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Susceptibility Testing of Clinical Isolates of *Methylobacterium* Species

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***Methylobacterium* species represent a relatively new genus which is being increasingly isolated from cases of opportunistic infections. This study reports on 3 reference strains and 15 clinical isolates of *Methylobacterium* species. Susceptibility tests were performed by the agar dilution and commercial broth microdilution methods at both 30 and 35°C. Readings were made at 24, 48, and 72 h. Incubation conditions of 48 h and 30°C were found to be optimum. Both the agar dilution and broth microdilution methods gave equivalent results. Drugs tested and their MICs for 90% of isolates (in micrograms per milliliter) were as follows: amikacin, ≤1; gentamicin, 1; ciprofloxacin, 1; trimethoprim-sulfamethoxazole, 2/38; ceftriaxone, 16; and ceftizoxime, 16. The majority of our isolates were resistant to six other beta-lactam drugs tested. Nine of the 15 *Methylobacterium* isolates were β-lactamase positive.**

The genus *Methylobacterium* was first proposed in 1976 (7) to accommodate gram-negative bacteria that have the ability to utilize methane and other more complex organic compounds as carbon and energy sources. Subsequently, the description of the genus *Methylobacterium* has been emended to include non-methane-utilizing bacteria (4); these authors also proposed that all pink-pigmented, facultatively methylotrophic bacteria be transferred to the genus *Methylobacterium*. This latter group of organisms includes previously named *Pseudomonas rhodos*, *Pseudomonas rodiora*, and *Pseudomonas mesophilica*. Nucleic acid hybridization studies verified that the pink-pigmented facultatively methylotrophic strains should not be included among the pseudomonads, and the molecular data support inclusion of these pink-pigmented bacteria in the genus *Methylobacterium* (12). These organisms and subsequently named species of *Methylobacterium* have been recognized as common environmental isolates from such habitats as leaf surfaces, leaf nodules of plants, soil, water, grass, sewage, air, and rice grains (3). During the last 8 years, there have been increasing numbers of reports of human clinical isolates (2, 3, 8–10).

We isolated our first clinical strain of *Methylobacterium* in 1987 in pure culture from continuous ambulatory peritoneal dialysis fluid. The patient was undergoing dialysis and had developed continuous ambulatory peritoneal dialysis peritonitis. Identification of the isolate was made by the Michigan Department of Public Health (Lansing) and confirmed by the Centers for Disease Control (Atlanta, Ga.). An additional 14 isolates have been recovered from other patients. This study was designed to investigate the in vitro susceptibility testing of these clinical isolates and three reference strains.

MATERIALS AND METHODS

Strains. Three *Methylobacterium* reference strains were included: *Methylobacterium extorquens* ATCC 43645, *M. radiotolerans* ATCC 27329, and *M. rhodinum* ATCC 14821. The 15 clinical isolates were recovered from blood (3 isolates), continuous ambulatory peritoneal dialysis or peritoneal fluid (3 isolates), thoracentesis fluid (2 isolates), respiratory secretions (2 isolates), wounds (2 isolates) stool (1 isolate), and other sources (2 isolates). The clinical isolates are from our laboratories, one strain was obtained from Ronald Masters (Reading Medical Center, Reading, Pa.), and two strains were obtained from James Hong (University of Maryland Cancer Center, Baltimore, Md.). All clinical isolates grew well on Sabouraud dextrose agar and buffered charcoal-yeast extract agar but poorly or not at all on MacConkey agar. Growth was better at 30 than 35°C, and no growth occurred at 42°C. All isolates produced pink colonies of gram-negative bacilli that were oxidase, catalase, and urease positive. Identification was made by conventional biochemical reactions (1) and by the Microbial Identification System (Microbial ID, Inc., Newark, Del.), which utilizes cellular fatty acid analysis with computerized high-resolution gas chromatography (5, 11). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control checks on the agar dilution and MicroScan panels (MicroScan Division, Baxter Healthcare Corporation, West Sacramento, Calif.).

Susceptibility testing. Agar dilution and broth microdilution procedures were performed at both 30 and 35°C. The agar dilution method for determining MICs was carried out as described by the National Committee for Clinical Laboratory Standards (6). Briefly, Mueller-Hinton agar (pH 7.3) (Difco Laboratories, Detroit, Mich.) was sterilized and cooled in a water bath at 48°C. Appropriate dilutions of each antimicrobial agent were added to 20-ml aliquots of the medium, and the mixtures were poured into petri dishes, cooled, solidified, and used within 48 h. The plates were stored at 4 to 6°C in sealed plastic bags until used. Plates were warmed and dried at 35°C for 30 min prior to inoculation. The direct suspension method was used to prepare inocula, and a Steers replicator was used to inoculate the suspension onto the agar surfaces. Four plates for each dilution of each drug and control plates with no antimicrobial agent were prepared. All tests were performed in duplicate, with one set of plates incubated at 30°C and the second set incubated at 35°C in air. The MIC was the lowest concen-

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TABLE 1. MIC results for *Methylobacterium* reference strains and 15 clinical isolates tested by agar dilution and broth microdilution procedures at 30°C

Antimicrobial agent	MIC (μg/ml)[a] for:									
	M. radiotolerans ATCC 27329		*M. rhodinum* ATCC 14821		*M. extorquens* ATCC 43645		Clinical isolates			
							50%		90%	
	AD	MB	AD	MB	AD	MB	AD	MB	AD	MB
Ampicillin	2	4	8	8	≤2	≤2	8	4	>32	>32
Piperacillin	128	>64	>128	>64	128	128	>128	>64	>128	>64
Cefazolin	8	4	32	16	8	8	32	16	>32	>16
Cefotetan	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Cefuroxime	>32	>16	>32	>16	32	32	32	>16	>32	>16
Ceftazidime	>32	>16	>32	>16	>32	>32	>32	>16	>32	>16
Ceftriaxone	≤2	≤4	8	≤4	≤2	≤2	4	≤4	16	16
Ceftizoxime	≤2	≤4	8	≤4	≤2	≤2	8	≤4	16	16
Gentamicin	≤0.5	≤1	≤0.5	≤1	≤0.5	≤0.5	≤0.5	≤1	1	≤1
Amikacin	≤1	≤2	≤1	≤2	≤1	≤1	≤1	≤2	≤1	≤2
Ciprofloxacin	1	1	2	1	1	1	≤0.5	1	1	2
Trimethoprim-sulfamethoxazole	0.25/4.75	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	1/19	1/19	1/19	0.25/4.75	2/38	2/38

[a] The ranges of most drug concentrations in the agar dilution (AD) procedure were extended 1 dilution above and below the respective ranges in the broth microdilution (MB) procedure. 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

tration of antimicrobial agent that completely inhibited growth, with a single colony or a faint haze of growth disregarded.

The broth microdilution method was performed with MicroScan dried Negative Combo Panels type 2. The procedure recommended by the manufacturer was used. Briefly, direct-suspension inocula from 48-h cultures were prepared by using the Prompt System (MicroScan). As with the agar dilution, four panels were prepared for each strain. Two panels were incubated at 30°C in air, and another two panels were incubated at 35°C in air. The MIC was the lowest concentration of antimicrobial drug that completely inhibited growth as detected by the unaided eye with the TouchScan (MicroScan).

β-Lactamase testing was carried out with the nitrocefin disk test (Cefinase; BBL Microbiology Systems, Cockeysville, Md.).

Antimicrobial agents. Standard powders and the companies from which they were obtained were as follows: amikacin, Bristol-Meyers Squibb, Syracuse, N.Y.; sulfamethoxazole and trimethoprim, Burroughs Wellcome Co., Research Triangle Park, N.C.; ceftizoxime, Fugisawa Pharmaceutical Co., Deerfield, Ill.; ceftazidime and cefuroxime, Glaxo Inc., Durham, N.C.; ceftriaxone, Hoffmann-La Roche Inc., Nutley, N.J.; piperacillin, Lederle Inc., Pearl River, N.Y.; cefazolin, Eli Lilly & Co., Indianapolis, Ind.; ciprofloxacin, Miles Pharmaceuticals, West Haven, Conn.; ampicillin, Pfizer Pharmaceuticals, Groton, Conn.; gentamicin, Schering-Plough Corp., Bloomfield, N.J.; and cefotetan, Stuart Pharmaceuticals, Wilmington, Del.

The antimicrobial powders were weighed, corrected for potency, and dissolved as directed by the manufacturers. Preparations at 20 times the highest desired concentration and serial twofold dilutions were made for the range of concentrations to be tested by the agar dilution procedure.

Quality control. All antimicrobial agents prepared in agar dilution plates and the MicroScan panels had quality control checks with the reference strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. All antimicrobial agents had MICs within the acceptable range for both species when tested in the agar dilution or the commercial broth microdilution preparations.

RESULTS

The optimum lengths of time for incubation at each temperature for agar dilution and broth microdilution methods were determined by visual observations at 24, 48, and 72 h. All *Methylobacterium* strains had grown to their optimum concentrations for reading by 48 h, and this time was selected for MIC readings. The temperature selected for measuring the MIC was 30°C. All strains grew best at this temperature, with several strains not growing at all at 35°C. Results from the duplicate sets of susceptibility tests at both temperatures and methods were always within 1 dilution. In the few incidents with two different endpoints, the greater concentration of antimicrobial drug was used as the MIC.

Results of testing the three reference strains of *Methylobacterium* species by both procedures are shown in Table 1. The results are very similar, with both systems having almost identical results. The MIC was outside the range of dilutions tested for many of the isolate-antimicrobial agent combinations. A few MICs were 1 dilution greater in the agar dilution procedure. These same trends were observed with clinical isolates, as seen in comparing the MICs for 50% of strains tested obtained by the two methods at 30°C (Table 1).

Our data show that the commercial MIC panels can be used for determining the susceptibility values for *Methylobacterium* species and that the endpoints are comparable to the agar dilution reference testing results as shown in Table 1.

A summary of the in vitro susceptibilities of our 15 clinical isolates of *Methylobacterium* species is shown in Table 2. The majority of isolates were resistant to the beta-lactam drugs with the exceptions of ceftriaxone and ceftizoxime (breakpoints, 32 and 64 μg/ml, respectively). All isolates were susceptible to these two antimicrobial agents (Table 2). Both aminoglycosides were extremely active in vitro, with 100% of the strains inhibited by 1.0 μg of amikacin per ml and 2.0 μg of gentamicin per ml. All strains were also inhibited by ciprofloxacin, but one isolate fell into the moderately susceptible range (2 μg/ml). All isolates were susceptible to trimethoprim-sulfamethoxazole.

TABLE 2. Susceptibilities of 15 clinical isolates of *Methylobacterium* species[a]

Antimicrobial agent	Range of dilutions (μg/ml)	Accumulative % of isolates inhibited at the following dilution (μg/ml):								
		0.5	1.0	2.0	4.0	8.0	16	32	64	128
Ampicillin	1.0–32		20	40	40	60	67	80		
Piperacillin	4.0–128				0	0	0	13	20	40
Cefazolin	1.0–32		0	0	13	33	47	73		
Cefotetan	1.0–32		0	0	0	6	27	33		
Cerfuroxime	1.0–32		0	0	0	0	20	60		
Ceftazidime	1.0–32		0	0	0	6	13	33		
Ceftriaxone	2.0–64			36	57	79	100	100	100	
Ceftizoxime	2.0–64			36	43	86	100	100	100	
Gentamicin	0.5–12	86	93	100	100	100				
Amikacin	1.0–32		100	100	100	100	100	100		
Ciprofloxacin	0.5–4.0	57	93	100	100					
Trimethoprim-sulfamethoxazole[b]	0.25/4.75–4/76									

[a] Tested by agar dilution at 30°C.

[b] At the following dilutions (in micrograms per milliliter) trimethoprim-sulfamethoxazole inhibited the indicated percentages of isolates: 0.25/4.75, 21%; 0.5/9.5, 21%; 1.0/19, 71%; 2/38, 100%; 4/76, 100%.

β-Lactamase testing with the nitrocefin disk testing method was performed, and all strains positive for β-lactamase correlated with an ampicillin MIC above 4 μg/ml.

DISCUSSION

The 15 clinical isolates of *Methylobacterium* species reported in this study were identified by using conventional physiological and biochemical schemes (1) and by using gas-liquid chromatography analysis of the cellular fatty acids (11) by the Microbial Identification System (5). Gilardi and Faur (2) have reported on a heterogeneous group of 21 clinical isolates of oxidase-positive, pink-pigmented, oxidative bacteria. The majority of their isolates would be classified as *Methylobacterium* species by current taxonomy. Individual case reports of similar organisms have also been reported recently (3, 8, 9). Twenty percent of our isolates were from blood cultures, as were the isolate reported by Smith et al. (9) and 11 of the 21 isolates reported by Gilardi and Faur (2). Isolates from blood, continuous ambulatory peritoneal dialysis fluid, and thoracentesis aspirates accounted for more than 50% of our strains.

The purpose of our study was to determine a reliable in vitro susceptibility testing method to guide therapy for opportunistic *Methylobacterium* infections. Gilardi and Faur (2) used a disk diffusion procedure and incubation at 30°C for 2 to 4 days. Smith et al. (9) used disk diffusion and macrodilution for their isolate. Rutherford et al. (8) also used disk diffusion for their isolate and incubation at 30°C for 48 h. We also found incubation conditions of 48 h and 30°C to be optimum for our MIC testing for both the agar dilution and the broth microdilution methods. Two of our isolates would not grow at 35°C in either system but grew well at 30°C.

The agar dilution and the commercial broth microdilution MIC panels gave very similar results. We conclude that in vitro susceptibility testing for *Methylobacterium* species can be performed in MicroScan panels incubated in air at 30°C for 48 h with results which are equivalent to results obtained by the agar dilution method as outlined by the National Committee for Clinical Laboratory Standards (6).

The in vitro results show differences in activity of the antimicrobial agents against the isolates of *Methylobacterium*. The expression of the data as MICs for 50 and 90% of strains tested fails to show the activities of the individual agents. However, the cumulative data (Table 2) clearly display the relative activities of the antimicrobial agents against the clinical isolates. All our isolates and the three reference strains were inhibited by low levels of gentamicin and amikacin, in agreement with the previous studies using disk diffusion methods on two clinical isolates (8, 9). Furthermore, all our isolates were susceptible to ciprofloxacin, although almost half the isolates were approaching the breakpoint (2 μg/ml). All strains were also inhibited by trimethoprim-sulfamethoxazole, in contrast to the isolate reported by Smith et al. (9). Among the beta-lactam drugs tested, only ceftriaxone and ceftizoxime inhibited all the strains. Ceftazidime and cefuroxime inhibited only 13 and 20% of the isolates, respectively. Ampicillin was found to be intermediately susceptible in one study (8) and resistant in two other studies (3, 9). In our larger study, the ampicillin MICs for 6 of our 15 clinical isolates were ≤4 μg/ml. These six strains were β-lactamase negative, whereas the nine strains for which the MICs were ≥8 μg/ml were β-lactamase positive. However, even beta-lactam drugs usually resistant to β-lactamase inactivation had very little activity against the *Methylobacterium* isolates. Mechanisms other than drug inactivation by β-lactamases may be involved in the resistance.

Our data are in contrast to the findings of Gilchrist et al. (3), who reported on four isolates from patients, for which they also utilized agar dilution susceptibility testing. These investigators found sparse or no growth of their isolates on Mueller-Hinton agar, whereas all our strains grew on this medium. Their MICs were higher than those we observed, and this discrepancy could be explained by differences in strains or by the effect of the medium on the activities of the drugs. These investigators used buffered yeast extract medium, which is usually the isolation medium for *Legionella* species and upon which *Methylobacterium* species also grow. The inclusion of reference strains by these investigators would have aided in explaining the difference.

β-Lactamase testing by a method other than use of the chromogenic cephalosporin nitrocefin would be desirable for *Methylobacterium* species. However, this is the most sensitive test. The red color which developed on the disks for strains producing β-lactamase was sufficiently intense to readily distinguish positive reactions from the pink pigment contributed by the inoculum.

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