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In Vitro Susceptibility Testing with Tobramycin

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Tobramycin (nebramycin factor 6) is an aminoglycoside antibiotic active in vitro against many gram-negative species and *Staphylococcus aureus*. The susceptibility of 191 recently isolated pathogenic bacteria to tobramycin was measured by both a routine broth dilution procedure and the FDA standardized disc technique using a 10-μg disc. Twenty-five isolates each of *Escherichia*, *Enterobacter*, *Klebsiella*, indole-negative *Proteus*, *Pseudomonas*, *Serratia*, and *S. aureus*, and 16 isolates of group D enterococci were tested. The greatest activity was seen with *S. aureus* and *Pseudomonas* species; nearly all isolates of both were inhibited by 0.20 μg or less per ml. Tobramycin was slightly less active against *Klebsiella* and *Enterobacter* and moderately active against *Escherichia* and *Proteus*, with most isolates of these genera being inhibited by 1.56 μg/ml. Neither *Serratia* nor the enterococci were particularly susceptible. Correlations between zones of inhibition around the 10-μg disc and minimal inhibitory concentrations were determined, and a zone diameter of 16 mm was recommended as the critical point for prediction of susceptibility.

Tobramycin (nebramycin factor 6) is a recently described aminoglycoside antibiotic isolated from the nebramycin complex (6, 9). Previous investigations have shown it to be similar to gentamicin in terms of its in vitro activity against *Staphylococcus aureus* and members of the *Enterobacteriaceae* and from two to four times more active against *Pseudomonas* (2, 3, 7, 10). Moreland et al. found it to be inhibitory for all of 25 isolates of Pseudomonas at 0.78 μg/ml while gentamicin was inhibitory for only 2 at the same concentration (Abstr., Eleventh Intersci. Conf. Antimicrob. Ag. Chemother., p. 42). The study reported here was intended to provide additional data regarding the in vitro activity of tobramycin against recently isolated pathogenic bacteria and to evaluate the degree of correlation between susceptibility data obtained by disc diffusion testing and by determination of minimal inhibitory concentrations using a routine broth dilution procedure.

MATERIALS AND METHODS

Broth dilution susceptibility studies. Minimal inhibitory concentrations (MIC) were determined in heart infusion broth (Difco) by a twofold serial dilution procedure. Concentrations of tobramycin ranged from 50 to 0.05 μg/ml. The standard material was a sterile solution containing 1,000 μg of tobramycin per ml (Eli Lilly & Co., compound 47663, control S1 36-OA). Tests were performed with 3-ml volumes of broth and 0.05-ml volumes of inocula. The latter were prepared from 1:1,000 dilutions of overnight broth cultures. The MIC was defined as the lowest concentration of drug which inhibited growth as measured visually by the absence of turbidity following overnight incubation at 37 C.

Disc diffusion testing. The disc tests were performed by using the standardized technique of Bauer et al. (1) as modified by the ad hoc Advisory Committee on Anti-infective Drugs (5). The medium was Mueller-Hinton agar (BBL) poured in disposable petri dishes to a depth of 4 mm; plates over 96 hr old were not used. Several colonies of the organisms to be tested, usually five to seven, were picked and inoculated into 5-ml volumes of Trypticase soy broth. These were incubated overnight at 37 C and then diluted in sterile saline to a density equal to that of a number 3 McFarland nephelometer tube. Nephelometric readings at 530 μunits showed all inocula to have an optical transmission of between 98 and 100%.

Plates were inoculated by streaking on three planes with a cotton swab previously dipped into a diluted culture and then rotated against the side of the tube. Standard 10-μg tobramycin susceptibility test discs (Eli Lilly & Co., lot P-69790) were pressed onto the inoculated plates. After overnight incubation at 37 C, the diameters of the resulting zones of inhibition, including the disc, were measured with mechanics calipers to the nearest 0.5 mm.

Cultures. Twenty-five isolates of *Escherichia*, *Enterobacter*, *Klebsiella*, indole-negative *Proteus*, *Pseudomonas*, *Serratia*, and *S. aureus*, and 16 isolates of group D enterococci were tested. All were recent clinical isolates recovered and identified by the Bacteriology Laboratory, Clinical Pathology Laboratories,

Medical College of Virginia. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were included in all test runs as standard control organisms.

RESULTS

MIC values obtained for tobramycin in this study were in good agreement with previously published results. Most gram-negative organisms (76%) were inhibited by 1.56 μg/ml and many, including 23 of 25 isolates of *Pseudomonas* species, were inhibited by 0.78 μg/ml (Table 1). *Serratia* and *Proteus* species were less susceptible, with only three isolates of the former and 16 of the latter being inhibited at 1.56 μg/ml. Mean MIC values, in micrograms per milliliter, for the different gram-negative genera were as follows: *Escherichia*, 1.29 ± 1.12; *Enterobacter*, 0.73 ± 0.75; *Klebsiella*, 0.41 ± 0.29; *Proteus*, 2.19 ± 1.53; *Pseudomonas*, 0.55 ± 1.32; and *Serratia*, 6.19 ± 3.98.

Tobramycin was highly active against *S. aureus* but poorly active against enterococci (Table 1). All 25 isolates of the former were inhibited by 0.39 μg/ml, but none of the 16 isolates of the latter was susceptible to less than 3.13 μg/ml and six were resistant to less than 12.5 μg/ml. Mean MIC values for these two groups of organisms were 0.17 ± 0.11 and 8.13 ± 3.30 μg/ml, respectively.

The broth dilution studies required 11 separate tests. In 10, the MIC of tobramycin against *S. aureus* ATCC 25923 was between 0.05 and 0.20 μg/ml and the mean value was 0.16 ± 0.21 μg/ml (Table 1). Similarly, the MIC for *E. coli* ATCC 25922 in 10 sets was either 0.78 or 1.56 μg/ml, and the mean value was 1.42 ± 0.68 μg/ml.

The diameters of the zones of inhibition produced by the 10-μg tobramycin disc were generally in the range of 18 to 23.5 mm for most susceptible gram-negative organisms (Table 2). Twenty isolates of *Klebsiella* and 18 of *Escherichia* gave zones of inhibition in the range of 18 to 20.5 mm. Somewhat larger zones were obtained with *Enterobacter* and *Proteus*; 16 isolates of the former and 19 of the latter had zones in the range of 21 to 23.5 mm. A wider spread of values was seen in the zones obtained with *Pseudomonas*; no zone was obtained with one isolate, 5 gave zones in the range of 15 to 17.5 mm, 14 gave zones in the range of 18 to 20.5 mm, and 5 gave zones of 21 mm or more. In spite of the higher MIC values obtained with *Serratia*, more than half of the isolates of this organism produced zones in the range of 21 to 23.5 mm.

Fifteen isolates of *S. aureus*, or 60%, gave zones of inhibition with diameters of 21 to 23.5 mm with the 10-μg disc; the remaining 10 isolates gave zones of 24 mm or larger. In contrast, no zones were obtained with eight enterococci, and the remaining eight produced zones with diameters of only 6 to 8.5 mm.

Some variation in zone size values was seen with the two control organisms. With *S. aureus* ATCC 25923, the zones ranged from 20 to 25.5 mm in diameter; in 6 of 11 tests the zones were greater than 24 mm in diameter. With *E. coli* ATCC 25922, zone sizes ranged from 18 to 23.5

TABLE 1. *Susceptibility of 191 bacterial isolates to tobramycin*[a]

Organism (no. tested)	Cumulative percentage of susceptible strains (concn of tobramycin, μg/ml)										
	0.05	0.10	0.20	0.39	0.78	1.56	3.13	6.25	12.5	25	50
Gram negative											
Escherichia sp. (25)	0	0	4	4	56	96	96	100			
Enterobacter sp. (25)	0	0	4	60	92	92	100				
Klebsiella sp. (25)	0	8	28	88	96	100					
Proteus sp.[b] (25)	4	4	4	4	20	64	92	100			
Pseudomonas (25)	0	20	84	92	92	92	96	100			
Serratia (25)	0	0	0	0	0	12	44	76	100		
Total gram negative	0.67	5.3	20.8	41.3	59.3	76.0	88.0	96.0	100		
Gram positive											
Staphylococcus aureus (25)	8	56	84	100							
Group D streptococci (16)	0	0	0	0	0	0	6	63	94	94	100
Controls (11 determinations)											
S. aureus ATCC 25923	36.4	72.7	90.9	90.9	100						
E. coli ATCC 25922	0	0	0	0	36.4	90.0	100				

[a] Minimal inhibitory concentrations measured in heart infusion broth at 37 C.
[b] Indole negative.

mm, with 9 of 11 values in the 18- to 20.5-mm range. Mean values for the controls were as follows: *S. aureus* ATCC 25923, 23.9 ± 1.7 mm; *E. coli* ATCC 25922, 20.9 ± 1.2 mm.

Correlation and regression analyses were performed on the MIC values and zone size data for the different genera and for all of organisms as a single set. In these calculations, MIC values were expressed as base 2 logarithms. Individually, data for most of the genera demonstrated reasonably good correlation (Table 3). However, nonsignificant correlations were calculated for group D enterococci, *Serratia*, and *Proteus*. In the case of the latter organism, this probably reflected the particular population being studied as 23 isolates were inhibited by 0.78 to 3.13 μg of tobramycin per ml and gave zone sizes of 18.5 to 23.5 mm. The data from *Serratia* were contradictory; 23 isolates required 3.13 μg/ml or more for inhibition, yet 19 of these 23 gave zone sizes of 18.5 mm or greater. The significance of the degree of interdependency between zone sizes and MIC for all isolates tested as measured by analyses of variance ($F = 70.94$, $P < 0.001$) indicated that zone size values could be used for prediction of levels of susceptibility for most clinical isolates (Fig. 1). As noted, the most important exception to this was *Serratia*.

TABLE 2. *Per cent distribution of zone size responses of 191 bacterial isolates to a 10-μg tobramycin disc*

Organism (no. tested)	Distribution (%) of zone size responses (zone sizes, mm)[a]								
	≤6	6–8.5	9–11.5	12–14.5	15–17.5	18–20.5	21–23.5	≥24	Mean
Gram negative									
Escherichia sp. (25)					8	72	20		19.8
Enterobacter sp. (25)					4	32	64		20.9
Klebsiella sp. (25)					4	80	12	4	19.8
Proteus sp.[b] (25)					4	20	76		21.5
Pseudomonas (25)	4				20	56	16	4	18.9
Serratia sp. (25)					16	36	48		20.3
Total gram negative	0.67				9.33	49.33	39.33	1.33	
Gram positive									
Staphylococcus aureus (25)							60	40	23.6
Group D streptococci (16)	50	50							7.2
Controls (11 determinations)									
S. aureus ATCC 25923						9.1	36.4	54.6	23.9
E. coli ATCC 25922						81.8	18.2		20.9

[a] Disc test performed by the standardized FDA procedure.
[b] Indole negative.

TABLE 3. *Statistical analysis of tobramycin susceptibility data for 191 bacterial isolates*

Organism	Correlation coefficient and P[a]	Value of T and P[a]	Regression coefficient	Regression intercept (log 2)	Variance of regression (F) and P[a]
Escherichia	−.6332[b]	−3.9236[b]	−.4000	7.9955	15.3945[b]
Enterobacter	−.5913[c]	−3.5168[b]	−.5238	10.1008	12.3676[c]
Klebsiella	−.4617[a]	−2.4962[a]	−.2439	3.2813	6.2308[a]
Proteus	−.0203[d]	−0.0974[d]	−.0167	1.0823	0.0095[d]
Pseudomonas	−.7434[b]	−5.3298[b]	−.3045	3.6593	28.4069[b]
Serratia	−.0301[d]	−0.1445[d]	−.0150	2.6269	0.0209[d]
Staphylococcus aureus	−.4183[a]	−2.2085[a]	−.2729	3.6275	4.8775[a]
Group D enterococci	.0198[d]	.0716[d]	.0149	2.8052	0.0051[d]
All	−.5234[b]	−8.4228[b]	−.2531	4.6428	70.9433[b]

[a] $P < 0.05$.
[b] $P < 0.001$.
[c] $P < 0.01$.
[d] Not significant.

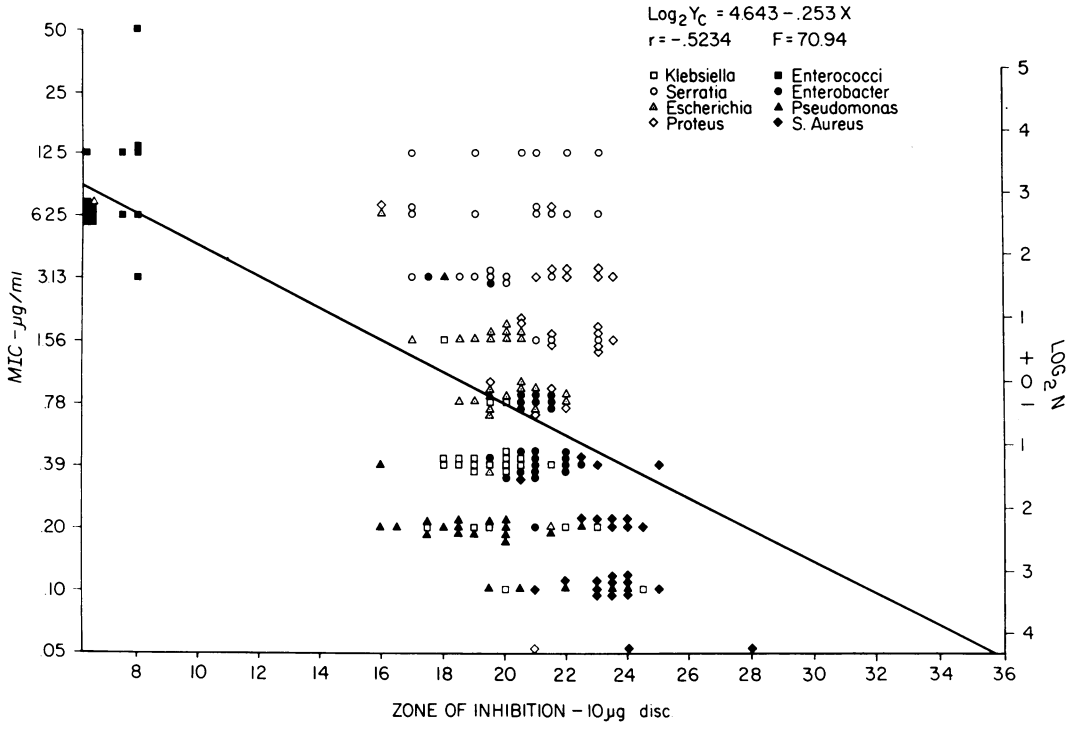

FIG. 1. *Linear correlation of 10-µg tobramycin disc results with MIC values determined by broth dilution studies.*

DISCUSSION

Preston and Wick (8) originally proposed a susceptibility threshold MIC of 8 µg/ml for in vitro testing with tobramycin. This value was based on data obtained with the ICS agar-dilution procedure (4) which, as these authors demonstrated, may have resulted in an elevation of MIC end points because of the presence of agar in the test media. Similar increases in MIC values of tobramycin when measured in solid media were reported more recently by Dienstag and Neu (3), who attributed the effect to binding by cations in the agar, particularly magnesium and calcium, which Dienstag and Neu also demonstrated as being inhibitory to the action of tobramycin. Similar elevation of MIC end points by agar also has been reported by Meyer et al. (7). Thus, any recommendation regarding "threshold" end points for tobramycin must be defined in terms of both the test procedure (broth or agar dilution) and the medium employed.

In the studies reported here with heart infusion broth, the threshold value appears to be in the range of 1.56 to 3.13 µg/ml. These concentrations approximate attainable blood levels in man. For example, in one group of volunteers receiving 100 mg of the drug intramuscularly three times a day for 10 days, peak serum levels rarely exceeded 4 µg/ml and, at 1 hr postinjection, they averaged only 5.03 µg/ml; in a second group receiving single 200-mg intramuscular doses, average levels in excess of 4 µg/ml could be measured only through 4 hr (R. S. Griffith, *personal communication*). These data, together with those reported here, support the use of 1.56 to 3.13 µg/ml as the threshold values of susceptibility for in vitro testing with tobramycin in broth. If these values are fitted to the regression line shown in Fig. 1, zone size values of 12 to 16 mm are obtained. Since most susceptible organisms included in this study gave zones of 16 mm or larger, 16 mm is recommended as the critical value for prediction of susceptibility with the standard 10-µg disc. This value is in agreement with those previously proposed by Preston and Wick (8) and more recently by Traub and Raymond (10), who used a Microtiter broth dilution procedure to measure MIC end points. This value cannot be used with isolates of *Serratia*.

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