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Detection of Cholera Toxin Gene in Stool Specimens by Polymerase Chain Reaction: Comparison with Bead Enzyme-Linked Immunosorbent Assay and Culture Method for Laboratory Diagnosis of Cholera

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Stool specimens obtained from 123 hospitalized patients with acute secretory diarrhea admitted to the Infectious Diseases Hospital, Calcutta, India, were examined for isolation of *Vibrio cholerae* O1 by direct or enrichment plating on selective media for cholera toxin (CT) by bead enzyme-linked immunosorbent assay (bead-ELISA) and for the CT gene by polymerase chain reaction (PCR). *V. cholerae* O1 was isolated either by direct culture or by enrichment culture from 70 stool specimens, all of which gave positive results by PCR. Eleven specimens which were culture negative and bead-ELISA positive also gave positive results by PCR. In addition, 13 more specimens which were negative by both the culture method and bead-ELISA were positive by PCR. With the combined results of both the culture method and the CT bead-ELISA, a confirmed laboratory diagnosis of cholera could be made from 81 stool specimens, while the combined results of the three methods, including PCR, yielded a positive result for 94 specimens examined. From these data, we conclude that PCR provides a more sensitive and specific assay for rapid diagnosis of cholera than currently available methods.

In recent years, there has been a concerted effort to reduce the turn-around time for the laboratory diagnosis of diarrheal etiologies. Most, if not all, the effort has centered around isolation of the etiologic agent followed by the same-day identification of the pathogen by manual or automated identification systems. Attempts have been aimed at detecting the causal agent or some signature antigen released by the causal agent directly in stools of diarrheal patients in order to obviate bacterial culture and thereby expedite laboratory diagnosis (2–4). A recent evaluation of a highly sensitive and simple bead enzyme-linked immunosorbent assay (bead-ELISA) for detection of cholera toxin (CT) in our laboratory showed that this assay could successfully detect CT directly in 84.7% of the culture-positive cholera stool specimens (5), thereby enabling a potential diagnosis of cholera to be made within 4 h of receipt of the specimen at the laboratory. However, what concerned us was the inability of the bead-ELISA to detect CT in 15.3% of the culture-positive stool specimens, which indicated the need for a more sensitive diagnostic technique.

Polymerase chain reaction (PCR) was subsequently exploited to provide a more sensitive and specific diagnosis of cholera. A set of oligonucleotide primers were developed, and amplification conditions for PCR to detect the CT gene of *Vibrio cholerae* O1 were optimized; the detection limit of the PCR was determined to be 1 pg of chromosomal DNA or broth culture containing three viable cells (7). In a preliminary study using 25 stool specimens, it appeared that PCR could successfully detect the CT gene directly in rice-watery stools of cholera patients (7). The present study is a continuation of our previous efforts and was conducted to compare the efficacy of culture methods (direct and enrichment) for detection of *V. cholerae* O1 with that of the bead-ELISA for detection of CT and that of PCR for detection of the CT gene directly in stool specimens of hospitalized patients with acute secretory diarrhea.

One hundred twenty-three stool specimens from patients with acute secretory diarrhea who had not received any antibiotic therapy prior to admission to the Infectious Diseases Hospital, Calcutta, India, were included in this study. Most of the patients included in this study had some to severe dehydration. The mean (± standard deviation) age of the patients was 21.4 (±16.3) years and the male/female ratio was 8:1. All the patients with severe dehydration were rehydrated with intravenous Ringer's lactate solution and then given oral rehydration by World Health Organization formula. Some dehydrated patients could be rehydrated effectively with oral rehydration salts only. Stool specimens were collected in sterile McCartney bottles upon admission of patients by inserting a sterile catheter into the patient's rectum, and the samples were transported to the laboratory within 1 h. On receipt at the laboratory, a portion of the specimen was immediately processed for bacteriological examination while another aliquot of 1 ml was heated at 95°C for 5 min and kept frozen at −20°C till used for the PCR assay. The period of storage of stool samples prior to the PCR assay varied between 2 and 4 months. The remaining portion of the stool specimen was transferred into a fresh sterile screw-capped tube and centrifuged at 8,000 × *g* for 15 min at 4°C, and the clear supernatant was stored at −20°C till used for the bead-ELISA.

To isolate *V. cholerae*, each specimen was examined by

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TABLE 1. Combined results of culture method, bead-ELISA, and PCR for the laboratory diagnosis of cholera

Possible combination of results[a]			No. of stool specimens with indicated result
Culture method	Bead-ELISA	PCR	
+	+	+	58
+	−	+	12
−	+	+	11
−	−	+	13
−	−	−	29

[a] +, positive; −, negative.

direct plating on thiosulfate citrate bile salts sucrose agar (TCBS) (Eiken Co., Tokyo, Japan) and by enrichment in alkaline peptone water (1% peptone, 1% NaCl [pH 8.5]) for 18 h at 37°C followed by plating on TCBS. All the stool specimens were concurrently examined for *Salmonella*, *Shigella*, and *Campylobacter* spp. and for other pathogenic vibrios by standard techniques (12). In addition, *Escherichia coli* strains from each specimen were individually investigated for their abilities to produce heat-labile toxin (LT) by the rapid GM1-ELISA (8) and for heat-stable toxin by the suckling mouse assay. Serogrouping for enteropathogenic *E. coli* was performed on all strains with commercially prepared antisera (Wellcome Reagents Limited, Beckenham, United Kingdom) by the slide agglutination test. The results were confirmed by performing tube agglutination with heat-killed bacterial suspension. PCR to amplify a 302-bp DNA fragment from the *V. cholerae* O1 CT gene was performed as described previously (7). Initially, only neat stool samples were examined by PCR assay. Stool samples which were negative by PCR assay were reexamined by diluting the sample 10 and 100 times with 10 mM Tris-HCl (pH 8.5)–1 mM EDTA. When required, identification of the amplified PCR product was achieved by Southern hybridization with a CT-specific oligonucleotide probe, as described previously (7). The procedure to perform the bead-ELISA to detect CT has been described previously (9).

The results of the isolation of *V. cholerae* O1 by either direct or enrichment culture, by the detection of free fecal CT by bead-ELISA, and by the detection of the CT gene by PCR in the 123 stool specimens are summarized in Table 1. Fifty-eight stool specimens yielded positive results by all three methods. Twelve stools from which a positive *V. cholerae* O1 isolation was made were negative for CT, while in 11 culture-negative stool specimens, CT could be detected by bead-ELISA. The 12 culture-positive bead-ELISA-negative and the 11 culture-negative bead-ELISA-positive specimens were positive by PCR. With the combined results of both the culture method and the CT bead-ELISA, a confirmed laboratory diagnosis of cholera could be made from 81 stool specimens. In addition to these 81 specimens, the PCR detected an additional 13 stool specimens which contained the CT gene. LT-producing enterotoxigenic *E. coli* (ETEC) was isolated from 1 of these 13 stool specimens, but the isolated ETEC strain did not give a 302-bp band by PCR, indicating that the stool specimen contained the CT gene.

Twenty-nine stool specimens were negative by all three tests. In these 29 PCR-negative stool specimens, *V. cholerae* non-O1, heat-stable toxin (ST)-producing ETEC, and ST-LT-producing ETEC and enteropathogenic *E. coli* were isolated from one, one, two, and one patients, respectively. These isolates did not give the 302-bp band by PCR. In the other 24 PCR-negative stool specimens, a bacterial etiology could not be assigned. These 29 PCR-negative specimens formed the in-built controls, showing that PCR is specific to the CT gene and amplifies only the CT gene when it is present in the specimens.

Of 94 PCR-positive specimens, 70 gave a positive result with undiluted stools, while 17 and 7 of the specimens gave a positive result only after 10- and 100-fold dilutions of the stool specimens, respectively, indicating the existence of inhibitor(s) of the PCR. The need to dilute stool specimens to obtain a positive result did not correlate with culture isolation results or with detection of CT in the stool specimens.

Cholera has assumed renewed global significance with the outbreaks of epidemics in vast areas of the South American continent for the first time in this century (10). For laboratory diagnosis of cholera, culture on selective media, especially after enrichment, is considered the "gold standard," although the sensitivity of the culture method is not 100%, as is evident in this study. The reason why 24 of the PCR-positive specimens which were negative for isolation of *V. cholerae* O1 in spite of enrichment is intriguing, because every specimen evaluated in this study was plated and introduced into an enrichment broth within 2 h of collection. It appears that there is a rapid loss in viability of cells in stools per se, possibly mediated by rapid changes in pH or some such hitherto unidentified factor(s).

The argument that some of the 24 PCR-positive culture-negative stool specimens gave false-positive results could be legitimately raised. In 11 of these specimens, however, the PCR result was not false positive, because the specimens were also positive for CT by the bead-ELISA. Additionally, to eliminate any doubt, the 13 PCR-positive culture-negative bead-ELISA-negative stool specimens were confirmed to give true positive results by Southern blot hybridization. The possibility of amplifying the LT gene of ETEC is not the case, as the selection of the amplimers, while standardizing the PCR assay, was made such that corresponding LT sequences were not amplified and its specificity has already been demonstrated (7).

It appears that in a few of the stool specimens some inhibitory factors which interfere with the performance of the PCR are present. Although several methods have been reported to remove such inhibitors (1, 11), it is shown in this experiment that suitable dilution of the specimens can eliminate such inhibitors; thus, 17 and 7 of the 94 PCR-positive specimens gave positive results only after 10- and 100-fold dilutions, respectively, of the specimens. The sensitivity would be reduced by dilution, but present results suggest that even 100-fold did not affect the sensitivity of PCR to detect the CT gene. We are still in the process of determining what this factor(s) may be. What was most encouraging was that the quality of template DNA in the boiled stool specimens remained suitable for PCR assay for several months; stool samples were stored for periods ranging between 2 and 4 months before being examined by PCR assay.

This study suggests that rapid diagnostic techniques (bead-ELISA and PCR) are sensitive and reasonably specific for use in an area endemic for cholera. These techniques would be less worthwhile in a low-frequency area for *V. cholerae* infections. Rapid tests for the diagnosis of cholera cannot replace bacterial isolation, because of the obligatory need to have the bacterial culture for determination of biotype, serotype, and antimicrobial susceptibility patterns. However, when used in conjunction with culture methods, the rapid tests can provide a significant increase in positive results, thereby diminishing the false-negative results. However, in some situations, PCR or bead-ELISA can be sub-

stituted for bacterial isolation as a diagnostic tool. A classic example was seen when we recently identified an outbreak of cholera which occurred on a passenger ship, despite our being unable to isolate *V. cholerae* O1 from the stool specimens of the passengers, by demonstrating the presence of anti-CT immunoglobulin G-absorbable fecal CT in the stools by the bead-ELISA (6). PCR can similarly be applied to culture-negative and/or bead-ELISA-negative stools to diagnose the disease in question.

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