Two-Tube Test for the Rapid Identification of Prompt Lactose-Fermenting Genera Within the Family Enterobacteriaceae

O. CLOSS

Department of Microbiology, The Haukeland Hospital, The Gade Institute, Bergen, Norway

Received for publication 24 May 1971

One hundred and fifty-nine prompt lactose-fermenting strains of Enterobacteriaceae were tested for H2S production, ornithine decarboxylase activity, motility, and citrate utilization by using two different sets of test media. It was shown that the three tests first mentioned could be carried out in a single tube, the results being as reliable as when each was done separately. Simmons medium was preferred for the detection of citrate utilization. Since these four tests require only two tubes, they are considered more convenient in routine diagnostics. A combined medium for the detection of H2S production and motility is described.

“If we are to identify speedily, which is one of the aims of the diagnostician, we must rely on the determination of selected characters” (7). For the identification of prompt lactose-fermenting strains of the family Enterobacteriaceae (LFE), four characters have been relied upon: H2S production, ornithine decarboxylase activity, motility, and citrate utilization (8; O. Closs and A. Digranes, Acta Pathol. Microbiol. Scand., in press).

As the number of specimens is increasing steadily in most bacteriological laboratories, one would hesitate to introduce any new identification procedure, however limited and however justified, that is more laborious than the one in use. Evaluation of the amount of work connected with identification is particularly pertinent when dealing with the more commonly isolated bacteria, which definitely include the LFE. Our problem has therefore been to find the most convenient and reliable way of performing the test for each of the four characters mentioned. This paper describes a study carried out to determine the accuracy and convenience of an improved set of tests, in comparison with the tests used in a previous study (O. Closs and A. Digranes, Acta Pathol. Microbiol. Scand., in press).

MATERIALS AND METHODS

Bacteria. One hundred and nineteen fresh isolates from routine clinical specimens as well as 41 of the strains previously investigated (O. Closs and A. Digranes, Acta Pathol. Microbiol. Scand., in press) were used. Primary isolation of LFE strains was done on lactose-bromothymol-blue-agar plates after overnight incubation.

Nomenclature and criteria for identification. LFE are divided into four genera: Escherichia, Citrobacter, Enterobacter, and Klebsiella (4, 6). The key to their identification by the H2S production, ornithine decarboxylase activity, motility, and citrate utilization system is shown in Table 1.

Media and reagents. To test for citrate utilization, Koser and Simmons media were prepared according to Cowan and Steel (1). Koser citrate was very lightly inoculated and was checked to ensure that the inoculation had not opalized the medium. After overnight incubation at 37°C, any tube showing opalescence was regarded as positive. Simmons medium was inoculated by streaking the loop once along the surface of the slant. Any change in color from green to blue was regarded as a positive reaction.

To test for ornithine decarboxylase activity (ODA), decarboxylase medium (Difco) with 0.3% agar and 1% ornithine added (7) was inoculated by stabbing once with the loop. If the bottom of the tube remained yellow after 1 day, the reaction was regarded as negative.

To test for H2S production, nutrient agar containing 0.2% lead acetate was inoculated by stabbing several times into the medium near the tube wall. Blackening of the medium along the stabs indicated a positive reaction.

To test for motility, the medium was prepared according to Edwards and Bruner (2). It was inoculated by stabbing once with a loop or straight wire. If the growth spread horizontally from the line of inoculation, the strain was recorded as motile even if the outgrowth occurred only at one or two points.

Combined medium for detection of H2S production and motility. The medium contained 80 g of gelatin (Difco) and 1,000 ml of distilled water. The gelatin
was soaked in hot water for 30 min, and the following were added: peptone (Difco), 10 g; beef extract (Difco), 3 g; agar (Difco), 4 g; NaCl, 5 g; sodium citrate, 2 g; sodium thiosulfate, 0.2 g; ferrous ammonium sulfate, 0.3 g. The medium was then sterilized at 121 C for 30 min. H2S-producing strains make the medium turn black in close correspondence with the growth.

**HOMo tube.** H2S production, ornithine decarboxylase, motility (HOMo) tubes were filled with 1.0 to 1.5 ml of ODA medium, and a layer of petroleum jelly 2 to 3 mm in thickness was poured on top. Care was taken not to get petroleum jelly onto the sides of the tube. After autoclaving for 20 min at 121 C, the medium was cooled, and 2 ml of sterile, warm motility-H2S medium was added by means of an automatic Oxford pipette. The tube was inoculated by a single stab by using a loop or a straight wire as shown in Fig. 1.

All reactions were read after incubation overnight at 37 C.

### RESULTS

One set of four tubes containing, respectively, lead acetate-agar, ODA medium, motility medium, and Koser citrate and another set consisting of the HOMo tube and Simmons citrate were used. The two sets of tubes were run simultaneously in the routine laboratory to decide which set was the easier one to work with. Our technicians found it a significant advantage that the two-tube version was quicker to inoculate and required less space in the racks. They also favored Simmons citrate because the medium was easier than Koser citrate both to inoculate and to read correctly.

The results obtained with the four-tube and the two-tube version are compared in Table 2. As shown, only minor discrepancies were found.

**H2S production.** One strain of *Citrobacter* was negative in the lead acetate medium after 1 day but was positive in the HOMo tube. After 2 days of incubation, the strain became weakly positive in the lead acetate medium. All of the other strains showed identical reactions in the two media.

**ODA.** This test showed the greatest discrepancies between the two methods. In general, the ODA tube tended to make more strains positive than the HOMo tube. Two strains of *Escherichia* and one strain of *Citrobacter* were positive in the former but not in the latter. However, none of the *Klebsiella* strains tested was positive in either tube after 1 day. The most important finding was that three *Enterobacter* strains, which were positive after 1 day in the ODA tube, were negative when tested in the HOMo tube after the same incubation period.

However, instead of producing a purely yellow color like the *Klebsiella* strains, the *Enterobacter* strains made the medium appear more greyish. In the light of this, the true result of the reaction was positive.

### TABLE 2. 
Reactions of the prompt lactose-fermenting genera of *Enterobacteriaceae* in the HOMoC scheme

| Genus      | H | O  | Mo | C  |
|------------|---|----|----|----|
| *Escherichia* | – | d  | d  | –  |
| *Citrobacter* | + | d  | +  | +  |
| *Klebsiella*  | – | –  | –  | +  |
| *Enterobacter* | – | +  | +  | +  |

* H, H2S production; O, ornithine decarboxylase activity; Mo, motility; C, citrate utilization; +, more than 90% positive; –, more than 90% negative; d, variable.

* More than 80% negative.

---

**Fig. 1. HOMo tube.**
could be assessed by further incubation. Two of the strains were incubated further and both became positive after 2 days. By comparison, none of the *Klebsiella* strains became ODA-positive in the HOMo tube when the incubation was prolonged. If it was necessary to test the reaction in the ODA tube, however, it was not possible to do this by prolonging the incubation period, because all strains then became uniformly positive.

**Motility.** Identical results were obtained with all strains, except two strains of *Escherichia*, which were found to be motile in the motility tube but not in the HOMo tube.

**Citrate utilization.** Although three strains of *Escherichia* were read as positive in Koser citrate, none was positive on Simmons agar. The three strains became negative in Koser medium when the inoculation was repeated, too heavy inoculation being the reason why the reaction was positive in the first instance. Strains utilizing citrate slowly, such as some of the *Citrobacter* strains, might easily be recorded as citrate-negative in both media after 1 day. Several strains needed 2 days to produce a clearly positive reaction.

With few exceptions, the identification of the strains according to the HOMoC scheme corresponded perfectly whichever set of tests was used. After 1 day, one H$_2$S-negative *Citrobacter* strain would have been falsely identified as *Enterobacter* if the four-tube version had been used. The three strains of *Escherichia* that were positive in Koser citrate would also have been identified as *Enterobacter*.

**DISCUSSION**

The principles governing the use of particular biochemical reactions for classification of bacteria are their practical value within the group studied, the ease with which the tests can be performed, and their reliability. On the basis of the work of Wolfe and Amsterdam (9) and Closs and Driganes (Acta Pathol. Microbiol. Scand., *in press*), the most useful and reliable characters for the identification of LEF are H$_2$S production to diagnose *Citrobacter*, lack of citrate utilization to diagnose *Escherichia*, and ODA and motility to distinguish between *Klebsiella* and *Enterobacter*.

A comparison between two sets of tests for the determination of these characters has shown highly corresponding results. Simmons and Koser media give nearly identical results. We would, however, regard Simmons medium as the more reliable, since it was easier to inoculate correctly and the reaction was perhaps somewhat easier to read. Strains utilizing citrate slowly, such as some of the *Citrobacter* strains, are difficult to detect after 1 day by both methods. This represented no problem in the present study since the slow citrate utilizers all belonged to the genus *Citrobacter*; being H$_2$S-positive they could not be misidentified as *Escherichia*.

The HOMo tube represents a simple way of reducing the number of tubes the technician has to handle while performing the routine diagnostic procedures. By layering different media on top of each other, separated by a layer of petroleum jelly, several tests can be carried out independently in a single tube. Reactions requiring aerobic conditions, such as the utilization of citrate, may be carried out on a slanted top layer. In our case, we found that nothing was gained by placing Simmons agar on top of the other two media as the tube then became more difficult to inoculate.

The combined H$_2$S-motility medium has given results that correspond extremely well with the reaction tests separately performed. Gershman (4) described a similar medium containing 0.05%
2,3,5-triphenyltetrazolium chloride. We also tried to add this substance to our medium but found it to be of no advantage as it made the medium become less clear. Initially we tried the combined medium of Hajna, which is referred to by Cowan and Steel (1), but found that too many strains of genera other than Citrobacter became H₂S-positive in this medium, which contains cystine.

When the ODA was carried out in the bottom layer of the HOMo tube, positive reactions tended to develop more slowly. Nonmotile Enterobacter strains might then be confused with strains of Klebsiella. Apparently, the anaerobic conditions in the HOMo tube make the test somewhat less sensitive. On the other hand, the test appeared less reliable when carried out in separate tubes without an air seal. False-positive reactions occurred, and all reactions became positive after prolonged incubation.

Carrying out the H₂S production, ornithine decarboxylase activity, motility, citrate utilization test in two tubes has been shown to give at least as reliable results as in four tubes, as previously described. Being more convenient, the two-tube version appears to be better suited for routine diagnostic work.

LITERATURE CITED
1. Cowan, S. T., and K. J. Steel. 1965. Manual for the identification of medical bacteria, 1st ed. Cambridge University Press, Cambridge.
2. Edwards, P. R., and D. W. Bruner. 1942. Serological identification of Salmonella cultures. Circ. Ky. Agr. Exp. Sta. no. 54.
3. Ewing, W. H., and P. R. Edwards. 1960. The principal divisions and groups of Enterobacteriaceae and their differentiation. Int. Bull. Bacteriol. Nomencl. 10:1-12.
4. Gershman, M. 1963. Modified motility-sulfide medium. J. Bacteriol. 86:1122-1123.
5. Hormaeche, E., and P. R. Edwards. 1960. A proposed genus Enterobacter. Int. Bull. Bacteriol. Nomencl. 10:71-74.
6. Johnson, J. G., L. J. Kunz, W. Barron, and W. H. Ewing. 1966. Biochemical differentiation of the Enterobacteriaceae with the aid of lysine-iron-agar. Appl. Microbiol. 14:212-217.
7. Steel, K. J. 1965. Microbial identification. J. Gen. Microbiol. 40:143-148.
8. Wolfe, M. W., and D. Amsterdam. 1968. New diagnostic system for the identification of lactose-fermenting gram-negative rods. Appl. Microbiol. 16:1528-1531.