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Disk Diffusion Testing with Polymyxin and Amikacin for Differentiation of *Mycobacterium fortuitum* and *Mycobacterium chelonei*

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Disk diffusion is one method of susceptibility testing of the *Mycobacterium fortuitum* complex to antibacterial agents. We utilized disks of polymyxin B (300 U), amikacin, and kanamycin to determine whether they could also be used for species identification when compared with standard biochemical methods. With the polymyxin disk, 100% of 75 *M. fortuitum* strains produced zones of inhibition, whereas none (0%) of 58 *Mycobacterium chelonei* subspecies *abscessus* and *chelonei* strains had any zone of inhibition. With the amikacin disk, 99% of *M. fortuitum* biovariant *fortuitum* had zones of ≥30 mm compared with 6% of *M. chelonei*. The rare *M. chelonei*-like organisms gave variable results, and 42% of the unnamed "third group" biovariant of *M. fortuitum* exhibited an unusual but diagnostic pattern of small zone sizes to amikacin and no zone to kanamycin. The kanamycin disk was otherwise not helpful, although it resulted in larger zone sizes for *M. chelonei* than did amikacin. Thus, disk diffusion susceptibilities which include these disks (especially polymyxin) will provide presumptive evidence of species as well as susceptibility data.

Identification to the species level of the pathogenic, rapidly growing mycobacteria is not done in most laboratories because this separation has been felt to be of no use clinically. Most isolates have been referred to only as the *Mycobacterium fortuitum* complex. However, recent studies have shown a distinct difference in antimicrobial susceptibility between *M. fortuitum* and *Mycobacterium chelonei* for docycycline, the sulfonamides, and amikacin (4, 11, 12), suggesting that correct identification of the species is important for predicting drug susceptibilities. The major biochemical tests that have been relied on to separate *M. fortuitum* and *M. chelonei* have been nitrate reduction and, more recently, iron uptake. Other tests that have been used include arylsulfatase isoenzymes (2), esterase activity (6), penicillinase, β-glucosidase, trehalose, fructose (5), seroagglutination (7), and lipid chromatography (7). Unfortunately, most of the latter tests are useful only in a large reference laboratory.

Casal and Rodriguez (3) described the use of a 20-μg pipemidic acid (Bio-Merieux) disk test to provide a simple and rapid means of distinguishing the two species. Results with 48 strains of *M. fortuitum* and *M. chelonei* showed complete correlation with the species identification by the standard biochemical tests. All the isolates of *M. fortuitum* were sensitive to the disk (mean zone diameter, 21.5 mm), and all the *M. chelonei* isolates were resistant (mean zone diameter, 6 mm). Unfortunately, this disk is not available in the United States.

Welch and Kelly (13) described the use of a 300-U polymyxin disk as a possible means to separate *M. fortuitum* and *M. chelonei*. Because this disk is more readily available, we elected to test it along with amikacin and kanamycin to determine if these disks would successfully identify as to species the pathogenic members of the rapidly growing mycobacteria.

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MATERIALS AND METHODS

A total of 138 clinical strains of rapidly growing mycobacteria were evaluated. Most of these isolates had caused clinical disease and had been referred to one of the authors for antimicrobial susceptibility testing. Acid-fast organisms were identified as members of the *M. fortuitum-M. chelonei* complex on the

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basis of growth within 7 days on solid agar with typical colony morphology, a positive arylsulfatase test after 3 days, and growth on MacConkey agar without crystal violet at 28°C (9). Organisms were identified as *M. fortuitum* or *M. chelonei* by using nitrate reduction and iron uptake tests and were identified as to biovariant (*M. fortuitum*) or subspecies (*M. chelonei*) according to the method of Silcox et al. (9). Among the 58 isolates of *M. chelonei*, 52 were *M. chelonei* subspecies *abscessus*, and 6 were *M. chelonei* subspecies *chelonei*. Of the 79 strains of *M. fortuitum*, 66 were *M. fortuitum* biovariant *fortuitum*, one organism was *M. fortuitum* biovariant *peregrinum*, and 12 belonged to the currently unnamed "third group" biovariant (8). Five isolates were *M. chelonei*-like organisms (1) from different geographic sites or with different antimicrobial susceptibilities that were felt to represent different strains.

Susceptibility testing. For susceptibility testing, organisms were grown or suspended in Middlebrook 7H9 broth to match the turbidity of ½ the no. 1 McFarland standard. The organisms were then swabbed onto a Mueller-Hinton agar plate that had been poured to an average depth of 4 mm and whose surface had been swabbed with 10% Middlebrooks OADC (oleic acid, albumin, dextrose, and catalase). Commercial antimicrobial disks of amikacin (30 μg), polymyxin (300 U), and kanamycin (30 μg) were added to the surface of the plates, which were then incubated at 35°C for 72 h. The zones of inhibition around each of the disks were then measured. A fine haze of growth around the polymyxin disk was ignored when there was marked inhibition of growth. MIC (minimum inhibitory concentration) determinations for amikacin and kanamycin were performed for most isolates by using both an agar dilution and a broth dilution method (10, 11). For agar dilutions, the organisms were tested on commercial Mueller-Hinton agar supplemented with 10% OADC (11), whereas for broth dilution the organisms were tested in Mueller-Hinton broth supplemented only with Ca^{2+} and Mg^{2+} (10). For both methods, an innoculum size of approximately 10^4 colony-forming units per well or spot was used, and plates were incubated for 72 h. The MICs were interpreted as the lowest concentration that produced no visible turbidity in broth or no visible growth or very fine haze on agar.

RESULTS

The polymyxin disk was the easiest and most accurate disk for distinguishing *M. fortuitum* and *M. chelonei* to species level, but gave variable results with the *M. chelonei*-like organisms (Table 1). Of 75 isolates from the three biovariants of *M. fortuitum*, 74 (99%) produced zones of 10 mm or greater to polymyxin. The other isolate had a zone of 9 mm. Thus, with any zone of inhibition, this disk identified 100% of the isolates of *M. fortuitum*. In contrast, 0 (0%) of 58 isolates of the *M. chelonei* subspecies *abscessus* or *chelonei* produced any zone of inhibition around the disk. The *M. chelonei*-like organisms gave variable results, with two isolates having no zones of inhibition and three isolates having zones of 10 to 14 mm.

The amikacin disk was only slightly less accurate than the polymyxin disk in separating *M. fortuitum* from *M. chelonei* (Table 2). Of 66 *M. fortuitum* biovariant *peregrinum* or *fortuitum* isolates, 65 (98.5%) had zones of 30 mm or greater to amikacin, and all 66 isolates showed MICs of amikacin of 2.0 μg/ml or less. Among the 12 isolates from the third group biovariant, however, 5 organisms (42%) had zones of 20 to 29 mm. With the latter isolates, MICs of amikacin were 4 to 8 μg/ml.

Among the *M. chelonei* isolates, only 3 (6%) of 52 had zone diameters of 30 mm or more to amikacin. All the isolates tested had MICs of amikacin of 4.0 μg/ml or greater. The five *M. chelonei*-like organisms behaved like the *M. fortuitum* isolates in that all had zones of 30 mm or more.

The kanamycin disk did not distinguish between the three biovariants of *M. fortuitum*, with one exception. Among biovariant *fortuitum* and *peregrinum* isolates, 64 (96%) of 66 had zones of 20 mm or more, and all isolates had zones of 10 mm or more. Among the third group biovariant, however, seven isolates had zones of 20 mm or more and five isolates (42%) had no zones of inhibition. Amikacin had smaller zones and higher MICs with the same five strains. Thus, resistance to kanamycin and zone diameters of less than 30 mm to amikacin in an isolate of *M. fortuitum* were diagnostic of the third group biovariant.

Among the 50 *M. chelonei* isolates that were tested against the kanamycin disk, 64% had zones of 20 mm or more and 28% had zones of 30 mm or more. The zone sizes for these isolates were greater for kanamycin than for amikacin. The five *M. chelonei*-like organisms had zone diameters of more than 30 mm to kanamycin.

DISCUSSION

These studies demonstrate that standard commercial antibiotic disks can be used in diffusion tests to provide rapid presumptive species identification within the *M. fortuitum* complex. The polymyxin disk was clearly the best of the three disks that were tested. Welch and Kelly (13) reported similar results using the polymyxin disk with a smaller number of isolates. They found a mean zone size of 16 mm for 17 isolates of *M. fortuitum*, with a range of 13 to 19 mm. Among their seven *M. chelonei* strains, the mean zone diameter was 6 mm, with a range of 6 to 7 mm. These results are virtually identical to those reported here.

After organisms are isolated, identification to species level of the *M. fortuitum* complex can be done in 3 days by using growth rates, nitrate reduction, and a 3-day arylsulfatase test. The

TABLE 1. Disk zone diameters to polymyxin B on Mueller-Hinton agar

Organism (no. of strains)	No. of strains for the following zone sizes (mm):				
	No zone	7–9	10–14	15–19	20–24
M. fortuitum biovariant *fortuitum* (62)	0	1	31	27	3
M. fortuitum biovariant *peregrinum* (1)	0	0	1	0	0
M. fortuitum biovariant third group (12)	0	0	8	4	0
M. chelonei subspecies *chelonei* (6)	6	0	0	0	0
M. chelonei subspecies *abscessus* (52)	52	0	0	0	0
M. chelonei-like (5)	2	0	3	0	0

nitrate reduction test can give variable results even if carefully controlled, however, and many of our early isolates from outside laboratories referred for susceptibility testing were misidentified when only this test was used to separate *M. fortuitum* from *M. chelonei* isolates. The test for iron uptake is a good confirmatory test to separate these two species, but it requires a positive control organism, and it is often 7 days before positive tests are apparent and 3 weeks before negative ones can be read out. The current disk test clearly has a time advantage over the iron uptake test and appears to be equally accurate.

All the isolates were tested on Mueller-Hinton agar. Similar results were obtained on 7H10 agar, although the zone sizes to amikacin were smaller. Previous studies have suggested that amikacin MICs tend to be higher on 7H10 agar than on Mueller-Hinton agar (11).

Isolates of *M. fortuitum* have been traditionally grouped into two biovariants, *fortuitum* and *peregrinum*. Pattyn et al. (8) noted a third unnamed group of *M. fortuitum* isolates which did not fit the biotype of the previous two groups. Silcox et al. (9) found 5 of these isolates among 170 clinical isolates of the *M. fortuitum* complex, and these authors and Swenson (unpublished observations) noted that several of these isolates were more resistant to amikacin than the *M. fortuitum* isolates belonging to biovariants *fortuitum* or *peregrinum*. The present study evaluated 12 additional isolates. Five (42%) of these isolates were intermediate in susceptibility to amikacin by disk and MIC determinations, and the same five isolates were also resistant to kanamycin. Thus, this would support the earlier observation of Silcox and Swenson about the unusual susceptibilities of this group of organisms.

Numerous factors influence disk zone size in diffusion tests, especially inoculum size and depth of the agar. Specific zone sizes as criteria for identification are useful only when the methods are standardized and reproducible. Variations among laboratories (especially for the two factors mentioned) is a potential source of error, especially for amikacin, for which we used a specific zone size. Dependence on a specific zone size was not necessary with polymyxin. However, this drug was influenced by inoculum size; with very heavy inocula the zone of inhibition for *M. fortuitum* could almost be obliterated except for a faint incomplete zone. With *M. chelonei*, the inoculum size made no difference, since the organisms were completely resistant regardless of whether a light or heavy inoculum was used.

Thus, we suggest that the polymyxin disk be

TABLE 2. Disk zone diameters to amikacin on Mueller-Hinton agar

Organism (no. of strains)	No. of strains for the following zone sizes (mm):				
	No zone	7–9	10–19	20–29	≥30
M. fortuitum biovariant *fortuitum* (66)	0	0	0	1[a]	65
M. fortuitum biovariant *peregrinum* (1)	0	0	0	0	1
M. fortuitum biovariant third group (12)	0	0	0	5[b]	7
M. chelonei subspecies *chelonei* (8)	0	2	2	4	0
M. chelonei subspecies *abscessus* (44)	2	1	22	16	3
M. chelonei-like (5)	0	0	0	0	5

[a] MIC, 1.0 μg/ml.
[b] MICs, 4.0 to 8.0 μg/ml.

the primary disk for species identification of *M. fortuitum* and *M. chelonei*, with care that too heavy an inoculum not be applied. Any zone of partial or complete inhibition would identify the organism as *M. fortuitum*, and no zone of inhibition would identify the organism as *M. chelonei*. The amikacin disk could be used as a confirmatory test.

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