Rapid Identification of *Bacteroides fragilis* with Bile and Antibiotic Disks

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A simple screening test is described for separating *Bacteroides fragilis* from other anaerobic gram-negative bacilli. The test utilizes filter paper disks impregnated with 25 mg of oxgall (Difco), tested in conjunction with antibiotic identification disks. The bile disks and antibiotic disks are placed on a supplemented brucella blood agar plate which has been inoculated by swabbing with a standardized cell suspension. After 24 h at 35°C in a GasPak jar, resistance to kanamycin and bile is taken as a presumptive identification of *B. fragilis*. Susceptibility to one or both disks indicates the need for further identification and additional biochemical tests are required. Those strains that produce insufficient growth within 24 h are not likely to be *B. fragilis*. The reliability of the bile disk method was tested by comparing results with 100 clinical isolates versus results with bile in thioglycolate broth, peptone-yeast extract-glucose broth, and tryptic soy agar. All four bile test methods gave equivalent results, but the broth media required much longer periods of incubation.

Bacteroides fragilis is the anaerobic gram-negative bacillus most frequently recovered from human infections. It actually represents a large group of organisms that share certain common characteristics and can be divided into five different subspecies. Recently, it has been suggested that each of the subspecies should be given species status (2), one of which would be designated *B. fragilis* (*B. fragilis* subsp. *fragilis*). There are no major differences in the antimicrobial susceptibility of the five subspecies of *B. fragilis* (1, 6, 11); the vast majority are relatively resistant to the penicillins and cephalosporins. To the physician, the recovery of *B. fragilis* from an infected patient suggests the need for high-dosage penicillin or other antimicrobial chemotherapy. In the present report, we refer to the entire group as *B. fragilis*, including all five subspecies (or species).

Because of its unique resistance to the penicillins, the presence or absence of *B. fragilis* will be a determining factor in selecting the most appropriate chemotherapy. A simple screening test for rapidly determining whether or not an isolate belongs to the *B. fragilis* group could provide early, clinically important information. Identification of the subspecies (or species) may be useful information that could be provided at a later date.

A few simple screening procedures which

would quickly identify most *B. fragilis* strains could significantly reduce the number of isolates that require additional, more extensive testing for identification. Consequently, the cost of anaerobic cultures could be held to a minimum.

In 1974, Vargo et al. (12) described a simple method for identification of *B. fragilis* based on the fact that its growth is stimulated by bile and that it is resistant to high concentrations of kanamycin. They recommended the use of tryptic soy agar with 2% (wt/vol) oxgall (Difco) in petri plates, inoculated with several colonies selected from a 48- to 72-h blood agar plate. At the same time, a blood agar plate is inoculated and a disk containing 1,000 µg of kanamycin is applied to the swabbed surface. After 24 h in GasPak jars, the blood agar is examined, and, if growth is satisfactory, the presence of a zone around the kanamycin disk is noted, and the bile agar plates are also examined for growth. Among the 190 isolates tested by Vargo et al. (12), *B. fragilis* was the only species that was able to grow on tryptic soy-bile agar and also was resistant to the kanamycin disk (zone, ≤11 mm).

The present report describes a modification of this approach by which the oxgall is incorporated into dried filter paper disks. In this way, the bile disks can be tested along with other antibiotic identification disks for preliminary identification, as outlined by Finegold et al. (4)

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and Sutter et al. (9, 10). With this system, most strains of *B. fragilis* can be reported within 24 to 48 h after the colonies are first recovered. Other isolates will require additional tests, selected on the basis of the antibiotic susceptibility pattern.

MATERIALS AND METHODS

Bile tests were performed with 100 clinical isolates, including 69 *Bacteroides* sp. (61 *B. fragilis*, 4 *B. melaninogenicus*, 1 *B. clostridiiformis*, and 3 unidentified species) and 31 *Fusobacterium* sp. (3 *F. mortiferum*, 2 *F. varium*, 17 *F. nucleatum*, 3 *F. necrophorum*, and 6 unidentified species). The isolates were maintained at -70°C in skim milk and were grown on brucella agar (Pfizer) with 5% defibrinated sheep blood and vitamin K_1 ($0.1\text{ }\mu\text{g/ml}$). To prepare the inoculum, a freshly prepared thioglycolate broth (BBL 135-C) was inoculated with two to three colonies from a 48-h blood agar plate. The thioglycolate broth was incubated aerobically for 18 to 24 h or until there was good growth.

Bile disks were prepared with a thick solution containing 1 g of oxgall (Difco) per ml of distilled water. The solution was sterilized at 121°C for 15 min and then delivered to sterile, dry filter paper disks (Schleicher & Schuell no. 740-E), using a 25- μl Oxford pipette. To accomplish this, the disks were first spread over a fine-mesh, stainless-steel screen which would allow circulation of air for rapid drying. Because of the viscous nature of the bile solution, the pipette tips had to be changed several times while loading a batch of disks. To reduce viscosity, the bile solution was warmed to 50°C . Preliminary studies indicated that the exact content of the bile disks is not very critical and other less-accurate methods of loading the disks with about 20- to 25- μl drops should be acceptable, i.e., a drop from a Pasteur pipette. The 25-mg bile disks were allowed to dry at room temperature for about 1 to 2 h with circulating air (created by a small fan) or they may be dried overnight at room temperature without a fan. Once dried, the disks were stored at -20°C in a desiccator. A small working supply was held with a desiccant in the refrigerator for as long as 3 months with no loss of potency.

Kanamycin identification disks were prepared by delivering 20- μl volumes of a concentrated aqueous solution (50,000 $\mu\text{g/ml}$). In this way, each disk contained 1,000 μg of kanamycin. Once dried, the disks were stored at -20°C , in a desiccator.

As a standard reference procedure, all isolates were tested with two different bile tube methods. Broth tests were performed in thioglycolate medium, as recommended by Dowell and Hawkins (3), and in prereduced, anaerobically sterilized peptone-yeast extract-glucose (PYG) broth, as recommended by Holdeman and Moore (5). Each test included two tubes, one with 2% (wt/vol) powdered oxgall (Difco), equivalent to 20% fresh bile, and the other, without bile, served as a growth control. Each tube was inoculated with 4 drops of an undiluted thioglycolate broth culture. The tubes were examined after 1, 2, and 5 days of incubation, noting whether growth in

bile was inhibited, unaffected, or stimulated, judged by comparison with the amount of growth in the control tube (8).

The bile plate method of Vargo et al. (12) was performed by incorporating 2% (wt/vol) oxgall (Difco) into tryptic soy agar (Difco). The bile plates were inoculated with several colonies selected from 24- to 48-h cultures on blood agar. Blood agar plates were inoculated at the same time to serve as growth controls. The bile agar and growth control plates were examined for the presence or absence of growth after 24 h of incubation and again after 48 h in GasPak jars (BBL).

For testing the bile disks and antibiotic identification disks, brucella blood agar plates were inoculated by swabbing with a standardized cell suspension. The inoculum was adjusted by adding a thioglycolate broth culture to a small volume of thioglycolate broth which had been boiled and cooled just before use, until the turbidity matched that of a MacFarland 0.5 standard. Kanamycin and bile disks were applied to one plate; a second plate would be needed if additional antimicrobial disks were to be tested for preliminary grouping of the anaerobes. The susceptibility plates were then incubated anaerobically, in GasPak jars. The plates were examined after 24 h and again after 48 h of incubation. The organisms were considered susceptible to kanamycin if there was a zone of inhibition ≥ 12 mm in diameter and resistant if the zone was <12 mm (12). Strains that were resistant to bile grew up to the edge of the bile disk, whereas those that were inhibited by bile gave zones of inhibition 17 to 30 mm in diameter (average of 23 mm). The bile disks were all surrounded by a large zone of hemolysis, and those organisms that grew within this zone of hemolysis often produced a cloudy precipitate in the agar medium. This cloudiness accentuated the growth, making it easier to visualize and thus giving the appearance of stimulated growth. We were unable to consistently distinguish between unaffected growth and stimulated growth with the bile disk technique.

RESULTS

A preliminary grouping of the more common anaerobic gram-negative bacilli can be accomplished when bile disks are tested in conjunction with antibiotic identification disks. The results of such screening tests will then guide the selection of additional tests which may be needed for final identification. More to the point, one can issue a preliminary report identifying an isolate as *B. fragilis* or as a gram-negative bacillus other than *B. fragilis* on the basis of the screening tests. Such a preliminary report could have considerable impact on the selection of appropriate chemotherapy (7, 11, 13).

Table 1 lists the usual susceptibility patterns expected with four different groups of anaerobic gram-negative bacilli. In the present study, all isolates of *B. fragilis* were resistant to bile and

TABLE 1. Usual patterns^a of susceptibility to bile and antibiotic disks with four groups of common anaerobic gram-negative bacilli^a

Organism	Patterns of susceptibility in: ^b					
	Bile (oxgall) (25 mg)	Kanamycin (1 mg) ^c	Penicillin (2 U)	Erythromycin (60 µg)	Colistin (10 µg)	Rifampin (15 µg)
<i>Bacteroides</i>						
<i>B. fragilis</i>	R	R	R/S	S	R	S
Other spp.	S ^d	R/S	S/R	S	V	S
<i>Fusobacterium</i>						
<i>F. mortiferum</i> & <i>F. varium</i>	R	S	S/R	R	S	R
Other spp.	S	S	S/S	S/R	S	S

^a Data based on Sutter et al. (9, 10) and Vargo et al. (12) as well as results of tests in the present report.

^b Expected results expressed as: R, resistant (zone, <10 mm); S, susceptible (zone ≥10 mm); V, variable results with different strains; S/R, usually susceptible, some resistant strains; R/S, usually resistant, some susceptible strains.

^c Vargo et al. (12) recommended that interpretive zone standards for kanamycin disks should be ≥12 mm for S and <12 mm for R.

^d *B. clostridioformis* may be stimulated or unaffected by bile but should be susceptible to kanamycin. Our strain gave a 16-mm zone.

TABLE 2. Comparison of results with four different methods for determining the effect of bile on the growth of anaerobic gram-negative bacilli

Broth test results	Strains (%) in each category ^a			
	Tryptic soy-bile agar plate		Bile disk test	
	Inhibited	Growth	Inhibited	Resistant
PYG (PRAS) ^b				
Inhibited	33	0	33	0
Unaffected	0	19	0	19
Stimulated	0	48	0	48
Thioglycolate				
Inhibited	33	0	33	0
Unaffected	0	32	0	32
Stimulated	0	35	0	35

^a One-hundred isolates were tested; and 67 which were unaffected or stimulated by bile include all 61 *B. fragilis*, 1 *B. clostridioformis*, 3 *F. mortiferum* and 2 *F. varium*. The 33 isolates that were inhibited by bile represent other species.

^b PRAS, Prereduced, anaerobically sterilized.

to kanamycin disks (no zone of inhibition), and all other isolates were susceptible to a least one of the two disks.

The present study was carried out to determine whether inhibition of growth by bile could be detected with a bile disk. The results of bile disk tests were identical to those obtained on tryptic soy-bile agar plates. Both of these screening tests compared excellently to the two "standard" broth procedures (Table 2). The inhibition of growth by bile was detected equally well with all four methods, but the broth tube methods were technically more difficult to perform. Tests in thioglycolate broth were essentially the same as those in PYG broth, although we had some difficulty in consistently deter-

mining whether growth was actually stimulated by the bile in the thioglycolate media. Thus, a larger proportion of strains appeared to be stimulated by bile in PYG broth than in thioglycolate.

Table 3 summarizes the incubation time (1, 2, or 5 days) required before sufficient growth occurred in the control tube or plate to permit an interpretation of the bile test. With the bile disk and plate methods, all tests could be read after 48 h and all tests with *B. fragilis* could be interpreted after 24 h of incubation. With the two broth methods, 5% of the *B. fragilis* isolates and half of the other species could not be interpreted after 48 h of incubation. After 24 h, a significant proportion of the *B. fragilis* isolates failed to yield sufficient growth to allow for interpretation of the broth tests, even in the prereduced PYG broth.

To further assess the value of the bile disk as a screening method for rapid identification of *B. fragilis*, in clinical specimens, the clinical microbiology laboratory screened all anaerobic gram-negative bacilli with bile disks in conjunction with the antibiotic identification disks listed in Table 1. In a 3-month period, 220 specimens were accepted for anaerobic and aerobic cultures. No growth was obtained from 62 of those selected specimens, and 158 specimens provided 268 isolates (110 aerobes and 158 anaerobes). Twenty percent of the anaerobes were gram-positive cocci, 16% were *Clostridium* sp., 7% were nonsporeforming gram-positive bacilli, and 57% were anaerobic gram-negative bacilli. *B. fragilis* accounted for 78% of the anaerobic gram-negative bacilli. In the 3-month period, all but two of the *B. fragilis* isolates were rapidly identified on the basis of tests with

TABLE 3. Period of incubation required before the effect of bile can be determined with four different methods and 100 anaerobic-gram negative bacilli

Method ^a	Tests (%) with adequate growth in controls					
	<i>B. fragilis</i> (61 strains)			Other spp. (39 strains)		
	Day 1	Day 2	Day 5	Day 1	Day 2	Day 5
Bile disk	100			51	100	
TSA plate	100			53	100	
PYG broth	41	95	100	20	51	100
Thioglycolate broth	18	94	100	10	46	100

^a TSA, Tryptic soy agar; PYG, peptone-yeast extract-glucose.

bile and antibiotic disks alone, leaving only 22% of the anaerobic gram-negative bacilli, which require further testing for identification. Two strains of *B. fragilis* gave typical susceptibility patterns, except they were resistant to erythromycin disks. All isolates that failed to grow satisfactorily in thioglycolate or on blood agar plates within the first 24 h of incubation were found to be species other than *B. fragilis*.

DISCUSSION

When the clinician considers the possibility that the patient may have an anaerobic infection, one of the first questions that arises is whether *B. fragilis* is involved. Chemotherapy of *B. fragilis* infections might not be the same as that appropriate for other more susceptible anaerobes. For that reason, a screening test for quickly deciding whether or not *B. fragilis* is present in an anaerobic infection could provide useful preliminary information to the clinician. In our experience, screening tests with bile and antibiotic disks are extremely simple, rapid, and reliable for separating *B. fragilis* from other anaerobic gram-negative bacilli. The results of tests with the bile disk are comparable to those obtained with other techniques for determining susceptibility to bile. In addition, tests with dry bile disks are more efficient and less time consuming than any of the other methods that have been studied in this report.

The cost of identifying anaerobic isolates can be reduced significantly if each isolate is first screened with bile and antibiotic identification disks. Those isolates that fail to grow rapidly in freshly prepared thioglycolate broth or those strains that fail to give satisfactory disk tests after the first 24 h of incubation are not likely to be *B. fragilis* and require additional testing for identification. In our experience and in the experience of Vargo et al. (12), *B. fragilis* is the only commonly encountered anaerobic gram-negative bacillus that is not inhibited by bile and is also resistant to high concentrations of kanamycin. Consequently, we would feel justifi-

fied in reporting an isolate to be *B. fragilis* if it: (i) grows rapidly in fresh thioglycolate broth, (ii) provides a heavy lawn of growth on susceptibility test plates within 24 h, (iii) is resistant to disks containing 1,000 µg of kanamycin, and (iv) gives confluent growth to the edge of a 25-mg bile disk. In addition, *B. fragilis* should be resistant to 2-U penicillin disks and to 10-µg colistin disks and is usually susceptible to 60-µg erythromycin disks and 15-µg rifampin disks. An occasional strain of *B. fragilis* may give aberrant results with one or more of the above criteria: such strains deserve additional testing before final identification is reported. If desired, a limited battery of biochemical tests can be used to characterize the subspecies (or species) of *B. fragilis* identified by such screening tests.

For interpretation of the kanamycin disk test, we followed the recommendations of Vargo et al. (12) that strains are susceptible if the zone is ≥ 12 mm in diameter and are resistant if the zone is less than 12 mm. However, Sutter et al. (9, 10) recommend a breakpoint of ≥ 10 mm for susceptibility with all of the antibiotic disks. It is worth noting that Vargo et al. recommended the larger zone standards on the basis of one isolate of *B. fragilis* with a kanamycin zone of 11 mm; all other kanamycin-resistant isolates gave zones ≤ 9 mm. All kanamycin-susceptible strains they reported gave zones 15 to 44 mm in diameter. All of our strains gave zones that were either less than 10 mm or greater than 12 mm. Consequently, our results would have been the same with either interpretive zone-size criterion. We prefer to consider the rare strain with a zone of 11 to 12 mm to be equivocal and, thus, deserving further tests before it is identified. Strains with zones ≤ 10 mm are resistant and those with zones > 12 mm are susceptible. It is possible that similar criteria could be applied to tests with other antibiotic disks, but more extensive experience will be needed before such interpretive standards can be established.

The reliability of the disk test is dependent on the careful standardization of the inoculum density. When a dense inoculum is used, the zones become much smaller and less distinct. For that reason, we have standardized the test by first adjusting the turbidity of thioglycolate broth cultures to match that of a MacFarland 0.5 standard. This was felt to be necessary in spite of the fact that a time delay is involved in order to initiate growth in the thioglycolate medium. The use of a MacFarland 0.5 turbidity standard was selected to allow us to incorporate the bile disk into the antibiotic disk scheme recommended by Sutter et al. (9). In this way, those isolates that are not reported as *B. fragilis* will be placed into one of several groups, and the appropriate biochemical tests needed for identification can be selected.

In our laboratory, over half of the anaerobes recovered are gram-negative bacilli and about 78% of those are *B. fragilis*. By first screening each isolate with bile and antibiotic disks, the number of isolates requiring additional biochemical tests for identification could be reduced significantly.

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