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Aerobically Incubated Thioglycolate Broth Disk Method for Antibiotic Susceptibility Testing of Anaerobes

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The anaerobic broth disk (AnBD) method of Wilkins and Thiel and a new modification, designated the thioglycolate broth disk method, were compared with an agar dilution technique. The thioglycolate broth disk method was incubated aerobically (AeTBD) or anaerobically (AnTBD). One hundred anaerobic bacteria representing 15 species were tested with clindamycin, chloramphenicol, erythromycin, penicillin, and tetracycline. Agreement of results by the two methods with minimal inhibitory concentration determinations were: AnBD, 95.2%; AnTBD, 91.5%; AeTBD, 94.5%. With clindamycin, chloramphenicol, and penicillin, the agreement of the AeTBD and agar dilution results was 100%, 100%, and 95%, respectively. Using the AeTBD method, only 1.1% of all tests gave false susceptible readings, whereas 4.4% gave false resistant readings. All susceptibility testing errors occurred with tetracycline, erythromycin, and, to a lesser extent, penicillin. For each method, the changes in designation of bacteria as being susceptible or resistant to an antibiotic between trials primarily involved strains with minimal inhibitory concentrations which were ± one dilution of the respective breakpoint value. The same situation was true for most bacteria that yielded false resistant readings within each trial. False resistant readings with tetracycline were determined to be unrelated to excess cation content of test media. These results reaffirm the reliability of the AnBD method and indicate that the AeTBD modification is equally reliable. The greater convenience and lower cost of the AeTBD method should make possible more widespread performance of susceptibility testing for anaerobic bacteria in hospital laboratories.

In recent years, physicians and clinical microbiologists have become increasingly aware of the significance of anaerobic bacteria in a variety of infections. Specimen collection, transport, and culturing methods have been developed for the maximal recovery and quantitation of anaerobic bacteria from clinical specimens (12). As a consequence, there has been a demand by physicians for antibiotic susceptibility testing of anaerobes. Several investigators have described techniques for susceptibility tests using anaerobic incubation (5, 9, 10, 13, 15). Probably the most widely used of these is the broth disk method of Wilkins and Thiel (16), developed at the Virginia Polytechnic Institute. Wilkins and Thiel reported 97% agreement between their broth disk method and a broth dilution procedure. Unfortunately, the Wilkins and Thiel broth disk method is impractical for small laboratories since it requires the use of anaerobic grade CO_2, a gassing apparatus, and prereduced broth.

We have modified the Wilkins and Thiel method by substituting thioglycolate broth and the use of aerobic incubation. A comparison of antibiotic susceptibilities using the Wilkins and Thiel broth disk method, our aerobic thioglycolate modification, and an agar dilution method is presented.

MATERIALS AND METHODS

Bacterial strains. Anaerobic bacteria used were those that had been routinely submitted to the Wisconsin State Laboratory of Hygiene for isolation and/or identification. They were identified by methods developed by Holdeman and Moore (6) and then stored at −70°C in chopped meat glucose broth (CMG).

One hundred anaerobic bacteria representing 15 different species were studied. Identity of the bacteria and number of strains of each (in parentheses) were: *Bacteroides* sp. (4), *Bacteriodes fragilis* subsp. *fragilis* (38), *Bacteroides melaninogenicus* (7), *Bacteroides ruminicola* (1), *Fusobacterium nucleatum* (5), *Fusobacterium necrophorum* (2), *Fusobacterium mortiferum* (4), *Clostridium perfringens* (10), *Eubacterium lentum* (5), *Peptostreptococcus anaerobius* (8),

Peptostreptococcus intermedius (6), *Peptococcus prevotii* (1), *Peptococcus asaccharolyticus* (3), *Veillonella parvula* (3), and *Veillonella alcalescens* (3).

Media. All media were prepared in our laboratory and stored no longer than 2 weeks at room temperature. The medium used for the agar dilution method was prereduced and contained brain heart infusion (BHI broth, Difco), 2.5% agar, supplemented with 0.0005% hemin, 0.002% menadione, and 0.5% yeast extract (BHI-S). Prereduced BHI-S broth was used for the anaerobic broth disk (AnBD) method of Wilkins and Thiel (16).

For the thioglycolate broth disk (TBD) modification, thioglycolate broth (BBL no. 11720) was used in 5-ml quantities in screw-capped tubes (16 by 125 mm). Tubed media was stored in the dark at room temperature for up to 1 week, boiled for 10 min within 2 h before use, and cooled rapidly to room temperature in a water bath. For strains of *B. melaninogenicus*, a 0.5-ml supplement of 9 parts of rabbit serum plus 1 part of hemin-menadione stock solution (500 μg of hemin, 50 μg of menadione) was added to 4.5 ml of the cooled thioglycolate medium.

Antibiotics. Five reference standard antibiotic powders were used. Clindamycin (The Upjohn Co., Kalamazoo, Mich.) was initially dissolved in distilled water, chloramphenicol (Parke Davis and Co., Detroit, Mich.) in 95% ethanol, erythromycin (Eli Lilly and Co., Indianapolis, Ind.) in absolute methanol, tetracycline (Bristol Laboratories, Syracuse, N.Y.) in 0.01 N HCl, and potassium penicillin G (Bristol Laboratories, Syracuse, N.Y.) in 0.1 M phosphate buffer, pH 6.0 (8). Antibiotics were stored in sterile distilled water at 1,000 μg/ml (penicillin G at 1,000 units/ml) at −70°C for up to 4 months.

Antibiotic disks (Difco) were of the high concentration type. They were stored with a calcium chloride desiccant at 4°C in screw-capped bottles. Disk stability was determined by measuring zone sizes using the *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 strains.

Determination of MICs. Minimal inhibitory concentration (MIC) determinations were determined for each antibiotic by an agar dilution method modified after Martin et al. (9). After thawing, organisms were inoculated into prereduced peptone yeast glucose broth, supplemented when necessary (e.g., *B. melaninogenicus*) with hemin and menadione, and incubated for 24 h at 37°C. The broth culture was diluted under 100% CO_2 to a turbidity equal to a no. 1 MacFarland standard, for inoculation by a Steers replicator. Serial twofold dilutions of antibiotics in 3 ml of distilled water were added into separate 27-ml amounts of molten (55°C) BHI-S agar, the contents were mixed well, and each was poured into a square Falcon "integrid" plastic dish (100 by 15 mm). Final antibiotic concentrations ranged from 0.1 to 50 μg/ml (penicillin in units/ml); for tetracycline the concentration ranged up to 100 μg/ml. Antibiotic dilutions and agar plates were prepared on the day of the test, and the plates were air dried for approximately 2 h before inoculation. An antibiotic-free control plate was inoculated at the beginning and end of the inoculation procedure. A control strain of *S. aureus* ATCC 25923 was included with each batch of cultures tested.

Inoculated plates were incubated in a GasPak (150) jar containing a tube of aqueous methylene blue. Anaerobic conditions were achieved by the evacuation replacement technique, using three consecutive evacuations and replacements with the gas combination 80% N_2, 10% CO_2, and 10% H_2. Plates were incubated at 37°C for 48 h except for slow growers such as *B. melaninogenicus*, which required 72 h.

The MIC was interpreted as the lowest antibiotic concentration that resulted in two or less tiny colonies or a faint haze.

AnBD method. In the Wilkins and Thiel AnBD method (16), high concentration commercial antibiotic disks are added to separate tubes containing 5 ml of prereduced BHI-S broth. The number of disks added per tube for each antibiotic is: clindamycin, four; chloramphenicol, two; erythromycin, one; penicillin, one; and tetracycline, one. This results in final test concentrations of 1.6 μg/ml for clindamycin, 12 μg/ml for chloramphenicol, 3 μg/ml for erythromycin, 2 units/ml for penicillin, and 6 μg/ml for tetracycline. These concentrations are considered to be easily achievable in blood under normal therapeutic regimens. Tubes are inoculated anaerobically with 1 drop of an overnight CMG culture, incubated overnight, and observed for turbidity. The majority of tests involve only a judgment of growth versus no growth, and resistance is indicated when the turbidity is 50% or greater than the control culture. Susceptibility is indicated when there is no turbidity or the turbidity is obviously less than 50% of that in the control tube. Results in which the turbidity is very close to 50% of that in the control tube are reported as indeterminate.

Thioglycolate broth disk (TBD) method. The Wilkins and Thiel (16) method was modified by substituting thioglycolate broth (BBL no. 11720) for BHI-S broth. The appropriate number of antibiotic disks were added into screw-capped tubes (16 by 125 mm) containing 5 ml of boiled and cooled thioglycolate broth. Uninoculated tubes were then kept at room temperature for 2 h to allow for diffusion of the antibiotics into the medium. This prediffusion step was used to overcome the diffusion inhibitory effect of the 0.07% agar present in thioglycolate broth (1), a step that is not necessary in the AnBD method. Preliminary data indicate that the prediffusion period must be at least 1 h to avoid some false resistant readings. Tubes were then inoculated with 2 drops of an overnight CMG culture using a Pasteur pipette, and the screw caps were tightened. The tubes were inverted twice and incubated at 37°C overnight. In our experience, this latter step resulted in more uniform bacterial growth and made the results easier to read.

With slow-growing organisms, incubation was extended to 48 h to achieve adequate growth. For the anaerobic TBD (AnTBD) method, screw caps were loosened, and tubes were incubated at 37°C in an anaerobic jar under 80% N_2, 10% CO_2, and 10% H_2 (evacuation replacement technique). For the aerobic

TBD (AeTBD) method, the cultures were tested by leaving the screw caps tight and incubating without an anaerobic atmosphere at 37°C. Interpretation of results for the AnTBD and the AeTBD methods was the same as for the AnBD method. For comparative purposes, indeterminate readings were recorded as resistant.

The agar dilution, AnBD, and AnTBD determinations were always run simultaneously on the same culture. The AeTBD determinations were run separately. In addition, all agar dilution, AnBD, and AnTBD tests were repeated once to determine reproducibility. Reproducibility was not determined for the AeTBD method since only one trial was made, but AeTBD results were compared separately to first and second trial agar dilution results.

RESULTS

Reproducibility of MICs. Table 1 lists the variation in MIC results that occurred with each antibiotic upon repeat testing. A fourfold variation in MIC values upon testing is considered acceptable (3). The percentage of determinations within the acceptable fourfold range varied from 87% with erythromycin to 98% for clindamycin. The cumulative data for all antibiotics shows that 93% of the determinations were within the acceptable limits of variation.

Of greater significance, however, is the reproducibility of the agar dilution method in designating strains as being susceptible or resistant. Table 1 shows that susceptibility designation reproducibility was 95% or greater for all antibiotics except tetracycline (87%). Eleven of 13 isolates whose reproducibility was unacceptable for tetracycline were *B. fragilis* subsp. *fragilis*. Overall, the susceptibility designations were acceptable in 96% of the repeat tests. Furthermore, if the latter figures are adjusted by accepting as being in agreement, the results for those strains having MICs within one dilution of the breakpoint value in both trials but opposite susceptibility designations, greater reproducibility is indicated. As a result, Table 1 shows that adjusted susceptibility reproducibility was 100% for all antibiotics except tetracycline (95%). Overall, the adjusted susceptibility designations were acceptable in 99% of the repeat tests.

Individual susceptibility patterns are not presented since no significant variation was noted from more extensive studies (9, 11).

Agreement of AnBD, AnTBD, and AeTBD methods results with MICs. The overall correlation of the AnBD method with simultaneous MIC determinations was 95.2% (Table 2). False resistant readings averaged 3.4%, whereas false susceptible readings averaged 1.4%. Furthermore, susceptibility designations of 14 out of 100 strains changed from the first to the second trial (data not shown), involving six strains for tetracycline, seven for erythromycin, and one for penicillin. Except for one strain tested with tetracycline, all bacteria involved in change in susceptibility designation between trials had MICs within ± one dilution of the breakpoint value. No such changes occurred with clindamycin or chloramphenicol.

The AnTBD method results (Table 2) were also shown to agree well with simultaneous MIC determinations, yielding an average correlation of 91.5%. False resistant readings and false susceptible readings averaged 7.6% and 0.9%, respectively, for this method. There were 34 instances of change of susceptibility designation between trials (data not shown); 23 of these occurred with erythromycin, seven with tetracycline, and four with penicillin. The number of strains involved in MIC changes that had MICs ± one dilution of the breakpoint value were 6 of 23 for erythromycin, 5 of 7 for tetracycline, and 1 of 4 for penicillin.

For the AeTBD method, 97 of the original 100 strains were retested once using aerobic incubation of tubes. As a result, the correlation of AeTBD results with MICs (Table 2) increased to 94.9% if compared with first trial MICs and

TABLE 1. *Reproducibility of agar dilution MICs and susceptibility designations determined in two trials*

Determinant	% Reproducibility for each antibiotic[a]					
	CC	C	E	Pen	Tet	All
Reproducibility of MICs within acceptable fourfold range	98	94	87	97	90	93
Reproducibility of susceptibility designations	100	100	95	99	87	96
Adjusted reproducibility of susceptibility designations[b]	100	100	100	100	95	99

[a] Abbreviations: CC, Clindamycin; C, chloramphenicol; E, erythromycin; P, penicillin; T, tetracycline; All, average for all antibiotics.

[b] Figures are adjusted by designating as being in agreement the results for those strains having MICs within a dilution of the breakpoint value in both trials but opposite susceptibility designations.

94.0% if compared with second trial MICs (average of 94.5%). False resistant readings dropped to an average of 4.4% as compared with 7.6% in the AnTBD method. The incidence of FSRs remained approximately the same as with the AnTBD method (1.1% versus 0.9%, respectively). Extent of growth in thioglycolate broth inoculated from CMG and incubated aerobically was very similar to that obtained in prereduced BHI-S broth, except for several strains of *Fusobacterium* species which obviously grew better in thioglycolate broth.

False susceptible readings are considerably more important clinically than are false resistant readings, but false susceptible readings were minimal and relatively similar for each method, ranging from a low of 0.9% (average) in the AnTBD method to a high of 1.4% (average) in the AnBD method.

For the AnBD and AnTBD methods, the percentage of agreement of results compared to MIC readings between trials (Table 2) varied less than 1%, indicating adequate reproducibility. Since the AeTBD method was only run once and the results were compared to MICs determined in two trials, the data is inadequate to establish reproducibility for the AeTBD method.

Distribution of errors. It is of interest to inspect the distribution of errors for each method according to antibiotic. Table 3 shows that with the AnBD method there were neither false resistant nor false susceptible readings to either clindamycin or chloramphenicol. False resistant readings were most common with tetracycline (totaling 24 for two trials), and 19 involved bacteria for which the MIC was ± one dilution of the breakpoint value. With erythromycin, three of six false resistant readings involved bacteria having MICs ± one dilution of the breakpoint value. This association was not observed for the four penicillin false resistant readings.

With the AnTBD method, Table 3 shows that there were only 1 and 2 false resistant readings for clindamycin and chloramphenicol, respectively, and no false susceptible readings for these two antibiotics. Again, false resistant readings were most common with tetracycline, totaling 38. Eighteen of these 38 false resistant readings involved bacteria having MICs ± one dilution of the breakpoint value. It is notable that a total of 26 false resistant readings occurred with erythromycin and only five of those involved bacteria having an MIC ± one dilution of the breakpoint value. With penicillin, one of nine false resistant readings had an MIC ± one dilution of the breakpoint value.

The noticeable improvement in AeTBD results as compared with AnTBD results was largely due to a decreased incidence of false resistant readings (Table 3). This is especially evident for erythromycin (total of 16 fewer false resistant readings in AeTBD method) and to a lesser extent for tetracycline (total of 11 fewer

TABLE 2. *Agreement of broth disk method results with minimal inhibitory concentration (MIC) determinations and types of error*

Method	No. of strains	% Agreement with MIC[a]	Type of error	
			% False resistant readings[b]	% False susceptible readings
AnBD:[b]				
First trial	100	95.8	2.6	1.6
Second trial	99	94.6	4.2	1.2
Avg		95.2	3.4	1.4
AnTBD:[b]				
First trial	100	91.6	7.6	0.8
Second trial	99	91.4	7.6	1.0
Avg		91.5	7.6	0.9
AeTBD:[b]				
First trial	97	94.9	3.9	1.2
Second trial	97	94.0	5.0	1.0
Avg		94.5	4.4	1.1

[a] Total for five antibiotics.

[b] Abbreviations: AnBD, Anaerobic broth disk; AnTBD, anaerobic thioglycolate broth disk; and AeTBD, aerobic thioglycolate broth disk.

TABLE 3. *Distribution of errors for broth disk methods according to antibiotic and the incidence of borderline MIC values among the errors*

Method	Ratio of no.[a] of false resistant readings for which MIC was within one dilution of breakpoint value to no.[a] of false resistant readings						Ratio of no. of false susceptible readings which MIC was within one dilution of breakpoint value to no. of false susceptible readings					
	CC[b]	C	E	P	T	All	CC	C	E	P	T	All
AnBD[c]	0/0	0/0	3/6	0/4	19/24	22/34	0/0	0/0	0/7	0/2	5/3	5/12
AnTBD[c]	0/1	0/2	5/26	1/9	18/38	24/76	0/0	0/0	2/3	1/2	1/4	4/9
AeTBD[c]	0/0	0/0	6/10	1/6	16/27	23/43	0/0	0/0	2/6	1/3	2/2	5/11

[a] Numbers are totals for two trials except for the AeTBD method which was run once and results compared separately to MICs from two trials.

[b] Abbreviations: see footnote, Table 1.

[c] Abbreviations: see footnote, Table 2.

false resistant readings). A total of three fewer false resistant readings occurred for penicillin with aerobic incubation. Strains with MIC values which were ± one dilution of the breakpoint values accounted for a total of 6 of 10 erythromycin false resistant readings, 1 of 6 penicillin false resistant readings, and 16 of 27 tetracycline false resistant readings in the AeTBD method.

The cumulative totals for all antibiotics (Table 3) show that the proportion of false resistant readings with MICs ± one dilution of the breakpoint values ranged from 31.6% (24 out of 76) of all false resistant readings in the AnTBD method to 64.7% (22 out of 34) of all false resistant readings in the AnBD method.

Table 3 shows that for all methods, some false susceptible readings were also associated with bacteria having MICs ± one dilution of the breakpoint values.

DISCUSSION

Agreement of the AnBD method and MIC results averaged 95.2%. This figure closely approximates those of Wilkins (16) and Blazevic (2) who obtained 97% and 95.6% correlations of the AnBD method results with MICs, respectively. In our experience, the AnBD method of Wilkins is a very reliable method of antibiotic susceptibility determination for the most commonly isolated anaerobes. Its major disadvantages are media expense and need for a gassing apparatus and anaerobic gas.

Results obtained with the AnTBD method approached the accuracy of AnBD results (average of 91.5% versus 95.2% agreement with MIC results, respectively). The data of Ingham et al. (7) and Eagle et al. (3) on the inactivation of erythromycin by CO_2-induced acidity suggested that fewer false resistant readings might be obtained by aerobic incubation of the tubes in the TBD method. Under anaerobic incubation, decreased activity of erythromycin was evident in the TBD method by the frequent observation of heavy bacterial growth at the top of the thioglycolate broth (where the pH could be assumed to be lowest due to dissolved CO_2) and no growth or diminished growth deep in the medium. As a result, this growth pattern was incorrectly recorded as indeterminate (resistant). This growth pattern only occurred in erythromycin tubes and only with strains of *B. fragilis* subsp. *fragilis* (11 total strains). Consequently, 97 of the original 100 strains were retested using the TBD method with incubation under aerobic conditions (AeTBD method). As a result, the correlation of AeTBD results with MICs increased to an average of 94.5% (Table 2). Surface growth was not observed with aerobic incubation, and the respective strains of *B. fragilis* subsp. *fragilis* were correctly recorded as being susceptible to erythromycin.

That CO_2 induced acidity did not appreciably decrease the activity of erythromycin in the AnBD method (despite the fact that the tubes were gassed with 100% CO_2) may be due to the absence of agar in the BHI-S broth and/or differences in buffering capacity of the two media. In thioglycolate broth, agar would have the effect of concentrating CO_2 in the uppermost portion of the medium therby appreciably lowering the pH in that restricted volume. In addition, the growth-enhancing effect of CO_2 may also contribute to enhanced surface growth in thioglycolate broth. In contrast, with BHI-S broth, convection currents in the fluid medium would more easily dilute the CO_2 and maintain a higher pH throughout. However, further work is necessary to test the validity of these speculations.

For each method, the changes in susceptibility designations between trials primarily involved strains having MICs which were ± one dilution of the breakpoint values. The same situation was true for most bacteria which yielded false resistant readings within each trial.

In this study, no explanation is apparent for the tetracycline, erythromycin, and penicillin false resistant readings which involved bacterial strains with MICs greater than ± one dilution of the breakpoint values. Consideration was made of inactivation of tetracycline through chelation by excess cation concentrations (14). Table 4 shows the concentration of Ca and Mg in the media used for each method studied. It is evident that BHI-S agar used for all MIC determinations had the highest Ca and Mg concentrations of all the media used. Consequently, MIC determinations with tetracycline should have been affected to a greater extent than the broth disk susceptibility determinations. Secondly, thioglycolate broth had a much higher Ca and Mg concentration than did BHI-S broth, yet the number of tetracycline false resistant readings obtained in both broths

TABLE 4. *Cation concentration of susceptibility test media as determined by atomic absorption spectrophotometry*

Cation	Cation concn (μg/ml)		
	BHI-S broth	BHI-S agar	Thioglycolate broth
Ca	1.80	44.1	39.2
Mg	2.43	39.6	34.2

by the respective methods was approximately the same (Table 3). Thus, the presence of excess cations in the test media was not associated with inactivation of tetracycline.

However, related to this problem is the report of Wilkins (16) that different batches of 30-μg tetracycline disks varied in content from 26.7 to 44.0 μg. This variation in disk content of antibiotic is great enough to lead to erroneous susceptibility determinations by any broth disk method when testing bacteria having MICs $\pm$ one dilution of the breakpoint value.

In summary, the AeTBD method was found to be a reliable method of antibiotic susceptibility testing for the most commonly isolated anaerobes from clinical specimens. With clindamycin, chloramphenicol, and penicillin, which are the three primary antibiotics used to treat anaerobic infections, the agreement of the AeTBD and agar dilution results was 100%, 100%, and 95%, respectively. The advantages of reliability, convenience (most hospital laboratories can easily prepare thioglycolate medium), and low cost make possible more widespread performance of susceptibility testing for anaerobic bacteria in hospital laboratories.

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