Citrate Mannitol Agar Medium for Separating *Escherichia coli* Paracolons from *Shigella*

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A culture medium is described which not only differentiates *Shigella* from *Escherichia coli* but also serves as the basis for an extremely simple test which specifically identifies *E. coli*. A test that will identify an organism as *E. coli* is more useful than one that merely groups this species with other non-*Shigella* organisms. The theoretical basis for specificity of the test for *E. coli* and other uses for the medium are discussed.

Citrate Mannitol Agar development resulted from the chance observation that, in mineral salts medium with a growth-limiting amount of fermentable sugar, *Escherichia coli* isolates produced alkaline reactions from citrate.

Gilvarg and Davis showed the importance of the citric acid cycle in *E. coli* (2). It was demonstrated that *E. coli* and some other organisms are unable to oxidize components of the citric acid cycle because of inability to transport them into the cell (3). However, I was unable to find a published example of another organism in which a second energy source was required before the original substrate could be attacked. After inquiry to Orville Wyss, however, I learned that Bernard D. Davis had made a similar observation with *E. coli*. Growth of *E. coli* under anaerobic conditions with glucose as second carbon source caused induction of a transport system which then allowed cells to metabolize citrate (Davis, personal communication).

Alkaline reactions from citrate are produced only under aerobic conditions. Aerobic attack of citrate in Citrate Mannitol Agar may have resulted from use of a lower carbohydrate concentration, since carbohydrate was required in a concentration which would produce visible growth, yet become growth-limiting, for *E. coli* to produce an alkaline reaction from citrate within 24 hr.

*E. coli* paracolons which ferment lactose slowly or not at all are commonly picked from differential plating media inoculated with stool samples. They have had a high nuisance value because of their frequency and because many tests are required for their cultural separation from other members of the *Enterobacteriaceae*. This paper describes a simple procedure which I believe is diagnostic for *E. coli*. The test is based on the only recognized example among bacteria, where a second energy source is required for induction of a transport system for a structurally different energy source (4). For more than 3 years in our laboratory, this test has invariably proved to be diagnostic among the *Enterobacteriaceae* for *E. coli*.

**MATERIALS AND METHODS**

Citrate Mannitol Agar (Difco) has the following composition (in grams): sodium citrate, 3.0; magnesium sulfate, 0.2; dipotassium phosphate, 0.3; ammonium chloride, 1.0; sodium chloride, 5.0; d-mannitol, 2.0; bromothymol blue, 0.04; cresol red, 0.02; agar, 15.0; and distilled water to 1,000 ml. Unadjusted pH is 6.8.

The ingredients were dissolved in distilled water with magnesium sulfate and potassium phosphate dissolved separately in small amounts of water before addition. This avoided formation of an insoluble magnesium phosphate precipitate. Stock solutions of the indicator dyes were dissolved in 0.01 N NaOH at concentrations of 4 mg/ml for bromothymol blue and 2 mg/ml for cresol red. The medium was boiled to dissolve the agar and tubed in amounts sufficient to make 1.5-inch (3.8 cm) butts and 3-inch (7.6 cm) slants. Tubes were autoclaved for 10 min at a pressure of 15 psi and were slanted. A small loop needle of inoculum from Triple Sugar Iron Agar slants with a *Shigella*-like appearance was routinely used to stab the slants of citrate mannitol medium and, without flaming, to streak the slant of a tube of Simmons Citrate Agar. It is essential that cultures be incubated with loose caps to produce an alkaline reaction.

**RESULTS AND DISCUSSION**

*E. coli* grew overnight on Citrate Mannitol Agar. Unlike other members of the family able to attack citrate, it produced an acid slant before a
pH reversal began to turn the slant alkaline. A blue color, indicating an alkaline reaction, would almost always develop first at the junction at the butt and slant, generally within 24 hr. The alkaline reaction would gradually spread to the tip of the slant. If the citrate was omitted from the medium, the alkaline reaction did not occur. A total of 3 or more days were required before the entire slant became alkaline. However, *E. coli* could be diagnosed approximately 90% of the time within 24 hr. At this time, pH reversal had usually begun at the butt-slant junction. Invariably, once pH reversal began in this area, the entire slant would eventually become alkaline. The difference in appearance between *E. coli* growth on Citrate Mannitol Medium and any other member of the family was so characteristic that inoculation of a Simmons Citrate Agar slant was hardly necessary. However, the latter medium was routinely inoculated for confirmatory evidence that the isolate was *E. coli*.

With other citrate-positive groups in the family, the entire slant uniformly became alkaline on both Citrate Mannitol Agar and Simmons Citrate Agar. None of these organisms ever caused a pH reversal beginning at the junction and migrating to the tip of the slant on Citrate Mannitol Agar as did *E. coli* isolates. *E. coli* isolates did not produce an alkaline reaction on Simmons Citrate Agar.

Citrate Mannitol Agar combines both advantages of two culture media which have been helpful in elimination of *E. coli* paracolons from *Shigella*. First, the selective advantage of ammonia as the sole nitrogen source as with Ammonium Salts Glucose Agar is gained. Second, this agar has the differential value of citrate utilization as with Christensen's Citrate Yeast Extract Agar. Since *E. coli* attacks mannitol as readily as glucose, mannitol was used because of its differential value within the *Enterobacteriaceae*. Ammonia is readily used by *E. coli* and only rarely by *Shigella* as a sole nitrogen source. Citrate is not attacked by *Shigella* under any conditions (1). Citrate is attacked by *E. coli* in Christensen's nonselective medium because it happens to contain growth-limiting amounts of additional energy sources other than the citrate, not because, as is commonly believed, the medium contains organic nitrogen. (Young *E. coli* cells, washed three times in saline, produced typical citrate reactions within 48 hr on sparsely inoculated slants of Citrate Mannitol Agar, even though butts were not stabbed and tubes were inverted to avoid effects of possible autolytic nitrogenous products in syneresis fluid.) The recommended 7-day incubation period for an alkaline reaction by *E. coli* on slants on Christensen's Citrate Agar indicates that energy sources present in addition to citrate are not in optimal concentration and that phosphate concentration is too high.

The phosphate concentration in Citrate Mannitol Agar is considerably less than in such media as Simmons Citrate Agar, Acetate Differential Agar, and Christensen's Citrate Agar. Considerable experimentation showed that, with this concentration, surface growth was not affected, that citrate reversal by *E. coli* was more rapid, and that acid butts were produced faster with mannitol-fermenting organisms. These advantages are related to the low buffering capacity of the medium. The citrate concentration used was the smallest amount required to produce alkaline slants with citrate-using organisms, whether or not they attacked the mannitol.

In addition to its differential uses within the family *Enterobacteriaceae*, the medium often has been of aid in identifying other organisms. Of a 50% sterile aqueous glucose solution, 0.15 ml is allowed to run down the tube opposite the slant before stabbing the butt and streaking the slant. This amount of glucose represents a 1% concentration for a 7.5-ml volume of medium. During the incubation period, a concentration gradient of glucose occurs as this sugar diffuses from a high concentration at the junction into the slant and butt. Growth indicates ability to use ammonia as the sole nitrogen source. Glucose could, of course, be added to Simmons Citrate Agar in a similar manner; however, with this highly buffered medium, reactions are not sharply defined and are delayed considerably.

Fermentative organisms such as *Aeromonas* species produce a yellow butt with a positive citrate reaction on the upper half of the slant. Organisms that produce acid from glucose oxidatively (such as *Pseudomonas aeruginosa* and *Herellea vaginicola*) produce a yellow band at the junction with no change in the butt and a positive citrate reaction on the upper half of the slant. Organisms such as *Alcaligenes dentrificans*, which do not attack sugars, produce a positive citrate reaction with no acid production.

**LITERATURE CITED**