APPLIED MICROBIOLOGY, July 1970, p. 168-169
Vol. 20, No. 1

Medium for Differential Count of the Anaerobic Flora in Human Feces

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Received for publication 16 April 1970

On reinforced clostridial agar with blood and 0.03% China blue, organisms of the bacteroides group grow with blue translucent colonies, whereas bifidobacteria grow with opaque brown colonies. This permits the differential counting of the predominant anaerobic organisms in human feces on one medium.

The majority of the fecal flora consists of gram-positive and gram-negative anaerobic rods. Both groups occur in numbers of about 10^9 to 10^{10} organisms per g of feces, thereby exceeding the numbers of coliforms and enterococci by a factor of 100 or more (3, 13). The gram-positive anaerobes mainly belong to various species of the genus *Bifidobacterium* (1, 8, 9). Among the gram-negative anaerobes, various species of the bacteroides group (including the genus *Fusobacterium*) are abundant. Various media for the isolation of these organisms have been described (2, 4, 7, 10). However, to estimate, quantitatively and separately, each of the two main groups remained a difficult task. By using a rich medium on which most cultivable organisms seem to grow well and by using the method of Kludas (5) with China blue, we arrived at a formula which seems to permit the differential counting of gram-positive and gram-negative anaerobes on one plate. Some types of *Bifidobacterium* can tentatively be identified by colony form alone or by colony form and microscopy.

Reinforced clostridial agar (RCA, Oxoid) with 1% glucose was used as base medium. After sterilization and cooling to 50 C, horse blood and China blue (ferri-ferrocyanide, G. Grübler and Co., Leipzig) were added to final concentrations of 7.5 and 0.03%, respectively (China blue-blood-RCA or CBRCA). The medium was inoculated by the method of Miles and Misra (6) with dilutions of pure cultures or feces suspensions. The cultures were grown in thioglycolate broth prepared with Todd Hewitt broth (Difco) to which 0.5% glucose, 0.04% agar, and 0.05% sodium thioglycolate had been added. An aqueous solution of 0.5% yeast extract, 0.1% peptone (Oxoid), 0.85% NaCl, and 0.05% cysteine (*p*H 7.0) was used as a diluent.

Feces suspensions (about 1 g in 19 ml of diluent) were homogenized on a shaker for 5 min and diluted by pipetting 0.5 ml in 9.5 ml of diluent by using a fresh pipette for each dilution step. Duplicate standard droplets (0.03 ml) of the last three or four dilutions were dropped on a plate. Plates were incubated for 72 hr at 37 C in a McIntosh anaerobic jar by using hydrogen with 5% carbon dioxide as a gas phase. The performance of the jars was checked regularly with *Clostridium sporogenes*.

A series of 20 samples collected in diluent in a weighed, screw-cap bottle was studied immediately and at 1, 2, 3, and 6 hr after collection. The differential counts did not differ significantly. No loss of viability was observed up to 6 hr.

With regard to viable count and colony size, the medium was compared with blood agar (nutrient agar with 7.5% sheep blood), Rogosa medium at *p*H 6.8 and 5.4 (12), penicillin medium (4), sodium azide medium (10), RCA with blood, neomycin RCA blood agar (2), and polymyxin RCA blood agar (7). Tests were performed with dilutions of feces and with the test organisms *B. bifidum* (nomenclature of Reuter, 11), *B. adolescentis*, *Lactobacillus acidophilus*, and two bacteroides isolates.

With test strains and feces suspensions, the CBRCA medium showed a higher viable count and a larger colony size than blood agar (Fig. 1) and most of the other media. The size of colonies generally exceeded 1 mm. The important feature is that the colonies of bifidobacteria are brown and opaque, whereas those of bacteroides are blue and more translucent. This was confirmed by microscopy of Gram stained slide preparations for hundreds of colonies from over 200 samples of feces.

Other gram-negative bacteria also give blue translucent colonies, as shown for strains of *Escherichia coli* and strains of the genera *Klebsiella*, *Enterobacter*, *Salmonella*, *Proteus*, and

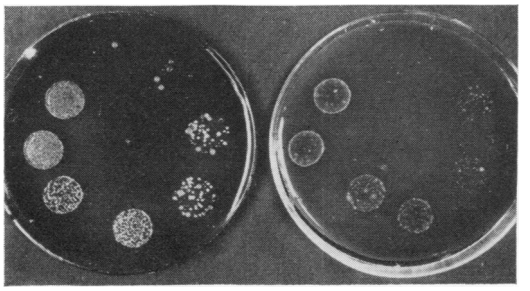

FIG. 1. *Dilutions of the same feces suspension on CBRCA (left) and on blood agar (right). Each dilution in duplicate; counterclockwise* 10^{-5}, 5×10^{-6}, 10^{-8} *and* 10^{-9}. *In the last dilutions, the opaque colonies of bifidobacteria can be distinguished from the translucent bacteroides colonies even without the aid of the color difference (brown-blue).*

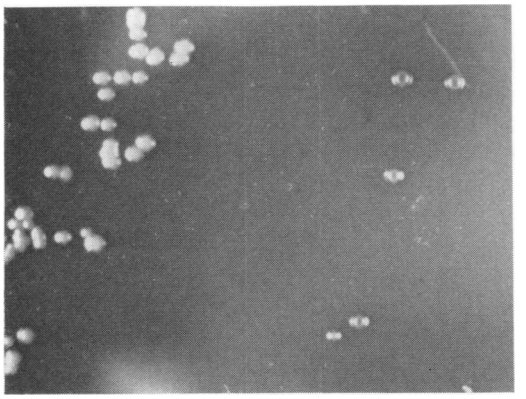

FIG. 2. *Colonies of Bifidobacterium bifidum (right) and B. adolescentis (left).*

Fusobacterium. Staphylococci and *Streptococcus faecalis* produce brown colonies. The colonies of the aerobic lactobacilli are cream-colored. However, this characteristic is not very important for fecal samples since, in the highest dilution showing growth, the choice is generally between the predominant groups of bacteroides and bifidobacteria. Within these groups, differences in colony morphology can be observed. *B. bifidum* at 72 hr shows small (2 mm) dark-brown hard granular colonies. *B. adolescentis* has large (4 mm) convex light-brown colonies of buttery consistency (Fig. 2). The medium was used for counting a number of consecutive samples of feces from children with various illnesses. A representative graph is given in Fig. 3.

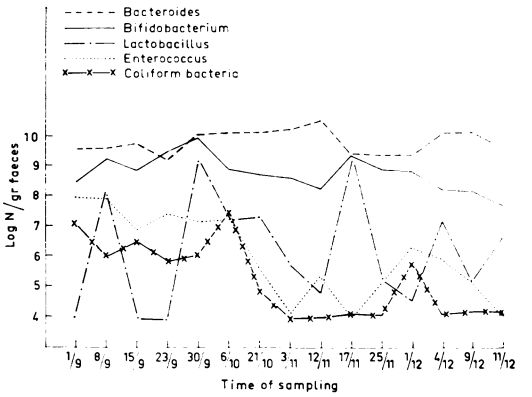

FIG. 3. *Serial count of weekly samples of feces from a child with a protein-losing enteropathy. Bifidobacteria and bacteroides were counted on the CBRCA, lactobacilli on Rogosa medium (pH 5.4), and enterococci and coliforms on routine media.*

LITERATURE CITED

1. Dehnert, J. 1957. Untersuchung über die gram-positive Stuhlflora des Brustmilchkindes. II. Mitteilung. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I Orig. 169:66–83.
2. Fuller, R., and M. Lev. 1964. Quantitative studies on some of the gram-negative anaerobic bacteria in the pig alimentary tract. J. Appl. Bacteriol. 27:434–438.
3. Haenel, H. 1961. Some rules in the ecology of the intestinal microflora of man. J. Appl. Bacteriol. 24:242–251.
4. Kalz, K. 1962. Die Einwirkung chemotherapeutischer Substanzen auf Bakterium bifidum (*Lactobacillus bifidus*). Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I Orig. 183:452–472.
5. Kludas, M. 1959. Züchtung von Laktobacillus bifidus in und auf einfachen Nährmedien. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I Orig. 174:71–75.
6. Miles, A. A., and S. S. Misra. 1938. The estimation of the bactericidal power of the blood. J. Hyg. 38:732–750.
7. Miller, L. G., and S. M. Finegold. 1967. Antibacterial sensitivity of *Bifidobacterium* (*Lactobacillus bifidus*). J. Bacteriol. 93:125–130.
8. Mitsuoka, T. 1969. Vergleichende Untersuchungen über die Bifidobakterien aus dem Verdauungstrakt von Menschen und Tieren. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I Orig. 210:52–65.
9. Orla-Jensen, S. 1924. La classification des bactéries lactiques. Lait 4:468–474.
10. Post, F. J., A. D. Allen, and T. C. Reid. 1967. Simple medium for the selective isolation of *Bacteroides* and related organisms, and their occurrence in sewage. Appl. Microbiol. 15:213–218.
11. Reuter, G. 1963. Vergleichende Untersuchungen über die Bifidus-Flora im Säuglings- und Erwachsenenstuhl. Zentralbl. Bakteriol. Abt. I Orig. 191:486–507.
12. Rogosa, M., J. A. Mitchell, and R. F. Wiseman. 1951. A selective medium for the isolation of oral and fecal lactobacilli. J. Bacteriol. 62:132–133.
13. Snyder, M. L. 1967. Further studies on Bacillus difficilis (Hall and O'Toole). J. Infec. Dis. 60:223–231.