Disk Diffusion Testing of Susceptibility of *Mycobacterium fortuitum* and *Mycobacterium chelonei* to Antibacterial Agents

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Although recent studies have suggested that some antibacterial agents have good activity against the rapidly growing mycobacteria *Mycobacterium fortuitum* and *Mycobacterium chelonei*, an easily applicable method for susceptibility testing of clinical isolates is not yet available. We evaluated a disk diffusion method with Mueller-Hinton agar and 48-h readings with 59 strains of *M. fortuitum* and 11 strains of *M. chelonei* and compared the results to agar dilution susceptibilities for nine antimicrobial agents. All isolates were susceptible to 16 μg of amikacin or kanamycin per ml with minimum zone diameters of 14 and 18 mm, respectively. Amikacin inhibited 100% of isolates of *M. fortuitum* at 2 μg/ml, whereas 10 of 11 (91%) of *M. chelonei* strains had minimum inhibitory concentrations of 4.0 μg/ml or greater. Doxycycline and minocycline had almost identical activities, inhibiting 44% of strains at 4.0 μg/ml, and both allowed easy differentiation between susceptible and resistant strains by disk diffusion. Although most isolates of *M. chelonei* grew better on 7H10 agar, this media gave two- to eight-fold higher minimum inhibitory concentrations than were obtained with Mueller-Hinton agar. Disk diffusion susceptibility testing appears to be a simple and reliable means of predicting susceptibility results for *M. fortuitum* and most isolates of *M. chelonei* by the agar dilution method.

The rapidly growing mycobacteria are resistant in vitro to all of the commonly used antituberculous agents (6, 7); as might be expected, the therapeutic application of these agents has generally been unsuccessful (1). Recent studies have demonstrated that some antibacterial agents, such as doxycycline and amikacin, have good activity against the above organisms at achievable serum levels (2, 3, 5, 6). Because susceptibility to some of these agents is variable, a simple and rapid method for testing the susceptibility of these mycobacteria is needed.

We have evaluated a disk diffusion susceptibility method, using commercial antibiotic disks, and compared the results to agar dilution susceptibilities for 70 strains of *Mycobacterium fortuitum* and *Mycobacterium chelonei* against nine antibacterial agents.

**MATERIALS AND METHODS**

Eleven isolates of *M. chelonei* and 59 of *M. fortuitum* were used in the study. The majority of isolates were provided by the Louisiana State Board of Health, the Mycobacteriology Laboratory of Charity Hospital of Louisiana (both of New Orleans), and the Houston City Health Department, Houston, Tex. Six isolates of *M. chelonei* came from patients with clinical disease (two ulcerative keratitis, two chronic cavitary pulmonary disease, one cutaneous abscess, and one disseminated disease) as did 10 isolates of *M. fortuitum* (five cutaneous abscesses, two osteomyelitis, one cavitary lung disease, and two meningitis). All isolates except one were obtained pretreatment. The exception was obtained from the sputum of a patient who had been on long-term tetracycline (tetracycline-resistant). Twenty-four isolates of rapidly growing mycobacteria (two *M. chelonei* and 22 *M. fortuitum*) were obtained from sputum but were not felt to be associated with disease. Sources and clinical significance of the remaining 30 isolates were not known.

Organisms were identified by the referral laboratories. Identification as to species of the *M. chelonei* isolates was confirmed on the basis of a negative nitrate reduction and negative iron uptake. These *M. chelonei* isolates also showed no zone of inhibition to a 300-U polymyxin disk by disk diffusion (8).

Agar dilution testing was performed with a Steers replicator and Mueller-Hinton agar (pH 7.2 to 7.4). Antibiotics were prepared by serial twofold dilutions of a concentrate prepared from standard diagnostic powders of nine antimicrobial agents: kanamycin, gentamicin, tobramycin, amikacin, streptomycin, erythromycin, tetracycline, doxycycline, and minocycline. Control strains of *Escherichia coli* ATCC 25922.
(American Type Culture Collection, Rockville, Md.). Staphylococcus aureus ATCC 25923, and Pseudomonas aeruginosa ATCC 28753 were inoculated simultaneously on the agar plates. Plates were incubated at 37°C and read at 48 h except for the bacterial control strains which were read at 24 h. The minimal inhibitory concentration (MIC) was read as the lowest dilution of antibiotic which produced complete inhibition of growth or a very fine haze. Most of the MICs were repeated on at least one other occasion and were found to be reproducible to within one dilution. MICs of some of these isolates have been reported previously (2).

Because not all M. chelonei isolates grew well on unsupplemented Mueller-Hinton agar and because most mycobacterial susceptibilities are tested on other media, simultaneous testing by agar dilutions was performed on 21 strains of M. fortuitum and 6 strains of M. chelonei with commercial 7H10 agar, and Mueller-Hinton agar to which OADC (oleic acid, albumin, dextrose, and catalase) had been added in a ratio by volume of 10:1. Comparative MICs were determined for doxycycline, gentamicin, and amikacin.

Disk diffusion susceptibility studies were performed with Mueller-Hinton agar plates of 4- to 5-mm thickness and commercial antibiotic disks (BBL Microbiology Systems, Cockeysville, Md.). The drug contents of the disks were 10 µg for amikacin, gentamicin, streptomycin, and tobramycin; 30 µg for kanamycin, minocycline, tetracycline, and doxycycline; and 15 µg for erythromycin. An experimental 30-µg amikacin disk (which is expected to soon replace the smaller 10-µg disk) was provided by Bristol Laboratories, Syracuse, N.Y. Organisms as prepared below were streaked on the plates with cotton swabs, and disk zone sizes were measured at 48 h. Confirmatory readings on some organisms (especially M. chelonei) were also made at 72 h.

Mycobacteria for susceptibility testing were prepared by two different methods. Approximately one-half of the isolates (28 M. fortuitum and 3 M. chelonei) were taken from 48-h cultures on Lowenstein-Jensen agar and suspended in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). The remaining isolates were grown in Trypticase soy broth at 37°C until a cloudy suspension was obtained. These isolates were shaken daily (Vortex mixer), and if necessary, 0.5-mm sterile glass beads were added to aid in fragmentation. Before sampling the cultures, any heavy particles that remained were allowed to settle out. Both groups were then diluted to match the turbidity of one-half the number one McFarland standard (1.0 × 10^6 colony-forming units per ml). Colony counts were performed, and only organisms in concentrations of 1.0 × 10^3 to 1.5 × 10^6 colony-forming units were used for susceptibility testing. This dilution was used for both susceptibility methods.

Regression line analysis was performed for each antibiotic by least-squares fit of the linear model that relates MICs and zone diameters [ln(MIC) = a + b · zone diameter].

RESULTS

Correlation between MICs and disk diffusion. (i) Aminoglycosides. As shown in Fig. 1, amikacin inhibited 100% of the 65 mycobacterial isolates at a concentration of 16.0 µg/ml. There was a species difference in the degree of susceptibility, however. All 54 isolates of M. fortuitum were inhibited by 2.0 µg of amikacin per ml, whereas 10 of 11 (91%) of M. chelonei strains had MICs of 4.0 µg/ml or greater. Zone diameters with the 30-µg amikacin disk averaged 5 to 7 mm larger than with the 10-µg amikacin disk. The smallest zone size with the larger disk was 20 mm for M. chelonei isolates and 31 mm for the M. fortuitum strains.

All isolates tested (32 M. fortuitum and 3 M. chelonei) were also susceptible to kanamycin, with MICs ranging from 4.0 to 16.0 µg/ml. They produced clear, distinct zones of at least 18 mm with the 30-µg disk, with 30 of 34 isolates falling between 18 to 35 mm. Because of this narrow range of susceptibility, however, no linear correlation was evident.

Gentamicin inhibited only 28% of 54 strains at a concentration of 4.0 µg/ml. Results with tobramycin were very similar to gentamicin for both MICs and disk zone diameters. Streptomycin had poor activity against the rapidly growing mycobacteria with 28 of 28 strains tested having MICs of 32 µg/ml or greater. By disk diffusion these isolates had zone diameters of 17 mm or less.

(ii) Erythromycin. Only 8/58 (14%) of strains were susceptible to 2.0 µg of erythromycin per ml. The outer zone margins around the susceptibility disk were not sharp and tended to fade gradually. Because of this phenomenon, a good control growth had to be present on the disk diffusion plate before a mycobacterial strain was interpreted as susceptible to erythromycin. In most instances confirmatory readings at 72 h were necessary to avoid a false interpretation.

(iii) Tetracyclines. The tetracyclines as a group had good activity. Organisms susceptible to one tetracycline were usually susceptible to all of them, although the MICs were one to two dilutions lower for doxycycline and minocycline than for tetracycline. Both doxycycline and minocycline inhibited 44% of strains at 4.0 µg/ml with a median MIC among susceptible strains of 0.5 µg/ml. As with erythromycin, the outer margins of the zones of inhibition were not sharp.

Comparison of MICs in Mueller-Hinton agar, 7H10 agar, and Mueller-Hinton agar plus OADC. As shown in Table 1, all of the isolates grew well on 7H10 agar, with most strains of M. chelonei growing better than on the Mueller-Hinton agar. With doxycycline, gentamicin, and amikacin, most isolates showed two- to fourfold higher MICs with 7H10 agar than with Mueller-Hinton agar. This phenomenon was most evident among strains of M. che-
DISCUSSION

In this study a disk diffusion method was evaluated for testing the susceptibility of *M. fortuitum* and *M. chelonei* to various antibacterial agents. These results were compared with MICs by a standard agar dilution procedure. Recently Welch and Kelly reported results of disk susceptibility testing with 24 strains of rapidly growing mycobacteria (8). They showed good correlation for amikacin and gentamicin between disk diffusion and MICs by agar dilu-
tions with zones sizes comparable to those observed in this study. Comparisons of disk diffusion with agar dilutions for other antibiotics were not provided, however.

These methods may be unsuitable for some isolates of M. chelonei. Six strains of M. chelonei obtained for the study failed to produce adequate growth on Mueller-Hinton agar to be evaluated by these susceptibility methods, whereas all of the isolates of M. fortuitum grew well. In addition, most strains of M. chelonei grew more slowly on Mueller-Hinton agar than the M. fortuitum strains. Optimal growth of all M. chelonei isolates occurred with 7H10 agar, but the MICs were two- to eightfold higher with this medium than with Mueller-Hinton agar. The addition of OADC to Mueller-Hinton agar enhanced the growth of M. chelonei without affecting the MICs to amikacin and gentamicin, suggesting that the addition of such enrichment factors may prove useful for testing the more fastidious or slow-growing isolates.

Several antimicrobial agents tested in this study have had little or no previous in vitro evaluation against the rapidly growing mycobacteria. Minocycline inhibited 44% of isolates and had MICs similar to doxycycline. Kanamycin was as active as amikacin (inhibiting 100% of isolates at 16.0 μg/ml), although the median MIC for kanamycin (8.0 μg/ml) was higher than for amikacin (1.0 μg/ml). Sanders et al. (6) found only two of nine isolates susceptible to 12.5 μg/ml of kanamycin. However, their tests were not performed on Mueller-Hinton agar, so some of their differences may reflect differences in method.

This disk diffusion method appears to provide a good screening method for susceptibility testing of clinically significant isolates of the rapidly growing mycobacteria. Isolates clearly resistant or susceptible by this method could be reported as such. Isolates which are intermediate or questionable could be retested by an agar dilution method. Agar or broth dilution tests are more reliable tests of susceptibility, but they are generally impractical for testing single isolates against a large number of antimicrobial agents.

**LITERATURE CITED**


