Semi-Microtechnique for the Biochemical Characterization of Anaerobic Bacteria

JOHN R. MORGAN,* P. Y. K. LIU, AND JOHN A. SMITH

Department of Medical Microbiology, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada

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A semi-micromethod is described for characterizing anaerobic bacteria by substrate utilization. Small volumes of individual substrates were placed in the wells of plastic microtiter trays. When heavy inocula and a colorimetric indicator were used, complete results were available after 30 h of incubation. Microtiter trays containing a range of substrates can be stored at -20°C . The method is accurate, economical, and convenient for use in a hospital microbiology laboratory.

The identification of anaerobic organisms by the fermentation of various carbohydrates is laborious and time consuming because large volumes of substrates are required in individual screw-cap tubes with prolonged periods of incubation. We describe a method achieving the same results by the use of plastic microtiter trays carrying small volumes of substrate into which are placed heavy inocula of anaerobic bacteria. The method is inexpensive and convenient, and the results are read after 30 h of incubation. Plates containing a number of substrates can be prepared in advance and stored at -20°C before use. The method was compared with a tube technique with respect to speed and accuracy. Recently, a micromethod was reported by Wilkins and Walker (5). Their technique, which is substantially different, is claimed to be useful for laboratories that identify large numbers of anaerobes. We have developed our technique for use in a hospital microbiology laboratory.

MATERIALS AND METHODS

The trays used were Cooke microtiter trays (ICN Canada Ltd.) and substrates were dispensed in 0.2-ml quantities, using an autopipette (Oxford Laboratories) with sterile, disposable glass tips.

Media. The basal medium consisted of CHO medium base (Difco) supplemented with vitamin K₁ and hemin to a concentration of 0.1 and 5 $\mu\text{g}/\text{ml}$, respectively (4). The carbohydrate substrates used were glucose, mannitol, lactose, sucrose, maltose, xylose, and arabinose. A 10% (wt/vol) stock solution of each carbohydrate was sterilized by filtration and added aseptically to the basal medium to obtain a final 0.6% concentration. The exception was glucose, which was incorporated into the basal medium at a final concentration of 0.6% and sterilized by autoclaving. The indicator used was 0.1% (wt/vol) brom-

othymol blue in distilled water. Esculin hydrolysis and nitrate reduction were tested with 1% esculin (Difco) and 1% nitrate (Difco).

Preparation of microtiter trays. The various substrates were distributed in each microtiter tray as illustrated in Fig. 1. Each well received a 0.2-ml volume of fluid. The controls consisted of a row of wells containing basal medium without substrate as a negative color control (row CHO, Fig. 1) and a row of wells (row 8, Fig. 1) that acted as a sterility control. To reduce evaporation before use, trays were wrapped inside sterile plastic bags in packs of five.

Inoculation and incubation. Pure cultures were grown for 24 to 48 h on fresh Columbia agar (Difco) enriched with 5% defibrinated sheep blood, vitamin K₁ (10 $\mu\text{g}/\text{ml}$), and hemin (5 $\mu\text{g}/\text{ml}$). Standard bacterial suspensions were prepared in basal medium to match the turbidity of a McFarland number one nephelometer standard. One drop was added to each well of a numbered row in the tray. Row 8 received sterile basal medium (Fig. 1).

Trays were incubated for 30 h at 36°C in a GasPak jar laid horizontally. Thus, if set up sufficiently early, results could be read on the next day. Anaerobic conditions were established by five evacuations and exchanges with a final atmosphere of 85% N₂, 10% H₂, and 5% CO₂. The catalyst was palladium-coated alumina pellets (BBL) rejuvenated in a hot-air oven at 160°C for 2 h.

Reading results. After 30 h of incubation, the trays were removed from the GasPak jar and to each well was added 1 drop of 0.1% bromothymol blue indicator, with the exception of those wells containing esculin and nitrate broth. A positive reaction was indicated by a yellow color, and a negative reaction was indicated by a color that matched the CHO negative color control, which was usually green. Equivocal color changes in the substrate wells were compared with the negative color control. The latter had been inoculated with bacteria, and any color change that occurred possibly represented the carry-over of fermentable substrate in the inocu-

FIG. 1. Arrangement of substrates in a microtiter tray.

lum and was interpreted as a negative result. Esculin hydrolysis was indicated by the development of a brown or black color in the well. An absence of color change was considered negative (3). Nitrate reduction was detected by adding 1 drop of reagent A (0.8% sulfanilic acid in 5 N acetic acid) and 1 drop of reagent B (0.5% alpha-naphthylamine-5 N acetic acid) to the nitrate broth. The appearance of a red color represented a positive result and its absence was considered presumptive negative. The latter was checked by adding a pinch of zinc dust, whereupon a pink or red coloration confirmed the negative result. Alternatively, a failure to produce a pink or red color indicated positive nitrate reduction (2, 3).

Conventional method. Sterile CHO medium base with 0.6% of the respective carbohydrate and 5 µg of hemin per ml was distributed in 8-ml quantities in sterile screw-cap tubes (16 by 25 mm) (1). Just before inoculation, tubes were steamed for 10 min and allowed to cool, and vitamin K₁ was added (0.1 µg/ml) (1). Individual substrates used included glucose, mannitol, lactose, maltose, sucrose, xylose, arabinose, and glycerol. Sterile disposable pipettes were used to introduce a few drops of the bacterial suspension to the bottom of the tube. Incubation was at 36°C.

Results were first read after 30 h of incubation. A green color in the tube was regarded as a negative result; conversely, a yellow color was recorded as positive. When decolorization of the indicator was observed, 1 ml of the culture was removed and tested separately with 1 to 2 drops of bromothymol blue. Tubes were read again on day 7 and the results were recorded.

Organisms. The anaerobic bacteria used in the study were from clinical specimens and reference strains obtained from the Center for Disease Control (CDC), Anaerobic Laboratory, Atlanta, Ga. The clinical isolates and the number of strains included: *Bacteroides* sp. (fermentative bacteroides that grow

in 20% bile), 24; *Clostridium perfringens*, 2; *Bacteroides melaninogenicus*, 5; *Eikenella corrodens*, 1; *Peptococcus* species, 2; *Peptostreptococcus* species, 2; *Bacteroides* species, 29; *Fusobacterium nucleatum*, 2; *Propionibacterium acnes*, 3; unidentified gram-negative rods, 2; and *Actinomyces viscosus*, 1. One of each of the following CDC strains was used: *Eubacterium limosum*, *F. mortiferum*, *B. fragilis*, *B. distasonis*, *B. melaninogenicus*, *C. perfringens*, *C. sporogenes*, *C. butyricum*, *C. subterminale*, *C. sordellii*, *C. cadaveris*, *C. tertium*, *C. histolyticum*, and *C. bifermentans*.

Shelf life of prepared microtiter trays. Six microtiter trays were prepared as described previously. Two trays were used to record the biochemical reactions of 11 anaerobic bacterial strains: *Bacteroides* sp. (fermentative bacteroides that grow in 20% bile), 3 strains; *Bacteroides* species, 5 strains; *Peptostreptococcus* species, 2 strains; and *B. melaninogenicus*, 1 strain. The remaining four trays were carefully sealed in plastic bags and stored at -20°C. After 2 weeks, two trays were removed, thawed at 36°C for 30 min, and inoculated with the same 11 anaerobes. This was repeated after a further 2 weeks with the last microtiter trays.

RESULTS

A comparison was made between the conventional tube method and the semi-micromethod with regard to any disparity of results and time taken for positive reactions to occur.

Table 1 is an analysis of the utilization of eight substrates recorded after 30 h and 7 days of incubation. A total of 74 strains fermented glucose after 7 days of incubation; 73 of 74 strains were positive by the semi-micromethod, whereas only 65 of 74 were positive by the conventional tube method at this time. Similar observations were made with lactose, sucrose, maltose, and glycerol (Table 1), for which substrate utilization was consistently seen earlier

TABLE 1. Comparison of the number of strains showing positive reactions, using eight substrates, by the semi-micromethod and a tube method when incubated for 30 h and 7 days^a

Substrate	No. of positive strains after incubation for:		
	30 h		7 Days
	Semi-micromethod	Tube method	Tube method
Glucose	73	65	74
Mannitol	0	0	0
Lactose	59	46	59
Sucrose	39	37	39
Maltose	64	52	64
Xylose	26	26	26
Arabinose	8	8	8
Glycerol	26	11	26

^a A total of 87 strains were tested.

by the semi-micromethod. No false-positive reactions were given by the semi-micromethod and, similarly, after 7 days of incubation, the conventional tube method results were typical of the anaerobic strains used. One false-negative reaction was observed with the semi-micromethod which, for a strain of *Peptostreptococcus*, gave a negative glucose utilization result which was positive by the tube method.

Esculin hydrolysis and nitrate reduction were tested only by the semi-micromethod. The validity of these results were judged from the CDC anaerobe manual (1). Of the organisms used, 32 hydrolyzed esculin and 7 reduced nitrate. No false-positive or false-negative results were encountered.

Trays that contained the various substrates and were stored for 2 and 4 weeks, respectively, showed no loss of reproducibility. When a panel of 11 anaerobic strains was used, the results were identical with those obtained with freshly prepared semi-microtrays.

DISCUSSION

The convenience, saving of space, and economy resulting from the miniaturization of any chemical or biochemical test are clearly desirable if they can be shown to be feasible. We have demonstrated the latter with regard to substrate utilization tests currently used in the identification of anaerobic bacteria. Thus, when small volumes of medium in microtiter plastic trays were used, the results compared favorably with those obtained by a conventional tube method that used relatively large volumes of medium. The use of inocula containing large numbers of viable organisms yielded readable results after 30 h of incubation. This meant that trays set up early in the working day could be read on the next afternoon. With the substrates and anaerobic bacteria used in this study, we found one discrepancy out of 87 strains and eight substrates. Complete results were readable after 30 h of incubation, whereas with a conventional method results were incomplete after this length of incubation period. The results obtained with the semi-micromethod agreed both with those obtained by 7 days of incubation with the conventional method and those given in the CDC reference anaerobic manual for the organisms used (1).

There are two possible explanations for the faster results obtained by the semi-micromethod, including the lower ratio of substrate to inoculum and the fact that the semi-microplates were incubated in an anaerobic jar whereas the screw-capped tubes were incubated in an aerobic incubator. Therefore, the

small amount of air introduced into the tube during inoculation could have slightly impaired the anaerobic conditions in the medium, resulting in slower growth.

We have found that the semi-micromethod is convenient at all stages of preparation, inoculation, and reading of results and that it offers many advantages over the more conventional screw-cap tubes. A GasPak jar accommodates nine microtiter trays so the saving of space is a desirable feature. The results of the shelf life experiment demonstrate that microtiter trays containing substrate can be stored at -20°C for at least 1 month ready for use.

During the preparation of this manuscript, Wilkins and Walker (5) reported a replicator method for identifying anaerobes that employed carbohydrate fermentation in microtiter plates. There are substantial differences between our technique and theirs, which possibly reflect the development of the method and its applicability in a research and reference laboratory (5, 6). The essential differences include the addition of agar to the substrate media, the use of an anaerobic glove box throughout the setting-up procedure, and the measurement of pH when the results are being read. Since we do not possess an anaerobic glove box, we were not restricted to the use of a solid medium which, otherwise, appears to offer no significant advantages. Furthermore, with the organisms used in our study, the inoculation of microtiter trays on an open bench did not appear to affect the results. It is tempting to speculate that the small volumes used speedily facilitates anaerobic conditions after the trays are placed in a GasPak jar. Since the preparation and inoculation of trays were performed on an open bench, the sealing of plates was contraindicated. The inclusion of sterility and negative color controls gave reassurance against cross-contamination either by motile organisms or volatile acid during the incubation period. We have tested fewer anaerobic species, which could explain why we found 30 h of incubation to be sufficient as compared with 3 days quoted by Wilkins and Walker (5). However, the anaerobes used in our study are representative of those commonly encountered in a clinical microbiology laboratory. The measurement of pH change by using pH electrodes is superior to the subjective reading of color change. However, we have found with a negative color control that distinguishing between positive and negative was not difficult after the addition of fresh indicator to each well.

The range of substrates used in our study is not as extensive as those of Wilkins and Walker, but were chosen as a useful adjunct to

other methods for the identification of anaerobes commonly isolated in hospitals. Further evaluation would be required to discover whether our method as described is applicable to the full biochemical identification of anaerobes. The method was developed for use in a hospital laboratory, and each tray represents a kit that can be stored ready for use for the biochemical characterization of seven anaerobic isolates. The results obtained when combined with Gram stain reaction, colonial morphology, and gas-liquid chromatography facilitate the identification of anaerobes in a hospital microbiology laboratory.

ADDENDUM

Before going to press, the nomenclature of the genus *Bacteroides* was modified to correspond with the revisions suggested by Cato and Johnson, 1976. (E. P. Cato and J. L. Johnson. 1976. Reinstatement of species rank for *Bacteroides fragilis*, *B. ovatus*, *B. distasonis*, *B. thetaiotaomicron*, and *B. vulgatus*: designation of neotype strains for *Bacteroides fragilis* (Veillon and Zuber) Castellani and Chal-

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