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New Vancomycin Disk Diffusion Breakpoints for Enterococci

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Since 1988, when the first vancomycin-resistant enterococcus was described, several descriptions of failures of disk diffusion breakpoints to detect low-level vancomycin resistance (MICs, 8 to 32 μg/ml) have been published. A four-laboratory collaborative study was undertaken to establish more accurate breakpoints for the disk test. Mueller-Hinton agar was used to perform dilution testing (in three laboratories) and disk diffusion testing (in all laboratories). Results were determined at 18, 24, and 48 h, and zones of inhibition were read using both transmitted and reflected light. One hundred organisms (35 *Enterococcus faecalis*, 55 *E. faecium*, and 10 *E. gallinarum* or *E. casseliflavus* isolates) were selected to represent vancomycin-susceptible and -resistant phenotypes. Interlaboratory agreement of agar dilution MICs was better at 24 h (91 to 94% within ±1 dilution) than at 18 h (76% within ±1 dilution). Therefore, 24-h agar dilution MIC results were used as the reference. For disk diffusion, it was critical to note the presence of a haze or colonies inside the zone when interpreting the test, since this correlated better with the results of the agar dilution test. The presence of a haze or inner colonies was best detected by reading the zones with transmitted light and incubating the plates for a full 24 h. When plotted against 24-h agar dilution MICs, breakpoints of ≤14 mm (resistant), 15 to 16 mm (intermediate), and ≥17 mm (susceptible) resulted in 58 minor errors (14.5% of total values) and 5 very major errors (2.2% of resistant values or 1.3% of total values). No major errors were seen. Results of repeat testing using a common lot of Mueller-Hinton agar showed 52 minor errors (13.3%) and 4 major errors (4.2% of susceptible values or 1.0% of total values) but no very major errors. It is recommended that any haze or colonies within the zone be taken into account when determining zones of inhibition and that an MIC test be performed for strains with intermediate zones if vancomycin is being considered for treatment.

Disk diffusion breakpoints for vancomycin susceptibility testing were included in the first National Committee for Clinical Laboratory Standards (NCCLS) document on disk diffusion testing (8). As with the breakpoints for some of the newer drugs, those for vancomycin were developed with inherently vancomycin-resistant gram-negative organisms and fully susceptible gram-positive organisms. In 1988 the first vancomycin-resistant enterococcus was reported (17). Since then, problems with the detection of some kinds of vancomycin resistance in enterococci have been described by several investigators using current vancomycin disk diffusion breakpoints (12, 13, 16). Prior to this, Barry et al. (1) suggested modifying the breakpoints for vancomycin, but only one gram-positive isolate (an *Enterococcus faecium* strain for which the vancomycin MIC was >4.0 μg/ml) was included in that study.

Since 1988 there have been many descriptions of isolations of enterococci resistant to vancomycin (2, 4–7, 12, 14, 15, 18). Although most of the resistance has been found in isolates from Europe, the fact that disk diffusion breakpoints may not detect resistance may mean that resistance in the United States is underdetected. Descriptions of the molecular basis for the resistance have shown that there are at least three different phenotypes of vancomycin resistance: transferable high-level resistance to both vancomycin and teicoplanin (3), nontransferable variable vancomycin resistance without accompanying teicoplanin resistance (3), and intrinsic low-level vancomycin resistance in *E. gallinarum* and *E. casseliflavus* (19).

Under the auspices of the NCCLS, a collaborative study was undertaken to determine whether different breakpoints for the disk diffusion test would be more accurate for detecting these kinds of vancomycin resistance. This is the report of that study.

MATERIALS AND METHODS

Participating laboratories. Four laboratories participated in the study, including the clinical microbiology laboratories at Massachusetts General Hospital, the University of Chicago, Johns Hopkins Hospital, and the Nosocomial Pathogens Laboratory Branch of the Hospital Infections Program of the Centers for Disease Control.

Organisms. The strains were chosen from the culture collections of the participating laboratories to represent a wide spectrum of resistance phenotypes; 26 susceptible strains were included. In addition, strains were provided by Cynthia L. Fowler (National Institutes of Health, Bethesda, Md.). One hundred organisms were tested: 55 *E. faecium*, 35 *E. faecalis*, and 10 *E. gallinarum* or *E. casseliflavus* isolates.

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Antimicrobial agents. Each laboratory used vancomycin powder that had been supplied to them by Eli Lilly & Company (Indianapolis, Ind.) and their in-house supply of antibiotic disks. Three laboratories used disks purchased from Becton Dickinson Microbiology Systems (BDMS, Cockeysville, Md.) and one used disks from Difco Laboratories (Detroit, Mich.).

Susceptibility tests. All four laboratories performed disk diffusion testing with unique lots of Mueller-Hinton agar (MHA) plates (Mueller-Hinton II; BDMS) by the method described by the NCCLS (9) except that readings were done at 18, 24, and 48 h and the zones were read by using both reflected light (by holding the plate against a black surface as recommended by the NCCLS) and transmitted light (by holding the plate so that the light shone directly through the plate). In addition to disk diffusion, three laboratories performed agar dilution testing (10) with MHA (BDMS or Difco). Results were read at 24 and 48 h in all laboratories and also at 18 h in three of the laboratories.

After the initial testing had been completed, the results had been analyzed, and tentative breakpoints had been chosen, all strains were retested by disk diffusion in all four laboratories with a common lot of MHA, which was kindly provided by George Evans (BDMS), and unique lots of antibiotic disks.

All data were sent to the Centers for Disease Control (Atlanta, Ga.) for analysis using Epi Info (Centers for Disease Control) and SAS (SAS Institute, Cary, N.C.) software.

RESULTS

Agar dilution. Agreement of agar dilution MICs among laboratories (i.e., percentage of strains within ±1 twofold dilution) at 24 h ranged from 91 to 94%, while agreement at 18 h was only 76% (data not shown). Increasing the incubation time to 48 h tended to raise the MICs 1 to 3 dilutions (data not shown). In general, very susceptible strains (MICs, ≤2 μg/ml) remained susceptible after increased incubation. However, MICs for *E. faecalis* or *E. faecium* strains which were borderline (MICs, 4 to 8 μg/ml) tended to increase to 8 to 32 μg/ml. MICs for more-resistant strains (MICs, >8 μg/ml) were also likely to be 1 to 2 dilutions higher at 48 h. MICs for strains of *E. gallinarum* or *E. casseliflavus* which were 4 to 8 μg/ml at 24 h did not change at 48 h. Because incubation beyond 24 h is not recommended by the NCCLS, the 24-h agar MICs were used as the reference for analysis of the disk diffusion test.

In order to facilitate comparison of MICs to disk diffusion data, the mode MIC from the three participating laboratories was used as the reference MIC for the 100 strains tested. In 14 cases, there was no mode; in those cases, the middle value was chosen as the reference MIC. When the 24-h agar dilution MICs obtained in the three laboratories (n = 300) were compared with the reference values chosen (n = 100), 97% of the MICs were within ±1 dilution of the mode; 100% of the values were within ±2 dilutions of the mode. The distribution of the reference MICs for the 100 strains tested is given in Table 1 by species.

Disk diffusion. Inspection of the data (not shown) revealed that some vancomycin-resistant strains produced individual colonies or haze within a larger zone of inhibition that was easier to detect with transmitted light than with reflected light, which the standard disk diffusion procedure specifies. Therefore, zone diameter readings were evaluated with transmitted light only. When 24-h disk diffusion readings for all four laboratories were plotted against the 24-h agar dilution reference MICs, the breakpoints that resulted in the fewest errors were ≤14 mm for resistant, 15 to 16 mm for intermediate, and ≥17 mm for moderately susceptible (Fig. 1). With these breakpoints there were 5 very major errors (2.2% of 232 resistant values, or 1.3% of total values), no major errors, and 58 minor errors (14.5% of total values). Extending the incubation time to 48 h slightly decreased the total number of very major errors (to 2, or 0.9% of resistant values) but increased the total number of minor errors (to 64, or 16.0% of total values). The five very major errors involved a total of four strains in one laboratory and one of the same strains in another laboratory. Those four strains were among those producing a haze inside a zone of inhibition.

When the isolates were retested using a common lot of agar (Fig. 2), there were no very major errors; however, there were four major errors (4.2% of susceptible values, or 1.0% of total values). On retesting, the five very major errors became either correct (four instances) or a minor error (one instance). The number of minor errors was also slightly less (52, or 13% of total values) on retesting. However, there was a shift in the kinds of minor errors seen. Even though zone diameters in the first testing (Fig. 1) were read using transmitted light, there was a tendency to report smaller zone sizes when strains were retested (Fig. 2). This occurred mainly in two of the four laboratories.

TABLE 1. Vancomycin reference MICs[a]

Organism	No. of strains at each MIC (μg/ml):										
	≤0.5	1.0	2	4	8	16	32	64	128	256	≥512
E. faecalis		4	5	1		3	3	1	1	1	16
E. faecium	3	5	5		3	3	9	9	3	7	8
E. casseliflavus or *E. gallinarum*				1	9						

[a] Mode MICs of 100 enterococcal strains tested on MHA and read after 24 h of incubation.

DISCUSSION

As the reference test for our study, we chose the agar dilution method using MHA read after 24 h of incubation. There are precedents pertaining to staphylococci in the NCCLS susceptibility testing standards (9, 10) both for reading MICs at 24 h and for reading disk diffusion zone diameters at 24 h using transmitted instead of reflected light. Just as with that group, the additional 6 h of incubation appears to improve the detection of vancomycin resistance in some enterococci. An additional 24 h of incubation may increase detection of resistance; however, it may not be practical for most clinical laboratories to delay the reading of susceptibility results until 48 h.

Choosing breakpoints that would detect as many resistant strains as possible yet would not result in too many false-resistant values was a difficult task. Data from Massachusetts General Hospital, where there is currently no vancomycin resistance among enterococci, showed that of the 6,530 unique isolates tested during 1986, 1987, and 1989, 80% had zone diameters in the 17- to 20-mm range (data not shown). The mode zone diameter for about one-third of the strains was 18 mm. Choosing a susceptible breakpoint too close to the mode or creating a large intermediate range would decrease the usefulness of the disk test for the clinical laboratory.

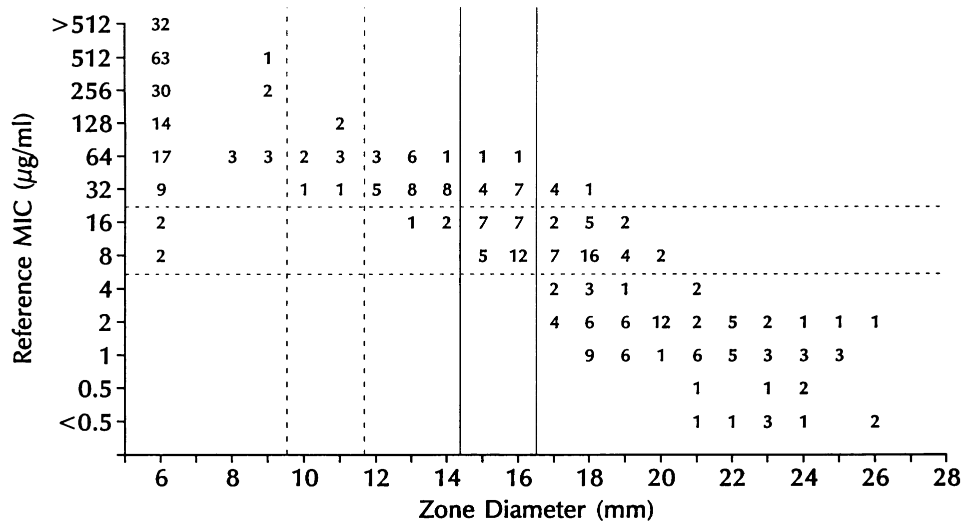

FIG. 1. Vancomycin breakpoints for enterococci with unique lots of MHA. Results of susceptibility tests performed at four institutions with 100 strains of enterococci are shown. MHA dilution MICs are compared with zone diameters obtained using a 30-µg vancomycin disk on four unique lots of commercially prepared MHA. Original breakpoints are indicated by the broken lines; new breakpoints are indicated by the solid lines.

Though the original testing of the strains resulted in five very major errors (Fig. 1), repeat testing with the common lot of agar produced no very major errors (Fig. 2), perhaps because of the increased awareness among all participants of possible haze or colonies inside the zones. Two of the laboratories tended to report smaller zone sizes when strains were retested on the common lot. Since smaller zones were not reported by all four laboratories, the shift was probably due not to the lot of medium used but instead to increased attention to haze or colonies inside the zones. This is supported by the fact that there were only major errors in the repeat testing. Interestingly, the haze or colonies noted on repeat testing were inside a second zone that was usually ≤14 mm; only rarely was the second zone >14 mm, and only once was it >16 mm.

There were some strains with intermediate values by disk diffusion testing that were characterized as resistant by MIC testing (Fig. 1). Because of this and because the mode zone diameter for susceptible strains is close to the upper breakpoint, we recommend that when vancomycin is being considered for use in treating serious infections, strains which test intermediate by disk diffusion testing be retested by an MIC method to be sure that the strain is indeed susceptible to vancomycin.

The most significant errors in detection of resistant strains were made with strains for which the MICs were intermediate but which had susceptible zone sizes. These errors were made mainly with strains of *E. gallinarum*. *E. gallinarum* and *E. casseliflavus* appear to be inherently resistant to low levels of vancomycin (MICs, 4 to 16 µg/ml) (19). The first

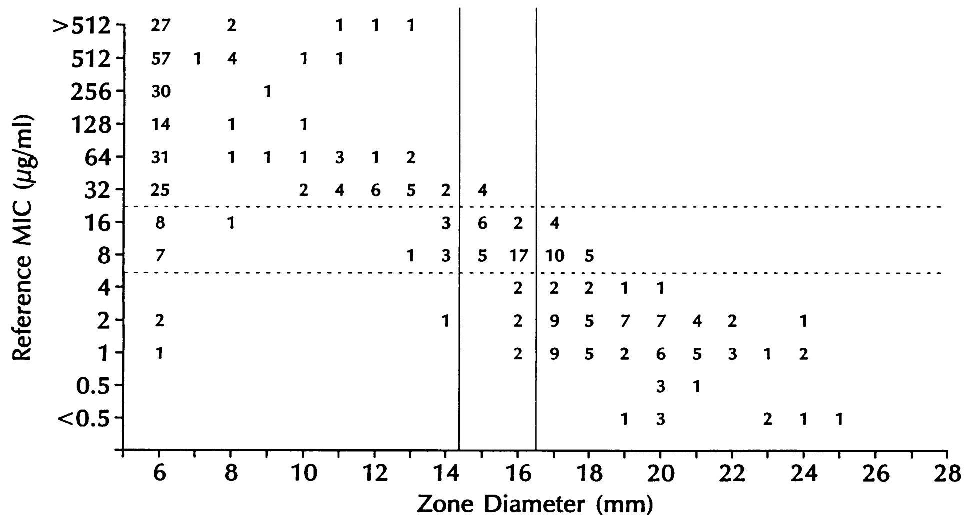

FIG. 2. Vancomycin breakpoints for enterococci with a common lot of MHA. Results of susceptibility tests performed at four institutions with 100 strains of enterococci are shown. MHA dilution MICs are compared with zone diameters obtained using a 30-µg vancomycin disk on a common lot of commercially prepared MHA.

vancomycin-resistant enterococcal strain identified in the United States was *E. gallinarum* (5). The clinical significance of these strains as well as those of *E. faecalis* and *E. faecium* with low-level resistance is unknown.

The failure of the current disk diffusion test to distinguish vancomycin-resistant from vancomycin-susceptible enterococci causes problems for clinical laboratories which rely on that standard method. It also points to an inherent problem with the disk diffusion test breakpoints that were set for some drug-microorganism combinations in the absence of resistant isolates. More recently, the NCCLS has dealt with this problem by indicating a susceptible breakpoint only in the absence of resistant strains, e.g., *Haemophilus influenzae* and broad-spectrum cephalosporins (9, 10). It is important that microbiologists be aware of usual patterns of susceptibility for commonly encountered pathogens and that they investigate any result that falls outside the usual range, even if that result is safely inside the susceptible breakpoints, as these enterococci with low-level resistance were.

The breakpoints established in this study are those that were included in the most recent update of the NCCLS tables (11) published in December 1991.

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