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Isolation of Sucrose Late-Fermenting and Nonfermenting Variants of *Vibrio cholerae* O139 Bengal: Implications for Diagnosis of Cholera

M. ANSARUZZAMAN, M. RAHMAN, A. K. M. G. KIBRIYA, N. A. BHUIYAN, M. S. ISLAM, AND M. JOHN ALBERT*

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

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The sucrose-containing selective medium thiosulfate-citrate-bile salt-sucrose agar missed a sucrose nonfermenting and four sucrose late-fermenting variant strains of *Vibrio cholerae* O139 Bengal from diarrheal stools. These strains were, however, correctly identified as *V. cholerae* O139 on a sucrose-deficient selective medium, taurocholate-tellurite-gelatin agar.

Until recently, *Vibrio cholerae* belonging to serogroup O1 was considered to be the sole agent of cholera. However, a second agent of cholera, namely, *V. cholerae* O139 Bengal, was identified when it caused large epidemics of clinical cholera in the Indian subcontinent from late 1992 through the end of 1993 (1). *V. cholerae* O139 exhibits a striking resemblance to *V. cholerae* O1, biotype El Tor, in its cultural, biochemical, physiological, and genetic properties (1). It can be cultured with the same selective media used for *V. cholerae* O1. The most commonly used selective medium for culturing diarrheal stools from suspected cholera patients is thiosulfate-citrate-bile salt-sucrose (TCBS) agar which contains 2% sucrose. *V. cholerae* ferments sucrose, producing characteristic yellow colonies on TCBS agar, whereas most other medically important vibrios are sucrose negative and produce green colonies on this medium (6). Isolates producing yellow colonies are further studied for the identification of various vibrios, including *V. cholerae* O1 and *V. cholerae* O139. An alternative selective medium that has been in use in the laboratories of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), is taurocholate-tellurite-gelatin agar (TTGA) (7). Since this medium is not commercially available, however, it has not been widely used. Unlike TCBS agar, it does not contain sucrose and is therefore not dependent on sucrose fermentation for recognition of vibrios. However, vibrios produce characteristic gray colonies with a black center. The colonies are also surrounded by a zone of opacity due to the production of gelatinase (for *V. cholerae* O1 and most strains of *V. cholerae* O139). These characteristics of the colonies are evident after 18 to 24 h of incubation in the medium at 37°C.

We have successfully used TTGA for investigation of the recent epidemic of *V. cholerae* O139 diarrhea in Bangladesh. In September 1993, we identified a 9-month-old boy as having cholera due to *V. cholerae* O139 by using TTGA for culturing his stool. However, for the purpose of a separate ongoing study, we subcultured 25 individual colonies resembling vibrios from TTGA to TCBS agar. After 18 h of incubation at 37°C, 24 colonies appeared yellow and one colony appeared green. One yellow colony and the green colony were subcultured onto Kligler's iron agar and incubated for 18 h at 37°C; slide agglutination on the growth was performed with *V. cholerae* O1- and *V. cholerae* O139-specific antisera (ICDDR,B). Both the yellow colony and the green colony agglutinated with the *V. cholerae* O139 antiserum only, thus confirming the earlier diagnosis of *V. cholerae* O139 infection. Both colonies also agglutinated with a *V. cholerae* O139-specific monoclonal antibody (ICL12) generated in our laboratory (8).

The yellow colony and green colony were tested in the API-20E biochemical system (API System, Montalieu-Vercieu, France). The yellow colony resulted in an identification code of 5146124, which corresponded to *V. cholerae*, and the green colony resulted in an identification code of 5144104, which corresponded to *Vibrio mimicus*. Inoculation of the green colony onto TCBS plates with decreased concentrations of sucrose (0.5, 1.0, and 1.5%; with medium prepared from individual ingredients) and incubation at 37°C for 18 to 24 h or inoculation in a conventional tube fermentation test with 1% sucrose and incubation of the test mixture at 37°C for up to 21 days did not result in the fermentation of sucrose. Inocula of both the yellow colony and the green colonies were studied in a battery of 25 biochemical tests (2, 4, 5), and they produced identical biochemical reactions, except that the green colony did not ferment sucrose.

The yellow colony and the green colony were tested for cholera toxin production by the adult rabbit ileal loop assay (10) and the Y1 adrenal tumor cell assay (9). The colonies were grown as shaker cultures in Casamino Acids-yeast extract broth (3) at 37°C for 24 h. Membrane-filtered (0.45-μm-pore-size membrane filter; Millipore Corporation, Bedford, Mass.) supernatants were found positive for cholera toxin (volume/length ratio of ileal loop, >1.0; and rounding of Y1 cells, >50%). The effect of the toxin on Y1 cells was neutralized by rabbit polyclonal antiserum to purified cholera toxin (Sigma, St. Louis, Mo.). Both colonies also generated a 302-bp amplicon by PCR with two primers specific for the *ctxA* gene of the *ctx* operon of *V. cholerae* O1 (5, 13).

The foregoing data thus suggested that the green colony was indeed a sucrose nonfermenting variant of *V. cholerae* O139. However, biochemically it resembled *V. mimicus*. Since *V. mimicus* also produces cholera toxin (6), it can be argued that the green colony was *V. mimicus*. However, this identification is unlikely, since (i) the colony reacted with *V. cholerae* O139-specific polyclonal antibody (titers in the tube test [2] for green and yellow colonies were both 1:2,560) and monoclonal antibody, (ii) antigenic studies have shown that *V. cholerae* O139 does not cross-react with *V. mimicus* (2, 8, 12) (this finding was again confirmed by further screening of 100 clinical isolates of *V. mimicus* with *V. cholerae* O139 antiserum which showed a lack of reactivity), and (iii) the organism was polymyxin B

* Corresponding author. Mailing address: Laboratory Sciences Division, ICDDR,B, GPO Box 128, Dhaka 1000, Bangladesh. Phone: 880 2 600171. Fax: 880 2 883116 or 886050. Electronic mail address: albert%cholera@external.ait.ac.th.

resistant and Voges-Proskauer reaction positive, whereas *V. mimicus* strains display the opposite characteristics (11).

To determine how frequently sucrose nonfermenting variants of *V. cholerae* O139 occur in clinical specimens, 365 selected watery diarrheal stool samples submitted to the clinical laboratory of ICDDR,B, during November-December 1993, were plated simultaneously on both TCBS agar and TTGA. With TTGA alone, 152 cultures of *V. cholerae* O1 and 117 cultures of *V. cholerae* O139 were identified from these specimens. However, with TCBS agar alone, all 152 *V. cholerae* O1 cultures and 113 *V. cholerae* O139 cultures were identified. The four *V. cholerae* O139 cultures that were missed initially on TCBS agar appeared as green colonies on the medium, and there were no yellow colonies. Multiple green colonies from these cultures agglutinated strongly with *V. cholerae* O139-specific polyclonal and monoclonal antibodies. Five randomly picked colonies from each of the corresponding cultures on TTGA were plated on TCBS agar and were incubated at 37°C for 18 to 24 h. All colonies appeared green, but all of them agglutinated with *V. cholerae* O139-specific polyclonal and monoclonal antibodies. Additional incubation of the plates for 48 h did not result in conversion of green colonies into yellow colonies. One colony from each of the original TCBS agar cultures and TTGA cultures and TCBS agar subcultures from the original TTGA cultures was studied for sucrose fermentation in the conventional tube fermentation test with 1% sucrose. Colonies from all these cultures remained negative for sucrose fermentation for up to 48 h but turned positive at 72 h. These colonies were also subcultured consecutively three times on TCBS agar. In all these subcultures, the colonies remained green after 24 h of incubation at 37°C. Also, original colonies stored in T1N1 soft agar (1% Trypticase, 1% NaCl, 0.7% agar [pH 7.4]) for up to a month at room temperature (25°C) and then subcultured on TCBS agar remained green after 24 h of incubation at 37°C. Additional studies suggested that these isolates were polymyxin B resistant and Voges-Proskauer reaction positive. Thus, these isolates were also deemed to be *V. cholerae* O139. Thus, these four strains differed from the first isolate in being late fermenters of sucrose, whereas the first isolate did not ferment sucrose for up to 21 days of incubation.

These data suggest that it is possible to encounter sucrose nonfermenting and late-fermenting strains of *V. cholerae* O139 in clinical specimens and these strains will appear as green colonies on TCBS agar. Reliance on appearance of only yellow colonies will miss these strains on TCBS agar; however, these strains will not be missed on TTGA. Therefore, TTGA is a medium superior to TCBS agar. However, unlike TCBS agar, TTGA is not commercially available, which will preclude its widespread use. Alternatively, if adequate attention is given to green colonies on TCBS agar, which will also identify another important diarrheal pathogen, *Vibrio parahaemolyticus* (a sucrose nonfermenter), the sucrose late-fermenting and nonfermenting variants of *V. cholerae* O139 will not be missed on this medium.

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