Disk Diffusion Susceptibility Testing of Nocardia Species

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The effectiveness of 13 antimicrobial agents against 51 clinical isolates of Nocardia was determined with use of agar dilutions and a disk diffusion method. Amikacin inhibited >90% of isolates and, like the other aminoglycosides, showed good correlation between minimal inhibitory concentrations and sizes of zones of inhibition around the disks. Both sulfisoxazole and trimethoprim-sulfamethoxazole were very active, although they required a 2- to 3-log lower inoculum for demonstration of susceptibility. Results with the two sulfa disks were variable, but they did allow distinction between sensitive and intermediate strains. All of the isolates of Nocardia were inhibited by 6.3 μg of minocycline; however, the degree of susceptibility could not be determined by zone diameters. Only two-thirds of these clinical isolates of Nocardia grew rapidly enough to be assayed by either susceptibility method.

Infection due to Nocardia is a serious clinical problem, especially in patients who are given corticosteroids or other immunosuppressive agents [1, 2]. Although sulfonamides are considered to be the drugs of choice to treat infections with Nocardia, they may fail to eradicate disease [3]. Among immunosuppressed patients or those with disseminated disease, the mortality rate remains as high as 80% [4]. As a result, interest in other antimicrobial agents for treatment of nocardia infections has increased [5, 6]. The choice of antibiotics in individual cases is often based on previously reported results of agar dilution sensitivities and on clinical response. A disk diffusion method for antimicrobial sensitivity testing of Nocardia is not currently available. This lack has been attributed to the slow growth pattern of Nocardia and the clumping of organisms during growth in broth culture media. Bach et al. suggested that a disk diffusion method was feasible for Nocardia and reported zone diameters for tetracycline and minocycline [7]. Further laboratory evaluation, however, has not been done. We have compared the results of agar dilutions with a disk diffusion method for sensitivity testing of Nocardia, employing a number of clinically useful antibiotics.

Materials and Methods

Nocardia species. Seventy-six clinical isolates of Nocardia were evaluated. Most were provided by Dr. Charles Stager of the Houston Health Department, Dr. Joe Steadham of the Texas State Department of Public Health, and Ms. Virginia Shorall of the Mycology Section of the Center for Disease Control (Atlanta, Ga.). Fifty-one isolates that produced easily discernible growth on agar plates after incubation for 48 hr were included in the study. These strains included Nocardia asteroides (48), Nocardia brasiliensis (two), and Nocardia caviae (one).

Antibiotics. Antibiotics were prepared by dissolving laboratory standard powders in sterile distilled water. These solutions were used fresh or after storage at −20°C for no more than 30 days. Antimicrobial agents tested included ampicillin, tetracycline, amikacin, and kanamycin (Bristol Laboratories, Syracuse, N.Y.); sulfamethoxazole, trimethoprim-sulfamethoxazole (TMP-SMZ; ratio, 1:20); and sulfisoxazole (Hoffman-La Roche, Nutley, N.J.); streptomycin and clindamycin (Upjohn, Kalamazoo, Mich.); mi-
nocycline (Parke-Davis, Detroit, Mich.); gentamicin (Schering, Bloomfield, N.J.); and tobramycin and erythromycin (Eli Lilly and Co., Indianapolis, Ind.).

Fresh, commercially available paper disks were used for the disk sensitivity testing. Drugs tested were ampicillin (10 μg), tetracycline (30 μg), kanamycin (30 μg), TMP-SMZ (1.25/23.75 μg), sulfisoxazole (250 μg), streptomycin (10 μg), clindamycin (2 μg), gentamicin (10 μg), tobramycin (10 μg), and erythromycin (15 μg). Two disks that are not commercially available, minocycline (30 μg) (Baltimore Biological Laboratories, Baltimore, Md.) and amikacin (10 μg) (Bristol), were also tested.

Culture methods. Organisms were initially inoculated into 125-ml Erlenmeyer flasks that contained trypticase soy broth (Difco, Detroit, Mich.) and 5-mm sterile glass beads. After incubation in a shaking water bath at 37 C, a fine homogeneous suspension of organisms was generally obtained. For a culture method that was more practical for laboratory use, most organisms were cultured by placing a large inoculum of organisms in trypticase soy broth in 20-ml tubes that contained glass beads. Tubes were incubated at 37 C and mixed for 15–30 sec daily on a vortex mixer as soon as growth was visible. An adequate homogeneous suspension of organisms was generally obtained by either method after incubation for 48–96 hr. In a few instances larger clumps were allowed to settle out or were removed by gentle centrifugation for 30 sec. The suspensions were then adjusted visually to match the Kirby-Bauer 1% barium sulfate standard. Serial 10-fold dilutions were made, and 0.01-ml aliquots were plated to determine the number of cfu. Only those dilutions that were shown to contain 10^7–10^8 cfu/ml were reported. Dilutions that matched the standard regularly contained the correct number of organisms.

Sensitivity testing. Agar dilution plates were made with use of twofold dilutions of antibiotic and Mueller-Hinton agar. Organisms were inoculated with a replicator that delivered ~0.001 ml, which resulted in a plate inoculum of 10^4–10^6 cfu [8]. This inoculum was used for the agar dilution plates of all antimicrobial agents except the sulfonamides and TMP-SMZ; for these, a plate inoculum of 10^4 cfu was used. Results were read after 48 hr. The MIC was interpreted as the lowest antinicrobial concentration that showed no growth, one or two colonies, or a very fine haze [9]. (In agar dilution tests in other laboratories, complete inhibition of growth has also been used as the MIC end point [5, 6, 10].) With repeated testing of one isolate with each series of tests on two to four occasions, there was no more than a twofold variation in the MIC (except for gentamicin on one occasion).

A modification of the Kirby-Bauer technique was used for disk sensitivity testing, with Mueller-Hinton agar poured to a depth of 5 mm in 150-mm plates [11]. After plates were streaked with a standardized dilution of Nocardia, antibiotic disks were applied and plates were incubated at 37 C. The diameters of clear zones around the disks were measured for all antimicrobial
agents except the sulfonamides, for which the sizes of zones representing ≥80% inhibition of growth were recorded [12]. Measurements of zone size were made at 24 and 48 hr; in a few cases confirmatory readings were also made at 72 hr. Two disks on separate plates were used for each antimicrobial agent, and 48-hr measurements were averaged to determine zone size for a given strain.

Regression line analysis. The coefficients of the linear model that relates MICs and zone diameters (zone diameter = a • log_{10}MIC + b) were estimated by least squares, with use of computer program BMD08V Stepwise Regression [13]. For better comparison with previous data on other organisms, this relation was rewritten in terms of x = log_{2}MIC + 9 [9]. The test for linearity was the same as that made by Ericsson and Sherris; that is, the variability of data at each MIC value was compared to the scatter of points around the least-squares regression line [9].

Results

Isolates of *Nocardia* exhibited great variation in rates of growth. About one-third of the organisms had easily measurable zones of inhibition after incubation for only 24 hr ("rapidly growing" strains). Another one-third of the organisms had barely readable results after 24 hr. The zone sizes produced by most antibacterial agents with these isolates differed by only 2–4 mm on the second reading at 48 hr, although those produced by sulfonamides and erythromycin often changed significantly. Another one-third of the isolates showed no visible growth at 24 hr, and zone sizes could be determined only after incubation for 48 hr ("slowly growing" strains). In general, these slowly growing strains had larger zone diameters than did the rapidly growing ones that had the same MIC by agar dilutions. For example, nine rapidly growing and five slowly growing strains were inhibited by 3.1 μg of minocycline/ml; the mean zone diameters of these two groups were 23.8 ± 3.6 and 29.4 ± 3.8 mm, respectively (P < 0.05). There were three rapidly growing strains and nine slowly growing strains of *Nocardia* with MICs of 0.39 μg of amikacin/ml; the mean zone diameters for these groups were 32.0 ± 2.6 and 41.8 ± 5.4 mm, respectively (P < 0.05). The differences were greatest with the lower MICs; only on rare occasions was the difference in zone size sufficient to produce confusion between sensitivity and resistance.

Sulfonamides. The agar dilution technique indicated that 33 of 37 isolates of *Nocardia* were sensitive to 50 μg of sulfisoxazole/ml and that >80% were sensitive to 25 μg/ml. Similar results by agar dilutions were achieved with sulfamethoxazole, although MICs were usually twofold lower for this drug than for sulfisoxazole.

![Figure 2](image-url)
Figure 3. Regression line for correlation of agar dilution and disk diffusion tests of sensitivity of 34 strains of Nocardia to trimethoprim-sulfamethoxazole (25 mg per disk). Thirty of 32 isolates sensitive to 12.5 mg/ml had zones of inhibition of ≥20 mm. SE = 8.9; correlation coefficient (r) = -0.695.

(figure 1). When TMP-SMZ at the ratio of 1:20 was tested, 94% of the strains were sensitive to 12.5 mg/ml and all were inhibited by 25 mg/ml. However, most of the isolates tested had a decrease in the MIC of twofold or less compared with the MIC of sulfamethoxazole alone; this small decrease suggested the lack of synergy against most of these isolates of Nocardia at this ratio. Zone diameters of ≥35 mm for sulfisoxazole and ≥20 mm for TMP-SMZ distinguished between sensitive and intermediate strains (figures 2 and 3). Only one-half of the strains showed a completely clear zone around the sulfisoxazole disk; almost no strains gave totally clear zones around the TMP-SMZ disk. There was only a fair degree of correlation between MICs and zone sizes for both sulfonamides.

Ampicillin. With determination by agar dilutions, 45.6% of the isolates of Nocardia were inhibited by concentrations of 6.2 mg of ampicillin/ml. Seventeen of 22 isolates with an MIC of 6.2 mg/ml and none of 26 more resistant strains had zone diameters of ≥25 mm (figure 4).

Aminoglycosides. Of the aminoglycosides

Figure 4. Regression line for correlation of agar dilution and disk diffusion tests of sensitivity of 48 strains of Nocardia to ampicillin (10 mg per disk). SE = 5.1; correlation coefficient (r) = -0.924.
tested, amikacin was the most active against *Nocardia*, with 47 (92%) of 51 isolates being inhibited by 6.2 μg/ml. Tobramycin and gentamicin inhibited 61% and 47% of isolates, respectively, at 6.2 μg/ml, as compared with 34% and 18% for kanamycin and streptomycin, respectively (figure 5). The aminoglycosides in general showed a high degree of correlation between MICs and zone sizes. Amikacin, kanamycin, and tobramycin exhibited a bimodal distribution of sensitive and resistant organisms, with few intermediate strains. This resulted in good distinction between sensitivity and resistance by zone size. Of 30 isolates of *Nocardia* sensitive to 6.2 μg of tobramycin/ml, 29 had zones of ≥25 mm, whereas none of 19 more resistant organisms had zones this large (figure 6). Similar discrimination was possible with kanamycin (figure 7) and amikacin (figure 8) with use of zone sizes of 28 and 25 mm, respectively. However, there was intermediate susceptibility of nocardia isolates to gentamicin (figure 9) and streptomycin, with some overlap between zone sizes of sensitive and intermediate strains by disk diffusion.

**Tetracycline.** Many of the strains of *Nocardia* showed large zones of inhibition with the

\[ y = -4.473x + 74.6 \]

![Figure 6. Regression line for correlation of agar dilution and disk diffusion tests of sensitivity of 49 strains of *Nocardia* to tobramycin (10 μg per disk). The bimodal distribution of sensitive and resistant strains is evident. SE = 5.1; correlation coefficient (r) = -0.938.](image)
tetracycline disk. With use of the agar dilution technique, however, 37 (97%) of 38 strains were tetracycline-resistant, with MICs of $\geq 6.2 \, \mu g/ml$. Minocycline was very active against all isolates of *Nocardia*. Almost 90% of organisms were inhibited by 3.1 $\mu g/ml$, and all were inhibited by 6.2 $\mu g/ml$. All isolates gave a zone of $\geq 20$ mm. It was not possible, however, to predict the degree of susceptibility on the basis of disk sensitivity tests (correlation coefficient, $-0.441$).

**Macrolides.** Erythromycin inhibited only two (5.4%) of 37 isolates of *Nocardia* at a concentration of 0.78 $\mu g/ml$. Both of these organisms had zones of inhibition with diameters of $>30$ mm, whereas the remaining more resistant strains had zones of $\leq 20$ mm. There was no sharp zone with the erythromycin disk, and frequently what appeared to be a large zone at 24 hr decreased by as much as 10–15 mm by 48 hr, with the appearance of many smaller but definite colonies within the original zone. The MIC correlated with the zone noted at the 48-hr reading.

**Clindamycin.** Clindamycin was relatively inactive against *Nocardia*, with 5.3% (two of 38) of the organisms being inhibited by 3.1 $\mu g/ml$. Both sensitive strains had zone diameters of $>15$ mm, and all the remaining isolates with MICs of $\geq 6.2 \, \mu g/ml$ had no measurable zone of inhibition around the disk.

**Discussion**

The aminoglycosides showed considerable activity against *Nocardia* by agar dilutions in vitro.
Two newer aminoglycosides, tobramycin and amikacin, were the most active. Of potential importance to the clinician was the finding that over half of the isolates of *Nocardia* sensitive to kanamycin, tobramycin, and amikacin by our methods were sensitive to 0.78 μg of antibiotic/ml. Substantially higher concentrations of these antibiotics are regularly achieved in serum during therapy.

The resistance pattern of *Nocardia* to the aminoglycosides was similar to that seen with the Enterobacteriaceae. Those isolates resistant to amikacin were all resistant to the other four aminoglycosides tested. Of the 19 organisms resistant to tobramycin, all but two were resistant to gentamicin, and all but three were resistant to kanamycin. Isolated resistance to gentamicin, kanamycin, and streptomycin was common. Unlike the Enterobacteriaceae, however, *Nocardia* were almost always sensitive to the sulfonamides and minocycline.

Results of testing the sensitivity of *Nocardia* to sulfonamides by agar dilution have been variable; inconsistencies have usually reflected lack of a standardized method and differences in inoculum size [5, 6, 10, 14]. With plate inocula of 10^5–10^6 cfu, isolates have been shown to be resistant to sulfonamides when the end point of complete inhibition of growth has been employed [5]. In an experimental model, however, mice infected with strains of *Nocardia* resistant by these criteria were cured by treatment with sulfonamides [7]. This discrepancy, combined with the observation that many patients respond well to treatment with sulfonamides, suggests that this inoculum size does not accurately distinguish sulfa-sensitive from sulfa-resistant isolates. With smaller inocula, however, most isolates of *Nocardia* have been shown to be inhibited by ≤25 μg/ml [6, 15–17]. With use of the agar dilution technique with a plate inoculum of 10^3 cfu, in vitro susceptibility has generally been correlated well with clinical response in human infection [17, 18].

When we employed an inoculum of 10^4–10^6 cfu, we were unable to demonstrate sensitivity of any of 14 isolates of *Nocardia* to 50 μg of sulfisoxazole/ml, and only one strain was inhibited by 12.5 μg of TMP-SMZ/ml. With a plate inoculum of 10^2 cfu, however, almost 90% of nocardia strains were sensitive to this concentration of sulfisoxazole, and almost 95% were sensitive to this concentration of TMP-SMZ.

There have been few clinical studies evaluating the sulfa disk for sensitivity testing of *Nocardia* [5, 18]. We found reproducible results with both the sulfisoxazole and TMP-SMZ disks, as well as distinction by zone size between sensitive and intermediate strains. Criteria for sensitivity to the sulfonamides for infections other than those of the urinary tract have not been established [9]. In this study, concentrations of 50 μg/ml for sulfisoxazole and 12.5 μg/ml for TMP-SMZ were chosen as dividing lines between sensitive and intermediate strains because clinically achievable blood levels could exceed the

![Figure 9. Regression line for correlation of agar dilution and disk diffusion tests for sensitivity of 51 strains of *Nocardia* to gentamicin (10 μg per disk). The distribution of points at each MIC was fairly uniform. Twenty of 24 sensitive isolates (MIC, ≤6.2 μg/ml) and none of the resistant isolates (MIC, ≥12.5 μg/ml) had zones of inhibition of ≥25 mm. SE = 4.6; correlation coefficient (r) = -0.911.](image)
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MIC of sensitive strains by at least three- to five-fold [19, 20].

There were too few organisms with MICs of \( \geq 100\, \mu g/\text{ml} \) of sulfoxazole for proper evaluation of resistance to that drug by the disk method. No strains resistant to TMP-SMZ were noted, and the presence of two organisms that had no demonstrable zone of inhibition around the disk but were sensitive by agar dilutions suggested that this TMP-SMZ disk did not distinguish sensitive from resistant strains. There were no organisms with zone diameters of 35 mm with sulfoxazole or 20 mm with TMP-SMZ that were resistant by agar dilutions. Hence isolates of Nocardia with these zone sizes may be safely considered to be sensitive, but those with zone sizes in the intermediate range or with no demonstrable zone around the disk should have sensitivities determined by agar dilutions. Similar problems with indications of "false resistance" by disk diffusion have been noted in tests of the sulfonamides with other organisms.

This study compared the results of standard agar dilution methods with a Kirby-Bauer disk diffusion method for testing of antimicrobial agents against Nocardia. The aminoglycosides (especially amikacin), minocycline, and the sulfonamides all showed good antimicrobial activity, with the aminoglycosides showing the best correlation between MICs and sizes of zones of inhibition. Unfortunately, one-third of our isolates failed to produce any visible growth on agar plates at 48 hr and could not be tested for sensitivity by either method.

Because rapid growth is important to both methods of sensitivity testing, development of a better growth medium than Mueller-Hinton agar may be required. Problems of slow growth were encountered by Bach et al. in use of the agar dilution method, and they were able to report sensitivities on only 20%–40% of their isolates [5]. Agar dilution studies similar to those now utilized for testing of mycobacteria may prove useful for these slowly growing isolates, if the timing can be standardized and if the antibiotics are sufficiently stable. Efforts to improve both sensitivity methods are needed if they are to provide a means for sensitivity testing of all clinical isolates.

Addendum

Since initiation of this study, we have evaluated 16 clinically significant isolates of Nocardia from 15 patients. All of these isolates grew rapidly enough to allow susceptibility testing by the disk diffusion method.

References

11. Bauer, A. W., Kirby, W. M. M., Sherris, J. C., Turck, M.


