

Isolation of *Bacteroides fragilis* from the Feces of Diarrheic Calves and Lambs†

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A selective medium was developed for the isolation of *Bacteroides fragilis* directly from ovine and bovine fecal samples. The medium (tryptose blood agar plus polymyxin B, triclosan (Irgasan), novobiocin, and nalidixic acid) permitted growth of *B. fragilis* and several other species of *Bacteroides* but inhibited growth of most other intestinal bacteria.

Bacteroides fragilis is an obligate anaerobe found in the intestinal tract of normal animals and humans. Recent work at this laboratory showed that *B. fragilis* with enterotoxin-like activity (BFEL) is prevalent in the feces of diarrheic lambs (7) and calves and that BFEL produces a conventional enterotoxin (submitted for publication). Enterotoxin activity was detected in the calf and lamb ligated intestinal loop tests (4, 8). Several isolates of enterotoxigenic *B. fragilis* appeared to be diarrheagenic after oral challenge inoculation of newborn lambs and calves. The development of a selective medium for the isolation of *B. fragilis* directly from ovine and bovine feces is reported herein.

The initial isolation of BFEL from feces was made on tryptose-blood agar (TBA) plates (Difco Laboratories, Detroit, Mich.) to which 8 µg of triclosan (Irgasan DP 300; 2,4,4'-trichloro-2'-hydroxydiphenyl ether; (CIBA-GEIGY Corp., Greensboro, N.C.) (5) had been added per ml of TBA (7). Triclosan was used because it inhibited the spread of *Proteus* sp. and the growth of some enteric bacteria on TBA but did not reduce the enterotoxin-like activity (subsequently shown to be due to *B. fragilis*) of lamb fecal specimens (7). The antibiotic susceptibility patterns of the four initial ovine isolates of BFEL and of frequently encountered contaminants constituted the basis for the development of a medium more effective than TBA plus triclosan for the isolation of *B. fragilis* from feces.

The MIC values of antibiotics (all except triclosan, obtained from Sigma Chemical Co., St. Louis, Mo.) were determined with the four ovine isolates and selected ovine fecal contaminants (one isolate of *Escherichia coli*, two isolates of *Proteus* sp., one isolate of a gram-positive coccus) in brain heart infusion broth with 0.5% yeast extract (BHI-YE; Difco Laboratories). The highest concentration of antibiotic desired was added to 10 ml of BHI-YE, and doubling dilutions were made until the lowest concentration used was reached. The following antibiotics were screened: ampicillin, cephalothin, triclosan, nalidixic acid, trimethoprim, polymyxin B, and novobiocin. The results in BHI-YE indicated that triclosan, nalidixic acid, novobiocin, and polymyxin B might be of value in a selective medium. The agar dilution method with TBA plates was then used to determine the MIC values of these antibiotics. Because of the protective effect of blood agar, the antibiotics were used at higher concentrations than in the broth. Triclosan was active against *Proteus* sp. and *E. coli*; nalidixic acid had some effect

against *Proteus* sp. and *E. coli*; novobiocin inhibited the gram-positive coccus and had some effect against *Proteus* sp.; polymyxin B was active against *E. coli*. Ampicillin was active against the gram-positive coccus, and some of the *B. fragilis* isolates were susceptible, although most human isolates of *B. fragilis* are resistant (1, 9).

The final selective medium (PINN) was made by adding 34 g of TBA base with yeast extract (Difco Laboratories) to 1 liter of water. After the medium was autoclaved and cooled to 53 to 54°C, 50 ml of defibrinated bovine blood was added. The following amounts of antibiotics were then added (per milliliter): 5 IU of Polymyxin B, 2 µg of triclosan, 30 µg of novobiocin, and 32 µg of nalidixic acid.

Growth of four ovine and four bovine isolates of BFEL was compared on TBA and PINN. Total counts were similar on the two media. The diameter of the colonies varied from 0.5 to 1.5 mm on PINN and from 0.5 to 2.0 mm on TBA.

Isolations of *B. fragilis* were obtained from diarrheic lambs and calves by spreading a small portion of feces with a sterile cotton swab on one-half of a PINN plate. A 3-mm inoculating loop was used to spread the specimen on one-quarter of the plate perpendicularly to the original inoculum; the loop was then flamed, and the remainder of the plate was spread. The plates were incubated anaerobically (GasPak anaerobe system; BBL Microbiology Systems, Cockeysville, Md.) for 48 h at 37°C. Colonies of *B. fragilis* were white to gray, convex, entire, and smooth. The mottled internal structure (often giving the appearance of concentric swirls) characteristic of *B. fragilis* was readily observed by microscopically magnifying the colonies 25× under intense, direct illumination. PINN medium suppressed the growth of most enteric bacteria and prevented the spread of *Proteus* species. Many of the plates appeared to have pure cultures of *B. fragilis*. The colonies were well isolated. Some plates had a few punctate colonies with mottled internal structure (more pronounced than *B. fragilis*) which were gram-positive bacilli. Sometimes a few convex colonies, smaller than *B. fragilis* and without the mottled internal structure, were present and were determined to be gram-positive cocci. Other miscellaneous contaminants were occasionally observed. Colonies typical in appearance to *B. fragilis* and composed of small gram-negative rods that were obligately anaerobic were transferred to TBA plates to ensure purity and then to kanamycin-esculin-bile agar (1). *B. fragilis* and some other species of *Bacteroides* turn kanamycin-esculin-bile agar black.

To presumptively identify the isolates to species, we performed catalase and indole tests and rhamnose, tre-

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halose, and mannitol fermentations (2, 3). All were incubated for 48 to 72 h, and those with poor growth were incubated for a total of 7 days. For catalase production, agar stabs of BHI-YE were melted on the day they were to be used. Autoclaved stock hemin solution (0.1 ml) containing 350 µg of hemin per ml of 0.01 N NaOH was added to 7 ml of agar, and the medium was solidified on a slant. After incubation, growth on the slants was exposed to air for 30 min, a loopful of bacterial growth was transferred to a glass slide, and 3% hydrogen peroxide was added. Evolution of bubbles was considered a positive test for catalase. For the indole test, isolates were grown in 2% tryptone (Difco Laboratories) semisolid agar, pH 7.2. Indole was extracted by shaking the agar vigorously with xylene and then holding the tube stationary for 1 to 2 min for the xylene to form a layer on top. Ehrlich reagent (0.5 ml) was added slowly down the side of the tube. If indole was present, a red ring formed between the xylene and the reagent. For the fermentation reactions, 0.6% (wt/vol) carbohydrates was added to thioglycolate medium without glucose or indicator (pH 7.2; Difco Laboratories). After good growth of the isolates, 1% bromothymol blue was added. Confirmatory identification of a number of isolates was done by the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg.

Growth of reference strains of various species of *Bacteroides* was evaluated on PINN medium. *B. fragilis* (VPI 2553 and 2393), *B. thetaiotaomicron* (ATCC 29148), and *Bacteroides* 3452A group (6) grew on PINN and produced colonies with the characteristic mottled internal structure. *B. ovatus* (ATCC 8483) grew on PINN, but the colonies had a granular internal structure rather than the mottled structure typical of *B. fragilis*. *B. distasonis* (ATCC 8503), *B. vulgatus* (ATCC 8482), and *B. uniformis* (VPI 5444A) did not grow on PINN.

A fecal sample was collected aerobically from each of 94 diarrheic lambs (1 to 2 days old). The samples were plated on PINN medium after storage aerobically for 2 weeks, followed by storage anaerobically at room temperature for 1 to 2 months (until BFEL was discovered) and then anaerobically at 4°C for 1 to 2 months. *B. fragilis* was isolated from 43 of the 94 samples, and no bacterial growth occurred with 31 samples. Some growth occurred with the remaining 20 samples, but none of the colonies resembled *B. fragilis*. Failure to isolate *Bacteroides* species from 51 samples may have been due to the prolonged aerobic and anaerobic storage of the samples before attempted isolation. The relatively young age (1 to 2 days) of the lambs at the time of sample collection may have contributed to the failure to isolate species of *Bacteroides* other than *B. fragilis* from any of the samples.

Fifty-three diarrheic calf fecal samples (collected aerobically) were plated on PINN as they were received at the laboratory. Colonies similar in appearance to *B. fragilis* were selected for identification. *B. fragilis* only was isolated from 32 samples, *B. fragilis* and *B. thetaiotaomicron* were isolated from 6 samples, *B. fragilis* and *Bacteroides* 3452A group were isolated from 3 samples, and *B. thetaiotaomicron* only was isolated from 3 samples. There was no growth with four of the samples, and in five samples there were no colonies that resembled *B. fragilis*.

In early studies, ovine fecal samples were plated directly on kanamycin-esculin-bile agar. In all cases, the medium turned black owing to esculin hydrolysis by *B. fragilis* or other intestinal bacteria. Gram-positive cocci and bacilli grew on the medium, and in our hands it was not useful for isolation of *B. fragilis* directly from feces.

On occasion, colonies of *B. fragilis* appeared slightly smaller on PINN than on kanamycin-esculin-bile agar. The addition of bile, a growth stimulant for *B. fragilis* (9), as 2% oxgall powder (Sigma Chemical Co.) to PINN medium sometimes resulted in slightly larger colonies of *B. fragilis* but increased both the number and the size of contaminant colonies.

PINN medium allowed the growth of several species of *Bacteroides* and a few other enteric bacteria but had the degree of selectivity needed for the isolation of *B. fragilis*. Isolates of *B. fragilis* can be distinguished from other *Bacteroides* species by the appropriate biochemical tests. For best results, PINN medium should be used within 3 weeks, since its inhibitory activity is gradually lost.

Results of this study indicated that *B. fragilis* could be readily isolated from diarrheic calves and lambs after direct plating of feces on PINN medium and selection of colonies that resembled *B. fragilis*. Some of the isolates of *B. fragilis* from calves and lambs had enterotoxin-like activity and others did not. Enterotoxigenic and nonenterotoxigenic isolates of *B. fragilis* were indistinguishable on PINN.

The role of enterotoxigenic *B. fragilis* in the etiology of the enteric disease complex is unclear and should be studied further. No attempt was made in this study to isolate *B. fragilis* from the feces of normal calves or lambs. PINN medium may be useful in a variety of future studies involving the isolation of *B. fragilis* from highly contaminated specimens.

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LITERATURE CITED

- Chan, P. C. K., and R. K. Porschen. 1977. Evaluation of kanamycin-esculin bile agar for isolation and presumptive identification of *Bacteroides fragilis* group. J. Clin. Microbiol. 6:528-529.
- Dowell, V. R., Jr., and T. M. Hawkings. 1978. Laboratory methods in anaerobic bacteriology, p. 55. CDC laboratory manual. Center for Disease Control, Atlanta, Ga.
- Finegold, S. M., and D. M. Citron. 1980. Gram-negative, non-sporulating anaerobic bacilli, p. 431-439. In E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
- Firehammer, B. D., and L. L. Myers. 1981. *Campylobacter fetus* subsp. *jejuni*: its possible significance in enteric disease of calves and lambs. Am. J. Vet. Res. 42:918-922.
- Furia, T. E., and A. G. Schenkel. 1968. New, broad, spectrum bacteriostat. Soap Chem. Spec. p. 47-50, 116-122.
- Johnson, J. L. 1978. Taxonomy of the bacteroides. I. Deoxyribonucleic acid homologies among *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. Int. J. Syst. Bacteriol. 28:245-256.
- Myers, L. L., B. D. Firehammer, D. S. Shoop, and M. M. Border. 1984. *Bacteroides fragilis*: a possible cause of acute diarrheal disease in newborn lambs. Infect. Immun. 44:241-244.
- Myers, L. L., F. S. Newman, G. R. Warren, J. E. Catlin, and C. K. Anderson. 1975. Calf ligated intestinal segment test to detect enterotoxigenic *Escherichia coli*. Infect. Immun. 11: 588-591.
- Vargo, V., M. Korzeniowski, and E. H. Spaulding. 1974. Tryptic soy bile-kanamycin test for the identification of *Bacteroides fragilis*. Appl. Microbiol. 27:480-483.