In Vitro Activity, Synergism, and Testing Parameters of Amikacin, with Comparisons to Other Aminoglycoside Antibiotics

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The activity of the new aminoglycoside antibiotic, amikacin, was evaluated in vitro against 219 clinical bacterial isolates. One hundred eighty-nine of the 219 strains had agar dilution minimal inhibitory concentration values of 8.0 μg/ml or less for amikacin. Comparative agar dilution studies were performed for gentamicin, kanamycin, and tobramycin. Gentamicin was the most active overall, but tobramycin and amikacin also had significant activity against most bacterial groups. The effects of divalent cations on the susceptibility of Pseudomonas aeruginosa to amikacin were evaluated, and the minimal inhibitory concentration values varied sixfold over a range of divalent cation concentrations from 0.2 to 8.75 mg%. The effects of media and inoculum size on disk susceptibility testing with amikacin were also evaluated. In addition, a synergistic interaction between carbenicillin and amikacin against P. aeruginosa was demonstrated. Amikacin appears to be a promising new broad spectrum antimicrobial agent.

The availability of the aminoglycoside antibiotic, gentamicin, over the past several years has greatly enhanced the therapeutic approach to serious gram-negative infections. Gentamicin is active against Staphylococcus aureus, members of the family Enterobacteriaceae and, very importantly, Pseudomonas aeruginosa (17). Disturbingly, however, reports of resistance to gentamicin among members of the family Enterobacteriaceae have begun to appear (11). More importantly, clinical isolates of P. aeruginosa resistant to gentamicin have been reported for some time (14), and now constitute 8% of such strains isolated in the University of Utah Hospital. Therefore, the search for new antimicrobial agents with a similar spectrum of activity has continued.

Amikacin is one of the more recently discovered agents which seems to have such activity. This antimicrobial is a semisynthetic aminoglycoside produced by acylation of kanamycin A (10). Amikacin has been reported to have activity against a variety of gram-negative bacteria, including P. aeruginosa, and also against S. aureus. There have also been indications in previous studies that amikacin has a higher therapeutic index than other aminoglycoside agents active against P. aeruginosa (2, 18).

In the present study, the activity of amikacin against a variety of bacteria has been analyzed, and a regression curve has been prepared. The effects of various media on susceptibility testing with amikacin have been determined as well as the effects of divalent cations and inoculum size. In addition, studies of synergy between amikacin and carbenicillin against P. aeruginosa have been performed.

MATERIALS AND METHODS

In vitro susceptibility determinations by standard methods. Agar and broth dilution antimicrobial susceptibility determinations were carried out as outlined by Ericsson and Sherris (4). Disk diffusion antimicrobial susceptibility determinations were performed as outlined in the Federal Register (5, 6). Stock solutions of amikacin, kanamycin (provided by Bristol Laboratories), gentamicin (Schering Corp.), and tobramycin (provided by Eli Lilly Co.) were filter sterilized, dispensed, and stored at −20 C. Serial twofold dilutions were carried out in Mueller-Hinton broth (MHB). Unless otherwise specified, the organisms to be tested were grown overnight in tryptic soy broth and adjusted to the density of 0.5 McFarland standard. The standardized inocula were diluted 1:10 and applied to the plates in the agar dilution method using the replicating device of Steers et al. (16). The standardized inocula were applied by the cotton swab technique for the disk diffusion studies.

A total of 219 bacterial strains were analyzed. The
group comprised of predominantly clinical isolates obtained from the Diagnostic Microbiology Laboratories at the University of Minnesota. Standard reference strains of E. coli (ATCC 25922), S. aureus (ATCC 25923), and P. aeruginosa (ATCC 27853) were also examined. The clinical isolates included 21 E. coli, 20 Klebsiella, 20 Enterobacter, 22 P. aeruginosa, 12 Serratia, 20 Acinetobacter (10 Mima, 10 Herellea), 10 Proteus mirabilis, 10 Proteus species (vulgaris, rettgeri, and morganii), 8 Citrobacter, 10 Salmonella, 10 Providencia, 9 Shigella, 9 Pseudomonas species, 12 S. aureus, 10 S. epidermidis, 10 group D streptococci, and 5 group A streptococci. All clinical isolates were identified by standard means.

MIC Versus MLC concentrations. Five strains each of E. coli, Klebsiella, Enterobacter, and P. aeruginosa were tested simultaneously by the standard broth and agar dilution testing as noted above for minimal inhibitory concentration (MIC) values. Minimal lethal concentrations (MLC) were determined by plating 0.01 ml of the tubes of the MIC to sheep blood agar plates, and reading 99.9% killing.

Microtiter broth dilution studies. For studies of antibiotic synergy and the effects of cations on susceptibility testing, a modification of the microtiter method of Gavan and Town (7) was used. Microtiter plates having 96 wells (U-type) were gas sterilized. Fifty-microliter diluters (Microtiter) and 50-μl micropipettes with sterile disposable tips (Eppendorf) were used.

For the studies of cation effects, MHB supplemented with varied amounts of MgCl₂ and CaCl₂ was prepared and amikacin solutions were made in each broth. Well no. 1 of each row was filled with 100 μl of each cation solution containing antibiotic. Wells 2 through 12 contained 50 μl of each broth preparation without antibiotic. Serial dilutions were made from wells 1 through 11 in each row (64 to 0.062 μg of amikacin per ml). Column 12 served as a growth control for each broth preparation. The concentrations of Mg²⁺ varied from 0.4 to 7.4 mg%, and the Ca²⁺ from 0.2 to 8.75 mg%. Overnight broth cultures of the P. aeruginosa strains to be tested were standardized against the 0.5 McFarland standard and diluted 1:100 in each MHB plus cation preparation to obtain an inoculum of 10⁸ to 10⁹ bacteria/ml. Each well was inoculated with 50 μl of the bacterial suspension. The plates were covered and incubated at 35 to 37°C overnight, and the results were recorded as growth or no growth using a Microtiter reader.

This technique was also used to assess the synergistic interaction between carbenicillin and amikacin against P. aeruginosa. Well no. 1 in each row of the microtiter plate was loaded with 100 μl of amikacin (128 μg/ml) in MHB. The remaining wells contained 50 μl of MHB. Serial dilutions were made from wells 1 through 11. Fifty microliters of carbenicillin (2,048 μg/ml) in MHB was added to wells 1 through 12 of row A (top row of plate). Serial dilutions were made from wells A through G in each vertical column, leaving the bottom row (row H) with no added carbenicillin. Column no. 12 on the far right of the plate served as the MIC determination for carbenicillin, and row H at the bottom of the plate served as the MIC determination for amikacin. Antibiotic combinations ranged from 512 μg of carbenicillin per ml plus 32 μg of amikacin per ml down to 8 μg of carbenicillin per ml plus 0.015 μg of amikacin per ml. Each well was inoculated with 50 μl of the P. aeruginosa strain to be tested. The inocula were prepared as above.

RESULTS

Amikacin MIC determinations. The amikacin susceptibility of 219 bacterial strains was tested by agar dilution methodology. All strains of the Enterobacteriaceae tested, including those presented in Fig. 1 through 7 as well as Citrobacter, Salmonella, and Shigella, had MIC values of 16 μg/ml or less. S. aureus strains were susceptible to 2 μg or less of amikacin per μl, and all but one strain of S. epidermidis were susceptible to this level of amikacin. The majority of the MIC values fell well within the therapeutic range of amikacin, and the median MIC value was 2.0 μg/ml. Of the 219 strains tested, 189 had MIC values of 8.0 μg/ml or less. Enterococci and group A beta hemolytic streptococci were notably resistant to amikacin. Pseudomonas species other than P. aeruginosa, especially P. maltophilia, were

![Fig. 1. Cumulative percentage of E. coli strains inhibited by increasing concentrations of amikacin, kanamycin, tobramycin, and gentamicin.](http://aac.asm.org/Downloaded_from)
also quite resistant to amikacin. However, most of the \textit{P. aeruginosa} strains were susceptible to concentrations of amikacin achievable in vivo. In addition, two strains of \textit{E. coli} and \textit{Klebsiella} that were highly resistant to gentamicin were very susceptible to amikacin in simultaneous testing.

Comparison of amikacin, kanamycin, tobramycin, and gentamicin. Agar dilution studies comparing amikacin, kanamycin, tobramycin, and gentamicin were performed for each of the 219 bacterial strains. The results comparing the percentage of strains of selected bacterial groups inhibited at various MIC values are presented in Fig. 1 through 7. The antibiotics having the greatest in vitro activity varied with the bacterial group. For \textit{E. coli}, gentamicin had the greatest activity, and amikacin was the least active of the four antibiotics (Fig. 1). Gentamicin was also most active against \textit{Klebsiella}, \textit{Enterobacter}, \textit{Serratia}, \textit{Acinetobacter}, and \textit{Proteus-Providencia} species (Fig. 2–6), and tobramycin was slightly more active than amikacin against these groups of organisms except for \textit{Serratia} and \textit{Proteus-Providencia}. In the latter cases, amikacin was more active than tobramycin (Fig. 4 and 6). For all these organisms, kanamycin was the least active antibiotic. In the case of \textit{P. aeruginosa}, tobramycin had the greatest in vitro activity. Gentamicin was nearly as active as tobramy-
Amikacin testing profile

**Acinetobacter**

- Fig. 5. Cumulative percentage of Acinetobacter strains inhibited by increasing concentrations of amikacin, kanamycin, tobramycin, and gentamicin.

**Proteus - Providencia**

- Fig. 6. Cumulative percentage of Proteus and Providencia strains inhibited by increasing concentrations of amikacin, kanamycin, tobramycin, and gentamicin.

**Pseudomonas aeruginosa**

- Fig. 7. Cumulative percentage of Pseudomonas aeruginosa strains inhibited by increasing concentrations of amikacin, kanamycin, tobramycin, and gentamicin.

Amikacin and amikacin was slightly less active (Fig. 7).

Amikacin regression analysis. A scattergram of MIC versus disk zone size values was constructed for the amikacin determinations, and a line of regression was calculated using a computerized version of the formula of least squares. A linear relationship was found for the amikacin regression analysis, and the points of MIC versus zone size were fairly evenly distributed along the line (Fig. 8).

Comparison of agar and broth dilution methods for MIC determination and evaluation of MIC versus MLC. Five strains each of E. coli, Klebsiella, Enterobacter, and P. aeruginosa (a total of 20 strains) were tested by standard agar and broth dilution methods simultaneously. The results reveal fairly close agreement for MIC values obtained by two methods with the exception of P. aeruginosa. The mean agar dilution MIC value for P. aeruginosa was 5.6 μg/ml, whereas the broth dilution value was only 0.9 μg/ml (Table 1) for amikacin. Here the low value of Ca²⁺ and Mg²⁺ in regular MHB is felt to be the cause of this difference for P. aeruginosa. When the Ca²⁺ and Mg²⁺ content is equivalent between broth and agar, this difference with P. aeruginosa is obviated.

MLCs were determined by plating 0.01 ml of the broth from each tube in the above experiments, and reading 99.9% killing. The plates were examined for the presence of bacterial colonies to determine what concentration of amikacin exerted a lethal effect on the strains...
Table 1. MIC versus MLC testing for amikacin in vitro

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mean MIC (µg/ml)</th>
<th>Mean MLC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agar</td>
<td>Broth</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>5.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>

tested. The MLC values were two- to threefold higher than the corresponding MIC values (Table 1).

Effect of divalent cations on amikacin MIC determinations. The differences between MIC values determined by broth and agar dilution methods for *P. aeruginosa* have been due to differences in the divalent cation content of the media for other aminoglycoside antibiotics (8). Therefore, the effects of Ca²⁺ and Mg²⁺ concentration on broth dilution MIC values for amikacin against *P. aeruginosa* were determined using a modification of the microtiter method.

The average MIC values for four strains of *P. aeruginosa* increased sixfold over a Mg²⁺ concentration range of 0.4 to 7.4 mg% (Fig. 9). The MIC values also demonstrated a fourfold variation over a range of Ca²⁺ concentrations from 0.2 to 8.75 mg%. Therefore, the concentration of divalent cations in media has a marked effect upon the amikacin MIC values for *P. aeruginosa* as has been observed with other aminoglycoside antibiotics. The most meaningful range would appear to be that comparable to the physiological condition in humans.

Amikacin standard curve and disk assay. An amikacin standard curve was prepared by impregnating standard blank filter paper disks with known quantities of the antibiotic. These disks were used on plates to determine the size of the zones of inhibition by the standard Bauer-Kirby method. The standard curve revealed a linear relationship between zone size and disk content over the range of 0.62 to 40 µg of amikacin. Six commercially available amikacin disks were examined simultaneously with the standard curve disks. All six had zone sizes of 24 mm which corresponds to exactly 10 µg of antibiotic.

Effects of media on susceptibility testing with amikacin. The standard disk diffusion
method was used with various agar media to determine the effects of this variable on susceptibility to amikacin, tobramycin, and gentamicin. The differences in zone size on Mueller-Hinton agar as compared to MHB plus agar (two sources) and tryptic soy agar were of limited significance for the three antibiotics tested against *P. aeruginosa* and *E. coli*. However, brain heart infusion agar gave significantly larger zone sizes for *P. aeruginosa* and significantly smaller zone sizes for *E. coli*. Nutrient agar gave unacceptably small zones in all cases (Table 2).

![Graph](image)

**FIG. 9.** Effect of divalent cation concentration on amikacin MIC determinations. Four strains of *P. aeruginosa* were tested by the microtiter broth dilution method for MIC determination. Tests were carried out in the MHB supplemented with varying amounts of Ca**2**+ and Mg**2**+. Results are presented as average MIC values for the four strains examined. Symbols: (○) increasing concentrations of Mg**2**+ in the presence of 0.2 mg% Ca**2**+; (□) increasing concentrations of Ca**2**+ in the presence of 0.4 mg% Mg**2**+.

**TABLE 2.** Effects of media on susceptibility testing with amikacin, tobramycin, and gentamicin

<table>
<thead>
<tr>
<th>Medium</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Escherichia coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amikacin</td>
<td>Tobramycin</td>
</tr>
<tr>
<td>Mueller-Hinton agar</td>
<td>17.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Mueller-Hinton broth plus agar (Difco)</td>
<td>19.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Mueller-Hinton broth plus agar (BBL)</td>
<td>19.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Tryptic soy agar</td>
<td>18.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Brain-heart infusion agar</td>
<td>20.0</td>
<td>21.5</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>11.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Effect of inoculum size on susceptibility testing with amikacin and tobramycin. Cultures of the standard *E. coli* strain were grown overnight in tryptic soy broth. The turbidity was adjusted to the 0.5 McFarland standard. The original culture was diluted serially and viable counts were determined by the pour plate method. Mueller-Hinton agar plates were swabbed with the standardized *E. coli* suspension and with the diluted inocula. Amikacin and tobramycin disks were placed on the surface, and zones of inhibition were determined after overnight incubation. The zone sizes decreased from 40 mm at 7.5 × 10⁵ bacteria/ml to 26 mm at 7.5 × 10⁶ bacteria/ml for amikacin. Tobramycin showed a parallel effect with zone sizes decreasing from 44 to 29 mm.

**Demonstration of synergy between amika-**
Enterobacteriaceae, KELLY 446
microtiter
amikacin against to assess the interaction of carbenicillin and amikacin against four strains of P. aeruginosa. The combinations ranged from a maximum of 512 µg of carbenicillin per ml plus 0.015 µg of amikacin per ml. An isobologram was constructed from the MIC values obtained (Fig. 10). The results were concave, indicating synergy.

DISCUSSION
Amikacin has been shown in previous studies to have activity against members of the Enterobacteriaceae, as well as P. aeruginosa (1, 9, 13, 18, 19). Our studies confirm these observations, and 86% of the 219 strains tested were susceptible to 8.0 µg or less of amikacin per ml. Of the Enterobacteriaceae, 97% were susceptible to 8.0 µg or less of amikacin per ml, and the resistant strains were all members of the Proteus-Providencia group. The strains of P. aeruginosa examined were all susceptible to 32 µg/ml or less, and 82% were susceptible to 8 µg/ml or less. The studies comparing amikacin to gentamicin, tobramycin and kanamycin are also in agreement with previous findings (1, 9, 13, 18, 19). In general amikacin is less active on a weight basis than gentamicin or tobramycin, but it is more active than kanamycin (Fig. 1 to 7). Although amikacin is less active than gentamicin or tobramycin in vitro, higher serum levels are achievable in man with amikacin, and its therapeutic efficacy may be equal to the other agents overall (2, 15).

The amikacin regression analysis, relating the agar dilution MIC values to disk zone sizes for a variety of clinical isolates (Fig. 8), demonstrates that the data points are distributed fairly evenly along the regression line except for some spreading in the high MIC range. This regression analysis can be useful for determining resistance breakpoints for amikacin. It also provides a means for estimating a range of MIC values from a given disk zone size.

Another aspect of aminoglycoside susceptibility testing that has relevance for amikacin is a comparison of broth to agar dilution method for MIC determinations. Our results indicated agreement within the limits of error of the methods for E. coli, Klebsiella, and Enterobacter. However, with Pseudomonas strains, the agar dilution values were sevenfold greater than the broth dilution values (Table 1). This problem will likely be minimized as MHB media becomes supplemented with physiological amounts of the critical divalent cations, Ca²⁺ and Mg²⁺. This discrepancy is related to the cation content of the two media, and it has been observed with other aminoglycoside antibiotics (8). Our studies of the effects of divalent cations on amikacin MIC values for P. aeruginosa confirm this impression since a four- to sevenfold rise in MIC values was observed over a range of Ca⁺⁺ and Mg⁺⁺ concentrations (Fig. 9).

Differences between Mueller-Hinton agar and various other media were encountered with the disk diffusion method for both P. aeruginosa and E. coli (Table 2). Pronounced differences were observed with nutrient agar media. Bodey and Stewart (1) also found pronounced media effects in amikacin susceptibility testing by the broth dilution method. Our results confirm their findings and extend them to the disk diffusion method as well.

In another study, by Yu and Washington (19), the effect of inoculum size on amikacin susceptibility testing by the broth dilution method was evaluated. They found significant differences in MIC with variations in inoculum size. Our studies confirm and extend these findings to the disk diffusion method. Marked differences in zone size were encountered with variations in the inoculum size. These findings again emphasize the need for strict adherence to the Bauer-Kirby procedure for amikacin susceptibility testing.

Previous studies have demonstrated a synergistic interaction between gentamicin and carbenicillin (3). The concave nature of the amikacin-carbenicillin isobologram (Fig. 10) suggests a synergistic interaction between these agents (3). This synergy is exemplified by an example of MIC values. At a carbenicillin concentration of 60 µg/ml, inhibition would have been expected in the presence of 1.6 µg of amikacin per ml if the agents had only additive effects. However, only about 0.04 µg/ml was required to produce growth inhibition, indicating a 40-fold increase in activity.

These studies, together with those previously reported, suggest that amikacin may provide a useful addition to the available antibacterial agents. It is our current clinical experience that organisms of the Enterobacteriaceae or Pseudomonas groups which are resistant to gentamicin may be susceptible to amikacin. It has activity against the Enterobacteriaceae and staphylococci which exceeds that of kanamycin, and it has the added advantage of activity against P. aeruginosa. It is also active in vitro against certain gentamicin-resistant Enterobacteriaceae in the present study, and it has been reported to be active against Pseudomonas and other organisms (12, 15). Our studies indicate that amikacin behaves in a manner
similar to other aminoglycoside antibiotics in in vitro testing, and it should provide no special problems. Amikacin is a promising new agent which deserves further clinical evaluation.

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LITERATURE CITED