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Rapid Fermentation Testing of Anaerobic Bacteria

PAUL C. SCHRECKENBERGER[1]* AND DONNA J. BLAZEVIC

Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455

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Rapid tests for glucose, maltose, lactose, sucrose, and starch fermentation were performed on 112 strains of anaerobic bacteria. The tests were incubated under aerobic conditions, and results were read within 4 h. An overall correlation of 89% was achieved between the rapid tests and the Virginia Polytechnic Institute method.

In a previous paper (7), we described a series of rapid biochemical tests for use with anaerobic bacteria. These tests were incubated in an aerobic environment, and the results could be read within 4 h. The tests utilized a large inoculum and relied upon preformed bacterial enzymes that reacted with a small amount of substrate to produce the desired end product. The present paper expands this concept to include the rapid detection of carbohydrate fermentation by anaerobic bacteria.

MATERIALS AND METHODS

Test organisms. All anaerobic bacteria used in the study were recent laboratory isolates saved in chopped meat broth at room temperature in the dark. All organisms had been previously identified by the Virginia Polytechnic Institute (VPI) methodology (3). There were 112 organisms used in the study, consisting of: 1 *Arachnia*, 10 *Bacteroides fragilis*, 10 *Bacteroides* species, 10 *Bifidobacterium*, 10 *Clostridium perfringens*, 10 *Clostridium* species, 10 *Eubacterium*, 6 *Fusobacterium*, 5 *Lactobacillus*, 10 *Peptococcus*, 10 *Peptostreptococcus*, 10 *Propionibacterium*, and 10 *Veillonella*.

Growth media. All prereduced media were purchased from Scott Laboratories (Fiskeville, R.I.) and contained hemin and vitamin K. The media were inoculated using the VPI Anaerobic Culture System (Bellco). Prior to testing, each strain was subcultured to a fresh blood agar plate prepared with Trypticase soy agar, 5% sheep blood, and 1% hemin-vitamin K solution (Scott Laboratories). The plates were incubated anaerobically in a vented GasPak jar evacuated and filled three times with 90% CO_2 and 10% H_2. If the culture was pure, a single well-isolated colony was picked and transferred to prereduced peptone-yeast-glucose broth or chopped meat-glucose broth. All further subculturing was carried out from this broth. In the case of the rapid tests, a fresh blood agar plate supplemented with hemin and vitamin K was inoculated and incubated anaerobically for 24 to 48 h and was the source of inoculum for all the rapid tests.

[1] Present address: Curriculum in Medical Laboratory Sciences, University of Illinois Medical Center, Chicago, Ill. 60612.

Conventional tests. Prereduced peptone-yeast-carbohydrate broths for glucose, maltose, sucrose, lactose, and starch were used as the conventional tests for determining carbohydrate fermentation. The tests were performed by the procedures outlined in the VPI manual (3). Each broth was inoculated with 4 drops of an actively growing culture from either peptone-yeast-glucose or chopped meat-glucose broth. Peptone-yeast broth without carbohydrate was included with each set of peptone-yeast-carbohydrate broths that was inoculated. Acid production from carbohydrate fermentation was determined by measuring the pH of the broth cultures after 7 days of incubation at 35 C. The criteria used for differentiating between weak and strong acid production was that established in the VPI manual; namely, pH 5.5 to 6.0 equals weak acid production, and pH below 5.5 equals strong acid production. However, if the peptone-yeast broth without carbohydrate had a final pH below 6.0, then 0.2 was subtracted from the final reading to determine the cutoff for a weak acid reaction, and 0.4 was subtracted to determine the cutoff for strong acid production.

Preparation of rapid tests. Rapid fermentation tests for glucose, maltose, sucrose, lactose, and starch were prepared by adding 1.0 g of the appropriate carbohydrate to a buffer-salt solution consisting of dipotassium phosphate (0.04 g), potassium dihydrogen phosphate (0.01 g), potassium chloride (0.8 g), bromthymol blue (0.2 ml of 1% aqueous solution), and distilled water (100 ml). A fermentation base medium was also prepared and consisted of buffer-salt solution without the addition of any carbohydrate. All the fermentation media were adjusted to a pH 7.2, dispensed in 0.5-ml aliquots, and frozen at −20 C until used.

Inoculation and incubation of the rapid tests. All rapid tests were performed by using a sterile loop (3 mm in diameter) and scraping up a loopful of growth from the surface of the agar medium and inoculating directly into the substrate that had been preheated to 35 to 37 C. All tests were incubated aerobically at 35 to 37 C and read after 4 h of incubation.

Interpretation of rapid tests. Carbohydrate fermentation was demonstrated by a change in the color of the indicator from blue (at the starting pH of 7.2) to green (that was interpreted as weakly acid) to yellow (that was considered acid, indicating fermen-

tation). A blue color at the end of 4 h was interpreted as negative. Questionable reactions were compared with the fermentation base medium, which was inoculated with the same organism but contained no carbohydrate. Fresh bromthymol blue solution (0.5 ml, adjusted with 0.1 N NaOH to give a deep-blue color) was added to some tubes at the end of incubation to aid in the interpretation of questionable reactions.

All media and reagents were checked with positive and negative controls to ensure their accuracy.

RESULTS

Table 1 shows the correlation between the rapid and conventional tests for carbohydrate fermentation. A total of 560 rapid tests were run on 112 organisms utilizing five different substrates. A total of 498, or 89%, of the tests were in complete agreement with the conventional tests. Thirty-eight of the rapid tests (7%) appeared to be either less sensitive or falsely negative when compared to the conventional tests, whereas the remaining 24 tests (4%) appeared to be more sensitive or falsely positive by comparison. Lactose gave the highest correlation (94%) of the substrates tested, with sucrose (90%), starch (88%), and maltose (87%) showing decreasing agreement.

Seventy-eight of the 112 organisms tested (70%) showed complete agreement between the rapid and conventional tests. Table 2 lists those organisms which accounted for the 62 discrepancies. The bifidobacteria were responsible for the highest number of discrepancies between the conventional and rapid methods, accounting for nearly 27% of the disagreements noted. In each case the discrepancy was due to a weakly acidic rapid test, whereas strong acid was produced in the conventional method. In fact, 17 of the 22 tests that resulted in this type of discrepancy were due to five strains of *Bifidobacterium*. There were seven strains of *Clostridium* species that were responsible for 12 discrepancies between the conventional and rapid methods. Four of the discrepancies were due to one strain of *Clostridium septicum* that strongly fermented glucose, maltose, and lactose and weakly fermented sucrose but failed to produce any acid in the rapid test system. Of the eight discrepancies produced by strains of *Peptostreptococcus*, three were attributed to one strain that produced weak acid from maltose in the conventional test but was negative in the rapid test. This strain also was negative for acid production from sucrose and starch in the conventional test but was weakly positive in the rapid test. Two strains of *Eubacterium* resulted in seven discrepancies. Both strains produced acid from glucose and maltose with the rapid test but were negative with the conventional test. One strain produced acid from sucrose, and the other strain produced weak acid, but both failed to ferment sucrose in the conventional test. One strain produced acid from starch in the rapid test but was negative in the conventional method. In summary, of the 112 organisms tested for carbohydrate fermentation, 9 organisms, including 5 *Bifidobacterium*, 1 *Clostridium*, 1 *Peptostreptococcus*, and 2 *Eubacterium*, accounted for 31 (50%) of the total number of discrepancies noted.

Reproducibility of the fermentation reactions was at times a problem. One strain of *Clostridium* species produced acid from starch in the conventional test but was negative for starch fermentation when the test was repeated. Another strain of *Clostridium* that first produced acid from lactose in the conventional test was negative when repeated. Three strains of *Propionibacterium* were negative for glucose fermentation with the conventional tests. When the tests were repeated, one of the negative strains became positive, another produced weak acid, and the third strain still remained negative. Two other strains of *Propionibacte-*

TABLE 1. *Results of conventional and rapid fermentation tests[a] performed on 112 strains of anaerobic bacteria*

Test or substrate	No. of tests[b]									No. of tests in agreement	% Tests in agreement
	a a	w w	– –	w a	– a	– w	a w	a –	w –		
Lactose	46	1	58	5	1	0	1	0	0	105	94
Sucrose	48	1	52	5	1	2	0	1	2	101	90
Starch	29	1	69	3	0	5	0	2	4	98	88
Glucose	62	10	25	4	1	1	5	4	0	97	87
Maltose	44	4	49	5	1	4	2	3	0	97	87
Total	228	17	253	22	4	12	8	10	6	498	89

[a] Rapid tests were incubated aerobically.

[b] a, Strong acid; w, weak acid, –, negative reaction. Top row of symbols is for the rapid tests; bottom row of symbols is for the conventional tests.

TABLE 2. *Organisms showing discrepancies between rapid and conventional fermentation tests*

Organism[a]	No. of organisms[b]																														Total
	Glucose						Maltose						Sucrose						Lactose						Starch						
	w	–	–	a	a	w	w	–	–	a	a	w	w	–	–	a	a	w	w	–	–	a	a	w	w	–	–	a	a	w	
	a	a	w	w	–	–	a	a	w	w	–	–	a	a	w	w	–	–	a	a	w	w	–	–	a	a	w	w	–	–	
Bifidobacterium (5)	3						5						4						5												17
Clostridium species (7)	1	1	1					1	1				1	1	2					1					1					1	12
Peptostreptococcus (5)									3									1							1					3	8
Eubacterium (2)					2						2						1	1											1		7
Peptococcus (5)					1						1																4				6
Fusobacterium (3)				2																		1							1		4
Lactobacillus (2)				1																							1				2
Propionibacterium (2)				1	1					1																					2
Bacteroides fragilis (2)										1															1						2
Bacteroides species (1)				1																											2
Total (34)	4	1	1	5	4	0	5	1	4	2	3	0	5	1	2	0	1	2	5		0	1			3	0	5	0	2	4	62

[a] Values in parentheses indicate number of strains showing discrepancies.

[b] a, Strong acid; w, weak acid; –, negative reaction. Top row of symbols is for the rapid tests; bottom row of symbols is for the conventional tests.

rium produced weak acid from glucose the first time the VPI test was run but produced strong acid when the test was repeated. All five strains of *Propionibacterium* mentioned were positive for glucose fermentation with the rapid test, both initially and upon repetition. Three strains of *Peptostreptococcus* were negative for glucose fermentation with the conventional test but were weakly positive when the test was repeated. The same three strains initially produced weak acid from starch but were negative when the conventional test was repeated. One strain of *Eubacterium* was negative for glucose fermentation with the conventional test but was weakly positive when the test was repeated. One strain of *Fusobacterium* that was negative for all the carbohydrates tested with the conventional tests was positive for glucose, maltose, and sucrose fermentation when the tests were repeated. Two strains of *Lactobacillus* that failed to ferment one or more sugars initially produced acid from the same sugars when the conventional tests were repeated. Reproducibility with the rapid fermentation tests was also somewhat of a problem, but no more so than that experienced with the conventional method. When a discrepancy occurred between the first and second runs of a test, the results obtained in the second run were used in the comparison.

The addition of fresh indicator to the rapid fermentation tubes after incubation and the use of a carbohydrate-free fermentation base for comparison greatly aided in the interpretation of the rapid fermentation reactions. Turbidity of the cell suspensions did not affect the color of the indicator solution.

DISCUSSION

The substrate used for the rapid fermentation tests was based on the formula of Kellogg and Turner (4), except that a smaller concentration of carbohydrate was used and bromthymol blue was substituted for phenol red as the indicator. The pK of phenol red is 7.9, with a pH range of 6.8 to 8.4. In the presence of a heavy inoculum the change in color from red to orange was indistinct. Bromthymol blue, with a pK of 7.0 and a pH range of 6.0 to 7.6, provided better discrimination between negative, weak acid, and strong acid reactions. Bromthymol blue is also the indicator recommended by the anaerobe laboratory at the Center for Disease Control for use in their fermentation base medium (2).

Reed and Orr (6) reported that certain anaerobes have the ability to reduce bromthymol blue. These findings were confirmed during the

course of our study, and it was therefore decided to include a fermentation blank with each set of carbohydrates that were inoculated. In cases where the indicator was reduced, as evidenced by a green color in the carbohydrate-free fermentation substrate, the final reactions were read after the addition of fresh indicator to each tube. In some cases the blue color of the original indicator could not be restored in the fermentation blank, due probably to the acidity of some inocula. However, interpretations could be made by comparing the color in the fermentation blank with the color achieved in the tubes containing carbohydrate. In spite of the problems encountered with organisms that were capable of reducing the bromthymol blue dye, it was decided to continue including the indicator with the substrate so that early detection of fermentation reactions could be made with those organisms not capable of reducing the dye. Indeed, many of the strong acid reactions, such as those that occurred with *Bacteroides fragilis* and *Clostridium perfringens*, took place within 2 h, with some reactions occurring as early as 30 min after inoculation. The carbohydrates used in this study (glucose, maltose, sucrose, lactose, and starch) were selected as being representative, and since each showed the same approximate degree of accuracy in our evaluation, it is reasonable to suspect that the equivalent results might be achieved with other carbohydrates as well.

The 1% concentration of carbohydrate used in the rapid tests was selected as a compromise between the 2% solutions used by Kellogg and Turner (4) and the 0.6% concentration used by the Center for Disease Control (2). The final concentration of starch was lowered to 0.5%, since this amount was easier to solubilize and did not adversely affect the outcome of the reactions. Substrate concentration, however, was not considered to be nearly as critical as inoculum size. Brown (1) modified the rapid fermentation procedure of Kellogg and Turner (4) and found results to be much improved when the inoculum size was tripled. Indeed, we found this to be true with our tests, especially with the slower growing anaerobes that produced a smaller amount of inoculum. When testing the more fastidious organisms it was necessary to use half the growth from one plate to obtain a loopful of inoculum sufficient to inoculate one tube of medium. In the case of the hardier organisms, however, this was not necessary, since one plate supplied enough growth to inoculate all five tubes of fermentation media plus the noncarbohydrate-containing fermentation control.

Each of the carbohydrates, with the exception of starch, was dissolved in the buffer-indicator solution without heating. The starch suspension was autoclaved for 15 min to obtain a soluble suspension. Heating the starch did not seem to produce any adverse effect on the substrate.

Although each test in the conventional method was inoculated with an actively growing culture, good growth did not always occur, thus producing results which were inconsistent with the defined characteristics of a given organism. For example, we observed five strains of *Propionibacterium* that failed to produce strong acid from glucose even though glucose fermentation is one of the main identifying characteristics of this organism (3). These strains were well characterized and were identified on the basis of Gram smear morphology and gas chromatography. However, even after repeated testing, one strain remained negative and another was only weakly positive, although all five strains produced strong acid in the rapid procedure.

The inconsistencies reported with the conventional fermentation tests may be partially explained by the fact that the prereduced carbohydrate broths contain, in addition to the particular carbohydrate, a complex of peptones and yeast extracts which may support a variety of reactions. Thus, the end product often becomes a balance between acid production from the breakdown of carbohydrate and alkali production from the breakdown of protein constituents. In the rapid test system multiple reactions were not likely to occur, since the system was devoid of any nutrients except for the single carbohydrate being tested. To aid in the interpretation of the VPI fermentation tests, peptone-yeast broth without carbohydrate was inoculated routinely with each set of carbohydrates. In spite of this precaution, interpretation of the reactions was often difficult. With some organisms the pH of the peptone-yeast broth would drop to as low as 5.5 and was often around 5.8, whereas a nonfermented carbohydrate in the same series might have had a pH above 6.0. Thus the boundary between calling a reaction negative, weak acid, or strong acid often became arbitrary.

In a recent study comparing three procedures for biochemical testing of anaerobic bacteria (5), an 84.6% correlation was obtained when 20 different biochemical tests were performed using the thioglycollate-based media used by the Center for Disease Control and the prereduced anaerobically sterilized media recommended by the Virginia Polytechnic Institute. This evi-

dence suggests that a certain amount of inconsistency is inherent among the conventional methods available for biochemical testing of anaerobic bacteria.

Recently two micromethod multitest system for identification of anaerobes (Minitek [Becton, Dickinson and Co.] and the API Anaerobe System [Analatab Products, Inc.]) have become commercially available. Comparisons of the latter system with conventional identification procedures have found it to be a reliable means of identifying most clinically significant anaerobic isolates (5, 8). The major differences between these systems and the procedure described here is anaerobic incubation for 48 h with the API and Minitek Systems as opposed to aerobic incubation for 4 h with our tests. Thus, although the API and Minitek are microtests and offer the convenience of a multitest system, they do not offer any significant improvement in incubation time, since many of the conventional test results can also be read after 48 h of incubation.

It should be stressed that the distribution of organisms selected for this study was somewhat skewed so that each genus of anaerobic bacteria isolated in our laboratory would be significantly represented in the study. Many of the routinely isolated anaerobes showed good correlation between the rapid and conventional tests. However, many of the infrequently isolated anaerobes showed considerable disagreement. As stated earlier, 8% of the organisms tested, representing five strains of *Bifidobacterium,* one *Clostridium septicum,* one *Peptostreptococcus* species, and two *Eubacterium* species, were responsible for 50% of the discrepancies noted. Yet, these organisms are not the most common isolates in the clinical microbiology laboratory. Indeed, if a comparison between rapid and conventional tests were to be made on a group of organisms selected on the basis of their frequency of isolation in the clinical laboratory, a much higher correlation might be expected.

Although fermentation is considered to be an anaerobic process, the results presented here indicate that fermentation of carbohydrates may be detected by using rapid tests that utilize a large inoculum and a small amount of substrate and which are incubated in an aerobic environment. Even though the anaerobic organisms fail to grow under aerobic conditions, the enzymes responsible for their fermentation pathways apparently continue to function even in the presence of oxygen. Further studies should be guided by the fact that the conventional methods presently in use for biochemical testing of anaerobic bacteria have certain inherent limitations and that ultimately the correct identification of the bacterium itself is the best standard from which to make a comparison.

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