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Micro-Media Systems Anaerobe Panel Versus Broth Disk Method in Anaerobe Antimicrobial Testing

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Frozen microtitration trays manufactured by Micro-Media Systems (MMS) for antimicrobial testing of anaerobic bacteria were evaluated and compared with the broth disk elution method of Virginia Polytechnic Institute (VPI) described by Wilkins and Thiel. A total of 224 clinical anaerobic isolates were tested. Susceptibility results were compared for carbenicillin, cefoxitin, chloramphenicol, clindamycin, penicillin, and tetracycline. Published procedures for the MMS and VPI methods were followed using aerobic inoculation. Growth performance in the MMS method was 74% compared with 89% in the VPI method. For each antibiotic, the MMS minimum inhibitory concentration was compared with the VPI test concentration. Of the 972 drug-organism combinations, 2.9% of the results were discrepant. Clindamycin and tetracycline accounted for 82% of the discrepancies. The MMS anaerobe susceptibility panels were easy to inoculate and interpret and compared well with the VPI broth disk susceptibility method.

Many laboratories rely on the broth disk elution technique (4, 11, 15) for antimicrobial testing of anaerobic bacteria. However, the broth disk method is limited since results are reported qualitatively (susceptible, intermediate, resistant). The awareness and interest in the quantitative measurement of antimicrobial susceptibilities are increasing (1, 10, 16). In the past, those laboratories interested in applying minimum inhibitory concentrations (MICs) to anaerobes were required to prepare their own microtitration trays and macrotube dilutions or perform the agar gel dilution method (13, 14). In busy clinical laboratories there is often neither the personnel, time, nor space to prepare or perform such methods. A frozen MIC microtitration tray for anaerobe susceptibility testing is presently available from Micro-Media Systems (MMS), Potomac, Md. The MMS MIC anaerobe panels have yielded results comparable to the agar gel dilution reference method and have shown good intra- and interlaboratory reproducibility (7). Thus, we evaluated the MMS MIC method by comparing it with the broth disk elution technique of Virginia Polytechnic Institute (VPI) described by Wilkins and Thiel (15) for routine clinical use.

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MATERIALS AND METHODS

Anaerobic bacteria. Susceptibility testing was performed on 224 anaerobic organisms isolated from clinical specimens processed at the University of Michigan Clinical Microbiology Laboratory, Ann Arbor. Organisms were isolated in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) by procedures outlined previously (3). Isolates were identified by using gas-liquid chromatographic analyses, Gram stain reactions, Minitek biochemicals (BBL Microbiology Systems, Cockeysville, Md.), and egg yolk agar reactions (Scott Laboratories, Fiskeville, R.I). The isolates were stored in chopped meat broth (GIBCO Diagnostics, Madison, Wis.) until the susceptibility tests were performed.

Antibiotics. The six antibiotics tested by the VPI method were cefoxitin, chloramphenicol, penicillin G, and tetracycline (Pfizer Disks, Inc., Barcefonte, P.R.) and carbenicillin and clindamycin (General Diagnostics, Warner-Lambert Co., Morris Plains, N.J.). The antimicrobial concentrations used in the MMS method and the one-point test concentrations used in the VPI method are listed in Table 1. The medium used to dilute the MMS antimicrobial agents was Wilkins-Chalgren agar broth formulation.

Susceptibility testing. All incubation phases were carried out in an anaerobic chamber. The stored isolates were plated on anaerobic blood agar plates (GIBCO) and incubated for 24 to 48 h. For the VPI method, five colonies from each isolate were inoculated into a prereduced, supplemented brain heart infusion broth (Difco Laboratories, Detroit, Mich.) which was incubated for 24 h. Published procedures were

TABLE 1. Antibiotic concentrations used for comparison of MIC and VPI methods

Antibiotic	MIC method range (μg/ml)	VPI method final test conc (μg/ml)
Carbenicillin	8–512	100
Cefoxitin	1–64	18
Chloramphenicol	0.5–32	12
Clindamycin	0.25–16	1.6
Penicillin G	0.06–4	2
Tetracycline	0.25–16	6

followed for the VPI method (6) except all antibiotic disk dispensing and inoculating procedures were done aerobically. The VPI susceptibility tubes were incubated for 24 h. For the MMS method, five colonies from each isolate were inoculated into 7 ml of an enriched thioglycolate broth (Carr Scarborough Microbiologicals, Inc., Atlanta, Ga.) which was incubated for 18 to 24 h. Following the recommended procedures of MMS, the thioglycolate broth was diluted with prereduced sterile saline and prereduced distilled water containing Tween 80. An MMS microtitration tray, prereduced for 4 to 24 h in an anaerobic chamber, was inoculated and incubated for 48 h. The trays were stacked three high and placed in a plastic bag to prevent desiccation. Purity checks were performed for both methods by plating 0.001 ml from the growth controls on an anaerobic blood agar plate.

Interpretation. The MMS MIC results were interpreted as the lowest concentration showing complete inhibition of growth for each antibiotic. With the VPI method, the absence of growth in each respective antibiotic tube compared with growth in the control tube was interpreted as susceptible. In contrast, growth in each respective antibiotic tube compared with equal growth in the control tube was interpreted as resistant. We were able to interpret all VPI results as susceptible or resistant and therefore had no intermediate VPI results.

At present, the National Committee for Clinical Laboratory Standards has no interpretative anaerobe MIC standards. Laboratories may choose to use the MMS MIC interpretative values, which have changed periodically, or may use their own interpretative ranges. We therefore decided not to compare the interpretative results of the MIC and VPI methods.

We compared the MIC result by using the one-point VPI test concentration (Table 1) as a guide. When a VPI result was interpreted as susceptible and the MIC was less than the VPI test concentration for the specific antibiotic, the results agreed. However, if the MIC was greater than the VPI test concentration and the VPI result was interpreted as susceptible, the results were considered discrepant. For a VPI result which was interpreted as resistant, the MIC result had to be greater than the VPI test concentration for agreement and less than the VPI test concentration for a discrepancy.

Selected strains of those organisms showing discrepancies were sent to the Centers for Disease Control (CDC), Atlanta, Ga., and were tested by the CDC broth microdilution method. The broth base medium formulation used by CDC was brain heart infusion broth supplemented with vitamin K and hemin.

RESULTS

The anaerobe isolates tested and their respective growth performances in both susceptibility methods are listed in Table 2. A total of 74% (166 of 224) of the organisms tested grew in the MMS MIC method compared with a total of 89% (200 of 224) in the VPI method. In both systems, the anaerobic cocci and the *Bacteroides* species

TABLE 2. Growth of anaerobe isolates in the VPI versus MIC method

Organism	No. of isolates tested	No. of isolates showing:			
		Growth by VPI and MIC methods	Growth by VPI method only	Growth by MIC method only	No growth by MIC and VPI methods
B. fragilis	44	44	0	0	0
B. thetaiotaomicron	26	26	0	0	0
B. distasonis	8	8	0	0	0
B. uniformis	5	5	0	0	0
B. vulgatus	6	6	0	0	0
B. ovatus	2	2	0	0	0
Other *Bacteroides* species[a]	18	4	5	2	7
Fusobacterium species	12	7	3	1	1
Clostridium perfringens	29	29	0	0	0
Other *Clostridium* species[b]	33	19	13	0	1
Anaerobic cocci	35	8	17	0	10
Others	6	4	0	1	1

[a] Includes the following: *B. bivius*, five isolates; *B. ruminicola*, three isolates; *B. melaninogenicus*, three isolates; and *Bacteroides species*, six isolates.

[b] Includes the following: *C. ramosum*, eight isolates; *C. innocuum*, five isolates; *C. sporogenes*, three isolates; *C. cadaveris*, three isolates; *C. butyricum*, two isolates; *C. difficile*, two isolates; *C. glycolicum*, one isolate; *C. sordellii*, one isolate; *C. paraputrificum*, one isolate; *C. septicum*, one isolate and *Clostridium* species, six isolates.

TABLE 3. Organism-drug discrepancies in the VPI versus MIC method

Drug[a]	Organism	No. of isolates tested	Result (μg/ml)	
			VPI	MIC
Clindamycin	*C. perfringens*	5	>1.6	1
	C. perfringens	3	>1.6	0.5
	C. perfringens	2	>1.6	≤0
	Other *Clostridium* species	3	>1.6	≤(
Tetracycline	*B. fragilis*	1	<6	16
	B. distasonis	1	<6	16
	B. distasonis	1	>6	0.5
	B. vulgatus	1	<6	16
	Anaerobic coccus	1	>6	4
	Anaerobic coccus	1	>6	≤0.06
	Other *Clostridium* species	1	<6	8
	Other *Clostridium* species	3	>6	≤0.06
Cefoxitin	*B. fragilis*	1	<18	32
	B. fragilis	1	<18	64
	B. thetaiotaomicron	1	<18	64
Carbenicillin	*B. fragilis*	1	>100	64
	B. fragilis	1	<100	256

[a] No discrepancies were found with chloramphenicol or penicillin G.

isolates, other than the *B. fragilis* group (*B. fragilis, B. thetaiotaomicron, B. vulgatus, B. distasonis, B. ovatus*, and *B. uniformis*), accounted for the majority of tests with insufficient growth. The clostridial isolates (excluding *Clostridium perfringens*) showed insufficient growth with the MIC method only. Organisms which failed to grow in both systems were excluded from further comparison.

The occurrence of discrepant drug-organism combinations was 2.9% (28 of 972) (Table 3). The most common and consistent discrepancy was the clindamycin-*Clostridium* combination which accounted for 46% (13 of 28) of all discrepant results. In all instances the MMS MIC value was ≤1 μg/ml, with a VPI result of >1.6 μg/ml. Tetracycline accounted for 36% (10 of 28) of the total discrepant results. The discrepancies were variable and occurred with a variety of organisms. Cefoxitin and carbenicillin produced the remaining discrepancies of 10% (3 of 28) and 7% (2 of 28), respectively. Both strains producing the discrepant carbenicillin results were nonviable for CDC evaluation. One isolate which produced a cefoxitin discrepancy was sent to CDC for evaluation. The CDC MIC agreed with the MMS result. Chloramphenicol and penicillin showed no discrepant results.

DISCUSSION

With the MMS MIC method, the growth performance of the anaerobe isolates was notably less than the growth performance with the VPI method. The slower-growing and more fastidious anaerobes showed the greatest difference, as collectively 62% of the *Bacteroides* species (non-*B. fragilis* group isolates), *Clostridium* species (non-*C. perfringens* isolates), and anaerobic cocci failed to grow in the MMS MIC method, compared with 23% which failed to grow in the VPI method. In contrast, the faster growing anaerobe isolates, the *B. fragilis* group and *C. perfringens* strains, all grew sufficiently for MIC interpretation. Several factors may be responsible. First, the inoculum size used in the VPI method is approximately one log higher (10^7 CFU) when compared with the MMS MIC inoculum size (10^6 CFU). The higher inoculum size for the VPI method was originally chosen for better anaerobe growth performance (15). Second, the broth media used in the two methods are different. Supplemented brain heart infusion medium was used in the VPI method, and Wilkins-Chalgren medium was used in the MMS microtitration trays. Third, a problem of dilution may have contributed to the insufficient growth in the MMS method. The turbidity in the thioglycolate inoculum broth appeared to be similar for the *B. fragilis* group strains and the clostridial isolates. However, after the inoculum was diluted according to MMS instructions, the final CFU taken from the microtitration plate was much less for the *Clostridium* isolates when compared with those obtained from the *B. fragilis* group strains. Further studies are needed to resolve these difficulties.

Of the 13 clostridial isolates showing clindamycin VPI MIC results of >1.6 μg/ml and MMS MICs of <1 μg/ml, 10 were *C. perfringens* strains. A clindamycin MIC result of >1.6 μg/ml for *C. perfringens* has seldom been reported. In contrast to the clostridial results, six *B. fragilis* group strains giving VPI results of >1.6 μg/ml for clindamycin had MMS MIC values of >16 μg/ml. We question the VPI clindamycin results for *C. perfringens* and believe several factors may have contributed to these values. First, the recommended concentrations used in the VPI broth disk method for all antibiotics except clindamycin equal the attainable serum levels of each antibiotic. To reach the clindamycin serum level a large number of disks are needed, which could result in oxidation and possible false-susceptible results. Thus, the recommended VPI

test concentration for clindamycin was cut in half (6). Second, the VPI inoculum size is higher when compared with other anaerobe susceptibility methods (4, 11). Last, *C. perfringens* is a rapidly growing anaerobe. These factors could account for the unusually high clindamycin values obtained for *C. perfringens* with the VPI method. MIC levels performed by CDC on two representative strains of *C. perfringens* further support the possibility of a methodological problem. The CDC MIC values for clindamycin were 0.5 and $\leq$0.01 μg/ml for the two strains.

Tetracycline, the second most common discrepant antibiotic, showed a number of different discrepancies occurring with a variety of organisms. Due to the variability of the tetracycline results in anaerobe susceptibility systems (7, 8, 9) and its questionable usefulness in anaerobic infections (5, 12), we question the need for this drug on the anaerobe MMS MIC panel. Doxycycline, a tetracycline derivative, has been shown to have a good spectrum of activity and appears to be effective against the *B. fragilis* group organisms (2). This drug would seem to be a reasonable alternative.

A purity plate, as outlined in Materials and Methods, is recommended as a check for insufficient growth in addition to the check for purity. When tested by CDC, a *Clostridium ramosum* isolate gave a three- to seven-well MIC increase for three of the antibiotics tested when compared with the MMS results. Growth in the MMS panel was present only in the control well, indicating a very susceptible organism. However, the corresponding purity plate had only three colonies present, suggesting insufficient growth of the isolate. Thus, the purity plate should be examined for sufficient growth to prevent the reporting of erroneous susceptible results.

We found that the MMS anaerobe panels were easy to inoculate and interpret. No additional time, personnel, or space was necessary for preparation, inoculation, and interpretation of the MIC panels when compared with the VPI broth disk method. The MMS MIC endpoints were clear and well defined for the antibiotics and organisms used in this study. In conclusion, the MMS anaerobe panel compared well with the broth disk method for routine clinical use.

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