World Journal of Microbiology & Biotechnology 10, 568–571

Cholera toxin gene polymerase chain reaction for detection of non-culturable *Vibrio cholerae* O1

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Cholera enterotoxin is a major antigenic determinant for virulence of *Vibrio cholerae* O1 which can enter into a viable but non-culturable (N-C) state, not detectable by conventional culture methods, yet remain capable of producing enterotoxin and potentially pathogenic. PCR was applied in the current study to detect the cholera toxin (*ctx*) gene of N-C cells, thus eliminating the necessity of culture. Sets of oligonucleotide primers were designed, based on the *ctx*AB operon of *V. cholerae* O1, to detect the presence of the *ctx* gene. DNA from both culturable and N-C cells of *V. cholerae* O1 was amplified by PCR using sets of primers flanking 302-, 564- and 777-bp fragments of the *ctx* gene. The PCR method employed was capable of detecting the *ctx* gene in N-C *V. cholerae* in aquatic microcosms and in diarrheal stool samples from three patients who had distinct clinical symptoms of cholera but were culture-negative for *V. cholerae* O1 and non-O1 and enterotoxigenic *Escherichia coli*. Forty cycles of a two-step reaction (30 s each at 94 and 60°C) were optimal and more time efficient than a three-step PCR described previously. The procedure, from the point of heating microcosms or broth culture samples to observation on gels, requires < 4 h to complete.

Key words: Cholera toxin, non-culturable, PCR, *Vibrio cholerae*.

Vibrio cholerae O1, the causative agent of cholera epidemics worldwide, continues to present a major threat for many developing countries, including those in Latin America (Williams 1992). From results of a series of microcosms and field studies, it is now known that *V. cholerae* enters into a viable but non-culturable (N-C) state under certain environmental conditions, whereby the cells cannot be propagated by conventional culture methods, yet remain capable of producing enterotoxin and retain pathogenicity (Tamplin & Colwell 1986; Colwell *et al.* 1989). Colwell *et al.* (1990) reported the recovery of *V. cholerae* O1 from stool samples of human volunteers fed with non-culturable cells and concluded that human passage can be effective in triggering outgrowth of dormant, i.e. N-C, *V. cholerae* in the aquatic environment. Temperature can play a major role in the entry of *Vibrio* species into the N-C state. Although a temperature shift can cause an apparent resuscitation of culturable cells (Nilsson *et al.* 1991), this recovery seems to be due, at least in part, to regrowth of a small number of cells which do not enter the N-C state (Weichart *et al.* 1992). Although the resuscitation mechanism is not yet clearly understood, the potential public health hazard presented by such vibrios may be significant. The inability to detect such cells, either in the natural environment or in clinical specimens, by conventional bacteriological methods is a serious problem, especially since any detection method that is devised must be capable of detecting low numbers of specific cells amongst large numbers of prokaryotic and eukaryotic cells and other organic material. PCR offers a powerful tool for detecting N-C cells (Brauns *et al.* 1991) by allowing a specific segment of DNA to be speedily amplified by a factor of 10^6 or more, thereby permitting detection of cells present in small numbers. Since PCR depends only on the presence of target DNA, it can be used to detect the cholera toxin (*ctx*) gene in a method unaffected by the culturability of the vibrio cells.

Oligonucleotide primers and amplification conditions for

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PCR detection of the *ctx* gene, a major antigenic determinant of *V. cholerae* O1, have been reported elsewhere (Shirai *et al.* 1991; Fields *et al.* 1992; Koch *et al.* 1993). We report here the detection of *ctx* gene by PCR in samples containing N-C cells of *V. cholerae* O1.

Materials and Methods

Bacterial Strain

A classical Inaba strain of *V. cholerae* O1 (569B; ATCC 25870) was used in this study. The strain was stored at −70°C in Trypticase soy (TS) broth containing 12% glycerol and maintained on TS agar slants (Difco) at 25°C.

Stool Samples

A total of 77 diarrheal stool specimens was collected from patients who had been admitted to the hospital of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) and who were suspected of having cholera. Three of the patients yielded specimens that were negative for culturable enteric bacterial pathogens but exhibited clinical symptoms of cholera. These three stool specimens were examined using PCR for detection of the *ctx* gene.

Each stool specimen (100 μl) was centrifuged at 13,000 × *g* for 2 to 3 min, resuspended in 100 μl distilled-deionized water and heated at 95°C for 10 min. The specimen was then recentrifuged at 13,000 × *g* for 2 to 3 min and the supernatant collected and used as DNA template.

Laboratory Microcosms

Microcosms of *V. cholerae* O1 were prepared and maintained in two 1-l acid-washed Erlenmeyer flasks to obtain viable but non-culturable cells. Filtered (0.2-μm pore) and autoclaved Potomac River water (300 ml) was added to each of the flasks. Washed cells of *V. cholerae* O1 were used as inoculum to minimize the amount of available nutrient in the flasks; the cells were grown for 18 h at 35°C in T_1N_1 (1% trypticase and 1% Nacl), harvested by centrifugation at 1,000 × *g* for 15 min, washed three times with sterile physiological saline (0.85% NaCl) and resuspended in saline at a concentration of about 10^8 c.f.u./ml. The microcosms were inoculated to obtain an initial cell concentration of about 10^6 c.f.u./ml and then maintained at 4°C, with rotary agitation (100 rev/min).

Enumeration Techniques

Specimens were collected aseptically for bacterial enumeration from the microcosms immediately after inoculation and every other day for 10 days. Bacterial cells were enumerated directly, using acridine orange direct counting (AODC) to obtain the total number of bacteria present in the microcosms (Hobbie *et al.* 1977). The number of culturable bacteria was determined by spread plating on TS agar. Viable bacterial counts were done using the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride-direct viable count (INT-DVC) method (Hasan *et al.* 1991). In brief, a 1-ml sub-sample from each flask was amended with nalidixic acid, yeast extract and an aqueous solution of INT dye (Sigma) to final concentrations (w/v) of 0.002%, 0.025% and 0.02% respectively. The samples were incubated for 4 h at 35°C, with rotary agitation (100 rev/min) and fixed with 2% (w/v) filtered formalin. The substrate-responsive, elongated cells showing intracellular deposits were counted, after staining with acridine orange.

PCR

PCR was carried out as described by Shirai *et al.* (1991) with modification as follows. Three sets of oligonucleotide primers, flanking 302-, 564- and 777-bp sequences within the *ctx* gene, were synthesized (Table 1). One ml samples, either from the microcosm containing N-C *V. cholerae* or from TS broth containing culturable *V. cholerae* O1 cells, were centrifuged. Each pellet was resuspended in 100 μl distilled deionized water and heated at 94°C for 5 min. To 49.5 μl of each suspension, 10 μl buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ 0.01% (w/v) gelatin), 10 μl of each primer, 20 μl of deoxynucleoside triphosphate mix (containing 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dTTP, and 0.5 mM dGTP) and 0.5 μl (2.5 units) of *Taq* DNA polymerase were added to a final volume of 100 μl. The mixture was overlaid with 50 μl of mineral oil and amplification was performed in a programmable Perkin-Elmer thermocycler. The samples were subjected to 40 cycles of amplification, each consisting of a 30-s denaturation at 94°C and a 30-s annealing/extension at 60°C.

Detection of Amplified DNA

Following amplification, 10 μl of the PCR products were analysed by electrophoresis on a 1.0% agarose gel in Tris/borate/EDTA (TBE) buffer for 1 h. The gels were stained with ethidium bromide in distilled water (0.5 μg/ml) and photographed.

Results and Discussion

The cells of *V. cholerae* O1 entered into the N-C state after incubation for 6 days, determined by plate count, with the majority of the cells remaining viable, i.e. responding to INT-DVC (Figure 1).

The PCR amplification assay, using primer set 1 (Table 1), permitted detection of the *ctx* gene in diarrheal stool samples from the three patients who had distinct clinical symptoms of cholera but who were culture-negative for *V. cholerae* O1 and non-O1 and *Escherichia coli* (Figure 2).

The same stool samples revealed the presence of *V. cholerae* O1 when tested by direct and indirect fluorescent antibody assays, (Xu *et al.* 1984; Hasan *et al.* 1994) (data not shown). These samples were also positive by the CholeraScreen (Colwell *et al.* 1992), a newly developed and highly specific monoclonal-antibody-(COLTA)-based coagglutination test (New Horizons Diagnostics Corp., Columbia, MD). Shirai *et al.* (1991), described four stool samples from acute cholera patients which were negative by culture but positive by PCR for the *ctx* gene; Although no other enteropathogens could be isolated from the four samples, they were confirmed positive by Southern blot hybridization (Shirai *et al.* 1991). It seems likely, that N-C *V. cholerae* O1 were present in these four samples. The N-C vibrios produced or collected in the present study were demonstrated by all the direct test methods used. (Colwell *et al.* 1992; Hasan *et al.* 1994).

The primers used by Shirai *et al.* (1991) had seven or eight mismatches between the *ctx* and *Escherichia coli* heat labile toxin (*elt*) genes, and intended to minimize positive

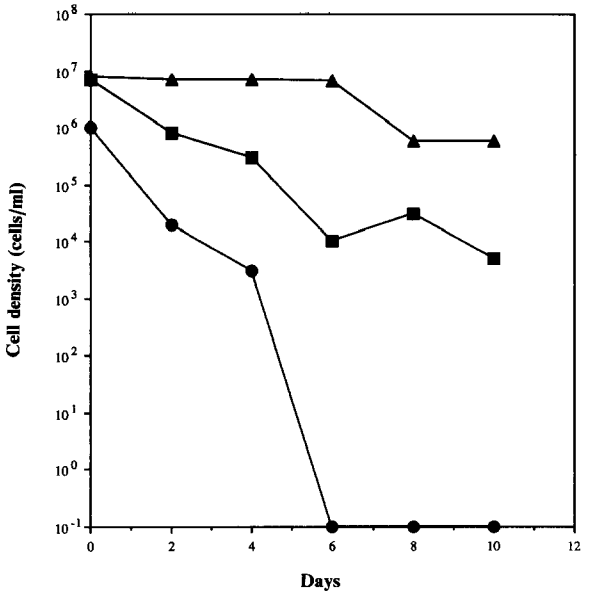

Figure 1. Cell counts for *V. cholerae* O1 microcosms incubated at 4°C. Acridine orange direct counts (▲), iodophenyl nitrophenyl tetrazolium-direct viable counts (■), and total viable culturable counts (●) were determined as described in the text.

signals from cells of *E. coli* with *elt*. However, in the present study, PCR amplification yielded a few unexpected products from two of the samples (Figure 2, lanes 3 and 4). These anomalies may have been the result of non-specific binding of the primers or the presence of closely related pathogens in the stool samples. In subsequent experiments, two different sets of primers (Table 1), positioned in regions of minimal homology between the *ctx* and *elt* genes, were used, ensuring that the 3′ base of each primer was specific for the *ctx* gene sequence (Mekalanos *et al.* 1983). These two primers (2 and 3, Table 1) are reported to give satisfactory results in amplification of the *ctx* gene when samples with mixed microbial populations are used (Fields *et al.* 1992; Koch *et al.* 1993). By employing these two primers, we were able to detect the *ctx* gene in microcosms of *V. cholerae* O1 which were in a N-C state (Figure 3).

Since available culture techniques cannot detect 'N-C' cells, it is important that methods to detect such cells be

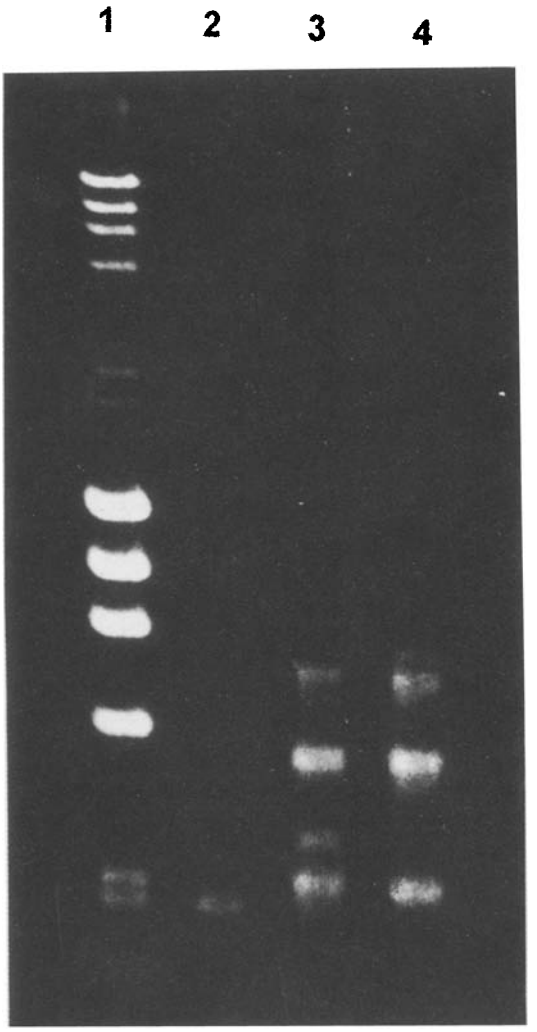

Figure 2. Analysis of PCR products of stool samples culture-negative for *V. cholerae* O1. Lanes: 1—molecular size markers (*Hin*dIII digest of Lambda DNA and *Hae*III digest of ΦX 174 DNA); 2—culture-negative stool sample 1; 3—culture-negative stool sample 2; 4—culture-negative stool sample 3.

developed. PCR allows amplification of the DNA from a single cell (Steffan & Atlas 1988). Although PCR may amplify DNA from dead cells and so give 'false-positive'

Table 1. Synthetic oligonucleotides primers used for PCR amplification of the *ctx* gene.

Primer set	Sequence	Corresponding position in the *ctx* operon*	Reference
1	5′-CTCAGACGGGATTTGTTAGGCACG-3′ 5′-TCTATCTCTGTAGCCCCTATTACG-3′	712 to 735 1013 to 990	Shirai *et al.* (1991)
2	5′-CGGGCAGATTCTAGACCTCCTG-3′ 5′-CGATGATCTTGGAGCATTCCCAC-3′	73 to 94 636 to 614	Fields *et al.* (1992)
3	5′-TGAAATAAAGCAGTCAGGTG-3′ 5′-GGTATTCTGCACACAAATCAG-3′	611 to 630 1388 to 1368	Koch *et al.* (1993)

* *ctx* operon nucleotide sequence reported by Mekalanos *et al.* (1983).

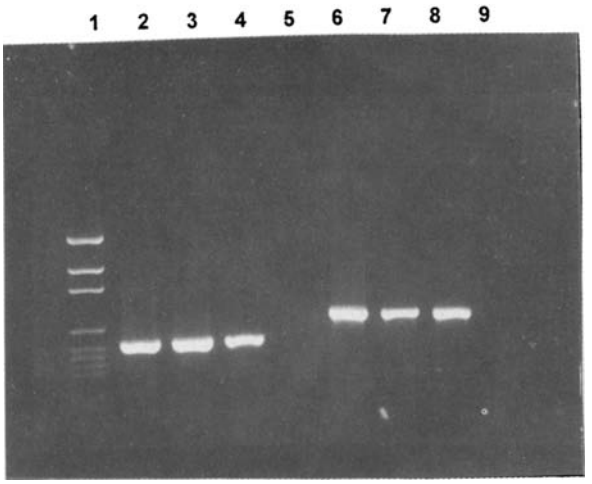

Figure 3. Analysis of PCR products of microcosm samples containing viable but non-culturable *V. cholerae* O1. Lanes: 1—molecular size DNA markers (pGEM; Promega Corp., Madison, WI); 2 and 6—positive control, with genomic DNA from *V. cholerae* O1; 3 and 7—samples from microcosm 1; 4 and 8—samples from microcosm 2; 5 and 9—negative controls for PCR. Lanes 2 to 5 with *ctx* primers set 2 and lanes 6 to 9 with set 3 (Table 1).

results, it has been postulated that such DNA is rapidly degraded (Brauns *et al.* 1991). Detection of the *ctx* gene in a specimen is, nevertheless, epidemiologically significant, because it indicates that toxigenic *V. cholerae* either are or were, present in the environment from which the specimen came. PCR can also be used to confirm the presence of toxigenic N-C *V. cholerae* when microcosms are employed.

Forty cycles of a two-step reaction (30 s each at 94 and 60°C) were found to be optimal, as well as more time efficient than the three-step PCR described by Shirai *et al.* (1991). The total time for the PCR method, from the point of heating the microcosm or culture broth samples to observation of the gels, requires < 4 h.

Acknowledgements

This research was supported by the National Oceanic and Atmospheric Administration (Grants NA-16RU0264-01 and NA86-AA-D-SG 010) the National Science Foundation (Grant BSR-9020268), and the Co-operative Agreement (CR-817791-02-1) between the Environmental Protection Agency and the University of Maryland.

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(Received 11 April 1994; accepted 17 May 1994)