this problem is to perform MIC tests.

It is difficult to make recommendations, since so few strains with vancomycin MICs of >4 μg/ml were included in this study. However, until more resistant organisms are studied, our tentative recommendation is to preferentially perform MIC tests with vancomycin and enterococci to circumvent the problem of the strains with MICs of 8 μg/ml. We further recommend that the disk diffusion breakpoints be changed to ≤14 mm for resistant and ≥15 mm for susceptible. Those microbiologists who use the disk test will need to recognize the problem of those strains with MICs of 8 μg/ml (in this study only *E. gallinarum*).

**LITERATURE CITED**


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Volume 27, no. 9, p. 2140, Abstract, line 8: "≤14 mm for susceptible and ≥15 mm for resistant" should read "≤14 mm for resistant and ≥15 mm for susceptible."