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Comparison of Anaerobic Susceptibility Results Obtained by Two Methods of Inoculum Preparation

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We evaluated the use of inocula prepared directly from blood agar plates in agar dilution susceptibility tests of anaerobic bacteria and compared the results with susceptibility results obtained from the National Committee for Clinical Laboratory Standards proposed thioglycolate broth cultures. The objectives were to evaluate the reproducibility of each of the two methods of inoculum preparation and to compare the MICs obtained by each method. The reproducibility studies were conducted on 14 stock strains. The mode MICs obtained by the direct agar method were identical to those obtained by the reference broth method 74% of the time and within ±1 $\log_2$ dilution 100% of the time. The degree of reproducibility of each of the two methods was identical (93% ±1 $\log_2$ dilution). MIC results obtained by the direct agar method agreed with the MICs obtained by the reference broth culture method in 92.9% of 1,125 MIC data pair determinations performed on stock cultures. The reproducibility of the direct agar method within ±1 $\log_2$ dilution step for 115 fresh clinical isolates was 93%, including 93.4% of the results with the *Bacteroides fragilis* group. Only two very major discrepancies (false-susceptible by the agar method) were identified among the 708 MIC data pairs on these clinical isolates. Preparation of inocula directly from growth on agar plates provides a rapid and reproducible method for agar dilution susceptibility testing of anaerobes.

Recently, several investigators have reported the successful application of a direct suspension method for the preparation of standardized inocula for antimicrobial susceptibility testing of aerobic and facultative bacteria (1–6, 12). This approach adds considerable flexibility to the set-up procedure for susceptibility testing by eliminating the conventionally required incubation for the preparation of a broth culture. Furthermore, it has been previously documented that inocula prepared by suspending bacteria from an agar culture yield the desired concentration of organisms more consistently than do broth cultures for certain fastidious organisms, e.g., *Haemophilus* spp., *Neisseria* spp., *Steptococcus pneumoniae*, and anaerobes (9–11). Recently, Murray and Niles (6), using a broth microdilution method for susceptibility testing of anaerobes, have shown that inocula could be prepared directly from agar media, yielding more timely test results.

The present study was undertaken to evaluate the performance of inocula prepared from agar plates by the National Committee for Clinical Laboratory Standards proposed reference agar dilution susceptibility tests of anaerobic bacteria (7).

MATERIALS AND METHODS

Bacterial strains. In the first phase of the study, 14 stock culture isolates were tested by the two methods on 14 different days. The organisms used were *Clostridium perfringens* ATCC 13124, *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, *Peptococcus variabilis* ATCC 14956, *Peptococcus prevotii*, *Propionibacterium acnes*, *Clostridium ramosum*, *Bifidobacterium longum*, *Bacteroides distasonis*, *Bacteroides melaninogenicus*, *Bacteroides bivius*, and *Veillonella parvula*. The organisms were maintained at −70°C. Before testing, bacterial suspensions were thawed and plated onto anaerobic blood agar plates.

Inocula. Inocula for standard antimicrobial susceptibility tests were prepared as described in the reference agar dilution procedure proposed by the National Committee for Clinical Laboratory Standards (7). Briefly, colonies of isolates were picked from the blood agar plate, transferred to thioglycolate broth supplemented with hemin (5 μg/ml) and vitamin K_1 (0.1 μg/ml), and incubated overnight at 35°C in an anaerobic chamber. The broth cultures were then diluted in brucella broth and adjusted to the turbidity of one-half of a no. 1 McFarland standard.

Inocula for direct antimicrobial susceptibility tests were prepared directly from agar plates. Isolated colonies were picked from a 24- to 48-h blood agar plate and suspended in brucella broth. The inoculum was adjusted to a turbidity equivalent of one-half a no. 1 McFarland standard.

Antimicrobial agents. The antibiotics tested were those most commonly recommended for therapy of anaerobic infections. The following laboratory standard antibiotic powders with the indicated concentrations were tested: penicillin G (0.015 to 32 U/ml) (Ayerst Laboratories, Montreal, Quebec, Canada); cefoxitin (0.06 to 64 μg/ml) (Merck Frosst Canada Inc., Pointe-Claire, Quebec, Canada); ticarcillin (0.06 to 128 μg/ml) (Beecham Laboratories, Inc., Pointe-Claire, Quebec, Canada); chloramphenicol (0.06 to 32 μg/ml) (Parke Davis Canada Inc., Brockville, Ontario, Canada); metronidazole (0.06 to 32 μg/ml) (Rhône Poulenc Pharma Inc., Montreal, Quebec, Canada); clindamycin (0.06 to 16 μg/ml) (The Upjohn Co., Kalamazoo, Mich.); and moxalactam (0.06 to 128 μg/ml) (Eli Lilly & Co., Indianapolis, Ind.).

Susceptibility testing. Agar dilution tests were performed in parallel with the inocula prepared by the direct blood agar plate and the standard broth methods. The MICs were determined by the proposed standard reference agar dilution procedure for antimicrobial susceptibility testing of anaerobic bacteria (7).

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TABLE 1. Intra- and intersystem reproducibility of the broth culture and agar plate methods of inoculum preparation of stock organisms[a]

Antibiotic	Reproducibility results by the following methods					
	Broth		Agar plate		Broth vs agar plate	
	No. of possible tests evaluated	% mode MIC ±1 $\log_2$ dilution	No. of possible tests evaluated	% mode MIC ±1 $\log_2$ dilution	No. of possible tests evaluated	% standard mode MIC ±1 $\log_2$ dilution
Penicillin G	165	89	163	89	163	88
Cefoxitin	162	94	160	98	160	96
Chloramphenicol	167	98	169	99	169	99
Clindamycin	160	93	162	93	162	93
Ticarcillin	160	93	161	92	161	93
Metronidazole	155	94	153	93	140	94
Moxalactam	166	89	170	91	170	88
Total	1,135	92.9	1,138	93.5	1,125	92.9

[a] 14 strains × 14 replicate testings.

Reproducibility study. Each of the 14 stock culture isolates were subjected to 14 replicate testings. For any given organism, both inocula were tested in parallel on the same day and with the same lots of materials. For the determination of discrepancies, MIC breakpoints were established for each of the antibiotics, above which organisms were considered to be resistant. These breakpoints were as follows: penicillin G, 16 U/ml; cefoxitin, 16 μg/ml; ticarcillin, 64 μg/ml; chloramphenicol, 16 μg/ml; clindamycin, 4 μg/ml; metronidazole, 8 μg/ml; and moxalactam, 16 μg/ml.

RESULTS

In the first phase of the study, the MICs of the 14 stock strains to the seven antibiotics were determined on 14 separate days. Data were examined for each bacterium-drug combination to determine the mode MIC and variation for each. Data were excluded if no growth or very poor growth occurred in the controls or if contamination was evident. Reasons for eliminated data were as follows. There was no growth of *C. ramosum* on three occasions and no growth of *Bacteroides distasonis* and *V. parvula* on one occasion each with inocula prepared from broth. *Bacteroides bivius* and *Propionibacterium acnes* did not grow in inocula prepared from either agar or broth in four and two tests, respectively. Finally, *Bacteroides melaninogenicus* grew only with inocula prepared from agar on three occasions and only with inocula prepared from broth on one occasion. Overall, the isolates did not grow with inocula prepared from agar 7 times and with inocula prepared from broth 14 times.

Of a total of 98 possible mode MIC determinations (14 strains × seven antibiotics), it was possible to evaluate only 88. Eight strains tested with clindamycin, one strain tested with penicillin, and one strain tested with metronidazole gave off-scale values, and these were excluded. The mode MIC values obtained by the direct agar plate method were identical to those obtained by the standard broth method 64 times (74%) and within ±1 $\log_2$ dilution 88 times (100%). The intrasystem reproducibility of MICs obtained by the two methods was quite comparable (Table 1). MICs falling on the modes and MICs falling on the modes or within ±1 $\log_2$ dilution of the modes varied from 55 to 80% and 89 to 98%, respectively, by the broth method and from 55 to 77% and 89 to 99%, respectively by the agar plate method. When data obtained by the direct method were examined for intrasystem variation, it was determined that MICs obtained by the direct agar method fell on the standard broth method modes from 49 to 76% of the time, with an overall agreement of 59%; MICs were on the mode or within ±1 $\log_2$ dilution of the modes 88 to 99% of the time, with an overall agreement of 92.5%.

Tables 2 and 3 show a direct comparison of the two methods of inoculum preparation in terms of comparability of paired MICs obtained in parallel tests. Although the MICs were essentially comparable (within the limits of reproducibility of the tests), there was a general trend for the direct method to give higher MICs. With the exception of moxalactam, 89.6% or more of the paired results with each antibiotic were within one dilution. There was variation in data related

TABLE 2. Distribution of paried MIC differences for the antibiotics tested against stock organisms

Antibiotic	No. of paired tests	No. of strains with the following $\log_2$ dilution differences[a]							Within ±1 $\log_2$ dilution (%)
		<−2	−2	−1	0	+1	+2	>+2	
Penicillin	164	5	5	25	114	13	2	0	152 (92.7)
Cefoxitin	162	3	7	27	107	14	3	1	148 (91.3)
Chloramphenicol	164	3	2	20	121	16	0	2	157 (95.7)
Clindamycin	164	0	5	12	135	10	2	0	157 (95.7)
Metronidazole	163	0	7	29	117	10	0	0	156 (95.7)
Ticarcillin	154	8	5	33	89	16	3	0	138 (89.6)
Moxalactam	163	4	10	32	89	19	5	4	140 (85.9)
Total (%)	1,134	23 (2.0)	41 (3.6)	178 (15.7)	772 (68.0)	98 (8.6)	15 (1.3)	7 (0.6)	1,048 (92.4)

[a] Values of <−2, −2, and −1 indicate higher MICs by the agar plate method than the broth method; 0 indicates equal MICs; +1, +2, and >+2 indicate lower MICs by the agar plate method than by the broth method.

TABLE 3. Distribution of paired MIC differences for stock culture isolates

Organism	No. of paired tests	No. of pairs showing a $\log_2$ dilution difference of[a]:							Within ±1 $\log_2$ dilution (%)
		<−2	−2	−1	0	+1	+2	>+2	
Control strains									
Clostridium perfringens	75	0	1	10	55	8	0	1	73 (97.3)
Bacteroides fragillis	69	0	2	11	56	0	0	0	67 (97.1)
Bacteroides thetaiotaomicron	68	1	3	15	42	7	0	0	64 (94.12)
Percent		0.4	2.8	17	72.16	7	0	0.4	96.22
Gram-positive strains									
Clostridium ramosum	77	9	10	10	40	5	3	0	55 (71.4)
Peptococcus variabilis	97	1	2	16	76	0	2	0	92 (94.8)
Bifidobacterium adolescentis	97	0	3	25	55	13	1	0	93 (95.9)
Bifidobacterium longum	97	2	2	17	70	6	0	0	93 (95.9)
Peptococcus prevotii	95	0	0	21	63	7	4	0	91 (95.8)
Propionibacterium acnes	83	2	4	4	57	14	2	0	75 (90.4)
Percent		2.56	3.8	17	66.1	8.2	2.2	0	91.4
Gram-negative strains									
Bacteroides vulgatus	97	0	1	10	82	4	0	0	96 (99)
Bacteroides distasonis	91	4	8	18	44	14	2	1	76 (83.5)
Bacteroides melaninogenicus	42	4	3	10	24	1	0	0	35 (83.3)
Bacteroides bivius	56	0	1	5	36	9	1	4	50 (89.3)
Veillonella parvula	90	0	1	6	72	10	0	1	88 (97.8)
Percent		2.1	3.7	12.9	72.3	10.1	0.8	1.6	91.7
Total (%)	1,134	23 (2.0)	41 (3.6)	178 (15.7)	772 (68.0)	98 (8.6)	15 (1.3)	7 (0.6)	1,048 (92.4)

[a] Values of <−2, −2, and −1 indicate higher MICs by the agar plate method than the broth method; 0 indicates equal MICs; +1, +2, and >+2 indicate lower MICs by the agar plate method than by the broth method.

to the individual test strains. Strains exhibiting the most variation were *C. ramosum*, *Bacteroides distasonis*, *Bacteroides melaninogenicus*, and *Bacteroides bivius*. These four strains were also those which had the fewest results for analysis because of their inability to grow well when the inoculum was prepared from a broth culture.

In the second phase of this study, 115 fresh clinical isolates were tested (Tables 4 and 5). Comparative susceptibility tests could not be performed with 13 isolates (*Clostridium* spp., 4; *Bacteroides bivius*, 4; *Bacteroides* spp., 2; *Fusobacterium* spp., 3). These isolates grew only with inocula prepared from blood agar plates. Altogether, 93% of the results with inocula prepared from agar and broth were within one twofold dilution. With the exception of penicillin and moxalactam, greater than 90% of the results were within one dilution. Interpretative errors between the broth and the agar methods were rare, i.e., very major discrepancies (false-susceptible by the agar method, 0.28%) and major discrepancies (false-resistant by the agar method, 1.27%). Some organism-antimicrobial agent combinations were more often associated with such interpretative errors. These combinations were the *Bacteroides fragilis* group-penicillin and moxalactam and *Clostridium* spp.-moxalactam. As with the stock strains, there was a trend toward higher MICs by the direct agar method when fresh clinical isolates were tested.

DISCUSSION

Results of this study indicate that a high degree of reproducibility was achieved by both methods of inoculum preparation in the agar dilution tests. Furthermore, in addition to being highly reproducible, the direct agar method gave results similar to those obtained by the standard broth

TABLE 4. Comparison of susceptibility test results with inocula prepared from broth cultures and from agar plates for clinical isolates

Antibiotic	No. of strains with an MIC ratio of[a]:							Percentage of results within ±1 $\log_2$ dilution[b]	No. of strains with discrepant results	
	≤0.125	0.25	0.5	1	2	4	≥4		Major[c]	Very major[d]
Penicillin	6	8	24	59	4	0	1	85.3	4	0
Cefoxitin	2	2	16	80	2	0	0	96.0	0	0
Chloramphenicol	0	3	22	76	1	0	0	97.0	0	0
Clindamycin	3	2	6	82	8	1	0	94.1	0	0
Ticarcillin	3	4	9	78	5	3	0	90.2	1	0
Metronidazole	0	1	5	95	1	0	0	99.0	0	0
Moxalactam	3	5	9	73	4	1	1	84.3	4	2
Total	17	25	91	543	25	5	2	93.07	9	2

[a] MIC ratio is the ratio of MICs with the broth inoculum to MICS with the direct inoculum.
[b] A total of 102 comparisons was made for each antibiotic, except moxalactam (95 comparisons).
[c] Susceptible by the standard method and resistant by the test method.
[d] Resistant by the standard method and susceptible by the test method.

TABLE 5. Comparison of susceptibility test results with inocula prepared from broth cultures and from agar plates for clinical isolates

Organism (no.)	No. of paired tests with MIC ratio of[a]:							Percentage of results within ±1 $\log_2$ dilution[b]	No. of strains with discrepant results	
	≤0.125	0.25	0.5	1	2	4	≥4		Major[c]	Very major[d]
B. fragilis group (53)	8	14	42	289	10	2	0	93.4	6	0
Bacteroides spp. (14)	3	5	14	71	4	0	1	90.8	1	0
Clostridium perfringens (12)	0	1	16	65	2	0	0	98.8	0	0
Clostridium spp. (15)	6	5	16	69	6	2	1	86.6	2	2
Propionibacterium acnes (5)	0	0	3	32	0	0	0	100	0	0
Fusobacterium spp. (3)	0	0	0	17	3	1	0	95.2	0	0
Total	17	25	91	543	25	5	2	93.07	9 (1.27)	2 (0.28)

[a] MIC ratio is the ratio of MICs with the broth inoculum to MICs with the direct inoculum.
[b] A total of 102 comparisons was made for each antibiotic, except moxalactam (95 comparisons).
[c] Susceptible by the standard method and resistant by the test method.
[d] Resistant by the standard method and susceptible by the test method.

method. Our results are in agreement with those observed by Sutter et al. in their evaluation of the reference agar dilution method of susceptibility testing of anaerobic bacteria (8); they reported that the MICs falling on the modes varied from 57 to 80% of all determinations and on the mode or within ±1 $\log_2$ dilution of the mode from 87 to 100%.

The greatest difficulty in obtaining reproducible results with stock strains involved tests with *C. ramosum*, *Bacteroides bivius*, *Bacteroides distasonis*, and *Bacteroides melaninogenicus*. These organisms tended to produce fairly poor growth in the thioglycolate medium after overnight incubation.

There was a trend toward lower MICs by the standard method of inoculum preparation; this skewing of results between methods was most clearly seen with *C. ramosum*, *Bacteroides distasonis*, and *Bacteroides melaninogenicus* and with the β-lactam antibiotics. Although we have no specific explanation for the greatest variations observed with these antimicrobial agents, we suspect an inoculum effect.

The comparability of data from the fresh clinical isolates obtained by the different inoculum preparation methods was well demonstrated by the data on paired MIC results. Altogether, 93% of 708 paired tests were within one twofold dilution, including 93.4 and 86.6% of the results with the *Bacteroides fragilis* group and *Clostridium* spp., respectively. Murray and Niles have compared results of anaerobic microdilution susceptibility tests with inocula prepared directly from agar media and overnight broth cultures (6); 93% of their results with these two inoculum preparations were within one twofold dilution, including 95.6 and 92.5% of the results with the *Bacteroides fragilis* group and *Clostridium* spp., respectively. The greatest variations in their tests were seen with metronidazole, whereas we noted the greatest variations with penicillin; these differences could possibly be explained by the differences in the media and methodologies used.

Although there was general agreement among MICs obtained with the two different types of inocula and interpretative errors were rare, MICs tended to be higher when the inoculum was prepared directly from the agar media. Such discrepancies could be attributed to differences in the inoculum density. In fact, Swenson and Thornsberry have shown that inocula prepared from broth suspensions of organisms from agar plates gave the most consistent counts and the highest numbers of organisms (9). We have made similar observations with our stock strains (data not shown) but have not tested our clinical isolates, since our primary goal was to compare MICs and not colony counts.

Altogether, 115 organisms could be tested from agar plates compared with only 102 organisms prepared from broth cultures. Similarly, Murray and Niles (6) could test only 87.2% of their isolates prepared from broth cultures, and they pointed out that their organisms grew better when the inoculum was prepared directly from agar plates.

In summary, our evaluation of agar dilution susceptibility tests of anaerobic bacteria performed with inocula prepared directly from 24- to 48-h agar media has resulted in favorable observations of MIC comparability, accuracy, and reproducibility. We also found the direct method to have definite advantages. The susceptibility results could be available at least 1 day faster, the purity of the culture could be confirmed at the same time the test was set up, and more organisms could be tested because of better growth of bacteria obtained from inocula prepared from agar plates. Our conclusion is that inocula for agar dilution susceptibility tests of anaerobic bacteria can be prepared from bacterial colonies taken from 24- to 48-h agar plates and can be expected to yield susceptibility results that are reproducible and comparable to those obtained with the broth cultures.

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