Three-Tube Method for Screening Stool Cultures for Salmonella and Shigella

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A three-tube method developed is described as a screening test for non-lactose-fermenting organisms isolated from stool cultures. To evaluate the method, 976 strains of gram-negative bacilli were tested. All strains of Salmonella and Shigella were correctly identified generically.

This report describes a three-tube method for the preliminary identification of gram-negative bacilli. Lysine decarboxylation, motility, indole production, hydrogen sulfide production, phenylalanine deamination, and the methyl red and Voges-Proskauer reactions can be tested by using this method which we have found to be useful for screening stool cultures for Salmonella and Shigella.

MATERIALS AND METHODS

The first tube which tests phenylalanine deamination, indole production, and hydrogen sulfide production was made in two steps. In the first step, modified peptone iron agar was made by mixing 20 g of tryptone (Difco), 0.5 g of ferric ammonium citrate (Fisher Scientific Co.), 1.0 g of dipotassium phosphate (Fisher Scientific Co.), and 15 g of agar (Difco) with 1,000 ml of water. Five milliliters of suspension was poured into glass tubes (150 by 18 mm) and autoclaved. The tubes were left in an upright position while the medium solidified. In the second step, phenylalanine iron agar (1) was made by adding 2 g of phenylalanine (Eastman Kodak Co., Rochester, N.Y.), 5 g of peptone (Difco), 3 g of yeast extract (Difco), 0.5 g of ferric ammonium citrate (Fisher Scientific Co.), and 15 g of agar to 1,000 ml of water. After autoclaving, 7 ml of the suspension was poured into the tubes which contained the solidified peptone iron agar, and allowed to harden in a slanted position.

The second tube, which was used for lysine decarboxylation and motility tests, was made by rehydrating 14 g of commercially available lysine decarboxylase media (Difco) in 1,000 ml of water. Two grams of agar was added, and the medium was heated in a boiling-water bath for 30 min. About 8 ml of the medium was poured into glass tubes (125 by 16 mm) and autoclaved. The medium was allowed to solidify in the upright tubes.

The third tube, utilized for the methyl red and Voges-Proskauer reactions, was made by dissolving 5 g of peptone, 5 g of dextrose, 5 g of dipotassium phosphate, and 2 ml of 1 N sodium hydroxide (Fisher Scientific Co.) in 1,000 ml of water. Approximately 8 ml of this solution was poured into glass tubes (125 by 16 mm) and autoclaved.

A single colony was picked from blood or eosin-methylene blue agar and emulsified in 2 ml of fresh beef heart infusion broth. One drop of bacterial suspension was inoculated with a Pasteur pipette into each of the three tubes. The surface of the agar in the first tube was then streaked with the pipette, and the butt was stabbed. The second tube was also stabbed with the Pasteur pipette. The second tube was not sealed; the medium contained 0.2% agar and was semisolid, thus providing in the bottom of the tube sufficient anaerobiosis for the decarboxylation reaction. All tubes inoculated were incubated for 18 to 24 hr at 37 C.

The first tube was colorless before the inoculation of organisms. With deamination of the phenylalanine, the surface of the slant turned dark brown. Production of H2S in the butt resulted in black discoloration. After these results had been recorded, 1 ml of Ehrlich solution was added, and the tube was left in an upright position for 10 min. If indole production has occurred, a pink color can be observed at the junction of the slant and butt.

The second tube was deep purplish in color due to the bromcresol purple indicator. Since the decarboxylation reaction requires anaerobiosis, all reactions were interpreted on the basis of changes in the lower two-thirds of the medium. A positive decarboxylase reaction is indicated by a change from deep purple to light purple. In a negative reaction, the color changes from purple to yellow or does not change at all. Motility is evidenced either by turbidity of the medium or by migration of growth into the medium from the line of inoculation. Methyl red and Voges-Proskauer reactions were performed on the medium in the third tube by using conventional techniques (4).
The results of each of these tests for a variety of gram-negative bacilli are listed in Table 1. Although many organisms can be fully identified with this system, it is impossible to distinguish between *Enterobacter aerogenes*, *E. hafniae*, *E. liquefaciens*, and *Serratia*. Distinctions also cannot be made among *Proteus morganii*, *P. rettgerii*, and *Providencia*, and among nonfermenting organisms such as *Pseudomonas*, *Herellea*, and *Mima*, without performing additional biochemical tests. *Salmonella*, *Shigella*, and other organisms totalling 976 strains of gram-negative bacilli (Table 2) which had been isolated from urine, sputum, stool, wound, blood, and other cultures were inoculated into the three tubes as described above. The tubes were read after overnight incubation by a technician who was not aware of the identity of the organisms. All of the organisms had been identified by using the routine methods of this laboratory (4) in accordance with standard taxonomic systems (1-3, 5). The identification of all strains of *Salmonella* and *Shigella* was confirmed by agglutination with specific antisera.

RESULTS AND DISCUSSION

As summarized in Table 2, all 23 strains of *Shigella* and all 56 strains of *Salmonella* were correctly identified by using the three-tube system. Furthermore, 960 (98.4%) of 976 strains tested were assigned to the correct group by using this method.

In our laboratory, stool cultures are inoculated onto blood agar and eosin-methylene blue agar, and into selenite broth and GN broth. Broths are subcultured onto eosin-methylene blue agar after 18 to 24 hr incubation at 37 C. Non-lactose-fermenting colonies from the eosin-methylene blue agar are agglutinated with polyvalent *Salmonella* and *Shigella* antisera. If agglutination occurs, a full battery of confirmatory biochemical tests are performed. However, when the slide agglutination test is negative, the organisms are inoculated into the three-tube system described in this report. If the reactions are inconsistent with *Salmonella* or *Shigella*, the organisms are not further identified. Biochemically atypical pathogens could conceivably be overlooked with this method although the agglutination test which is performed on all non-lactose-fermenting isolates would detect many of these atypical organisms, and they would be further identified with a full battery of biochemical tests. *Salmonellae* which ferment lactose could not be detected by this method or by most other methods which depend on lactose fermentation as a preliminary screening test. This method seems to provide a realistic approach to the screening of stool cultures for *Salmonella* and *Shigella*, combining accuracy with simplicity.

| Organisms            | Phenylalanine | H₂S | Indole | MR | VP | Lysine | Motility |
|----------------------|---------------|-----|--------|----|----|--------|----------|
| Shigella             | -             | -   | +, +   | +  | -  | -      | -        |
| Escherichia coli     | -             | -   | +      | +  | -  | d      | +, -     |
| Edwardsiella         | -             | +   | +      | +  | -  | +      | +        |
| Salmonella           | -             | +   | -      | -  | +  | +      | +        |
| Arizona              | -             | +   | -      | +  | -  | -      | -        |
| Citrobacter          | -             | +   | -      | +  | -  | +      | -        |
| Klebsiella           | -             | -   | +, -   | +  | +  | +      | +        |
| Enterobacter cloacae | -             | -   | -      | +  | -  | +      | -        |
| E. aerogenes         | -             | -   | -      | +  | -  | +      | -        |
| E. hafniae           | -             | -   | +, -   | +, -| +, -| +, +   | -        |
| E. liquefaciens      | -             | -   | +, -   | +, -| +, -| +, +   | -        |
| Serratia             | -             | -   | -      | +, +| +  | +      | +        |
| Proteus vulgaris     | +             | +   | +      | +  | -  | +      | +        |
| P. mirabilis         | +             | +   | -      | +  | -  | -      | +        |
| P. morganii          | +             | -   | +      | +  | -  | +      | -        |
| P. rettgerii         | +             | -   | +      | +  | -  | +      | -        |
| Providencia          | +             | +   | -      | +  | -  | +      | -        |
| Pseudomonas          | -             | -   | -      | +  | -  | +      | -        |
| Mima polymorpha      | -             | -   | -      | +  | -  | +      | -        |
| Herellea vaginicola  | -             | -   | -      | +  | -  | +      | -        |
| Alcaligenes sp.      | -             | -   | -      | +  | -  | +      | -        |

* Modified from: W. H. Ewing, Enteric Bacteriology Laboratories, National Communicable Disease Center, Atlanta, Ga. 30333. Symbols: + > 90% (+) in 1 to 2 days, - < 90% (-) in 1 to 2 days, d = different biochemical types, +, - = majority of cultures (+), -,+ = majority of cultures (-), NC = no change.
METHOD FOR SCREENING STOOL CULTURES

Table 2. Organisms used in the study

| Organisms            | No. of organisms | Correctly identified by three-tube composite media |
|----------------------|------------------|---------------------------------------------------|
|                      | No. | %   | No. | %   |
| Shigella             | 23  | 2.4 | 23  | 100 |
| Escherichia coli     | 222 | 22.8| 219 | 98.6|
| Edwardsiella         | 1   | 0.1 | 1   | 100 |
| Salmonella           | 56  | 5.7 | 56  | 100 |
| Citrobacter          | 15  | 1.5 | 15  | 100 |
| Klebsiella           | 121 | 12.3| 118 | 97.5|
| Enterobacter cloacae | 98  | 10.0| 98  | 100 |
| E. aerogenes         | 111 | 11.4| 109 | 98.2|
| E. hafniae           |     |     |     |     |
| E. liquefaciens      |     |     |     |     |
| Serratia             |     |     |     |     |
| Proteus vulgaris     | 2   | 0.2 | 2   | 100 |
| P. mirabilis         | 109 | 11.2| 105 | 96.3|
| P. morganii          | 104 | 10.7| 100 | 96.2|
| P. rettgeri          |     |     |     |     |
| Providencia          |     |     |     |     |
| Pseudomonas          | 114 | 11.7| 114 | 100 |
| Herellea             |     |     |     |     |
| Mimae polymorpha    |     |     |     |     |
| **Total**            | 976 | 100 | 960 | 98.4|

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