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Emergence in a Burn Center of Populations of Bacteria Resistant to Gentamicin, Tobramycin, and Amikacin: Evidence for the Need for Changes in Zone Diameter Interpretative Standards

BARBARA H. MINSHEW,[1]* HELEN M. POLLOCK,[2] FRITZ D. SCHOENKNECHT,[2] AND JOHN C. SHERRIS[3]

Departments of Surgery,[1] Laboratory Medicine,[2] and Microbiology and Immunology,[3] University of Washington Medical School, Seattle, Washington 98195

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From July 1974 through June 1976, a number of isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* from the Burn Center exhibited a shift to smaller zone diameters with gentamicin than did isolates from the general hospital population. Although many had zone diameters ≥13 mm and would have been considered susceptible by this breakpoint, they were found to have minimal inhibitory concentrations (MICs) of ≥8 μg of gentamicin per ml by agar dilution testing. Zone diameters and MICs of gentamicin, tobramycin, and amikacin were subsequently compared for 168 isolates from both the Burn Center and general hospital. The results revealed many isolates that fell into presently used gentamicin- and tobramycin-"susceptible" categories by disk diffusion tests but were resistant by MIC. The data indicated that criteria for gentamicin disk diffusion testing should include an intermediate or indeterminate category, and that the limits of the intermediate category for tobramycin and amikacin should be expanded.

For many years, gentamicin has been widely used for the treatment of serious gram-negative infections, and resistance to this antibiotic by several species of the *Enterobacteriaceae* and by *Pseudomonas aeruginosa* has now been reported by a number of investigators. Resistance to the newer aminoglycoside antibiotics, tobramycin and amikacin, is also becoming a matter of concern as the use of these drugs is increased (3, 6, 8, 11, 16-20).

A favorable clinical response with antibiotic therapy depends, among other factors, on the susceptibility of the infecting organism, and much effort has been expended in determining in vitro criteria for the susceptibilities of bacterial isolates to the newer aminoglycosides. Unfortunately, the results of laboratory tests with these antibiotics are subject to some technical variables, and the susceptibility of *P. aeruginosa*, in particular, depends on the medium used and its cationic content (2, 4, 9, 10, 13, 21). Many of the earlier studies designed to determine criteria for susceptibility by the diffusion test were performed before the difficulty with the cationic content of media was recognized. More recent studies have brought the validity of the present laboratory definition of resistance to gentamicin into question (15, 22, 23, 26).

During a 2-year period, from July 1974 through June 1976, we encountered a number of isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *P. aeruginosa* from the Burn Center at the Harborview Medical Center in Seattle that had smaller gentamicin zone diameters with the standard disk diffusion test than strains isolated from other parts of the hospital. Some of them had zone diameters that exceeded the presently recommended diffusion test susceptibility breakpoint (≥13 mm) by 1 to 3 mm for gentamicin, yet were found to be resistant to gentamicin by agar dilution testing (≥8 μg/ml). This report deals with the gentamicin, tobramycin, and amikacin susceptibilities of these and other isolates from both the Burn Center and general hospital patients, and includes studies by agar dilution and standard disk diffusion techniques using media of defined magnesium and calcium content. The results indicate that some currently recommended criteria for the interpretation of disk diffusion tests with these antibiotics require reevaluation.

MATERIALS AND METHODS

Bacteria. The distributions of populations of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* were analyzed with respect to zone diameters for isolates

from the Burn Center and the general hospital at Harborview Medical Center during the 2-year period, July 1974 through June 1976. The numbers of strains, their sources, and the antibiotics against which they were tested are shown in Table 1.

For the correlation of minimal inhibitory concentrations (MICs) and disk diffusion susceptibilities, isolates from the Burn Center and representative strains from general hospital patients were selected. In general, only one isolate from a single burn patient was tested unless the antibiograms or the specimen sources were different. Isolates from the Burn Center included 16 *E. coli*, 42 *K. pneumoniae*, and 53 *P. aeruginosa*. Most of these strains had zone diameters with gentamicin disks within 3 mm of the currently recommended breakpoint. Among the strains tested from nonburn patients, 14 *E. coli* and 13 *K. pneumoniae* were strains isolated at Harborview Medical Center, and 9 *K. pneumoniae* were from University Hospital. The 21 strains of *P. aeruginosa* were clinical isolates from a collection of strains stocked by the clinical Microbiology Laboratory at University Hospital.

Disk diffusion susceptibilities. The standard disk diffusion susceptibility test was performed on all isolates (15) . For those strains that were studied for a comparison of MIC and disk diffusion susceptibility, the zone diameters of two disks for each antibiotic were measured to the nearest 0.1 mm by two readers. The four values were averaged. Standard strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were included daily for quality control and gave satisfactory performance (Table 2). Mueller-Hinton agar (Difco lot control no. 620275) was analyzed by atomic absorption spectroscopy (21) and contained magnesium and calcium concentrations of 24 and 77 mg/liter. These concentrations were within the limits suggested for the susceptibility testing of aminoglycoside antibiotics on this medium (21).

Agar dilution susceptibilities. Standard agar dilution MICs (28) were performed with the lot of Mueller-Hinton agar described above. A Steers replicator was used to inoculate the agar surface (24).

Criteria for susceptibility and resistance. Unless otherwise stated, the presently recommended criteria for susceptibility and resistance were taken to be those shown in Table 3. The gentamicin and tobramycin diffusion test breakpoints are those recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (1). The amikacin breakpoints are those recommended by Bristol Laboratories.

The gentamicin MIC breakpoints have been adapted from the NCCLS standards (1) to conform to the dilution schedule recommended by the International Collaborative Study Group (7). The same MIC breakpoints were used for tobramycin as for gentamicin. The amikacin MIC breakpoints have been adapted from those recommended by Bristol Laboratories (≤20 μg/ml, susceptible; >20 μg/ml, resistant).

RESULTS

Comparison of burn and nonburn isolates. During the first year the Burn Center was open, it was noted that *E. coli* and *Klebsiella* isolates from this source more frequently ap-

TABLE 3. *Breakpoints for sensitive, intermediate, and resistant categories*

Antibiotic	Diffusion test[a] (zone diam in mm)			MIC (μg/ml)		
	S	I	R	S	I	R
Gentamicin ..	≥13		≤12	≤4		≥8
Tobramycin ..	≥14	12–13	≤11	≤4		≥8
Amikacin	≥14	12–13	≤11	≤16		≥32

[a] Disk content was 10 μg/ml for each of the three antibiotics.

TABLE 1. *Number of isolates analyzed for distribution of zone diameters*

Organism	General hospital (nonburn)			Burn Center	
	7/74–7/75[a]	7/75–7/76[a]	4/76–5/76[b]	7/74–7/75[a]	7/75–7/76[b]
E. coli	1,173	1,081	142	282	69
K. pneumoniae	456	489	64	116	205
P. aeruginosa	487	583	52	152	203

[a] Analyzed for zone diameters with 10-μg gentamicin disks.
[b] Also analyzed for zone diameters with 10-μg tobramycin and amikacin disks.

TABLE 2. *Correlation of MICs and zone diameter of control strains with aminoglycoside antibiotics*

Antibiotic	*P. aeruginosa* ATCC 27853		*E. coli* ATCC 25922	
	MIC (μg/ml)[a]	Zone (10-μg disk)[b]	MIC (μg/ml)[a]	Zone (10-μg disk)[b]
Gentamicin	3.0 (1.0–4.0)	20.1 ± 1.1	1.1 (1.0–2.0)	21.2 ± 1.1
Tobramycin	1.2 (1.0–2.0)	21.3 ± 0.9	1.4 (1.0–2.0)	19.4 ± 0.7
Amikacin	3.6 (2.0–8.0)	19.2 ± 0.8	2.0 (2.0)	19.2 ± 0.7

[a] Geometric mean of the MIC (and range); each value represents seven individual tests for *P. aeruginosa* and nine tests for *E. coli*.
[b] Mean zone diameter in millimeters ± standard deviation; each value represents the mean of 28 observations for *P. aeruginosa* and 36 observations for *E. coli*.

proached the borderline of resistance to gentamicin by the current diffusion test standards (resistant ≤ 12 mm; susceptible ≥ 13 mm) than those from the general hospital population. Figure 1 compares the distribution of gentamicin zone diameters for these species and for *P. aeruginosa* by source of isolates for the year 1974–75. There is evidence for a bimodal distribution of susceptibilities of *E. coli* and a pronounced shift to the left of *K. pneumoniae* among isolates from the Burn Center. The more susceptible populations of all three species from the Burn Center appeared to correspond to those of strains isolated from the general hospital, but strains in the more resistant population were rarely encountered outside the Burn Center. This difference is reflected in the means of the zone diameters of isolates from general hospital patients (Table 4).

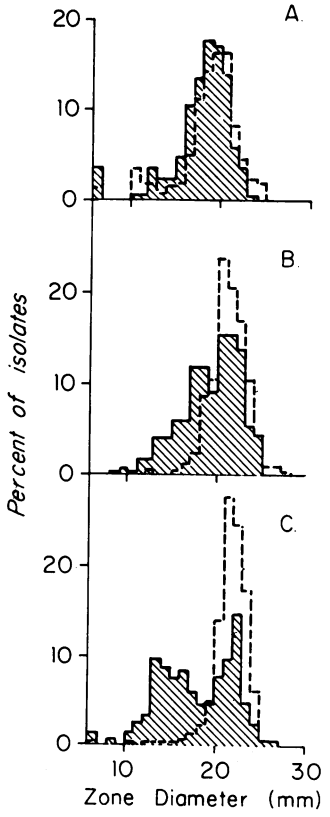

FIG. 1. *Comparison of population distributions of zone diameters with gentamicin. Bacterial isolates (1974–75) of (A) P. aeruginosa, (B) K. pneumoniae, and (C) E. coli from (▨) burn patients, and (□) nonburn patients were tested for disk diffusion susceptibility to gentamicin. The numbers of organisms included in the analyses and their dates of isolation are listed in Table 1 for Fig. 1 and 2.*

TABLE 4. *Comparison of means[a] of zone diameters with gentamicin for bacterial populations isolated from burn and general hospital patients*

Organism	Year	Burn patients	Nonburn patients
E. coli	1974–75	16.2 ± 4.3[b]	21.2 ± 1.9
	1975–76	18.8 ± 3.1	20.9 ± 1.6
K. pneumoniae	1974–75	19.3 ± 3.4	20.3 ± 2.3
	1975–76	16.2 ± 4.1	20.3 ± 2.0
P. aeruginosa	1974–75	17.5 ± 2.7	18.7 ± 2.7
	1975–76	12.3 ± 4.3	18.3 ± 2.8

[a] Population means of isolates from burn and nonburn patients were significantly different ($P < 0.001$) as determined by Fisher's *t* test.
[b] Mean and standard deviation.

During the next year, July 1975 to July 1976, the mean gentamicin zone diameters for *K. pneumoniae* and *P. aeruginosa* were reduced further for isolates from the Burn Center (Table 4). The means of the zone diameters of isolates from the nonburn populations fell by less than 1 mm over the 2-year period; those of the Burn Center isolates remained significantly lower throughout. These results indicate that a population with increased resistance was more prevalent in the Burn Center than in the rest of the hospital.

From July 1975 to July 1976, all gram-negative bacilli isolated from burn patients were also tested against tobramycin and amikacin as well as gentamicin by the diffusion test. The results with *E. coli*, *K. pneumoniae*, and *P. aeruginosa* are compared with those for nonburn isolates tested during April and May 1976 (Fig. 2). Populations of increased resistance to each of the antibiotics had developed in the Burn Center; this was particularly marked with *P. aeruginosa*, where populations of both high- and low-level resistance appeared to have been selected.

Relationships between zones of inhibition and MICs. Studies were performed to reevaluate present diffusion test breakpoints because of the apparent appearance of low-level resistant organisms, many of which fell into presently used "susceptible" categories. Selected isolates of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* from the Burn Center and general hospital were tested against gentamicin, tobramycin, and amikacin by both the agar diffusion and agar dilution methods.

The results are shown in Fig. 3, 4, and 5 as scattergrams. Also shown in the figures are regression lines relating the results of the two methods and lines representing the presently recommended susceptibility breakpoints for the diffusion and dilution tests as described in Materials and Methods.

A number of strains of *E. coli*, *Klebsiella*,

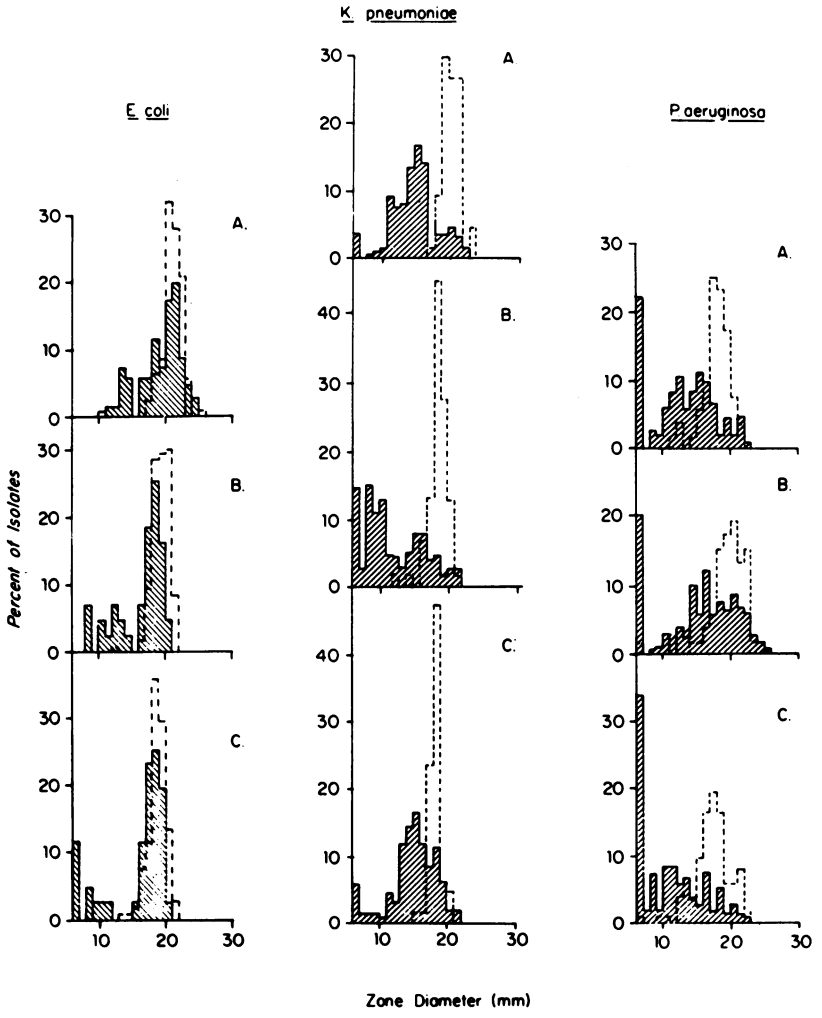

FIG. 2. *Comparison of population distributions of zone diameters with aminoglycoside antibiotics for E. coli, K. pneumoniae, and P. aeruginosa. Isolates (1975-76) from (▨) burn patients and a representative sample of isolates from (□) nonburn patients were tested for disk diffusion susceptibility to (A) gentamicin, (B) tobramycin, and (C) amikacin.*

and *Pseudomonas*, most of which were from the Burn Center, yielded gentamicin zones of ≥13 mm despite MICs in the ranges of 16 to 64 μg/ml (Fig. 3). There were no organisms with MICs of 8 μg/ml or less that fell into the presently accepted resistant category by the disk diffusion test (≤12 mm).

The application of presently recommended tobramycin diffusion test breakpoints to the data shown in Fig. 4 again shows that several isolates from the Burn Center with zones in the susceptible diffusion test range had MICs of 8 to 32 μg/ml. As with gentamicin, the presently recommended diffusion test breakpoint for resistance included only strains with MICs of ≥8 μg/ml, and in all but one case of ≥16 μg/ml.

The results with amikacin are shown in Fig. 5. In this case, the manufacturer's proposed diffusion test breakpoint for susceptibility corresponds to an MIC of ≤16 μg/ml in all but one case. The disk diffusion resistant category, however, includes a number of strains with MICs of 8 and 16 μg/ml, which would be considered susceptible by the agar dilution method.

The distribution of MIC and zone diameter values shown in Fig. 3 through 5 permitted valid regression line analyses. The lines for tobramycin and amikacin were similar, with slopes of −1.9 and −2.1. The gentamicin line,

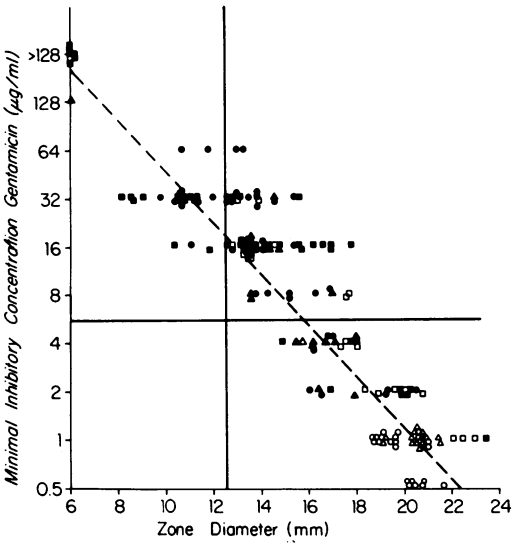

FIG. 3. *Correlation of agar dilution MICs and disk diffusion zone diameters for gentamicin. The correlation coefficient (r) was −0.90. The results of susceptibility tests for 168 organisms are plotted to the nearest 0.1 mm:* (□) *P. aeruginosa,* (○) *K. pneumoniae, and* (△) *E. coli. Isolates from burn patients are represented as closed symbols. For the purpose of analysis, the values for pairs of tests with either zone diameters of 6 mm or MICs of ≥128 μg/ml were not included for Fig. 3–5. The regression lines were fitted by the least-squares method with the $\log_2$ of the MICs as the independent variable.*

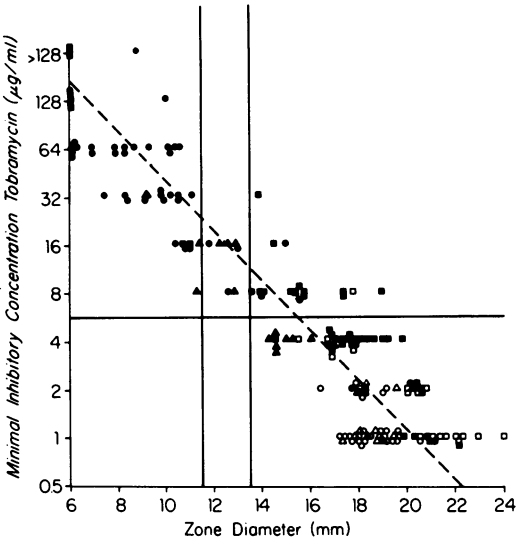

FIG. 4. *Correlation of agar dilution MICs and disk diffusion zone diameters for tobramycin (see legend Fig. 3). The correlation coefficient (r) was −0.91.*

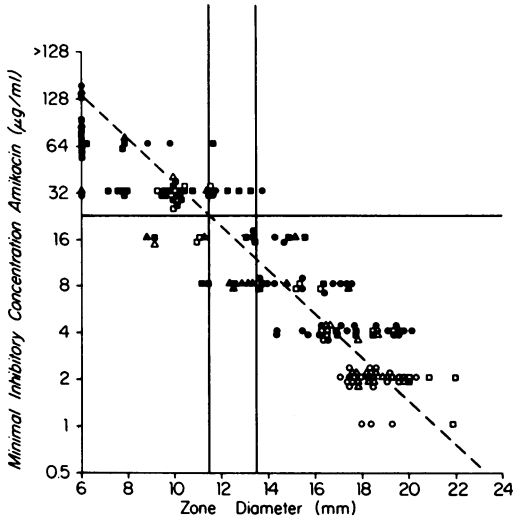

FIG. 5. *Correlation of agar dilution MICs and disk diffusion zone diameters for amikacin (see legend Fig. 3). The correlation coefficient (r) was −0.90.*

with a slope of −1.6, was steeper, suggesting poorer diffusibility and, therefore, a lower sensitivity of the diffusion test with this antibiotic.

The results of tests with the standard *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 25922) are shown in Table 2. The diffusion test results were within published limits, although the *Pseudomonas* figure was toward the upper end of the range suggested by NCCLS (16 to 21 mm) (1).

DISCUSSION

Populations of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* with increased resistance to the newer aminoglycoside antibiotics emerged in the Burn Center during a 2-year period. Gentamicin was widely used during this time, both parenterally and by subeschar clysis. Many of the Burn Center isolates showed increased resistance to tobramycin and amikacin, as well as to gentamicin, although initially only the latter antibiotic was being widely used, and amikacin was rarely used. Some cross-resistance would be expected in view of similar mechanisms of action of the three antibiotics and overlapping patterns of enzymatic modification (5).

Although a specific clinical study of the significance of these more resistant isolates was not made and the mechanisms of resistance have not yet been determined, several pieces of evidence suggest that they should be categorized as resistant or, at least, of equivocal or intermediate susceptibility for gentamicin and

tobramycin. Not only did many Burn Center isolates constitute a more resistant population of each of the species, but many had MIC values greater than the recommended breakpoints for susceptibility and above the usual blood levels achieved with recommended therapeutic doses (3, 12).

An analysis of the susceptibility test results for the strains with increased resistance from the Burn Center suggests the need for a revision in zone diameter interpretative breakpoints with gentamicin and tobramycin and, probably, also with amikacin. A number of isolates from the Burn Center showed zone diameters with gentamicin and tobramycin in the susceptible range, despite MICs above the levels generally accepted for categorization as susceptible. The present gentamicin breakpoint of ≥13 mm with a 10-μg disk for susceptible strains was tentatively recommended by Kirby and Standiford (14) from studies comparing broth dilution and disk diffusion tests. This recommendation was subsequently adopted by the Food and Drug Administration (15) and NCCLS (1). At that time, however, few if any gentamicin-resistant strains had emerged, and the discriminatory capacity of the breakpoint could not be fully evaluated. Subsequently, it was reported (25, 27) that some strains of *P. aeruginosa* showing gentamicin zones in the resistant range had MICs of less than 4 μg/ml; conversely, others reported encountering strains with agar dilution MICs of over 4 μg/ml that showed zones of up to 15 to 16 mm (22, 23, 26). These disparities are explainable, at least in part, by the demonstrated effect of divalent cation content on the susceptibility of *Pseudomonas* to gentamicin. Broth media are usually low in divalent cations relative to agar media and yield substantially lower MICs (4, 9, 10, 13, 21). This effect is much less marked with members of the *Enterobacteriaceae*, although total ionic strength may influence the activity of the antibiotic (2). Thus, gentamicin MICs, particularly of *Pseudomonas*, are manipulatable variables and must be related to medium constitution and the performance of control strains. The MICs and parallel diffusion tests in this study were determined on unsupplemented Mueller-Hinton medium with contents of magnesium and calcium within the ranges recommended by Reller et al. (21). Both the standard *E. coli* and the *P. aeruginosa* strains yielded zone diameters within published control limits (Table 2).

In our gentamicin diffusion tests, the present breakpoint of ≥13 mm for susceptibility correctly classified the majority of strains from the general hospital but included many Burn Center isolates with MICs of ≥8 μg/ml. There was no single breakpoint that could discriminate between all strains giving MICs of 4 and 8 μg/ml, i.e., between susceptible and resistant (Fig. 3). A breakpoint of 19 mm for susceptible would remove all strains with MICs ≥8 μg/ml from the susceptible category, but would also eliminate from this category all strains with MICs of 4 μg/ml and many with MICs of 2 μg/ml. The distribution of *Pseudomonas* strains among the susceptible general hospital population would be bisected by this breakpoint (see Fig. 1 and 2). A breakpoint of ≥17 mm for susceptibility would have properly allocated all but six isolates of *Pseudomonas* with MICs of 8 or 16 μg/ml and one *E. coli* and one *Klebsiella* with MICs of 8 μg/ml. It would, however, move approximately 15% of the more susceptible population of *Pseudomonas* (Fig. 1 and 2) and a number of strains with MICs of 2 and 4 μg/ml out of the susceptible category (Fig. 3). These results indicate the need for an intermediate or indeterminate category for gentamicin, as has been established for other antibiotics (15). This includes strains that should be retested and/or tested by a dilution procedure, using cation-supplemented broth or Mueller-Hinton agar, if they are causing infections that will be treated systemically.

The presently recommended tobramycin breakpoint for susceptibility also categorized a number of Burn Center isolates as susceptible despite MICs of 8 and 16 μg/ml. For the most part, nonburn isolates were correctly classified. The presently recommended breakpoint for resistance excluded from the resistant category all but one strain with MICs of 8 μg/ml. An increase in the breakpoint to ≥17 mm would have excluded from the susceptible category all isolates with MICs of ≥16 μg/ml and all but four pseudomonads with MICs of 8 μg/ml (Fig. 4). In this case, less than 10% of the more susceptible general hospital population of pseudomonads (Fig. 2) and several strains with MICs of 2 and 4 μg/ml would be moved from the susceptible category (Fig. 4).

The recommended amikacin breakpoint between susceptibility and resistance appears suitable if an MIC of ≤16 μg/ml is an appropriate figure for the susceptible category. In a discussion of the microbiology and clinical pharmacology of amikacin (Review of microbiology and clinical pharmacology, J. Infect. Dis. 134(Suppl.):S355–S460), Nauman noted that strains having MICs of 4 to 16 μg/ml required increased dosages of antibiotic for adequate therapy. Quinn also commented that, in his

experience, strains with MICs of 8 to 16 μg/ml exhibited zone diameters of 13 to 16 mm by disk diffusion. On these bases, an intermediate zone including organisms in this range would be useful, as suggested by Quinn. Drasar et al. (6) defined strains with amikacin MICs of 16 μg/ml as resistant. In this study, a susceptible disk diffusion breakpoint of ≥16 mm would leave eight strains with MICs of 8 μg/ml in this susceptible category; three isolates with MICs of 4 μg/ml would be excluded (Fig. 5). Moreover, less than 10% of the general hospital pseudomonads (Fig. 2) would be removed from the susceptible category. It is apparent from Fig. 5 that the ability to discriminate between susceptible and resistant strains is made difficult by the broad range of zone sizes given by strains possessing the same MIC. It appears that a reasonable solution to this problem involves the provision of a broader intermediate or indeterminate range (15) for the disk diffusion test with amikacin.

The results reported here cannot be taken as representative of the proportions of aminoglycoside resistant *E. coli*, *K. pneumoniae*, and *P. aeruginosa* strains that will be found by others. The emergence of these organisms was most likely occasioned by the particular epidemiological situation that existed in a Burn Center, where both a strong selective pressure toward resistance and unusual opportunities for transmission existed. Furthermore, many of the isolates may have been of the same strain infecting different patients. For these reasons, the precise proportions of the more resistant strains have no particular significance. What is significant, however, was the failure to detect resistance in a number of these strains with the standard disk diffusion test interpreted by the current criteria.

We consider that the use of an intermediate or indeterminate category is particularly important with aminoglycosides because the selection of low-level resistant variants results in resistant and susceptible population distributions that tend to overlap. Strains falling in the indeterminate range should be retested and/or examined by a dilution test in either cation-supplemented Mueller-Hinton broth or on Mueller-Hinton agar if systemic treatment with the antibiotic is considered.

In the case of *E. coli* and *Klebsiella*, decisions as to appropriate intermediate or indeterminate ranges are reasonably straightforward because results of tests with these species are essentially uninfluenced by differences of medium divalent cation contents, and because there is generally good batch-to-batch medium performance reproducibility. Intermediate ranges of 13 to 16 mm with gentamicin and tobramycin and of 12 to 15 mm with amikacin eliminated the great majority of major interpretative errors in our study. Even with the modified breakpoints, however, occasional isolates with gentamicin or tobramycin MICs of 8 μg/ml would have been allocated to the susceptible range. There is need for further study to determine whether these particular MICs represent the technical variability of the dilution tests (±1 dilution) or the inherent error of the diffusion test. Further increases in the zone diameter breakpoints for allocating an organism to the susceptible categories for these antibiotics would not appear feasible at present, because it would result in moving a considerable proportion of the susceptible population to the intermediate category.

In the case of *P. aeruginosa*, the problem of setting indeterminate criteria for aminoglycoside antibiotics is more complex because of the greater influence of medium variability with this species. In this study, the indeterminate or intermediate ranges suggested above for *E. coli* and *Klebsiella* were suitable for *P. aeruginosa* as well. After this study was completed, however, we encountered batches of Mueller-Hinton agar from our supplier that yielded mean zone diameters with gentamicin for the *P. aeruginosa* control strain lower than the results presented here (Table 2). This change was accompanied by a comparable shift to the left (to smaller zone sizes) of the population distribution of *P. aeruginosa* from those shown in Fig. 1 and 2. The population distributions of *E. coli* and the means of the *E. coli* control strain were relatively unaffected. With such batches of medium, the application of the proposed intermediate ranges for *E. coli* and *Klebsiella* to *P. aeruginosa* results in an unacceptable number of strains of *P. aeruginosa* from the susceptible population being classified as intermediate or indeterminate. Pending better performance standardization of Mueller-Hinton medium for *P. aeruginosa*, the problem appears to be soluble only by the application of breakpoints that relate results with an individual strain to the mean values for the control *P. aeruginosa* strain on the same batch of medium. This is essentially the approach adopted by Garrod and Waterworth (9). Our data indicate that strains of *Pseudomonas* yielding zones of 3 to 6 mm below the mean for the control should tentatively be regarded as of indeterminate susceptibility to the aminoglycosides. Our experience also indicates that it is wise to maintain plots of population distribu-

tions of zone diameters for clinical isolates of all commonly encountered species. Deviations from the more usual distributions of organisms can be detected by these plots, and a clue can be given to the occurrence of low-level variants.

We consider that further studies in more than one center are indicated to reevaluate the presently used breakpoints for the newer aminoglycoside antibiotics and to determine whether the proposed modifications fit the experience of others. Such studies should use agar dilution, or cation-supplemented broth dilution procedures, and batches of media that give diffusion test results at approximately the midpoints of published control limits with the standard *E. coli* and *P. aeruginosa* strains. We believe that the establishment of more stringent performance standards for susceptibility testing media should have high priority, especially with regard to *Pseudomonas* and the aminoglycosides.

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