

## Tryptic Soy Bile-Kanamycin Test for the Identification of *Bacteroides fragilis*

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A simple and practical test for the identification of *Bacteroides fragilis* is described. It utilizes two well-known properties of this species, i.e., stimulation of growth by bile and resistance to kanamycin. The test media are a tryptic-soy bile agar plate and a supplemented blood agar plate on which a kanamycin 1,000- $\mu\text{g}/\text{ml}$  disk is placed. Incubation is for 24 h at 37 C in GasPak. The results of screening 190 strains, mostly clinical isolates, indicate that *B. fragilis* can be easily and reliably distinguished from other *Bacteroides* and from *Fusobacterium* species by its growth on tryptic-soy bile agar and resistance to kanamycin.

It has been demonstrated with the improved methods now available that anaerobic bacteria are present in specimens from a wide variety of human infections (5, 7, 9, 15; also Swenson et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 84, 1973), and that their isolation rates are high (25 to 60%) when the specimens have been collected properly.

The species isolated most often from human infections is *Bacteroides fragilis* (1, 5, 7, 13, 15; also E. H. Spaulding et al. Abstr. 1st Int. Congr. Bacteriol., p. 50, 1973). This species not only has a high overall in vitro resistance to antibiotics (6, 11, 14, 16), but it also differs from most other anaerobes in that the majority of strains are resistant to penicillin (3). Therefore, it is important to make a definite species identification of *B. fragilis* whenever this organism is isolated from clinical specimens.

The present report describes a test based upon two well-established properties of *B. fragilis*, i.e., stimulation of growth by bile and resistance to kanamycin. This species grows poorly or not at all on tryptic-soy agar (Difco) without blood or other supplement, but incorporation of bile produces consistently good growth. Unlike most *Fusobacterium* strains, (but like most other *Bacteroides*), *B. fragilis* shows no zone of inhibition in agar diffusion tests with commercial 1,000- $\mu\text{g}$  kanamycin disks. The combined results of these two tests provide a practical and reliable means for distinguishing this species from other gram-negative, anaerobic bacilli.

### MATERIALS AND METHODS

**Test strains.** One hundred fifty well-characterized (4) clinical isolates of *Bacteroides* and *Fusobacterium*

species were screened. They included stock cultures, fresh clinical isolates, and nine *Fusobacterium* strains kindly sent to us by V. R. Dowell, Jr. Subsequently, an additional 40 strains of anaerobic gram-positive bacilli were also tested.

**Culture media.** Brain heart infusion agar (BBL) supplemented with 0.5% yeast extract (Difco) was used for the preparation of blood agar plates (BAP). After autoclaving, 0.5  $\mu\text{g}$  of menadione per ml (vitamin K) and 5% sheep blood were added.

TS bile agar consisted of tryptic-soy agar (Difco) containing 2% oxgall (Difco) equivalent to 20% bile. The TS bile agar plates were used the same day they were prepared or after overnight storage at room temperature. The freshly prepared plates were more satisfactory if they were first stored for a few hours at 37 C to remove surface moisture. Studies on the effect of prior storage upon TS bile agar plates showed that reliable results could be obtained with plates which had been stored at room temperature up to and including 5 days; the results after storage for 7 and 10 days were less reliable.

**Antibiotic disk.** Commercially prepared (BBL) kanamycin (kana.) 1,000- $\mu\text{g}/\text{ml}$  disks were used.

**Procedure.** All strains were tested as pure BAP cultures after 48 to 72 h of incubation in GasPak jars. In most instances, several colonies were picked with a platinum inoculating loop and the inoculum was placed at the center of a BAP. This procedure was repeated with the TS bile agar plate, using the same colonies as inoculum.

A sterile swab moistened in broth was used to spread the inoculum evenly over the surface of each plate by swabbing in three different directions. The swabs were moistened with prereduced anaerobically sterilized brain heart infusion broth supplemented with yeast extract, menadione, and hemin (4), but no attempt was made to keep it reduced. Subsequently, aerobically prepared brain heart infusion broth without supplements was found to produce the same results. However, the substitution of physiological saline resulted in lighter growth and should not be

used for this purpose. One kanamycin disk was placed on the BAP and both plates were incubated at 37 C in a GasPak jar containing freshly heated catalyst.

After a 24-h incubation period the plates were examined for growth. Zone sizes were measured with the cover removed from the plate using a vernier caliper.

## RESULTS

Most of the strains produced good growth on the BAP in 24 h. However, an additional 24 h of incubation was required by three strains of *B. melaninogenicus* and one strain each of *B. oralis* and *B. corrodens*. Growth on TS bile agar appeared as a confluent lawn or as separated but congruent colonies.

The results with strains of *Bacteroides* are shown in Table 1. All 91 strains of *B. fragilis* grew on TS bile agar and were unquestionably resistant to kanamycin. The eight strains of anaerobic *B. corrodens* also grew on TS bile agar, as did two of five strains of *B. clostridiiformis*, but all were inhibited by kanamycin with zone sizes ranging from 17 to 44 mm. The remaining strains of *Bacteroides* did not grow on TS bile agar; their resistance to kanamycin varied.

The results with strains of *Fusobacterium* are shown in Table 2. *Fusobacterium mortiferum*, *F. varium*, and one of two strains of *F. symbiosum* grew on TS bile agar. However, all of them were inhibited by kanamycin with zone sizes of 17 mm or larger. The remaining strains of *Fusobacterium* did not grow on TS bile agar and all but one showed zones of inhibition on the BAP.

TABLE 1. Growth on TS bile agar and inhibition by kanamycin 1,000- $\mu$ g disks

<i>Bacteroides</i> species	No.	Growth on TS bile agar	Zone diameter (mm) kanamycin disk
<i>B. fragilis</i> .....	91	+	NZ (88) <sup>a</sup> 9(2), 11(1) <sup>b</sup>
<i>B. corrodens</i> .....	8	+	28 to 44
<i>B. clostridiiformis</i> .....	5	v <sup>c</sup>	17 to 30
<i>B. melaninogenicus</i> .....	7	-	NZ
<i>B. oralis</i> .....	2	-	NZ
Other <i>Bacteroides</i> spp. ...	9	-	NZ (2), 18 to 39 <sup>d</sup>

<sup>a</sup> No zone of inhibition with 88 strains.

<sup>b</sup> Nine mm diameter zone with two strains, 11 mm with one.

<sup>c</sup> Variable; two positive, three negative.

<sup>d</sup> Two strains showed NZ, the remaining seven produced zones of 18 to 39 mm.

Repeat tests with 14 strains of *B. fragilis*, two strains of other *Bacteroides* species, and six strains of *Fusobacterium* several weeks later showed good reproducibility; there were no discrepancies in the identification of *B. fragilis*.

The guidelines in Table 3 are suggested for the interpretation of results.

Anaerobic gram-positive bacilli have a tendency to be decolorized easily and appear gram negative in smear preparations. Consequently, a gram-positive bacillus may be erroneously identified as *B. fragilis* if it produces the fragilis-type pattern in the TS bile-kanamycin test. This possibility was examined with 40 well-characterized (4) clinical isolates. Reactions typical of *B. fragilis* were obtained with two of five strains of *E. cylindroides*, four of seven other eubacteria (*E. alactolyticum*, *E. lentum*, *E. limosum* and *E. tenue*), one of five strains of *Clostridium ramosum*, and one unidentified *Clostridium* strain (Table 4). Among these, *E. cylindroides* and *C. ramosum* are recognized as decolorizing readily (4).

The TS bile-kanamycin test is intended to be used routinely with fresh isolates of anaerobic gram-negative bacilli from clinical specimens. When employed in this manner in our laboratory it has so far identified correctly (*B. fragilis* or not) all of the cultures tested. The only noteworthy difference between fresh isolates

TABLE 2. Growth on TS bile agar and inhibition by kanamycin 1,000- $\mu$ g disks

<i>Fusobacterium</i> species	No.	Growth TS bile agar	Zone diameter (mm) kanamycin disk
<i>F. mortiferum</i> .....	11	+	17 to 28
<i>F. varium</i> .....	3	+	18 to 22
<i>F. symbiosum</i> .....	2	v <sup>a</sup>	20, 22
<i>F. gonidiaformans</i> .....	5	-	24 to 29
<i>F. naviforme</i> .....	2	-	30, 31
<i>F. nucleatum</i> .....	1	-	31
<i>F. russii</i> .....	2	-	NZ <sup>b</sup> , 36
<i>Fusobacterium</i> spp. ....	2	-	27, 28

<sup>a</sup> Variable; one positive, one negative.

<sup>b</sup> One strain showed no zone of inhibition.

TABLE 3. Guidelines for interpretation of test results

TS bile agar	Kanamycin disk zone size	Identification
+	< 12 mm	<i>B. fragilis</i>
+	≥ 12 mm	not <i>B. fragilis</i>
-	NA <sup>a</sup>	not <i>B. fragilis</i>

<sup>a</sup> NA, Not applicable since inability to grow on TS bile agar indicates that the isolate is not *B. fragilis*.

TABLE 4. Growth on TS bile agar and inhibition by kanamycin 1,000- $\mu$ g disk gram-positive bacilli

Species	Total	TS bile agar		
		No growth no.	Growth	
			No.	Kanamycin disk-blood agar
<i>Bifidobacterium</i> (3 spp.)	4	1	3	17 to 26
<i>Clostridium ramosum</i>	5	1 <sup>a</sup>	4	NZ(1), 15 to 18 <sup>b</sup>
<i>Clostridium</i> (5 spp.)	9	2	7	NZ(1), 15 to 26
<i>Eubacterium cylindroides</i>	5	3	2	NZ(2)
<i>Eubacterium</i> (4 spp.)	7	1	6	NZ(4), 15, 31
<i>Lactobacillus</i> spp.	2	0	2	18, 23
<i>Propionibacterium</i> (3 spp.)	9	5	4	24 to 31

<sup>a</sup> Eleven colonies present, but there was heavy growth on the corresponding blood agar plate.

<sup>b</sup> No zone of inhibition with one strain, 15 to 18 mm zone diameter with the other three strains.

and laboratory-adapted cultures is that some freshly isolated strains do not grow quite so heavily on the bile plate as they will after one additional subculture. When this happens it may be desirable to repeat the test.

#### DISCUSSION

The capacity to grow in the presence of 10 to 20% bile, and even be stimulated by it, is a major criterion for the differentiation of *B. fragilis* from other gram-negative anaerobes (4, 9, 10). However, the study of Shimada et al. (8) was apparently the first in which this property was examined in detail as a means for the rapid identification of anaerobic gram-negative bacilli in the clinical microbiology laboratory. They evaluated several bile and/or desoxycholate-containing media. In their experience liquid media were better than desoxycholate agar or blood agar, and the best results were obtained in prereduced anaerobically sterilized peptone yeast glucose broth. They recommended both 20% bile broth and 0.1% desoxycholate broth for routine testing.

Tryptic-soy agar was selected by us as the basal medium for the bile test, after comparison with two other media, because it produced sparse or no growth of the test strains when used without supplement and showed the greatest stimulation of *B. fragilis* when bile was added. No attempt was made to compare 20% bile with a 10% concentration.

Selective inhibition by antibiotics as an aid in identifying gram-negative anaerobic bacilli has been the subject of reported studies by Finegold's group (2, 11). They observed that all of 100 *B. fragilis* strains were resistant to 1,000- $\mu$ g disks of kanamycin on blood agar, and we utilized this property for the second part of our test.

To the extent that they can be compared, our

bile and kanamycin results correspond closely to those reported from the Finegold laboratory (2, 8, 11), although we used somewhat different end points for measuring inhibition by bile. With very few exceptions our end point was complete inhibition of growth, whereas their criterion included partial reduction in the amount of growth as compared with the control (8). However, there are some discrepancies between their kanamycin results and ours. In their schema for separating *Bacteroides* from *Fusobacterium* the only *Bacteroides* species inhibited by a 1,000- $\mu$ g kanamycin disk is *B. corrodens* (11), whereas in our study all five strains of *B. clostridioformis*, as well as seven other *Bacteroides* strains, showed inhibitory zones ranging from 17 to 44 mm in diameter (Table 1). In reverse manner, one of our two *F. russii* strains was kanamycin resistant (Table 2).

Inclusion of the BAP is essential. Although it may appear that this test could be simplified by carrying out both parts on the TS bile plate, the BAP is needed for at least three purposes. First, it is used to check the viability of isolates that do not grow on TS bile agar. Second, *B. corrodens*, *B. oralis*, and *B. melaninogenicus* strains may grow rather slowly. When the BAP growth is light and there is none on the bile agar this indicates that additional incubation is required. Finally, the BAP aids in the interpretation of equivocal results such as the following two examples. If the growth on the BAP is good but only a few isolated colonies are seen on TS bile agar, that strain can be considered bile negative; if growth on the BAP is good and a moderate number of isolated colonies are seen on the TS bile agar plate (this may occur with fresh isolates of *B. fragilis* ss. *thetaitoamicon* that may not be stimulated by bile), the test should be repeated.

Both test plates can be inoculated from a single colony on a primary isolation culture provided the colonies are large and well separated. *B. fragilis* colonies are usually of adequate size after 48 h of incubation, but other frequently encountered species of gram-negative bacilli such as *B. melaninogenicus* grow characteristically as small colonies. A small colony should first be subcultured for purity so that several colonies can be inoculated without the risk of testing a mixed culture.

Mixed cultures are typical of anaerobic infections. About 75% of the anaerobe-positive specimens in a recent study in this laboratory contained facultative species as well as anaerobes (E. H. Spaulding et al. Abstr. 1st Int. Congr. Bacteriol., p. 50, 1973). Another characteristic of anaerobic infections is the presence of multiple anaerobes in clinical specimens. In our study the average was 2.3. Similar figures were reported in a recent study of anaerobic infections of the female genital tract (12). Therefore most primary anaerobic cultures need to be subcultured first to ensure that a pure culture of each colony type is being used as inoculum for the TS bile-kanamycin test.

When more than one isolate is to be tested at the same time, the bile and kanamycin plates can, in the interest of economy, be divided in half and used for two tests. However, the half-bile plates should be swabbed in two directions to ensure adequate spreading of the inoculum.

We caution against the use of outdated (older than 5 days) TS bile agar plates, for this can result in failure to identify *B. fragilis*. Whenever the test culture fails to grow on the TS bile plate but grows well on blood agar without an inhibitory zone, the age of that bile agar plate should be checked.

Our results indicate that gram-positive bacilli will seldom be incorrectly identified as *B. fragilis*. At least 18 different species were represented among the 40 strains we tested. Although one or two strains belonging to eight different species behaved like *B. fragilis*, only *E. cylindroides* and *C. ramosum* have a strong tendency to overdecolorize and therefore be considered as gram-negative. Furthermore, it appears that these two species are uncommon isolates from clinical specimens. In a study of 750 such isolates recovered in this laboratory, only five were identified as *E. cylindroides* and one as *C. ramosum*. (E. H. Spaulding et al., Abstr. 1st Int. Congr. Bacteriol., p. 50, 1973).

*B. fragilis* accounted for 12.5% of the anaerobes isolated from clinical specimens in the study just referred to. This high frequency,

coupled with its high level of penicillin resistance as compared with that of most non-sporulating anaerobes demands that clinical microbiology laboratories test all appropriate isolates to determine whether or not they are *B. fragilis*.

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