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Single-Disk Diffusion Testing (Kirby-Bauer) of Susceptibility of *Proteus mirabilis* to Chloramphenicol: Significance of the Intermediate Category

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The significance of the intermediate category of the single-disk diffusion test (Kirby-Bauer) of antibiotic susceptibility has never been clearly defined. Thirty-two percent of 756 clinical isolates of *Proteus mirabilis* were of intermediate susceptibility to chloramphenicol, a higher percentage than for any other species. The breakpoint separating susceptible and intermediate isolates nearly bisected the frequency distribution of zone diameters of *P. mirabilis* but not that of the other species tested. By serial broth dilution testing, the minimal inhibitory concentrations (MICs) of chloramphenicol of 50 individual isolates of *P. mirabilis* were 3.9 to 22.1 μg/ml (geometric mean, 8.0), whereas the MICs of susceptible *Escherichia coli*, *Klebsiella*, and *Enterobacter* strains were 2.0 to 3.9 μg/ml (geometric mean, 2.9). Seventy percent of isolates of *P. mirabilis* with MICs of 7.8 to 15.6 μg/ml were classified as susceptible by disk testing. We conclude that existing Kirby-Bauer breakpoints do not accurately discriminate *P. mirabilis* isolates that are marginally susceptible to chloramphenicol. These data underscore the difficulty of applying a single set of breakpoints to all species and suggest that species-specific breakpoints would more accurately predict the MIC equivalent of given zone diameters.

The significance of classifying clinical isolates as intermediate by the Kirby-Bauer single-disk diffusion test of antibiotic susceptibility is unclear. Is the level of susceptibility of these isolates marginal, in that unusually high doses of antibiotic are required for effective therapy, or is it indeterminate, reflecting the variability inherent in the disk diffusion method? Fortunately, only a small proportion of clinical isolates of most bacterial species fall into this intermediate category (1, 9).

In reviewing the results of disk diffusion testing of 5,667 gram-negative bacteria isolated from clinical specimens in 1978, we found that 32% of isolates of *Proteus mirabilis* were classified as intermediate, a percentage higher than for any other species. The studies described in this paper show that, in contrast to other gram-negative bacteria, most strains of *P. mirabilis* are marginally susceptible to chloramphenicol, and suggest that criteria based on species-specific breakpoints would more accurately categorize them.

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MATERIALS AND METHODS

Computer-generated plots. Computer analysis of the results of disk diffusion testing was done according to methods described previously (8).

Bacterial isolates. Bacterial isolates used in these studies were recent isolates from the Clinical Microbiology Laboratory of The Miriam Hospital. Species identification was performed with the API 20E System (Analytab Products). The organisms used for in vitro study were all single isolates from individual patients.

Disk diffusion. Single-disk diffusion was performed according to Bauer et al. (2-4). Four to five colonies of each organism were inoculated into 4 ml of tryptic soy broth (Scott Laboratories, Fiskeville, R.I.) and incubated for 3 to 4 h. A suspension of each organism was then standardized against a turbidity standard obtained by adding 0.5 ml of 1.175% $BaCl_2 \cdot 2H_2O$ solution to 99.5 ml of 0.36 N (1.0%) H_2SO_4. Large (150-mm) petri plates filled with 72 ml of Mueller-Hinton agar (Scott Laboratories) to a depth of 4 to 5 mm were streaked evenly in three planes with a cotton swab, with the excess inoculum being removed by rotating the swab against the side of the culture suspension tube. Plates were then incubated at 35 ± 1°C for 18 h. Zones of inhibition were recorded after incubation for 18 h. Antibiotic disks were purchased from Pfizer, Groton, Connecticut.

In a separate study, standardized suspensions of bacterial isolates were diluted with sterile saline to result in dilutions of 1:2, 1:5, 1:10, 1:100, and 1:1,000. These dilutions were utilized exactly as above.

Broth dilution MIC. Minimum inhibitory concen-

trations (MICs) of antibiotics were determined by making serial twofold dilutions of antibiotic in 2-ml volumes of Mueller-Hinton broth (Difco). Chloramphenicol standard powder was a gift of Parke, Davis and Co., Detroit, Mich. Samples (50 μl) from each tube were then distributed into the corresponding cups of a microtiter plate (Cooke Engineering Co.). An equal volume of diluted overnight cultures was added to each cup to make a final inoculum of approximately 5×10^4 organisms per ml. All determinations were made in duplicate. The plates were read after 24 h of incubation at 35 ± 1°C.

Agar dilution MIC. MICs were also determined for 47 isolates of *P. mirabilis* by making serial twofold dilutions of antibiotic in 18-ml volumes of Mueller-Hinton agar (Difco), inoculated with a Steers replicator, as described by Washington (11).

RESULTS

Disk diffusion testing of clinical isolates. Table 1 shows that 32% of 756 isolates of *P. mirabilis* were classified as intermediate by disk diffusion testing, a percentage higher than for any other species. Figure 1 shows that the distribution of zone diameters of *P. mirabilis* isolates around the 30-μg chloramphenicol disk was unimodal and was nearly bisected by the breakpoints separating susceptible and intermediate categories.

Susceptible strains of *Escherichia coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter* showed mean zone diameters of approximately 22 to 24 mm. In contrast, the mean zone diameter of *P. mirabilis* strains, seen in Fig. 1, was 18.7 mm.

Reproducibility of diameters of zones of inhibition on repeated testing. Among 16 isolates of *P. mirabilis* that were each tested twice by the single-disk diffusion method, 5 had identical zones, 7 varied within 1 mm, and 4 showed a change in diameter of zone of inhibition greater than 1 mm. The changes in zone diameters resulted in three isolates moving from the intermediate to the susceptible category and one isolate moving from susceptible to intermediate.

TABLE 1. *Susceptibility to chloramphenicol of clinical isolates from The Miriam Hospital, January to December 1978, by disk diffusion testing*

Organism	No. tested	Percent		
		Resistant	Intermediate	Susceptible
Escherichia coli	1,824	3	0	97
Klebsiella pneumoniae	961	3	2	95
Proteus mirabilis	756	4	32	64
Pseudomonas aeruginosa	730	96	2	2
Enterobacter cloacae	338	2	3	95
Acinetobacter anitratus	307	66	22	12
Proteus, indole positive[a]	251	12	12	76
Citrobacter sp.	187	4	1	95
Enterobacter aerogenes	160	1	1	98
Serratia sp.	153	32	9	59

[a] Includes *P. vulgaris*, *P. rettgeri*, and *Morganella morganii*.

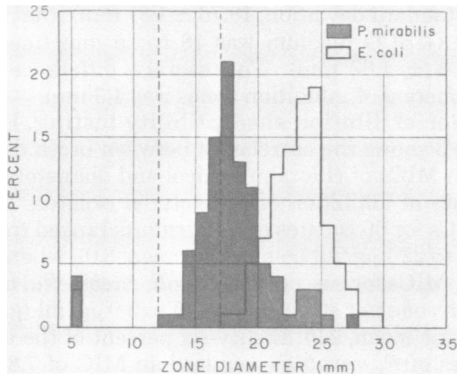

FIG. 1. *Comparison of histograms of zone diameters of P. mirabilis and E. coli, displaying their overlapping distributions.*

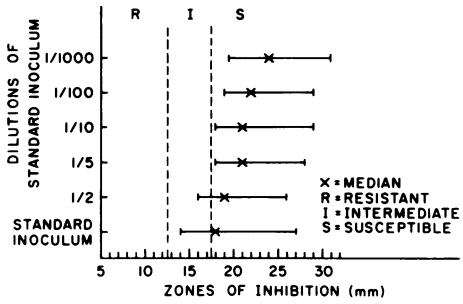

FIG. 2. *Illustration of the effects of inoculum size on the zones of inhibition around the 30-μg chloramphenicol disk for 50 isolates of P. mirabilis.*

Effects of inoculum on disk diffusion testing with chloramphenicol. Figure 2 illustrates the effects of inoculum size on the zone of inhibition around the chloramphenicol disk. With standard inocula, 16 (32%) of 50 strains of *P. mirabilis* tested gave zones in the intermediate range. A twofold dilution of the standard inoculum shifted 9 of the 16 strains into the susceptible range, and a fivefold dilution shifted all into the susceptible range.

Effects of using Mueller-Hinton agar from different manufacturers. Using Mueller-Hinton agar from different manufacturers (Scott, lot L 3110, and GIBCO, lot 0162808) had little effect on zone size. The zone for the Scott medium ranged from 17 to 25 mm in size (mean

± standard deviation, 19.76 ± 1.81 mm); that for the GIBCO medium was 18 to 25 mm (mean, 21.04 ± 1.67 mm). The average difference in diameters of inhibition zones was 1.3 mm.

Serial dilution susceptibility testing. Figure 3 shows the correlation between broth dilution MICs of chloramphenicol and diameters of zones of inhibition of 71 bacterial isolates. The MICs for 50 isolates of *P. mirabilis* ranged from 3.9 to 22.1 μg/ml (geometric mean, 8.0), whereas the MICs for susceptible *E. coli*, *Klebsiella*, and *Enterobacter* strains were 2.0 to 3.9 μg/ml (geometric mean, 2.9). Eighty-six percent of the isolates of *P. mirabilis* required an MIC of 7.8 to 15.6 μg/ml; of these isolates, 30% fell into the intermediate category and 70% fell into the susceptible category by Kirby-Bauer criteria. In contrast, all strains of *E. coli*, *Klebsiella*, and *Enterobacter* that required MICs of 2.0 to 3.9 μg/ml were classified as susceptible by disk diffusion testing, and those requiring MICs of 15.6 to 250 μg/ml were classified as resistant. Repeat broth dilution MICs determined for four isolates by using a heavier inoculum (5×10^5 colony-forming units per ml) showed a "one-tube" increase in MIC for all.

By agar dilution testing, the MICs for *P. mirabilis* isolates were slightly lower (geometric mean, 6.4) than by broth dilution, but they varied within 1 tube dilution value of the broth dilution values for all but two isolates.

DISCUSSION

Bacteria classified as intermediate according to the Kirby-Bauer method of single-disk diffusion testing of antibiotic susceptibility may be neither truly resistant nor fully susceptible. Barry defined an organism as susceptible "if it is inhibited by a concentration of antimicrobic that is less than that normally obtained in the blood of patients treated with doses of the drug that are normally given for the type of infection and type of microorganisms in question" (1). When unusually high concentrations of an antibiotic can be achieved at the site of infection, infections due to such bacteria may be treated effectively. Alternatively, the intermediate category can be considered a "buffer zone" which minimizes the significance of minor technical variables within the practical working system. The unusually high proportion of strains of *P. mirabilis* that fall into the intermediate zone for chloramphenicol challenges the concept of an intermediate category.

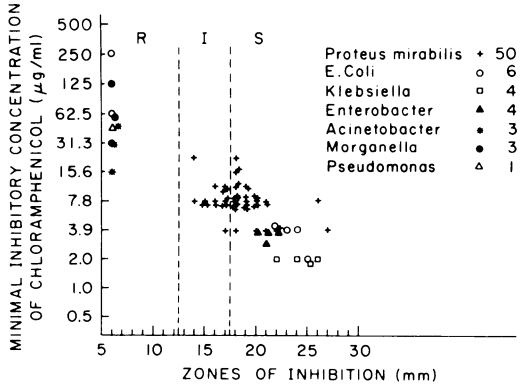

FIG. 3. *Correlation between broth dilution MICs of chloramphenicol and zones of inhibition with 30-μg chloramphenicol disks.*

At recommended dosages of 50 mg/kg per day, most patients achieve mean serum levels of chloramphenicol between 8 and 14 μg/ml (9). The upper limit of MIC for bacteria considered susceptible has ranged from 8 μg/ml (5) to 12.5 μg/ml (6). Interpretation is difficult when, as seen above, the majority of isolates of *P. mirabilis* are inhibited by 7.8 to 15.6 μg/ml. Also, the inoculum of 5×10^4 colony-forming units per ml used in this study was lighter than that used by many authors. With a heavier inoculum, a significant number of isolates of *P. mirabilis* might be expected to become more resistant to chloramphenicol, as we showed for four test strains. Our data suggest, therefore, that *P. mirabilis* isolates form a single population of organisms which are marginally susceptible to chloramphenicol. Strains of *P. mirabilis* are clearly not as susceptible as *E. coli*, *Klebsiella*, or *Enterobacter*, yet not as resistant as *Acinetobacter*, *Morganella*, *Pseudomonas*, or multidrug-resistant *E. coli*. Ericsson and Sherris have suggested that different organisms be categorized into groups according to degree of sensitivity (5).

Turck et al. were first to note that a problem existed in testing the susceptibility of *P. mirabilis* to chloramphenicol by using the single-disk diffusion method (10). They reported that of 80% of *P. mirabilis* strains interpreted as sensitive by disk diffusion, only 36% were inhibited by 10 μg/ml or less. In fact, the majority of *P. mirabilis* strains which they tested had MICs of 10 and 25 μg/ml, suggesting a borderline susceptibility to chloramphenicol similar to our observations. Turck et al. found that *Proteus* strains formed a broad unimodal, rather than a bimodal, distribution of sensitive and resistant organisms which, they suggested, accounted for the poor correlation between disk and serial dilution susceptibility test methods. Unlike Turck and his co-workers, however, we found that the distribution of zone diameters of inhibition around the chloramphenicol disk was no broader than

that of other species; rather, we found that mean zone diameters were several millimeters less, so that the distribution bracketed the cutoff separating the intermediate and susceptible zones. Thus, it would appear that most *P. mirabilis* isolates are marginally susceptible to chloramphenicol in comparison with other species and that this is reflected both in the distribution of zone diameters of inhibition and in MICs of chloramphenicol. A consequence is that relatively minor changes in inoculum may result in the same strain being reported variously as intermediate or susceptible by disk testing, as shown above.

These data emphasize the difficulty of applying the same zone size interpretive standards to all bacterial species. Within limits of experimental variation (±1 MIC), all the *P. mirabilis* isolates tested probably required the same MIC of chloramphenicol (7.8 μg/ml), indicating only moderate susceptibility. Since other bacterial species often have similar unimodal populations which vary ±1 MIC around a mean value, the "regression line" of MIC versus zone diameter is based not on a continuum of values but on groups of points at particular areas on the line, each representing only one or two species (5). Given that zone diameters vary within ± 2 mm, one would expect that different species that have mean MICs that are close in value would have zone diameter distributions that overlap. Within this region of overlap, isolates with the same zone sizes might have different MICs. To test this hypothesis, we collected several additional strains of *E. coli* with zone sizes of 19 to 21 mm, where the distribution of *P. mirabilis* zones overlaps that of *E. coli* (Fig. 1). All 10 isolates of *E. coli* had MICs of 3.9 μg/ml; in contrast, 13 of 15 *P. mirabilis* strains with zone diameters of 19 to 21 mm had MICs of 7.8 μg/ml.

We suggest, therefore, that the accuracy of zone size in predicting the MIC for an isolate would be greater if the interpretive standards used were based on values for a single species. When a single set of breakpoints is used for all species, the probability of error in predicting susceptibility of a clinical isolate will depend on the percent of isolates of that species used in constructing the regression line (7). Breakpoints of 14 to 21 mm would classify all but four of the *P. mirabilis* isolates as intermediate, and no marginally susceptible isolates would be classified as fully susceptible, as occurs with existing standards. The current breakpoints (13 to 17 mm) for *E. coli*, *Klebsiella*, and *Enterobacter* isolates should correctly identify most of these as fully susceptible. However, the zone size distributions of other *Proteus* species, *Acinetobacter*, and *Serratia* (unpublished data) suggest that a reexamination of interpretive standards for these species may also be warranted.

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