Biochemical Characterization of Anaerobic Bacteria by an Impregnated Disk Method

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An impregnated disk method was used to characterize 20 clinical isolates of anaerobic bacteria by utilization of substrate. The procedure was easy to perform, reliable, and economical.

Because of our increased awareness of the high incidence of anaerobic infections, much interest has been shown recently in rapid micro methods for identification of anaerobic bacteria (5, 6). The procedure described here is an impregnated disk method (4).

Twenty pure clinical isolates were previously identified by our clinical laboratory affiliates using a method similar to that of Dowell and Hawkins (2).

The various organisms tested were prepared by using 24- to 48-h cultures grown on brain heart infusion agar supplemented with 10 μ g of vitamin K₁ per ml, 5 μ g of hemin per ml, and 5% defibrinated sheep blood under an atmosphere of 85% N₂, 10% H₂, and 5% CO₂ in a vented GasPak at 35°C. The atmosphere in the GasPak was supplied by an external gas mixture (Matheson Gas Products). Each GasPak was flushed three times with gas before being sealed. Growth was suspended in fluid carbohydrate medium base (Difco) supplemented with vitamin K₁ (0.1 μ g/ml) and hemin (5.0 μ g/ml). The cell suspension was adjusted to 70% transmission at 520 nm (Beckman DB-GT grating spectrophotometer).

Sterile liquefied 1.25% purified agar (Difco) was placed in petri dishes and allowed to harden into a confluent surface. The surface of each plate was gently inoculated with a sterile cotton swab saturated with the suspension of the organism to be tested. The uniformly swabbed plates were dried under a nitrogen atmosphere.

On the dry inoculated surfaces were imbedded up to five disks prepared as follows. Carbohydrate medium base, supplemented as indicated previously, was used as the basal medium for carbohydrate tests. Specific carbohydrate substrates were prepared individually as 10% (wt/vol) stock solutions, filter sterilized,

and added to the basal medium to yield a final concentration of 0.6% (5). Carbohydrates tested include arabinose, fructose, glucose, glycerol, lactose, maltose, mannitol, sucrose, trehalose, and xylose. Disks were prepared by placing 0.05 ml of basal medium containing a carbohydrate substrate on 6.5-mm-diameter disks in 0.01- or 0.02-ml increments (1). Control disks were prepared by adding basal medium without carbohydrate.

Indole-nitrate broth (BBL) (3) was reconstituted and while hot was applied as above to the disks used for the nitrate test and then dried under nitrogen. For the esculin hydrolysis test, peptone-yeast extract broth made according to the method of Holdeman and Moore (3) excluding resazurin solution and including 0.5% esculin (Difco) were applied to the disks as indicated above and dried under nitrogen. Dried disks were stored under nitrogen at -10°C for as long as 2 months without variation in test results as compared with freshly made disks.

Inoculated plates with disks applied were incubated at 35°C in a GasPak jar under the atmospheric conditions already described. Plates were read in duplicate at 12, 24, 30, 36, and 48 h and then once daily to 7 days.

Since medium diffuses out uniformly from the disks, growth appeared circumscribed evenly about the disks. One drop of 0.1% bromothymol blue was placed on each disk used for carbohydrate tests after growth became visible. A positive reaction was considered to be a change from a blue-green color to a yellow color (Table 1). A weak positive reaction was one in which the color changed to yellow slowly and not as deeply as the positive reaction. When growth was visibly circumscribed about the esculin-impregnated disk, one drop of 1% ferric ammonium citrate was added to the disk. A black precipitate surrounding the esculin-im-

TABLE 1. Comparison of substrate utilization by the disk or tube method

Organism	Substrate																			
	Ara ^a		Fru		Glu		Gly		Lac		Mal		Man		Suc		Tre		Xyl	
	D ^b	T ^c	D	T	D	T	D	T	D	T	D	T	D	T	D	T	D	T	D	T
<i>Bacteroides fragilis</i> (isolate 1)	- ^d	-	+ ^e	+	+	+	+	(+) ^f	+	+	+	+	-	-	+	+	-	-	+	+
<i>B. fragilis</i> (isolate 2)	-	-	+	+	+	+	+	+	+	+	+	+	(+)	-	+	+	-	-	+	+
<i>B. melaninogenicus</i>	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-
<i>Clostridium butyricum</i> (isolate 1)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
<i>C. butyricum</i> (isolate 2)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
<i>C. butyricum</i> (isolate 3)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
<i>C. butyricum</i> (isolate 4)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
<i>C. bifermentans</i>	-	-	(+)	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
<i>C. perfringens</i> (isolate 1)	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-	-
<i>C. perfringens</i> (isolate 2)	-	(+)	+	+	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-	-
<i>C. perfringens</i> (isolate 3)	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-	-
<i>C. sporogenes</i> (isolate 1)	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. sporogenes</i> (isolate 2)	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Fusobacterium mortiferum</i>	-	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-
<i>F. nucleatum</i> (isolate 1)	-	-	(+)	(+)	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
<i>F. nucleatum</i> (isolate 2)	-	-	(+)	(+)	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
<i>Lactobacillus acidophilus</i> (isolate 1)	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-
<i>L. acidophilus</i> (isolate 2)	-	-	+	+	+	+	-	(+)	+	+	+	+	-	-	+	+	+	+	+	+
<i>L. brevis</i>	+	+	+	+	+	+	-	-	-	-	(+)	(+)	-	-	-	-	-	-	+	+
<i>Propionibacterium acnes</i>	-	-	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-

^a Ara, Arabinose; Fru, fructose; Glu, glucose; Gly, glycerol; Lac, lactose; Mal, maltose; Man, mannitol; Suc, sucrose; Tre, trehalose; Xyl, xylose.

^b Disk method.

^c Tube method.

^d Negative result.

^e Positive result.

^f Weak positive result.

pregnated disk was considered a positive reaction, whereas lack of a color change was a negative result (see Table 3).

Nitrate reduction was tested by the addition of one drop of a solution of 0.8% sulfanilic acid in 5 N acetic acid followed by one drop of a solution of 0.5% alpha-naphthylamine in 5 N acetic acid. A red color was indicative of a positive reaction.

Negative results were further checked by the addition of a small amount of zinc dust to the disk and to the immediately surrounding agar. After the addition of zinc, the appearance of a pink color confirmed the negative reaction.

Disk assay results were confirmed by a tube method similar to that of Dowell and Hawkins (2). Ten milliliters of carbohydrate basal medium with 0.6% of the tested carbohydrate, 5 µg of hemin per ml, and 0.1 µg of vitamin K₁ per ml were placed in screw-cap tubes under an N₂ atmosphere. Carbohydrates were the same as those used for the disk procedure.

Media and substrates used for nitrate reduction and esculin hydrolysis were made as above but were dispensed under an N₂ atmosphere into screw-cap tubes. Tests were performed as above when growth was visible in the tubes.

The disk assay was shown to yield results

TABLE 2. Comparison of incubation times necessary before all carbohydrate tests could be read

Organism	Tube	Disk
<i>Bacteroides fragilis</i> (isolate 1)	5 d ^a	24 h
<i>B. fragilis</i> (isolate 2)	6 d	30 h
<i>B. melaninogenicus</i>	5 d	30 h
<i>Clostridium bifermentans</i>	7 d	36 h
<i>C. butyricum</i> (isolate 1)	48 h	24 h
<i>C. butyricum</i> (isolate 2)	48 h	24 h
<i>C. butyricum</i> (isolate 3)	48 h	24 h
<i>C. butyricum</i> (isolate 4)	48 h	24 h
<i>C. perfringens</i> (isolate 1)	5 d	30 h
<i>C. perfringens</i> (isolate 2)	5 d	36 h
<i>C. perfringens</i> (isolate 3)	4 d	30 h
<i>C. sporogenes</i> (isolate 1)	7 d	36 h
<i>C. sporogenes</i> (isolate 2)	7 d	36 h
<i>Fusobacterium mortiferum</i>	3 d	24 h
<i>F. nucleatum</i> (isolate 1)	48 h	24 h
<i>F. nucleatum</i> (isolate 2)	48 h	24 h
<i>Lactobacillus acidophilus</i> (isolate 1)	36 h	24 h
<i>L. acidophilus</i> (isolate 2)	48 h	24 h
<i>L. brevis</i>	36 h	24 h
<i>Propionibacterium acnes</i>	36 h	24 h

^a d, Days.

consistent with the tube procedure for most organisms tested (Table 1). These results were generally obtainable within 24 to 48 h after

TABLE 3. Results of esculin hydrolysis and nitrate reduction tests by the disk and tube methods

Organism	Esculin hydrolysis		Nitrate reduction	
	Disk	Tube	Disk	Tube
<i>Bacteroides fragilis</i> (isolate 1)	+ ^a	+	- ^b	-
<i>B. fragilis</i> (isolate 2)	+	+	-	-
<i>B. melaninogenicus</i>	-	-	-	-
<i>Clostridium bifermentans</i>	+	+	-	-
<i>C. butyricum</i> (isolate 1)	+	+	-	-
<i>C. butyricum</i> (isolate 2)	+	+	-	-
<i>C. butyricum</i> (isolate 3)	+	+	-	-
<i>C. butyricum</i> (isolate 4)	+	+	-	-
<i>C. perfringens</i> (isolate 1)	-	-	+	+
<i>C. perfringens</i> (isolate 2)	-	(+) ^c	-	-
<i>C. perfringens</i> (isolate 3)	-	-	-	-
<i>C. sporogenes</i> (isolate 1)	+	+	-	-
<i>C. sporogenes</i> (isolate 2)	+	+	-	-
<i>Fusobacterium mortiferum</i>	+	+	-	-
<i>F. nucleatum</i> (isolate 1)	-	-	-	-
<i>F. nucleatum</i> (isolate 2)	-	-	-	-
<i>Lactobacillus acidophilus</i> (isolate 1)	+	+	-	-
<i>L. acidophilus</i> (isolate 2)	+	+	-	-
<i>L. brevis</i>	-	-	-	-
<i>Propionibacterium acnes</i>	-	-	+	+

^a Positive result.^b Negative result.^c Weak positive result.

inoculation for the impregnated disk procedure, whereas the tube procedure took longer, occasionally as long as 7 days until results were obtained (Table 2).

Results from esculin hydrolysis and nitrate reduction were also quite consistent with the tube procedure (Table 3). All tests were repeated in duplicate at least three different times for all reported organisms.

The procedure appears to be inexpensive, reliable, and easy to perform by a general hospital microbiology laboratory of any size.

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