

Rapid Methods for Biochemical Testing of Anaerobic Bacteria

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Rapid biochemical tests for nitrate, indole, gelatin, starch, esculin, and *o*-nitrophenyl- β -D-galactopyranoside were performed on 112 strains of anaerobic bacteria. All tests were incubated under aerobic conditions, and results were recorded within 4 h. The tests for nitrate, indole, and starch showed a 95% or greater correlation when compared to the standard biochemical tests. Tests for esculin and gelatin showed an agreement of 86 and 77%, respectively. PathoTec test strips for nitrate, indole, esculin, *o*-nitrophenyl- β -D-galactopyranoside, Voges-Proskauer, and urease were also tested and showed encouraging results.

The development of simple, rapid, and sensitive biochemical tests for use with the aerobic and facultative bacteria has greatly influenced the speed with which these bacteria may be identified. The main emphasis in the past has been directed toward those bacteria belonging to the family *Enterobacteriaceae*, as evidenced by the many kits which have become available commercially during the past few years (1, 2, 9, 10, 13, 14, 16). The present study was carried out to determine the feasibility of using rapid biochemical tests for the identification of anaerobic bacteria.

MATERIALS AND METHODS

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 (6). 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*.

All pre-reduced media were purchased from Scott Laboratories (Fiskeville, R.I.) and were inoculated using the VPI anaerobic culture system (Belco). Prior to testing, each strain was subcultured to a fresh blood agar plate (BAP) 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% CO₂ and 10% H₂. If the culture was pure, a single well-isolated colony was picked and transferred to pre-reduced peptone yeast glucose (PYG) broth or chopped meat glucose broth. All further subculturing was carried out from this

broth. In the case of the rapid tests, a fresh BAP 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. In addition, about 10% of the positive tests were retested with inoculum taken from pre-reduced supplemented brain heart infusion agar (BHIA) plates with 5% sheep blood added, a roll streak tube, and a PYG broth culture which was 24 to 48 h old. The standard tests used followed the procedures recommended by VPI (6) and were inoculated with 4 drops of an actively growing culture from either PYG or chopped meat glucose broths.

The rapid biochemical tests used were: nitrate reduction, indole production, hydrolysis of gelatin, esculin, and starch, and the ONPG test for measuring hydrolysis of *o*-nitrophenyl- β -D-galactopyranoside (ONPG) by beta galactosidase. All media were dispensed into tubes (13 by 100 mm). The nitrate test was prepared by dissolving 0.9 g of nitrate broth (Difco) in 100 ml of distilled water. The broth was dispensed in 0.5-ml portions into borosilicate tubes and autoclaved. The tubes were stored at 4 C no longer than 2 weeks. Tryptone broth was prepared by dissolving 1.0 g of tryptone broth (Difco) in 100 ml of distilled water and dispensing in 0.5-ml portions. The tubes were autoclaved and frozen at -20 C until used. The starch substrate was prepared by suspending 0.05 g of soluble starch in 100 ml of physiological saline (0.85% NaCl). The suspension was autoclaved, and the resultant solution was dispensed in 0.5-ml portions and stored at 4 C until used. The ONPG broth was made according to the formula of Cowan and Steel (4), dispensed in 0.5-ml portions, and frozen at -20 C. Esculin hydrolysis was determined by using 0.5 ml of enterococcosel broth (Gibco). The test for gelatin hydrolysis was performed on pieces of undeveloped X-ray film which had been cut into small strips (approximately 0.5 by 5.0 mm). The strips were placed into a 0.5-ml saline suspension of the test organism. All tests were performed with a sterile loop and by scraping up growth from the surface of the agar medium and inoculating directly into the broth substrate. When inoculum was taken

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from PYG broth, 2 drops of a 24- to 48-h culture were pipetted directly into the rapid test medium. All tests were incubated aerobically at 35 C and read within 4 h. Tests for gelatin hydrolysis which were negative after 4 h were reincubated and read after 24 h.

Reduction of nitrate was detected by adding 1 drop of reagent A (0.8% sulfanilic acid in 5 N acetic acid) and one drop of reagent B (0.5% alpha-naphthylamine in 5 N acetic acid) to the nitrate broth. The development of a red or pink color was considered positive, whereas the absence of color was considered negative. All negative tests were rechecked by adding a pinch of zinc dust to the nitrate broth. The absence of a red or pink color after the addition of zinc was considered positive. Indole production was detected by adding 2 drops of Kovac reagent to the tryptone broth. A positive reaction was denoted by a deep pink color in the surface layer. Starch hydrolysis was detected by adding 1 drop of a 1:4 dilution of Gram iodine solution (Kopeloff modification) to the starch broth. The development of a deep blue to black color was indicative of no starch hydrolysis. Any change in color as compared to an uninoculated control was considered positive. Esculin hydrolysis was indicated by the development of a brown or black color in the substrate broth. No change in color was considered negative. The gelatin tests were observed for the removal of the green gelatin emulsion from the X-ray strips. In a positive test the gelatin is removed, exposing the blue transparent strip. In a negative test the strip remains green. The ONPG tests were observed for the development of a yellow color, which was considered positive. In a negative reaction the substrate remained colorless.

PathoTec strips for nitrate, indole, esculin, urease, Voges-Proskauer (VP), and ONPG were tested with the same 112 organisms. The tests were performed according to the manufacturer's instructions for *Enterobacteriaceae*, including 4 h of incubation in an aerobic environment. Inoculum was taken from a 24- to 48-h anaerobic BAP. About 10% of the tests were performed in triplicate with organisms from BHIA, a roll streak tube, and PYG broth. When the PYG broth was used, 0.3 ml of a 24- to 48-h culture was transferred to a test tube (13 by 100 mm), and a PathoTec strip was added.

To determine the effect of atmosphere on both the rapid biochemical tests and the PathoTec test strips,

about 10% of those organisms giving a positive reaction for a given test were retested simultaneously under aerobic and anaerobic conditions. A double volume of substrate was inoculated with 24- to 48-h growth from an anaerobic BAP. The suspension of bacteria and substrate was then divided equally between two test tubes, and one was incubated aerobically at 35 C and the other anaerobically in an evacuation jar (90% CO₂, 10% H₂) also at 35 C.

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

RESULTS

Table 1 shows the results of the rapid tests compared with the standard tests as performed according to the VPI method. There was a 99% correlation with the indole test. One *Clostridium* species was positive with the standard test but negative with the rapid test. There was a 97% correlation with the test for starch hydrolysis. One *Eubacterium* and two *Lactobacillus* were positive with the standard test and negative with the rapid test. Nitrate gave a 95% correlation; both strains of *Bacteroides* *corrodens*, all three strains of *Eubacterium* *lentum*, and one *Veillonella* were positive with the standard method but were negative with the rapid tests. The rapid esculin test showed an 86% correlation with the standard method. There were 14 organisms which were positive with the standard test and negative with the rapid test. These included one *Bacteroides fragilis*, four *Bifidobacterium*, one *Clostridium perfringens*, five *Clostridium* species, two *Fusobacterium*, and one *Peptostreptococcus*. Two *Clostridium perfringens* were positive with the rapid test but negative with the standard method. Gelatin had the lowest correlation (77%). However, there were 13 instances where the standard test gave a weak positive reaction, and the rapid test was negative. A weak positive reaction in the VPI system is defined as liquefaction in less than one-half the time required for

TABLE 1. Results of standard and rapid tests performed on 112 strains of anaerobic bacteria^a

Test or substrate	No. of tests				No. of tests in agreement	% Tests in agreement
	Rapid + STD -	Rapid - STD +	Rapid + STD +	Rapid - STD -		
Indole	0	1	22	89	111	99
Starch	0	3	28	81	109	97
Nitrate	0	6	31	75	106	95
Esculin	2	14	32	64	96	86
Gelatin ^b	5	21	9	77	86	77

^a Rapid tests were incubated aerobically. STD, standard.

^b Incubation was for 24 h.

an uninoculated control. A weak reaction of this nature is of questionable significance in the classification of anaerobic bacteria, and so if we were to eliminate these 13 organisms from our comparison then 86 of 99 organisms would be in agreement (87%). Of the remaining organisms, three *Bacteroides fragilis*, one *Bacteroides* species, and one *Fusobacterium* were positive with the rapid test but negative with the standard test. One *Bacteroides* species, six *Clostridium perfringens*, and one *Clostridium* species were positive with the standard test and negative with the rapid test. Three strains of *Clostridium* were positive within 4 h with the rapid gelatin test. The remaining positive reactions were obtained after 24 h of incubation.

Table 2 shows the comparison between the PathoTec test strips and the standard method. Indole showed a 96% correlation; three species of *Fusobacterium* and one *Clostridium* species were positive with the standard method and negative with the PathoTec strips. Esculin showed an 89% agreement. There were eleven organisms, including four *Clostridium* species, one *Clostridium perfringens*, three *Bifidobacterium*, one *Peptostreptococcus*, and two *Fusobacterium*, which were positive with the standard test but negative with PathoTec. One *Clostridium perfringens* was positive with PathoTec but negative with the standard method. There were 21 organisms which were falsely negative for nitrate reduction by the PathoTec strips. These included two *Bacteroides corrodens*, two *Propionibacterium*, four *Veillonella*, three *Eubacterium lentum*, and ten *Clostridium perfringens*. In addition to the indole, esculin, and nitrate strips, PathoTec strips for urease, VP, and ONPG were also tested. The urease and VP strips were negative for most of the organisms tested. One strain of *Bacteroides corrodens* was urease positive. The ONPG strips compared in every instance with the rapid ONPG tests used routinely in our laboratory.

Concerning the effect of atmosphere, only the nitrate test seemed to prefer an anaerobic environment. All six organisms which had been falsely negative with the rapid nitrate test when

incubated aerobically were positive when the test was incubated anaerobically. Thirteen of the 21 organisms, which were falsely negative for nitrate reduction by the PathoTec strips when incubated aerobically, were positive under anaerobic conditions. Four strains of *Clostridium perfringens*, two *Bacteroides corrodens*, and two *Eubacterium lentum* remained negative even under anaerobic incubation. Atmosphere seemed to have no effect on the tests for esculin hydrolysis. The tests for indole, starch, gelatin, and ONPG all seemed to prefer an aerobic environment as measured by the intensity and speed of the color development.

Inoculum taken from the BHIA seemed to work slightly better than inoculum taken from the BAP. However, inoculum taken from the roll streak tube or PYG broth was less satisfactory.

DISCUSSION

Rapid testing of anaerobic bacteria is not new and has been reported as far back as 1941 by Reed and Orr (12). Clarke and Cowan (3) used several species of *Clostridium* in their studies of rapid techniques in 1952. Kaufman and Weaver (8) also used several species of *Clostridium* in their report of a combined media for detection of gelatin hydrolysis and indole production. In 1972, Sutter and Carter (15) evaluated four indole-spot tests for use in anaerobic bacteriology.

The results presented here show that rapid testing of anaerobic bacteria is possible, and in most cases can be carried out within 4 h under aerobic conditions. An exception to this rule may be the test for nitrate reductase. Our findings are consistent with others (11, 17) who have reported that nitrate reductase seems to have a diminished activity when exposed to oxygen. On the other hand, indole production has been shown to decrease when facultative organisms are incubated anaerobically (7). Fay and Barry (5) found the opposite to be true when testing for indole production with the obligate anaerobes. The discrepancy with our results may be explained by the fact that in

TABLE 2. Results of standard tests and PathoTec strips performed on 112 strains of anaerobic bacteria

Test or substrate	No. of tests				No. of tests in agreement	% Tests in agreement
	Rapid + STD -	Rapid - STD +	Rapid + STD +	Rapid - STD -		
Indole	0	4	19	89	108	96
Esculin	1	11	35	65	100	89
Nitrate	0	21	16	75	91	81

their system growth was allowed to occur under the anaerobic environment, and thus the increased production of indole may be attributed to an increase in cell mass rather than increased enzyme activity. In our system growth was unlikely, and thus the increased indole production under aerobic conditions can probably be attributed to an increased activity of the enzymes involved.

There was a wide range of colors produced with the test for starch hydrolysis. For this reason each test was compared to a negative control. Various colors, from light blue to brown to yellow, were obtained upon addition of the diluted iodine solution. These colors represent various degrees of starch hydrolysis, but, for the purpose of comparison, any color deviation from the negative control was interpreted as a positive reaction.

A special precaution might be mentioned concerning the test for gelatin hydrolysis. Prior to reading the test, the tubes containing the gelatin strips should be shaken vigorously to remove any loose gelatin which might be adhering to the strips.

Ten of the 21 organisms which showed a false negative reaction with the PathoTec nitrate strips were classified as *Clostridium perfringens*. The false negatives encountered with these and other species of anaerobic bacteria such as *Veillonella*, *Bacteroides corrodens*, and *Eubacterium lentum* suggest that the sensitivity of these strips might have to be adjusted before the nitrate strips can be recommended for use with the anaerobic bacteria.

The effect of inoculum size seems to be consistent with our experience in rapid testing of facultative bacteria. A larger inoculum produces a stronger reaction in a shorter period of time. It is important to note that at all times it is assumed that one is working with a pure culture. Whenever contamination is suspected, a single well-isolated colony must be picked to fresh media to initiate a pure culture. Inoculum taken from a BAP or BHIA generally produced the best results. However, in some instances better growth was achieved on BHIA, and in these instances a stronger reaction was elicited in the rapid test system from this medium. The poorer correlation resulting from the roll tube might be explained by the fact that it was generally difficult to scrape up growth from the roll tube. In the case of the PYG broth, it may be that a 24- to 48-h culture is too old and that enzyme activity has already begun to diminish due to the presence of acids and possibly toxic end products in the culture medium. Also, the

color of the broth may disguise or otherwise compromise the color of the end product in the biochemical reaction. For example, inoculum from PYG broth could not be used to determine the ONPG reaction, since the deep color of the broth obscured the yellow color produced in a positive reaction.

In summary, rapid biochemical testing may be a useful adjunct to the identification of anaerobic bacteria. The fact that these tests can be run under aerobic conditions within 4 h and without the use of elaborate equipment suggests their desirability over the present systems for biochemical testing.

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