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Rapid Detection of *Vibrio cholerae* O139 Bengal from Stool Specimens by PCR

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In a previous study using pure bacterial cultures in a PCR assay, a primer pair corresponding to a unique chromosomal region of *Vibrio cholerae* O139 Bengal generated an amplicon from only *V. cholerae* O139 Bengal. PCR with the same primer pair was used to screen 180 diarrheal stool specimens. All the 67 *V. cholerae* O139 culture-positive stool specimens were positive by PCR, and the remaining specimens, which contained either other recognized enteric pathogens or no pathogens, were all negative by PCR.

Vibrio cholerae O139 Bengal emerged as the second etiologic agent of cholera in the Indian subcontinent in late 1992 (1). After causing epidemics of cholera in the countries of the Indian subcontinent, it spread to several neighboring countries (8, 11). Imported cases of cholera due to *V. cholerae* O139 were also reported in many developed countries (6, 7). *V. cholerae* O139 infection can be diagnosed by conventional bacteriologic techniques, such as culturing stool on a selective medium, followed by biochemical testing of colonies and confirmation by a slide agglutination test with specific antiserum (4). Alternatively, colonies can be identified by hybridization with specific DNA probes (13). However, these methods are superseded by a rapid immunodiagnostic test called Bengal SMART (15), which uses a monoclonal antibody specific for *V. cholerae* O139. The Bengal SMART test has been evaluated with stool specimens only, and a diagnosis is available in approximately 20 min. However, we were interested in the development of a molecular diagnostic test, such as PCR, which can be used for both clinical and environmental monitoring of specimens.

The available data suggest that *V. cholerae* O139 probably arose from an O1 El Tor strain by genetic recombination which conferred a new serotype specificity on the El Tor strain (5). All the genes of the *rfb* complex which encode the O antigen in *V. cholerae* O1 El Tor have been found to be deleted in *V. cholerae* O139 (16). In their place, there is a new chromosomal region which encodes the lipopolysaccharide and capsular polysaccharide in *V. cholerae* O139 (9). The capsular polysaccharide contains a unique sugar, a 3,6-dideoxyhexose called colitose (12). A similar dideoxyhexose, tyvelose, is present in *Salmonella enteritidis* (17). A primer pair complementary to the gene encoding an enzyme involved in the biosynthesis of tyvelose in *S. enteritidis* generated a product of ~720 bp from *V. cholerae* O139 in an arbitrary PCR. The sequence of the PCR product did not show any homology with the previously published sequence for *S. enteritidis* but showed similarities with glycosyltransferases from other bacterial species. Based on the sequence of the PCR product, a new pair of *V. cholerae* O139 primers, O139-1 (5′-GCG TTA TAG GTA TCA TCA AGA GA-3′) and O139-2 (5′-GTC ATT ATT AAA ACT GCT CCA TT-3′), was designed. These primers were found to be specific for detection of *V. cholerae* O139 in a PCR assay, as they produced an amplicon of 417 bp from all tested pure cultures of *V. cholerae* O139 strains but not from pure cultures of other bacteria, including *S. enteritidis* and *V. cholerae* O1 (10). Later studies suggested that these primers also did not produce an amplicon from *Escherichia coli* serotype O157:H7, the causative agent of hemorrhagic colitis and hemolytic uremic syndrome (3). We used these primers in a PCR assay for the direct detection of *V. cholerae* O139 from diarrheal stools, and the results are reported in this communication.

The PCR conditions for stool specimens were initially optimized by using a stool specimen which was culture positive for *V. cholerae* O139. The watery stool was centrifuged at 900 × *g* for 5 min, and the supernatant, after boiling for 5 min, was used as the template for PCR. The concentration of reagents and PCR conditions given below were found to be optimal and were used for screening of all test stools. PCR was performed in a 50-μl volume of a final mixture containing 10 μl of template; 50 mM KCl; 20 mM Tris-HCl (pH 8.4); 2.5 mM $MgCl_2$; 12.5 pmol each of both primers; 100 μM (each) dATP, dCTP, dGTP, and dTTP; and 2.5 U of *Taq* polymerase (Gibco BRL, Gaithersburg, Md.). The conditions used were 5 min at 94°C for initial denaturation of DNA and 40 cycles, each consisting of 30 s at 94°C (denaturation), 30 s at 50°C (annealing), and 1 min at 72°C (extension), with a final round of extension for 10 min at 72°C using an automated DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The amplified fragments were separated by electrophoresis on a 1% agarose gel and visualized under UV light after staining with ethidium bromide. Initially, heated supernatants from undiluted stool specimens prepared as described above were subjected to PCR. When warranted by suspicion of cholera and presence of inhibitory substances, supernatants from 20% stool suspensions in phosphate-buffered saline (PBS), pH 7.3, and further 10-fold serial dilutions of the 20% suspensions in PBS were also tested by PCR. To avoid bias, all tests were done on coded samples. Positive and negative controls were run along with test samples with each batch of PCRs. To find out the detection limit of the PCR assay, aliquots of a *V. cholerae* O139-negative watery stool were spiked with varying dilutions of an 18-h culture of a clinical isolate of *V. cholerae* O139 grown in Luria broth at 37°C. The resulting concentrations of *V. cholerae* in the stool

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TABLE 1. Analysis of diarrheal stools by culture or ELISA for enteric pathogens and by PCR assay for *V. cholerae* O139[a]

Enteric pathogen(s)	No. of stool samples positive by:	
	Culture or ELISA	PCR for *V. cholerae* O139
V. cholerae O139	63	63
V. cholerae O139 + *Shigella flexneri* sp.	1	1
V. cholerae O139 + *V. cholerae* O1 El Tor Ogawa	1	1
V. cholerae O139 + *Salmonella* group B	1	1
V. cholerae O139 + *Aeromonas caviae*	1	1
V. cholerae O1 El Tor Ogawa	29	0
V. cholerae O1 El Tor Inaba	4	0
Vibrio parahaemolyticus	4	0
Aeromonas hydrophila	3	0
Enterotoxigenic *E. coli*	2	0
Edwardsiella tarda	2	0
Campylobacter jejuni	2	0
Rotavirus	4	0
Shigella dysenteriae type 1	5	0
Shigella flexneri spp.	5	0
Rotavirus + *Salmonella* group C_1	2	0
Plesiomonas shigelloides + *Salmonella* group D	3	0
Aeromonas spp. + *Plesiomonas shigelloides*	2	0
Rotavirus + *Aeromonas* spp.	2	0
No pathogen	44	0
Total	180	67

[a] All enteric bacterial pathogens were sought by culture, and rotavirus was sought by enzyme-linked immunosorbent assay (ELISA) (2).

aliquots varied between 10^8 and 10^3 CFU per ml. The aliquots were subjected to PCR as the test samples were.

A total of 180 watery stool specimens were tested (Table 1). Fresh stools were analyzed for enteric pathogens by standard methods as described previously (2). Of the 67 stool specimens from which *V. cholerae* O139 was cultured, 15 were stored at −70°C for 1 to 2 years before PCR was performed and 52 were tested while they were still fresh. The remaining 113 stool specimens which did not contain *V. cholerae* O139 were tested while they were still fresh; 69 of these stool specimens had other recognized pathogens. Of the 67 stool specimens culture positive for *V. cholerae* O139, 4 contained copathogens.

After the initial screening of the undiluted test stool specimens by PCR, the codes were broken. Of the 67 stool specimens positive for *V. cholerae* by culture, 63 were positive by PCR. An example of the analysis of the PCR product by gel electrophoresis is shown in Fig. 1. The four specimens that were culture positive but PCR negative were further tested in the PCR assay using a 1:5 initial dilution in PBS. One specimen became positive at the 1:5 dilution, another became at the 1:10 dilution, and another two became positive at the 1:100 dilution. These specimens obviously had inhibitors which disappeared upon dilution of the samples. Four specimens that were positive when tested undiluted were diluted and tested to find out the dilutions up to which they remained PCR positive. Three specimens remained positive up to a dilution of 10^{-4}. This suggested that the specimens contained high concentrations of *V. cholerae* O139. The experiment in which a *V. cholerae* O139-negative stool was spiked with *V. cholerae* O139 to determine the sensitivity of the PCR assay showed that the detection limit was 10^5 CFU of bacteria per ml, which is same as when pure *V. cholerae* O139 culture was tested (10). Thus, if the results for

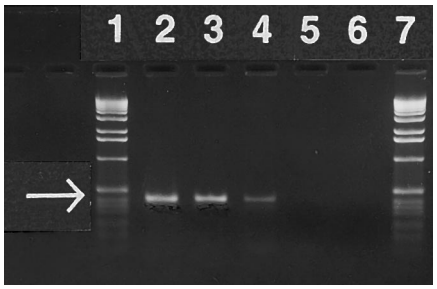

FIG. 1. Agarose gel electrophoresis of PCR-amplified product with *V. cholerae* O139-specific primers. Lanes 1 and 7, 1-kb DNA ladder used as molecular size marker (Gibco BRL); lanes 2 and 3, *V. cholerae* O139 culture-positive stools; lane 4, pure *V. cholerae* O139 culture; and lanes 5 and 6, *V. cholerae* O139 culture-negative stools. Lanes 2, 3, and 4 show the expected 417-bp band, indicated by an arrow.

undiluted stool specimens only are considered, the sensitivity of the PCR assay was 94% (63 of 67 culture-positive specimens positive by the PCR assay), with a specificity of 100%. However, when the results of the specimens tested after diluting the samples are also included, a sensitivity of 100% was achieved. Therefore, for practical purposes, when a suspected cholera specimen is screened, a sample sufficiently diluted to remove potential inhibitors should also be tested before the specimen is declared negative for the pathogen. The PCR assay which was found to be sensitive and specific when pure bacterial cultures were used was thus found to be equally suitable for direct use on stool specimens. Fresh stool samples as well as samples stored frozen for up to 2 years were positive by the PCR assay. This suggests that samples can be frozen and tested later at a convenient time.

Since *V. cholerae* O139 possesses a *ctx* gene identical to that of *V. cholerae* O1 (14), a PCR assay based on the *ctx* gene of *V. cholerae* O1 has been used to detect *V. cholerae* O139 as well (4). However, a diagnosis based solely on a *ctx* gene-based PCR assay is unable to distinguish cholera toxin-positive *V. cholerae* O1 and O139 from cholera toxin-positive non-O1, non-O139 *V. cholerae*. The surface antigen-based PCR assay described in the present report is able to diagnose *V. cholerae* O139 unequivocally. The addition of this PCR assay to the battery of tests already available should prove to be useful for diagnosing *V. cholerae* O139 in routine and outbreak situations and for monitoring environmental contamination with *V. cholerae* O139. In outbreak situations, a quick diagnosis of cholera is essential for mobilization of resources for treatment and containment of the outbreak. The mainstay of therapy in cholera is prompt replacement of lost fluids and electrolytes. Suitable antimicrobial agents have a role in adjunct therapy to reduce the duration of diarrhea, the shedding of cholera organisms, and the volume of fluids and electrolytes needed for therapy. For choosing an appropriate antimicrobial agent however, the specimen still needs to be cultured to determine the susceptibility of the organism.

We are currently working on the development of a similar PCR assay for diagnosis of *V. cholerae* O1 infection and a multiplex PCR assay for detection of either vibrio serogroup. These assays will enable us to diagnose cholera due to either serogroup O1 or serogroup O139.

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