ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1980, p. 288-291
0066-4804/80/02-0288/04$02.00/0
Vol. 17, No. 2

Vaspar Broth-Disk Procedure for Antibiotic Susceptibility Testing of Anaerobic Bacteria

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A modification of the Wilkins-Thiel broth-disk procedure for antibiotic susceptibility testing of anaerobic bacteria is described. This method utilizes an aerobically prepared medium overlaid with molten vaspar. Specialized anaerobic techniques or prereduced media are not required.

The broth-disk method described by Wilkins and Thiel (7) is used in many clinical laboratories to determine the susceptibility of anaerobic bacteria to antibiotics. This method is relatively simple for those laboratories that are equipped with either oxygen-free CO_2 or an anaerobic chamber, but many laboratories do not have such equipment. We have developed a broth-disk procedure that can be performed without specialized equipment.

The modifications are the manner in which oxygen is excluded from the medium and the use of a newly formulated broth medium. Instead of autoclaving the medium in rubber-stoppered tubes with a headspace of N_2, we have chosen to use the very old technique of pouring molten vaspar over the medium. The vaspar solidifies and thus prevents further diffusion of oxygen into the broth. Sufficient anaerobiosis is thus maintained for growth of most clinical isolates. Because the vaspar forms a solid plug over the broth, the tubes may be inverted for mixing the inoculum and the antibiotic without exposing the medium to oxygen.

Kurzynski et al. (3) have also modified the Wilkins-Thiel broth-disk procedure (7) by using thioglycolate broth, a semisolid medium, to reduce the diffusion of oxygen into the medium. The diffusion of antibiotics into the medium is also slowed by the agar (0.1%) in thioglycolate broth, thus necessitating a 2-h preincubation of the antibiotic disks in the medium.

The Wilkins-West (W-W) medium (Table 1) which we developed for the vaspar procedure supports optimal growth of the microorganisms being tested without requiring additional additives. In other aerobically prepared media, such as Wilkins-Chalgren medium (5) without agar and Schaedler and thioglycolate broths, several anaerobes, primarily the anaerobic cocci, *Eubacterium lentum*, some fusobacteria, and *Bacteroides melaninogenicus*, grow poorly. W-W broth is the result of several modifications of the Wilkins-Chalgren medium (5) which resulted in better growth of these organisms.

We compared the growth of 266 strains, representing 64 species (Table 2), in the final formulation of W-W broth medium (Table 1) with the growth of these same strains in prereduced brain heart infusion (BHI) broth, the medium used in the Wilkins-Thiel broth-disk test (7). Of the organisms, 92% grew in the W-W medium; 81% of the organisms tested grew as well or better than in prereduced brain heart infusion. Organisms which did not grow in the medium are noted in Table 2. However, except for *B. melaninogenicus* subsp. *intermedius* some strains of the same species grew well in W-W medium.

W-W medium could be stored for 2 weeks at room temperature without detectable deleterious effects on the growth of the 15 species (Table 2) we tested as long as the medium was boiled before use. The broth-dilution minimal inhibitory concentrations (MICs) (6) of penicillin and tetracycline determined in W-W medium also were reproducible over the 2-week period. After 2 weeks there were some erratic results with

TABLE 1. *Formulation of Wilkins-West broth medium*[a]

Component	Final concn
Trypticase (BBL 11921)	1.0%
Gelysate (BBL 11870)	1.0%
Yeast Extract (Difco 0127-05)	0.5%
Glucose (Fisher D-16 73412)	0.3%
Arginine HCl (Sigma A-5131)	0.5%
Na pyruvate (Sigma P-2256)	0.5%
Na carbonate, monohydrate (Fisher 78410)	0.1%
Tween 80 (J. T. Baker)[b]	0.025%
L-cysteine HCl (Sigma C-7880)	0.05%
Hemin (Sigma H-2375)[c]	5.0 μg/ml
Vitamin K_1 (Sigma V-3501)[c]	0.5 μg/ml

[a] Dissolve the ingredients in distilled water by boiling. The pH should be 7.2; if not, adjust with 0.1 N NaOH or 0.1 N HCl. Dispense 10 ml aliquots into screw-capped tubes (16 x 25 mm). Autoclave at 121°C for 15 min.
[b] Added as a 2.5% (vol/vol) solution.
[c] Added as stock solutions (8).

TABLE 2. *Species of anaerobic bacteria tested for growth in Wilkins-West broth and for susceptibility to four antibiotics by the Vaspar broth-disk method, the Wilkins-Thiel broth-disk method, and an agar dilution method*

Species[a]	No. of strains tested for		Species[a]	No. of strains tested for	
	growth[b]	antibiotic susceptibility		growth[b]	antibiotic susceptibility
Acidaminococcus fermentans	2/2	2	*Eubacterium aerofaciens*	2/3	3
			Eubacterium alactolyticum	2/3	3
Actinomyces israelii	1/1	1	*Eubacterium lentum*[c]	2/3	7
Actinomyces meyeri	1/1	1	*Eubacterium limosum*	10/10	2
Actinomyces naeslundii	4/4	3	*Eubacterium moniliforme*	4/4	2
Actinomyces odontolyticus	1/1	1			
Actinomyces viscosus	3/3	2	*Fusobacterium gonidiaformans*	2/2	1
			Fusobacterium mortiferum[c]	5/5	5
Bacteroides asaccharolyticus	1/3	3	*Fusobacterium naviforme*[c]	1/2	1
Bacteroides bivius	2/2	2	*Fusobacterium necrophorum*[c]	3/3	3
Bacteroides disiens	3/3	1	*Fusobacterium nucleatum*	8/10	6
Bacteroides distasonis	4/4	2	*Fusobacterium varium*	2/2	2
Bacteroides eggerthii	4/4	2			
Bacteroides fragilis[c]	14/14	14	*'Gaffkya' anaerobia*	1/1	1
Bacteroides fragilis 'subsp. a' (2)	4/4	2	*Lactobacillus fermentum*	1/1	1
			Lactobacillus plantarum	1/1	1
Bacteroides melaninogenicus subsp. *intermedius*	0/6	6			
Bacteroides melaninogenicus subsp. *melaninogenicus*[c]	2/3	3	*Peptococcus asaccharolyticus*[c]	11/11	4
			Peptococcus indolicus	1/1	1
Bacteroides oralis	3/6	5	*Peptococcus magnus*[c]	7/7	7
Bacteroides ovatus	3/3	3	*Peptococcus prevotii*[c]	11/11	4
Bacteroides putredinis	1/3	3			
Bacteroides thetaiotaomicron	9/9	6	*Peptostreptococcus anaerobius*	8/8	7
Bacteroides uniformis	2/2	2	*Peptostreptococcus micros*	3/3	1
Bacteroides vulgatus	7/7	5			
Bacteroides 'subsp. 3452A' (2)	4/4	4	*Propionibacterium acnes*	8/9	2
			Propionibacterium avidum	2/2	2
Bifidobacterium adolescentis	2/2	2	*Propionibacterium granulosum*	2/2	1
Bifidobacterium infantis	1/1	1			
			Streptococcus constellatus	4/4	3
Clostridium bifermentans	2/2	1	*Streptococcus intermedius*[c]	11/11	4
Clostridium butyricum	2/2	2	*Streptococcus morbillorum*	5/5	2
Clostridium chauvoei	1/1	1			
Clostridium clostridiiforme[c]	2/2	2	*Veillonella parvula*[c]	6/6	3
Clostridium difficile	2/2	1			
Clostridium innocuum	5/5	1			
Clostridium limosum	2/2	1			
Clostridium malenominatum	1/1	1			
Clostridium perfringens	10/10	8			
Clostridium ramosum[c]	10/10	6			
Clostridium septicum	4/4	1			
Clostridium sporogenes	2/2	2			
Clostridium tetani	2/2	1			

[a] All strains were from the culture collection of the VPI Anaerobe Laboratory and were identified by either W. E. C. Moore or L. V. Holdeman by previously described methods (1).

[b] The number of strains tested which grew in Wilkins-West broth medium/the total number of strains tested.

[c] To determine the effect of storage on the suitability of W-W medium for susceptibility testing, the MIC of either penicillin or tetracycline was determined for one strain each of these species.

MICs of tetracycline for *Fusobacterium naviforme*, *F. necrophorum*, and *Propionibacterium granulosum*.

The vaspar modification of the Wilkins-Thiel broth-disk test (7) was carried out in the following manner. The medium components (Table 1) were dissolved and dispensed aerobically into screw-capped tubes (16 by 25 mm; 10 ml per tube) before autoclaving (121°C, 15 min). W-W medium was either used the day it was prepared or was placed in a boiling water bath for 10 min with screw caps loosened to purge it of dissolved oxygen. Without attempting to exclude air, sterile antibiotic disks were added aseptically to the tubes. Since these tubes contain 10 ml of medium instead of the 5 ml used in the Wilkins-Thiel procedure (7), twice as many disks were added per tube. Table 3 gives for both the Wilkins-Thiel and the vaspar broth-disk methods the number of disks used per tube and the test

concentration obtained for a number of antibiotics which are used for treating anaerobic infections. The antimicrobial agents which we used in this study are penicillin G, clindamycin, chloramphenicol, and tetracycline. Immediately after the addition of the antibiotic disks, we inoculated the tubes of medium with 0.1 ml of an overnight culture grown in prereduced chopped meat broth. A control tube without antibiotic disks was included with each antibiotic series. About 2 ml of sterile molten vaspar was poured over the broth in each tube to form a layer about 0.5 inches (ca. 0.127 cm) deep. The vaspar hardened in approximately 2 min, and the tubes were then inverted gently several times to mix the inoculum and elute the antibiotic from the disks into the medium. The inoculated tubes were incubated aerobically at 37°C for 18 to 24 h. Susceptibility was defined as in the Wilkins-Thiel test (7) as either absence of turbidity or less than 50% of the turbidity of the control culture.

The accuracy of the vaspar method was determined by comparing results with agar dilution MICs (4) and the Wilkins-Thiel broth disk test (7) on 183 strains representing a total of 64 species (Table 1) of anaerobic bacteria. Twenty-one strains (Table 1) did not grow in the vaspar medium; 16 strains did not grow when tested by the agar dilution MIC procedure. These results were not included in the comparison. We considered the agar dilution method and the broth-disk methods to be in agreement when the MIC of a resistant strain was higher than the concentration of antibiotic in the broth-disk test or the MIC for a susceptible strain was equal to or lower than the concentration of antibiotic in the broth-disk test. Of the results with the vaspar method, 95% agreed with the MICs and 96% agreed with the Wilkins-Thiel method (Table 4). Some of the disagreements (23%) with the MIC procedure were within a twofold dilution of the antibiotic concentration in the broth disk tube, and this is within the range of error of an MIC test.

When cultures grown in the W-W medium were used as inocula, susceptibility results were equivalent to those obtained when cultures in prereduced chopped meat were used. There were only three disagreements in 208 tests (99% agreement). Organisms which did not grow were those which also did not grow or grew poorly in the W-W medium when chopped meat broth was used as the inoculum (Table 2). In addition, *B. bivius* and *B. disiens* did not grow.

To determine whether the susceptibility results were reproducible from one test series to another with the vaspar procedure, one strain each of 16 different species were tested with four antibiotics on different days for a total of 244 separate tests. Results were identical in 234 (96%) of the tests. The variations occurred mainly when the MIC of the antibiotic for the organism was within one twofold dilution of the antibiotic concentration in the broth-disk tube.

We compared for 20 strains (all different species) growth and susceptibility results from the vaspar procedure and the thioglycolate procedure (3). Several species (*E. lentum*, *F. necrophorum*, *P. magnus*, *P. prevotii*, and *Veillonella parvula*) grew better in W-W broth than in thioglycolate broth. A 48-h incubation period was often needed to detect resistance with the thioglycolate method (3) for *B. melaninogenicus*

TABLE 3. *Test concentrations of antibiotics for the Wilkins-Thiel (7) and the Vaspar broth-disk methods.*

Antibiotic	Labeled disk content	No. of disks per tube		Calculated test concn per ml
		Wilkins-Thiel method	Vaspar method	
Penicillin G	10 units	1	2	2 units
Cefoxitin	30 μg	3	6	18 μg
Cefamandole	30 μg	3	6	18 μg
Carbenicillin	100 μg	5	10	100 μg
Chloramphenicol	30 μg	2	4	12 μg
Erythromycin	15 μg	1	2	3 μg
Tetracycline	30 μg	1	2	6 μg
Metronidazole	30 μg	1	2	6 μg

TABLE 4. *Comparison of antibiotic susceptibility results obtained with the vaspar broth-disk, Wilkins-Thiel broth-disk, and agar dilution methods*

Antibiotic	Vaspar vs. Agar Dilution			Vaspar vs. Broth-disk		
	No. of strains tested	No. of disagreements	Percent agreement	No. of strains tested	No. of disagreements	Percent agreement
Penicillin G	154	11	93	162	10	94
Clindamycin	154	6	96	162	7	96
Chloramphenicol	154	3	98	162	4	98
Tetracycline	154	10	94	162	7	96
Average for all antibiotics tested			95			96

subsp. *intermedius*, *E. lentum*, *F. necrophorum*, *P. prevotii*, and *P. granulosum*.

The vaspar broth-disk procedure for antibiotic susceptibility testing of anaerobes requires equipment no more specialized than a Gas-Pak jar (BBL Microbiology Systems). Prereduced media or anaerobic chambers are not necessary. This procedure may be useful for those clinical laboratories which perform susceptibility tests on a small number of anaerobic isolates and which do not possess specialized anaerobic equipment.

We acknowledge the excellent technical assistance of Sarah L. Chalgren, Frances Kahle, David MacDonald, and Shahaireen Pellett.

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