Micromethod System for Identification of Anaerobic Bacteria

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A micromethod multitest system prepared by Analytab Products, Inc. and conventional tests employed at the Center for Disease Control for identification of anaerobes were compared. All procedures were conducted in an anaerobic glove box. A total of 104 cultures, including 18 reference strains and 86 diagnostic cultures, were examined. Ninety-one percent of the total tests performed with the two systems were in agreement. Greater than 90% agreement between the two systems was obtained with 12 of the 17 differential tests compared. The tests for nitrate reduction and H_2S production gave the poorest agreement, 77.8 and 80.8%, respectively. Only 66% of the 86 diagnostic cultures could be presumptively identified with the micromethod system supplemented only with microscopy and colonial characteristics. However, when appropriate supplementary tests and gas-liquid chromatography were used with the micromethod system, 85% of the 86 strains could be identified. When Ehrlich reagent, instead of Kovac reagent, was used with the micromethod to test for indole, the agreement in identification was raised to 93%.

There has been a recent trend in clinical microbiology toward the development of simple, prepared systems for rapid identification of bacteria. Such systems could be particularly helpful to those working with anaerobic bacteria. At present, a large number of differential tests are required for identification of anaerobes, and these are quite costly (3, 4).

Recently, a commercially available micromethod multitest system was described for identification of *Enterobacteriaceae* (6, 8) (API system, Analytab Products, Inc.). In this study, we explored the possibility of using a similar system for identifying anaerobic bacteria.

MATERIALS AND METHODS

Bacterial strains. Reference strains from the American Type Culture Collection, well-characterized Center for Disease Control (CDC) strains, and cultures referred to the CDC Anaerobe Unit for identification were used to test the micromethod and conventional systems.

Micromethod system. The structure and form of the micromethod system (Analytab Products, Inc., New York, N.Y.) were the same as the API system for *Enterobacteriaceae* (6, 8). However, the substrates were different. The system included tests for neutral

red and nitrate reduction; H_2S, urease, and indole production; hydrolysis of gelatin and esculin; and fermentation of glucose, mannose, fructose, galactose, mannitol, lactose, sucrose, maltose, salicin, glycerol, xylose, arabinose, and starch. The exact composition of the substrates was not disclosed.

Conventional system. Conventional media were prepared as previously described (3), then immediately introduced into an anaerobic glove box similar to that described by Aranki et al. (1), and maintained under anaerobic conditions for at least 48 h before use (1). All of the tests in the micromethod system, except those for neutral red reduction and the fermentation of galactose and fructose, were included in the conventional system. Depending upon the type of organism suspected (3, 4), the following supplementary tests were also performed: (i) gram-positive sporulating bacilli—catalase, lecithinase, lipase, motility, action on milk, toxicity, and toxin neutralization; (ii) gram-negative bacilli—action on milk, fermentation of trehalose and rhamnose, growth in 20% bile, penicillin sensitivity (2 U disk); (iii) gram-positive nonsporulating bacilli—catalase, action on milk; (iv) anaerobic cocci—catalase, action on milk.

The end products of bacterial metabolism in peptone-yeast extract-glucose broth were determined for all organisms by gas-liquid chromatography (GLC) (7).

Procedure. Cultures to be examined were introduced into the glove box and streaked on prereduced blood-agar medium (PR-TSYEA) (2) to obtain isolated colonies. A gas mixture of 85% N_2, 10% H_2, and 5% CO_2 was used to fill the chamber and flush the

entry lock (1). The micromethod systems were introduced into the glove box shortly before use. One to four colonies of each organism to be tested were picked from a plate of PR-TSYEA medium and emulsified in 4 ml of Thioglycollate medium (Difco) without dextrose or indicator. By using a capillary pipette, the systems were inoculated with these bacterial suspensions and then incubated in a incubator at 37 C within the glove box for 48 h. Appropriate conventional media were inoculated with a capillary pipette from a 24-h culture in Thioglycollate medium (BBL, 135 C) (3). Final readings of the conventional tests, with the exception of those for catalase and indole production, nitrate reduction, and esculin hydrolysis, were made after 7 days incubation. Conventional tests for nitrite, indole, catalase, gelatin hydrolysis, and esculin hydrolysis were performed as previously described (3) outside the chamber. Cultures were exposed to air for at least 30 min before addition of hydrogen peroxide for detection of catalase.

After 48 h of incubation, the micromethod systems were removed from the glove box, and a final reading of the tests was made. Tests for nitrate reduction were determined as previously described (6, 8). Indole production was determined by the addition of one drop of Kovac reagent to the well for the indole test. If the indicator in the carbohydrate media, bromocresol purple, was reduced by a culture, one drop of an 0.04% aqueous solution of bromocresol purple was added to each carbohydrate test before it was read.

Organisms were identified on the basis of the classification schemes of Dowell and Hawkins (3) and Holdeman and Moore (4). One of us identified the organisms submitted for identification on the basis of results obtained with conventional tests and GLC. Another attempted to identify the organisms with the micromethod system (i) on the basis of colony and microscopy morphology and micromethod system results, and (ii) on the basis of the information in i plus the results of the appropriate supplementary tests, including GLC listed above. Failure to subspeciate members of the *Bacteroides fragilis* group other than *B. fragilis* ssp. *thethaiotaomicron* was not considered an error, since rhamnose and trehalose fermentation, the results of which are required for such subspeciations, were not tested with the micromethod system. Fermentation of these two carbohydrates is tested with conventional media routinely.

RESULTS

Table 1 shows the results obtained with the 18 reference strains by using the micromethod system. The reactions agree quite well with those obtained by conventional methods in our laboratory and those reported by other investigators. (4) However, there was only 33% agreement between the two methods for the nitrate reduction test.

The results obtained with a total of 104 strains, including the 18 reference strains and 86 diagnostic cultures, are shown in Table 2. The

overall agreement between the micromethod and conventional systems was good, 91.2% on the basis of total tests performed. Greater than 90% agreement was obtained with all of the tests, except those for indole production (88.4%), esculin hydrolysis (86.6%), starch fermentation (82.7%), H_2S production (80.8%), and nitrate reduction (77.8%).

Three strains identified as *Clostridium sporogenes* with the conventional technique gave positive tests for indole by the micromethod technique. When the micromethod tests were repeated, with Ehrlich reagent as used in the conventional technique (3) (toothpicks were used to mix the xylene and the liquid culture for the extraction procedure), results were negative. Also, two *B. fragilis* ssp. *thetaiotaomicron*, one *Clostridium cadaveris*, and one *Clostridium* sp. were negative for indole with the micromethod system when tested with Kovac reagent but were positive when retested with Ehrlich reagent.

Differing reactions for esculin hydrolysis were obtained with 14 strains; nine were positive with the conventional technique but negative by the micromethod, and five hydrolyzed esculin in the micromethod system only.

A total of 86 of the 104 strains tested for fermentation of starch showed the same results with both systems. The differing reactions included 17 positive with the micromethod only and one positive with the conventional test only.

Differing results for H_2S production were obtained with 20 organisms. Eleven of these were positive in the conventional medium but negative in the micromethod system, and 9 were positive in the micromethod system only. Twenty-two strains exhibited a positive test for nitrate reduction with the micromethod system but were negative with the conventional test.

Only one strain, a *Bacteroides* sp., showed differing results for urease production with the two systems. Eight strains differed in respect to gelatinase production. In general the carbohydrate fermentation tests (glucose, mannitol, lactose, sucrose, maltose, salicin, glycerol, xylose, arabinose, and mannose) agreed quite well, 90% or better. Seven strains failed to ferment glucose in the micromethod system; one strain each of *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Fusobacterium russii*, *Propionibacterium granulosum*, and *Clostridium* sp., and two *Lactobacillus* sp.

Micromethod system results for fermentation of galactose and fructose and reduction of neutral red are not shown in Table 2. Neutral red was reduced by most of the strains tested; however, some strains of *B. fragilis* were weak

TABLE 1. *Biochemical reactions of eighteen reference strains of anaerobic bacteria with the micromethod system*

Reference strains	Glucose	Mannose	Mannitol	Lactose	Sucrose	Maltose	Salicin	Glycerol	Xylose	Arabinose	Starch	H$_2$S	Esculin	Urea	Indole	Nitrate	Gelatin
Bacteroides clostridiiformis ssp. *clostridiiformis* ATCC 25537	A	A	–	A	A	A	A	(–)	A	A	–	–	+	–	–	+	–
B. fragilis ssp. *distasonis* ATCC 8503	A	A	–	A	A	A	(–)	–	a	–	(a)	–	+	–	–	–	–
B. fragilis ssp. *fragilis* ATCC 25285	A	A	–	A	A	A	–	–	A	–	A	–	+	–	–	(+)	–
B. fragilis ssp. *fragilis* ATCC 23745	A	A	–	A	A	A	–	–	A	–	A	–	+	–	–	(+)	–
B. fragilis ssp. *ovatus* ATCC 8483	A	A	A	A	A	A	(A)	–	A	A	A	–	+	–	(–)	(+)	–
B. fragilis ssp. *thetaiotaomicron* ATCC 8492	A	A	–	A	A	A	A	–	A	A	A	–	+	–	(–)	(+)	–
B. fragilis ssp. *vulgatus* ATCC 8482	A	A	–	A	A	A	–	–	A	A	A	–	–	–	–	(+)	–
B. oralis ATCC 15930	a	a	(a)	a	(–)	a	(a)	–	–	–	a	–	+	–	–	(+)	(+)
Clostridium perfringens BP6K, CDC stock strain	A	A	–	A	A	A	–	A	–	–	A	+	–	–	–	+	+
C. ramosum ATCC 25582	A	A	A	A	A	A	A	–	–	–	–	–	+	–	–	(+)	–
Fusobacterium mortiferum ATCC 25557	A	A	–	A	A	A	A	–	–	–	–	(+)	+	–	–	(+)	–
F. novum ATCC 25550	A	A	–	A	A	A	A	(–)	A	A	A	–	+	–	(–)	–	–
F. nucleatum ATCC 25586	(a)	–	–	–	–	–	–	–	–	–	(a)	+	–	–	+	(+)	–
F. russii ATCC 25533	(–)	–	–	–	–	–	–	–	–	–	–	(+)	–	–	–	(+)	–
Lactobacillus catenaforme ATCC 25536	a	a	–	–	a	a	a	–	–	–	a	–	+	–	–	(+)	–
Propionibacterium acnes CDC 554	A	A	–	–	–	–	–	a	–	–	–	–	–	–	+	+	+
P. acnes CDC 605	a	a	–	–	–	–	–	a	–	–	–	–	–	–	(+)	+	+
P. granulosum ATCC 25564	(–)	(–)	–	–	(–)	–	–	–	–	–	–	–	–	–	–	(+)	–

^{a} Symbols: A, acid (definite yellow color); –, negative reaction; a, weak acid (brownish yellow color); +, positive reaction; ( ), different reaction obtained with conventional biochemical method.

reducers, and some *Peptostreptococcus* sp. and all *Propionibacterium acnes* strains failed to reduce the indicator.

Table 3 shows a comparison of the conventional and micromethod systems in the identification of 86 diagnostic cultures. Only 66% of the strains were presumptively identified with the micromethod system supplemented with microscopy and colonial characteristics only. However, when appropriate supplementary tests and GLC were used with the micromethod system, 73 (85%) of the organisms were identified. There was disagreement between the two

systems in the identification of seven clostridia, three fusobacteria, two bacteroides, and one eubacterium. Differences in the tests for indole accounted for disagreement in the identification of seven strains. Thus when Ehrlich reagent, instead of Kovac reagent, was used with the micromethod system to test for indole, there was agreement in the identification of 80 strains, bringing the overall agreement between the two systems to 93%.

DISCUSSION

With the exception of the tests for NO$_3$

TABLE 2. *Results of conventional and micromethod system tests performed on 104 strains of anaerobic bacteria*[a]

Substrate or test	No. of tests				No. tests in agreement	Tests in agreement (%)
	Micro + Conv −	Micro − Conv +	Micro + Conv +	Micro − Conv −		
Glucose	1	1	91	5	96	92.3
Mannose	3	5	63	33	96	92.3
Mannitol	1	1	13	89	102	98.1
Lactose	2	5	73	24	97	93.3
Sucrose	1	1	33	63	96	92.3
Maltose	2	4	69	29	98	94.2
Salicin	3	5	27	69	96	92.3
Glycerol	5	5	16	78	94	90.3
Xylose	1	1	36	66	102	98.1
Arabinose	1	0	14	89	103	99.0
Starch	17	1	39	47	86	82.7
H₂S	9	11	25	59	84	80.8
Esculin	5	9	57	33	90	86.6
Urea	1	0	1	102	103	99.0
Indole	5	7	14	78	92	88.4
Nitrate	22	1	28	53	81	77.8
Gelatin	3	5	25	71	96	92.3

[a] A total of 1,768 tests were performed; of these 1,612 (91.2%) were in agreement.

reduction, H₂S production, starch fermentation, esculin hydrolysis, and indole production, the agreement between the micromethod and conventional tests was quite good, 90% or better. In some cases the micromethod gave results previously reported for the species in question (3, 4), whereas the conventional method did not. In some instances the reaction in question was known to be a variable one, e.g., glucose fermentation by *F. nucleatum*.

Discrepancies in H₂S production were possibly due to the nature of the substrate used in the micromethod system; it is not known whether the substrate was comparable to the conventional lead acetate medium for detection of H₂S. Poor results were obtained with the micromethod nitrate reduction test. False-positive reactions were obtained more frequently than false-negative reactions, and almost all of the discrepancies were due to the lack of red-color development after addition of zinc powder. These results may also be due to the substrate used in the micromethod system.

Although only 66% of the anaerobes tested could be presumptively identified on the basis of the results with the micromethod system supplemented only with microscopy and colonial characteristics, the percentage of cultures identified was higher (85%) when certain supplementary tests and GLC were performed. Some of the supplementary tests, such as those for catalase, lecithinase, lipase, motility, etc.,

are quite simple to perform. Others, such as the determination of metabolic products by GLC, require equipment that is somewhat expensive. Unfortunately, the micromethod system does not contain all of the tests for speciation or subspeciation, or both, of some organisms according to present classification schemes (4).

Certain modifications of the micromethod system appear to be indicated. For example, the test for reduction of nitrate needs to be improved or possibly eliminated from the system. Tests for fermentation of fructose and galactose are not usually used for identification of anaerobes in this country and could be omitted. On the other hand, tests for fermentation of trehalose and rhamnose should be added to the system to permit subspeciation of the *B. fragilis* group as recently proposed (4). In view of our results, the tests for indole should be performed with Ehrlich reagent, instead of Kovac reagent, after extraction with xylene. Also, attempts should be made to adapt some of the conventional supplementary tests such as those for catalase, lecithinase, lipase, and growth in 20% bile to the micromethod system.

Although we did not systematically study the rapidity of the tests in the micromethod system, many of the biochemical reactions in the micromethod system were essentially complete after 24 h of incubation. This was expected since Kaufman reported that rapid results were obtained with various clostridia when small vol-

TABLE 3. *Comparison of conventional and micromethod systems for identification of 86 diagnostic cultures of anaerobic bacteria*

Microorganism	Conventional	Micromethod plus morphology	Micromethod plus supplementary tests[a] and GLC[b]
Bacteroides fragilis			
ssp. *fragilis*	18	18	18
ssp. *thetaiotaomicron*	2	0	0
Bacteroides sp.........	2	2	2
Fusobacterium			
nucleatum	2	2	2
F. necrophorum	3	1	1
Clostridium perfringens	10	7	10
Clostridium sp.........	11	7	10
C. tertium	5	3	5
C. sporogenes	3	0	0
C. ramosum	2	2	2
C. butyricum	2	1	1
Propionibacterium			
acnes	5	5	5
Lactobacillus sp.	4	0	4
Peptostreptococcus sp.	3	2	3
Miscellaneous[c]	14	7	10
Totals	86	57 (66%)	73 (85%)

[a] See test.

[b] Gas-liquid chromatography.

[c] One strain each of *B. fragilis* ssp. *distasonis*, *B. fragilis* ssp. *vulgatus*, *C. septicum*, *C. sordellii*, *C. tetani*, *C. cadaveris*, *C. paraputrificum*, *C. innocuum*, *P. avidum*, *Propionibacterium* sp., *Bifidobacterium* sp., *Eubacterium lentum*, *F. mortiferum*, and *Peptococcus* sp.

umes of test media and heavy inocula were used (5).

Although we used exclusively an anaerobic glove box in this study, it should be possible to use other techniques for providing anaerobic conditions. In this regard, good results were obtained with a limited number of organisms by incubating the micromethod systems in GasPak jars (BBL), Brewer jars (BBL), and in heat-sealed plastic bags (CEDANCO) using a Gas-Pak hydrogen-CO_2 generator and pallidinized alumina catalyst to remove oxygen (Starr et al., unpublished data).

LITERATURE CITED

1. Aranki, A., S. A. Syed, E. B. Kenney, and R. Freter. 1969. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. Appl. Microbiol. **17**:568–576.
2. Dowell, V. R., Jr. 1972. Comparison of techniques for isolation and identification of anaerobic bacteria. Amer. J. Clin. Nutr. **25**:1335–1343.
3. Dowell, V. R., Jr., and T. M. Hawkins. 1973. Laboratory methods in anaerobic bacteriology. CDC laboratory manual. Center for Disease Control, Atlanta, Ga.
4. Holdeman, L. V., and W. E. C. Moore. 1972. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg, Va.
5. Kaufman, L., and R. H. Weaver. 1960. Rapid methods for the identification of clostridia. J. Bacteriol. **76**:119–125.
6. Smith, P. B., K. M. Tomfohrde, D. L. Rhoden, and A. Balows. 1972. API System: a multitube micromethod for identification of *Enterobacteriaceae*. Appl. Microbiol. **24**:449–452.
7. Thompson, F. S., and V. R. Dowell, Jr., 1971. Identification of acid and alcohol products of anaerobic bacteria by gas liquid chromatography. Center for Disease Control, Atlanta, Ga.
8. Washington, J. A., P. K. W. Yu, and W. J. Martin. 1971. Evaluation of accuracy of multitest micromethod system for identification of *Enterobacteriaceae*. Appl. Microbiol. **22**:267–269.