Direct Detection of *Vibrio cholerae* in Stool Samples

PABLO VARELA,¹ GUIDO D. POLLEVICK,¹ MARTA RIVAS,² ISABEL CHINEN,² NORMA BINSZTEIN,²
ALBERTO C. C. FRASCH,^{3*} AND RODOLFO A. UGALDE³

Tecnología Genética, 1405 Buenos Aires,¹ Instituto Nacional de Microbiología "Carlos G. Malbrán," 1281 Buenos Aires,² and Instituto de Investigaciones Bioquímicas, Fundación Campomar, 1405 Buenos Aires,³ Argentina

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A direct method to detect *Vibrio cholerae* in stool samples was developed by using a PCR procedure that did not require a DNA purification step. Dilution (1/100) of stool samples prevented inhibition of the reaction by contaminants, and two consecutive PCRs, the second one with a nested primer, achieved the desired sensitivity. Comparison of the results obtained from stool swab samples processed by the two-step PCR and by an enzyme-linked immunosorbent assay using GM1 as the capture molecule showed that the former is more sensitive and gave positive results even when *V. cholerae* was not culturable or dead.

Cholera is a diarrheal disease caused by *Vibrio cholerae*, a bacterium that produces a potent toxin that causes alterations in the digestive tract (4). Since severe, watery diarrhea and epidemics were found to be always associated with the isolation of toxin-producing strains, the identification of cholera toxin (CT) production is an important step in the diagnosis of cholera. Toxin production does not correlate with serotype, since some strains of *V. cholerae* O1 may not produce CT (7) and *V. cholerae* non-O1 may be enterotoxigenic (1). The CT locus is a single transcription unit made up of sequences encoding toxin subunits A (*ctxA*) and B (*ctxB*) (5). Subunit A is responsible for the toxic activity of CT, and subunit B is involved in binding of the toxin to the ganglioside GM1 of the target cell (5).

To determine whether *V. cholerae* isolates are toxigenic, immunological tests for the detection of CT are used: a latex agglutination test (VET-RPLA; Denka Seiken, Tokyo, Japan) and an enzyme-linked immunosorbent assay (ELISA) using GM1 ganglioside receptor as the capture molecule of the toxin (GM1-ELISA) are the most commonly used procedures. These methods have some disadvantages, namely, low sensitivity (VET-RPLA) and the requirement of 2 to 3 days to obtain the results (GM1-ELISA). A recent study reports the use of an enzyme-labeled oligonucleotide probe to detect the CT gene. This method requires the previous isolation of suspect colonies from *V. cholerae* selective plating medium (18). Although this assay is more satisfactory for application in clinical laboratories than other hybridization tests, which use a radioisotope-labeled DNA fragment probe, its low sensitivity makes it impractical for field applications (18).

PCR allows detection in a few hours of organisms present in low numbers by amplifying specific DNA segments. In the case of *V. cholerae*, specific primers for amplifying *ctxA* or *ctxB* genes were used (3, 15). We have previously demonstrated that 43 strains of *V. cholerae* isolated in Argentina can be identified by PCR (16). In the present study, we developed an assay to directly detect toxigenic *V. cholerae* by PCR in stool specimens from patients with no previous cultures. Inhibition of the PCR by substances present in the samples was prevented by diluting the samples and applying a nested PCR protocol. Results

obtained by nested PCR are compared with those obtained by GM1-ELISA.

MATERIALS AND METHODS

Specimens. A total of 62 stool specimens, 46 from Salta and 16 from Jujuy provinces, were collected by culturing rectal swabs from patients with clinical symptoms of cholera diarrheal disease. Before the 62 samples were shipped, they were tested at the regional laboratories and found to be positive for *V. cholerae* O1 by standard methods (9). The 46 swabs from the Salta province were maintained in Cary Blair medium (Difco Laboratories, Detroit, Mich.) and the 16 samples from the Jujuy province were maintained in gelatin medium (1% NaCl, 0.5% K₂HPO₄, 3% gelatin, 0.7% agar) (12). All samples were kept at room temperature, until their arrival at the Instituto Nacional de Microbiología "Carlos G. Malbrán." The transit time from collection of stools to processing by PCR and GM1-ELISA ranged from 5 to 40 days.

Bacteriological and enterotoxin assays. Stool samples were enriched by culturing in alkaline peptone water (APW) (1% Bacto Peptone, 0.5% NaCl, 0.07% Na₂CO₃, 0.01% KNO₂; pH 8.6) for 4 h at 37°C. Grown cultures were plated on thiosulfate-citrate-bile-sucrose agar (TCBS) (Difco). Biochemical and serological characterizations of suspicious colonies were conducted by standard techniques (2, 9). Enterotoxigenicity analysis was done by the GM1-ELISA (13) (standard ELISA). Samples (200 µl) of APW cultures were transferred to 10-ml portions of AKI medium (6) and incubated for 18 h at 37°C. Aliquots were analyzed by GM1-ELISA for CT detection (quick ELISA).

Direct application of PCR to stool samples. Rectal swabs were squeezed in a microcentrifuge tube, boiled for 5 min, and centrifuged at 16,000 × g for 5 min to remove debris. PCR assays were carried out with 2-µl portions of supernatants either directly or diluted 1/100. Purification of genomic DNA was carried out as described elsewhere (14). Oligonucleotides homologous to a region spanning *ctxA* and *ctxB* cistrons (external primers) were designed. They differ in sequence from those of the heat-labile toxin of enterotoxigenic *Escherichia coli* (11). The sequences of the primers are 5' GTGGAAT GCTCCAAGATCATCG 3' (external forward primer, positions 1129 to 1151) and 5' ATTGCGGCAATCGCATGAG GCGT 3' (external reverse primer, positions 1625 to 1647). A third internal oligonucleotide (internal reverse primer) was designed to perform a second PCR amplification together with the external forward primer (heminested PCR). The sequence

* Corresponding author. Mailing address: Instituto de Investigaciones Bioquímicas, Fundación Campomar, Antonio Machado 151, Av. Patricias Argentinas 435, 1405 Buenos Aires, Argentina. Phone: (54-1) 88-4016. Fax: (54-1) 8652246.

FIG. 1. Specific amplification of *ctx* gene from *V. cholerae*. Agarose gel analysis of products obtained after the first round of PCR amplification with external primers (A) and second round of PCR amplification with the third internal primer (heminested PCR) (B). Lanes: M, ϕ X174 phage DNA digested with *Hae*III; 1 to 4, toxigenic *E. coli* DNA; 5 and 6, nontoxigenic *V. cholerae* DNA; 7, toxigenic *V. cholerae* DNA.

of this primer is 5' GATATGCAATCCTCAGGGTATCC 3' (reverse primer, positions 1558 to 1580) (11). PCRs were performed in 50- μ l portions of a final mixture containing 50 mM KCl, 10 mM Tris (pH 8), 2.5 mM MgCl₂, primers (1 μ M), 100 μ M each of the four deoxynucleoside triphosphates (deoxynucleotides), and 2 U of *Taq* polymerase (Bethesda Research Laboratories, Gaithersburg, Md.). The conditions used were 5 min at 94°C for initial denaturation of DNA and 30 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, using an automated thermal cycler (DNA Thermal Cycler; Perkin-Elmer Cetus, Norwalk, Conn.). After the first PCR with the external primers, 5- μ l samples of this PCR mixture were withdrawn and used in a second round of amplification with the internal reverse primer under the conditions described previously. Samples (15 μ l) of the PCR mixtures were subjected to 1.6% agarose gel electrophoresis.

Sensitivity of heminested PCR procedure. A stock culture of *V. cholerae* O 425 was titrated by dilution and CFU count as described previously (15). Serial dilutions of this culture in phosphate-buffered saline were boiled for 5 min and then centrifuged at 16,000 $\times$ g for 5 min. Samples (5 μ l) of supernatants were used for heminested PCR assay.

RESULTS

Specificity of PCR for detection of CT gene. In order to test the specificity of PCR, genomic DNAs from toxigenic and nontoxigenic *V. cholerae* strains and from toxigenic *E. coli* strains were subjected to single and heminested PCR amplifications (Fig. 1). After a single PCR amplification step, only toxigenic *V. cholerae* showed a 519-bp amplification product (Fig. 1A). Heminested PCR gave a product of the expected size, about 452 bp, from DNA from toxigenic *V. cholerae* (Fig. 1B). No products were obtained with DNA from nontoxigenic *V. cholerae*, and a low background level was observed with DNA from toxigenic *E. coli*. Serial dilutions of *V. cholerae* stocks in heminested PCRs were performed as described in Materials and Methods. Under our assay conditions, DNA from a single organism was detected (data not shown).

Direct application of PCR to rectal swab samples. The direct application of PCR methods to stool specimens has been complicated by the fact that inhibitors of the reaction are usually present in these samples (17). Consequently, a previous

FIG. 2. Direct PCR amplification from stool specimens. Agarose gel electrophoresis was performed with products obtained after the first PCR amplification (A) and heminested PCR amplification (B) with diluted (1/100) rectal swab supernatants. Lanes: M, ϕ X174 phage DNA digested with *Hae*III; 1, stool specimen from an individual infected with nontoxigenic *V. cholerae* non-O1; 2 and 3, stool specimens from individuals infected with *V. cholerae* El Tor Ogawa and CT positive.

DNA purification step was required in order to obtain amplification products (8). To circumvent this problem, undiluted and diluted (1/100) stool samples were tested in one-step and heminested PCRs. Twenty-seven stool samples positive for CT by GM1-ELISA and bacteriological assays were tested. When undiluted stool samples were used in a one-step PCR, only 40% generated the expected amplified products. Negative samples could not be further amplified in the second heminested PCR. On the other hand, when stool samples were diluted 1/100, only 10% were positive after the first PCR, but 100% were positive after the second heminested reaction. Examples of the results obtained are shown in Fig. 2. We conclude that the high sensitivity obtained with heminested PCR allows the dilution of the samples, thus overcoming the effect of inhibitory substances and avoiding the need for any DNA purification step.

Comparison of results of heminested PCR and ELISA on stool samples. Since excellent results were obtained with stool samples diluted 1/100 and heminested PCR, these conditions were used and the results were compared with those obtained by GM1-ELISA. Forty-six swab stool samples were collected from patients in the region where cholera is endemic (Salta province). After the samples were shipped (about 1,800 km), only 26 samples (57%) were positive by quick ELISA. Of the 20 remaining negative samples, 12 were bacteriologically positive for *V. cholerae* O1 (CT positive). On the other hand, 40 of the 46 samples (87%) were positive for CT by heminested PCR. To determine whether the negative results observed in the six remaining samples were due to substances inhibiting PCR, purified *V. cholerae* DNA was added to the stool samples and then processed by PCR. All these samples were positive (results not shown), thus suggesting that negative results were not due to substances inhibiting PCR and that it is highly possible that no detectable amount of DNA was present in these samples. Rectal swabs collected by the same procedure from individuals infected with nontoxigenic *V. cholerae* non-O1 were negative by GM1-ELISA and heminested PCR (Fig. 2, lane 1).

A second group of rectal swabs from Jujuy province (1,900 km from Buenos Aires) were transported in gelatin medium, which turned out not to be appropriate for the survival of *V. cholerae* for long periods of time. Only 2 of the 16 samples were positive by quick GM1-ELISA, whereas the other 14 samples did not have culturable bacteria, since they failed to grow under laboratory conditions (even when they were found to be positive for *V. cholerae* O1 at regional laboratories). In contrast, 15 of the 16 samples were positive by heminested PCR. These results show that even when no culturable bacteria are present in stool samples, the heminested PCR procedure allows the detection of CT-positive samples.

DISCUSSION

To summarize, we have demonstrated that PCR can be directly applied to stool swabs without any DNA purification or bacterial amplification steps. This was achieved by simply boiling the samples to liberate the DNA and diluting the samples to avoid the inhibition by substances present in the stool samples. By using heminested PCR, it was possible to detect DNA from a single organism. Thus, the method described is sensitive and, furthermore, specific, since no products were obtained with nontoxicogenic *V. cholerae* and only a low background level was obtained with toxigenic *E. coli*.

Although other PCR-based methods have been reported, they required previous enrichment of stool samples on appropriate culture media (3, 10). Time is crucial in a disease like cholera in order to take actions, such as the examination of the patient's relatives, early treatment, and implementation of biosecurity measures, to avoid spreading of the disease. The appropriate control strategies require presumptive diagnostic confirmation as soon as possible, so the time consumed by enrichment (8 h at least) becomes a limitation of these methods in epidemic situations. On the other hand, the heminested PCR method described here allows direct processing of the samples, so results are obtained only 7 h or less after collection of the samples. An additional advantage of avoiding a culture step is that the result does not depend upon the presence of culturable bacteria, allowing the successful detection on samples collected or transported under nonoptimal conditions and/or from patients receiving antibiotic treatment. Under these conditions, bacteria are no longer culturable and consequently, the GM1-ELISA results were negative.

This approach could also be applied to other kinds of samples (i.e., samples not from humans). Water and food samples could be collected from different regions and sent to laboratories with a low risk of false-negative results if *V. cholerae* DNA were present. Thus, the approach described here may have applications in epidemiology. A small amount of DNA is required to identify different isolates of *V. cholerae*, and they could be sent by mail. Different primers, including random primers in random amplified polymorphic DNA assays, are currently being applied to boiled samples in our laboratory for this purpose.

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