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Detection of toxigenic *Vibrio cholerae* from environmental water samples by an enrichment broth cultivation–pit-stop semi-nested PCR procedure

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J. THERON, J. CILLIERS, M. DU PREEZ, V.S. BRÖZEL AND S.N. VENTER. 2000. A pit-stop semi-nested PCR assay for the detection of toxigenic *Vibrio cholerae* in environmental water samples was developed and its performance evaluated. The PCR technique amplifies sequences within the cholera toxin operon specific for toxigenic *V. cholerae*. The PCR procedure coupled with an enrichment culture detected as few as four *V. cholerae* organisms in pure culture. Treated sewage, surface, ground and drinking water samples were seeded with *V. cholerae* and following enrichment, a detection limit of as few as 1 *V. cholerae* cfu ml^{-1} was obtained with amplification reactions from crude bacterial lysates. The proposed method, which includes a combination of enrichment, rapid sample preparation and a pit-stop semi-nested PCR, could be applicable in the rapid detection of toxigenic *V. cholerae* in environmental water samples.

INTRODUCTION

Cholera is a highly epidemic diarrhoeal disease which continues to devastate many developing countries where socio-economic conditions are poor, sanitary systems and public hygiene are rudimentary, and safe drinking water is not available. In 1992–93, 800 000 cholera cases were reported by 21 countries in the Western Hemisphere, mostly in coastal areas, with more than 8000 cases resulting in death (Tauxe *et al.* 1994). In July 1994, 14 000 deaths from cholera were reported in refugee camps in Rwanda (Siddique *et al.* 1995) while in April 1997, a total of 1521 deaths were recorded during a cholera outbreak among 90 000 Rwandan refugees residing in temporary camps in the Democratic Republic of Congo (Nabeth *et al.* 1997). Cholera is usually transmitted to humans by ingestion of contaminated water and foods. It has been determined on the basis of human volunteer trials that, depending on the health of a given individual, the ingestion of approximately 10^4–10^6 *V. cholerae* O1 organisms is likely to produce clinical cholera (Cash *et al.* 1974).

The major virulence factor produced by *V. cholerae* is the cholera enterotoxin (CT). Cholera toxin belongs to enterotoxins that consist of two subunits (Spangler 1992). Subunit A is responsible for adenylate cyclase activation, inducing tremendous loss of fluids during illness. The B subunit is involved in binding the toxin to the epithelial cell surface receptors in the small intestine (Guidolin and Manning 1987). The chromosomal genes encoding the A and B subunits are designated *ctxA* and *ctxB*, and are expressed as a single transcriptional unit (Mekalanos *et al.* 1983; Guidolin and Manning 1987). Toxin production, however, does not correlate with serotype, as some strains of *V. cholerae* O1 may not produce CT (Kaper *et al.* 1981).

Conventional microbiological methods for identifying *V. cholerae* involve cultural, biochemical and immunological assays which often take several days to complete (Farmer and Hickman-Brenner 1992). Nucleic acid-based methods, such as the polymerase chain reaction (PCR), have the potential to improve these detection times. It has been shown that PCR can detect microbial species by amplification of gene sequences unique to them, and the sensitivity of PCR is such that theoretically, only a single intact nucleic acid template is needed to amplify the target sequence sufficiently to visualize by electrophoresis (Saiki *et al.* 1988). PCR assays based on amplification of target DNA sequences in the *ctx* gene of *V. cholerae* have been reported (Kobayashi *et al.* 1990; Shirai *et al.* 1991; Fields *et al.* 1992; Varela *et al.* 1993). These investigators used PCR to identify toxigenic *V. cholerae* in stools of patients with cholera, or as an alternative to other *V. cholerae* diag-

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nostic methods such as enzyme-linked immunosorbent assay or DNA colony hybridization. PCR assays to determine the presence of enterotoxigenic *V. cholerae* in foods have been described by Koch *et al.* (1993) and Falklind *et al.* (1996), while Keasler and Hall (1993) and Shangkuan *et al.* (1995) used multiplex PCR to detect and biotype *V. cholerae* O1.

The goal of this research was to develop a rapid and sensitive detection protocol for low levels of toxigenic *V. cholerae* in environmental water and drinking water sources. Such a protocol has subsequently been developed based on the technique of semi-nested PCR following cultivation of the organisms in an enrichment broth. The sensitivity of the pit-stop semi-nested PCR procedure was determined using seeded environmental water samples collected from natural sources.

MATERIALS AND METHODS

Bacterial strains

Vibrio cholerae strain NCTC 5941, obtained from the National Collection of Type Cultures, UK, was used as the reference strain. This strain was reconfirmed by cultural, morphological and biochemical tests according to standard procedures (Farmer and Hickman-Brenner 1992). Various other bacterial strains used to test the specificity of the PCR are listed in Table 1. The strains were cultivated and maintained in nutrient broth (Difco) at 37 °C, unless the Culture Collection instructions specified otherwise.

Table 1 Bacterial strains examined

Micro-organism	Source or strain	No. of strains
Aeromonas hydrophila	RW	1
Bacillus cereus	CSIR	1
Citrobacter freundii	CSIR	1
Enterobacter aerogenes	ATCC 49469	1
Escherichia coli	CSIR	3
Escherichia coli	ATCC 13086	1
Escherichia coli	ATCC 14824	1
Escherichia coli	ATCC 25922	1
Klebsiella pneumoniae	ATCC 49472	1
Legionella pneumophila	ATCC 33153	1
Proteus mirabilis	ATCC 12453	1
Pseudomonas aeruginosa	ATCC 27853	1
Pseudomonas diminuta	CSIR	1
Salmonella typhi	ATCC 49469	1
Salmonella typhimurium	SAIMR	1
Shigella boydii	SAIMR	1
Shigella dysenteriae	NCTC 1311	1
Shigella flexneri	CCRC 10772	1
Shigella sonnei	ATCC 8574	1
Vibrio cholerae	NCTC 5941	1
Vibrio cholerae	ATCC 25870	1
Vibrio metschnikovii	CSIR	1
Vibrio parahaemolyticus	SAIMR	1
Vibrio cholerae (non-O1)	RW	16

NCTC = National Collection of Type Cultures, Public Health Laboratory Service, London, UK.
SAIMR = South African Institute for Medical Research, Johannesburg, South Africa.
ATCC = American Type Culture Collection, Rockville, MD, USA.
CCRC = Culture Collection and Research Center, Hsinchu, Taiwan.
RW = Rand Water, Johannesburg, South Africa.
CSIR = Council for Scientific and Industrial Research, Pretoria, South Africa.

Preparation of DNA for PCR

For specificity determination, DNA was extracted from bacterial strains by a rapid boiled-lysate technique. Briefly, bacterial colonies from an overnight cultivation on nutrient agar plates were suspended in 1 ml sterile distilled water to a concentration of approximately 10^6 organisms ml^{-1}. The bacteria were lysed by heating for 10 min at 100 °C. After centrifugation at 10 000 *g* for 5 min to remove the cellular debris, the supernatant fluid containing the bacterial lysate was used in the PCR immediately, or following storage at −20 °C. For sensitivity determinations, serial 10-fold dilutions of the culture suspension were prepared and the cell suspensions were lysed by boiling at 100 °C for 10 min as described above. Viable plate counts to determine the cfu ml^{-1} were performed in triplicate by plating each of the 10-fold dilutions onto nutrient agar plates and incubating for 18 h at 37 °C before counting colonies.

Enrichment of *Vibrio cholerae* in samples

Serially-diluted bacterial cells were recovered by centrifugation at 10 000 *g* for 5 min. The bacterial cells were then resuspended in 1 ml CDC medium (1% w/v peptone, 0·5% w/v NaCl, pH 8·4) (Farmer and Hickman-Brenner 1992) and the tubes were incubated at 37 °C in a shaking incubator. At time 0, 2, 4 and 6 h after seeding, the numbers of cells ml^{-1} were assessed by viable plate counts and template DNA was prepared from each tube. To obtain template DNA for the PCR assays, the bacterial cells were collected by centrifugation at 10 000 *g* for 5 min, washed twice in distilled water and then resuspended in 1 ml sterile

distilled water before heating in a water-bath at 100 °C for 10 min. After centrifugation in a microcentrifuge for 5 min, 10 μl of the supernatant fluid were used in the pit-stop semi-nested PCR method described below.

Preparation of seeded environmental water samples for PCR

Treated sewage, surface, ground and drinking water samples were collected from various localities in and near Pretoria, South Africa. The water samples were pre-tested for the presence of amplifiable *Vibrio* spp. DNA by PCR and found to be negative. A suspension of *V. cholerae* cells was prepared in the respective sterile environmental water samples and then used to seed two dilution series consisting of sterile and non-sterile treated sewage, lake, river, ground and tap water as diluent. The water was sterilized by autoclaving at 121 °C for 15 min at a pressure of 15 psi. The bacterial cells from each dilution were recovered by centrifugation at 10 000 *g* for 5 min and then resuspended in 1 ml CDC medium. Following incubation at 37 °C for 6 h, template DNA for pit-stop semi-nested PCR analysis was prepared as described above for enrichment samples. Positive as well as negative, uninoculated controls were included in each experiment.

Selection and synthesis of primers

Oligonucleotide primers CTX2, CTX3 and CTX15 from *V. cholerae ctxA* sequences were used in a pit-stop semi-nested PCR assay. Primers CTX2 (5′-CGGGCAGATT CTAGACCTCCTG-3′) and CTX3 (5′-CGATGATCTT GGAGCATTCCCAC-3′) have been described previously by Fields *et al.* (1992). An internal primer, CTX15 (5′-GAGTATGGAATCCCACCTAAAGC-3′), was designed on the basis of the published sequence of the *ctxAB* operon from *V. cholerae* 2125 (Mekalanos *et al.* 1983). The first PCR step, performed with primers CTX2 and CTX3, amplified a 564 bp region of the *V. cholerae ctxA* gene, while the size of the final PCR product obtained with primers CTX2 and CTX15 was 347 bp. The primers were synthesized by MWG Biotech (Ebersberg, Germany) with automatic DNA synthesizers.

DNA amplification

The PCR was carried out by performing different experiments. Boiled cultures of bacterial species were amplified to test the specificity of the semi-nested PCR primer, while the sensitivity of the pit-stop semi-nested PCR assay was determined and compared with a single-step PCR by amplifying a serially-diluted culture of *V. cholerae*. Also, the sensitivity for detecting *V. cholerae* cells in seeded environmental water samples was determined.

Pit-stop semi-nested PCR. The reaction mixtures used for both PCR steps contained 1 × PCR buffer, $MgCl_2$ at 1·5 mmol l^{-1}, each deoxynucleoside triphosphate at a concentration of 0·1 mmol l^{-1}, and 1 U Taq DNA polymerase (all these were purchased from Promega) per 50 μl reaction mixture. For the first PCR step, 0·22 μmol l^{-1} of primer CTX2, 0·32 μmol l^{-1} of primer CTX3, and a sample volume of 10 μl were used. The reaction tubes were placed in a Perkin-Elmer GeneAmp 2400 thermal cycler (Perkin-Elmer, Cetus, Norwalk, CT, USA). The following conditions were used: heat denaturation at 94 °C for 3 min, followed by 10 cycles consisting of heat denaturation at 94 °C for 1 min, primer annealing at 61 °C for 1 min, and DNA extension at 72 °C for 1 min. The second PCR step was performed using a total volume of 50 μl. A 1 μl aliquot of the first PCR product was used as the template. Primer concentrations of 0·22 μmol l^{-1} of primer CTX2 and 0·32 μmol l^{-1} of primer CTX15 were used. The cycle profile consisted of the same heat denaturation, primer annealing, and DNA extension conditions as those used for the first PCR step, but the number of cycles was 20. After the last cycle, the samples were kept at 72 °C for 7 min to complete synthesis of all strands. For control purposes, reaction mixtures containing distilled water and all other reagents but no template were included.

Single-step PCR reactions. PCR reactions were performed using 10 μl bacterial lysate as template DNA, buffer, deoxynucleoside triphosphate mixture, primers CTX2 and CTX3 or primers CTX2 and CTX15, at the above-mentioned primer concentrations, and Taq DNA polymerase in a final reaction volume of 50 μl. The reactions were then subjected to 30 cycles of amplification under the cycle conditions described above.

Electrophoretic detection of amplicons

The amplification products were separated by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining (0·5 μg ml^{-1}) and u.v. transillumination. A 100 bp DNA ladder (Gibco BRL, Life Technologies) was included on each gel as a molecular size standard.

RESULTS

Specificity of PCR

A novel protocol for detection of low numbers of *V. cholerae* in water samples was developed based on the techni-

que of semi-nested PCR. Any non-specific amplicons produced during the first PCR step should not be able to function as target DNA during the second PCR step due to a lack of complementarity with the inner primer sequence, thereby making confirmation of the product by other procedures such as hybridization unnecessary. The specificity of the oligonucleotide primer pair CTX2 and CTX3 had been previously demonstrated by Fields *et al.* (1992). In the present study, however, specificity testing was performed due to the development of a novel assay as well as to modifications of the amplification conditions, compared with those described by Fields *et al.* (1992). Thus, to confirm and to validate amplicon integrity in the present study, restriction enzyme digestions were performed with *Rsa* I on aliquots of the CTX2–CTX3 amplicons generated in PCR using crude cell lysates from toxigenic *Vibrio* spp. The patterns of the restriction fragments obtained experimentally were identical to those predicted from published nucleotide sequences of the targeted area of the *ctxA* gene. As expected, a 10 bp fragment, however, was not visible on the gel (results not shown). In order to study the specificity of the semi-nested CTX15 primer, crude lysates of a panel of different bacterial strains (Table 1) were subjected to PCR using the CTX2 and CTX15 primers. Only toxin-producing *Vibrio* strains yielded a single amplicon of the expected length, corresponding to 347 bp. All other strains tested were negative by testing with this primer pair, including non-toxigenic *Vibrio* strains. Pit-stop semi-nested PCR on these bacterial extracts also resulted in the amplification of a 347 bp amplicon from only toxigenic *Vibrio* strains.

Sensitivity of PCR with pure cultures

To determine the sensitivity of the PCR detection systems, *V. cholerae* NCTC 5497 cells were serially diluted 10-fold in sterile distilled water and the DNA was extracted by the boiling method described above. The bacteria present in the 10-fold dilutions were enumerated by dilutional plating. Lysate supernatant fluids were subjected to PCR amplification, and the products were visualized on 2% agarose gels stained with ethidium bromide. With the single primer pair, CTX2 and CTX3, the 564 bp fragment could be visualized in ethidium bromide-stained gels in reaction mixtures that contained $1{\cdot}3 \times 10^7$ cfu ml^{-1} *V. cholerae*, which corresponds to $1{\cdot}3 \times 10^5$ cfu per PCR reaction. The sensitivity was improved by the pit-stop semi-nested PCR. In this assay, samples were subjected to a 10-cycle PCR amplification with primers CTX2 and CTX3, and 1 μl of the first round PCR product was subjected to another 20 cycles of amplification with oligonucleotides CTX2 and CTX15. The application of this procedure rendered possible the detection of $1{\cdot}3 \times 10^5$ cfu ml^{-1} without loss of specificity (1300 cfu per reaction). The results of gel electrophoresis analysis following CTX2–CTX3 PCR, and after pit-stop semi-nested PCR, are presented in Fig. 1. The sensitivity following enrichment of pure cultures was also investigated. Serially-diluted enumerated *V. cholerae* cells were recovered by centrifugation, resuspended in CDC enrichment broth and incubated at 37 °C. Pit-stop semi-nested PCR reactions were subsequently performed on crude lysates after 0, 2, 4, and 6 h of enrichment. Although *ctx*-positive signals were produced after 2 h, the

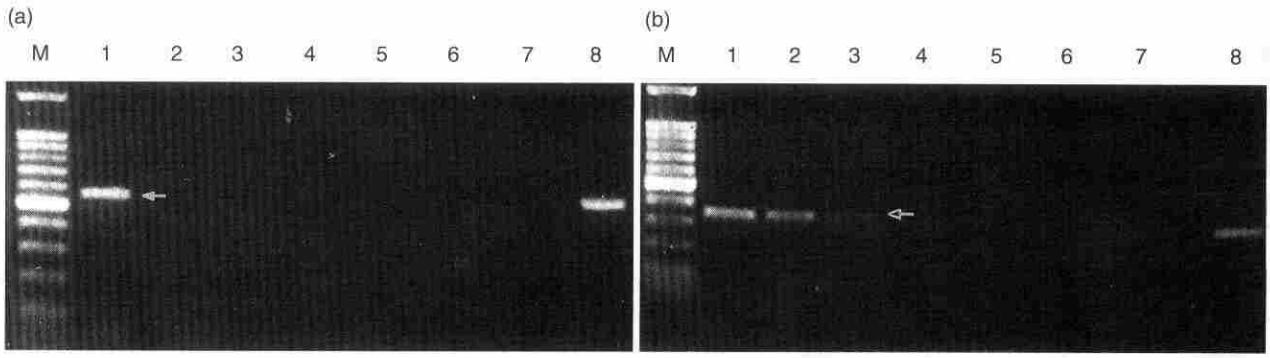

Fig. 1 Sensitivity of the PCR assay for the detection of toxigenic *Vibrio cholerae* by a single (a) and with a pit-stop semi-nested (b) PCR, evaluated with serially-diluted whole cells from toxigenic *V. cholerae*. Lane 1: $1{\cdot}3 \times 10^5$ cfu; 2: $1{\cdot}3 \times 10^4$ cfu; 3: $1{\cdot}3 \times 10^3$ cfu; 4: $1{\cdot}3 \times 10^2$ cfu; 5: $1{\cdot}3 \times 10^1$ cfu; 6: $1{\cdot}3 \times 10^0$ cfu; 7: DNA negative control; 8: positive toxigenic *V. cholerae* control. Lane M: 100 bp ladder as a molecular size standard. The 564 bp fragment of the single and the 347 bp fragment of the pit-stop protocol are indicated by closed and open arrows, respectively

detection limit was greatly enhanced after a 6 h enrichment. The results obtained demonstrated that 390 cfu ml^{-1} (3·9 cfu per reaction) could be detected after 6 h of incubation.

Examination of seeded environmental water samples

The above experiments indicated that crude lysates of toxigenic *V. cholerae* pure cultures readily served as template for PCR. Although amplification from pure culture lysates was relatively simple, the true test of a PCR-based method for detection of water-borne *V. cholerae* is its robustness and sensitivity in terms of its application to water samples from diverse water sources. To test the performance of the proposed method, consisting of enrichment for 6 h in CDC broth and detection of toxigenic *V. cholerae* using the pit-stop semi-nested PCR, treated sewage as well as surface, ground and drinking water samples were artificially inoculated with toxigenic *V. cholerae*.

Application of this PCR detection assay to these diverse environmental water samples (Fig. 2) yielded the following detection limits. While as few as 1 cfu ml^{-1} (0·01 cfu) of toxigenic *V. cholerae* organisms could be detected in seeded ground water, 92 cfu ml^{-1} (0·92 cfu) and 510 cfu ml^{-1} (5·1 cfu) could be detected in seeded lake and river water samples, respectively. In the case of seeded tap water and treated sewage samples, 960 cfu ml^{-1} (9·6 cfu) and 13 cfu ml^{-1} (0·13 cfu) toxigenic *V. cholerae* organisms could be detected, respectively. No amplified products were detected in unseeded water samples. All seeded control environmental water samples gave positive amplification, indicating that humic acids, micro-organisms and other interfering substances, which may be present in the water samples, did not greatly influence the PCR assay. When the detection limits were determined using the duplicate sterile water samples, the detection limits were found to be either the same as determined for the non-sterile samples, or increased 10-fold.

DISCUSSION

In order to evaluate the public health threat posed by toxigenic *Vibrio cholerae* in source and drinking water, a rapid, accurate method for the detection of these organisms within large populations of other bacteria is essential. The standard method for *Vibrio* detection involves isolation on a selective TCBS medium followed by a battery of biochemical and physiological tests. However, several problems are encountered with culturing methods, including the presence of viable but non-culturable cells, loss of viability of bacteria after collection, difficulties in isolation from biocontaminated samples and the time required for culture and confirmation, which can be several days (Wright *et al.* 1993). To avoid these problems, different methods based on molecular biology techniques have been developed, with those based on DNA amplification being the most rapid and sensitive (Garret *et al.* 1993; Ramamurthy *et al.* 1993). Nevertheless, amplified products are seldom detected by direct visualization in ethidium bromide-stained agarose gels, but rather by Southern blot or dot-blot hybridization (Koch *et al.* 1993; Wright *et al.* 1993; Nair *et al.* 1995). Although membrane hybridization is useful in research because it provides excellent sensitivity, these methods are generally time-consuming and labour-intensive. These detection methods are therefore considered impractical for routine laboratory use.

The PCR has become a powerful tool with which to explore microbial activities and identities in environmental microbiology (Mahbubani *et al.* 1990; Pillai *et al.* 1991; Bej and Mahbubani 1992; Koenraad *et al.* 1995; Juck *et al.*

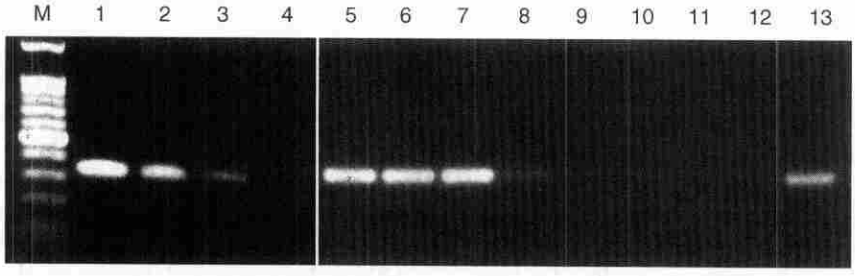

Fig. 2 Analysis of the limit of detection upon seeding environmental water samples with toxigenic *Vibrio cholerae* following enrichment in CDC broth using the pit-stop semi-nested PCR protocol. Lanes 1–4: well water seeded with 1×10^2 (lane 1), 1×10^1 (lane 2), 1×10^0 (lane 3), 1×10^{-1} (lane 4) cfu ml^{-1}; 5–10: tap water seeded with $9{\cdot}6 \times 10^5$ (lane 5), $9{\cdot}6 \times 10^4$ (lane 6), $9{\cdot}6 \times 10^3$ (lane 7), $9{\cdot}6 \times 10^2$ (lane 8), $9{\cdot}6 \times 10^1$ (lane 9), $9{\cdot}6 \times 10^0$ (lane 10) cfu ml^{-1}; lane 11: DNA negative control; lane 12: water negative control; lane 13: positive toxigenic *V. cholerae* control. Lane M: 100 bp ladder as a molecular size standard

1996; Sandery *et al.* 1996; Catalan *et al.* 1997). Due to the ability of PCR to amplify specifically a gene or a segment of gene directly from a sample, an important factor in evaluating any DNA-based test is the specificity of the DNA sequence chosen for the genes and strains of interest. The ability to produce cholera toxin is an important step in the diagnosis of cholera because only toxin-producing strains have been associated with severe, watery diarrhoea and epidemics (Finkelstein 1988). Various cholera toxin gene PCR assays, using primers that amplify regions of either *ctxA* or regions covering both *ctxA* and *ctxB*, have been described (Kobayashi *et al.* 1990; Shirai *et al.* 1991; Fields *et al.* 1992; Keasler and Hall 1993; Koch *et al.* 1993; Varela *et al.* 1993). The respective PCR assays were applied to the identification of toxigenic *V. cholerae* from bacterial colonies, faecal and food samples.

In this report, a modified semi-nested PCR assay (pit-stop semi-nested PCR) for the detection of toxigenic *V. cholerae* in environmental water sources has been developed and evaluated. The pit-stop semi-nested PCR, based on amplification of the *ctxA* sequence, was used with a rapid and simple DNA preparation procedure to detect small numbers of toxigenic *V. cholerae* cells. In contrast to conventional nested and semi-nested PCR procedures (Arnheim and Erlich 1992), which are performed using two rounds of successive amplification consisting of 30 cycles each with an outer and an inner primer pair, respectively, this pit-stop semi-nested PCR is performed in a total of 30 cycles. Not only did the pit-stop semi-nested PCR increase the specificity of the assay, but also the sensitivity. Only toxigenic *Vibrio* spp. strains screened by the PCR assay resulted in visualization of the predicted 347 bp amplified product in ethidium bromide-stained gels. Direct detection of *V. cholerae* by a single, first round PCR assay was possible with 130 000 bacteria. However, the modified pit-stop semi-nested PCR system yielded a positive signal from 1300 bacteria.

The use of PCR for identifying specific organisms obtained from environmental samples has been problematic. Detection of low copy number targets may require a large number of PCR cycles. Increasing cycles often leads to the formation of non-specific amplification products that result from mispriming within non-target sequences encountered in the sample, or by 'primer-dimer' formation (Starnbach *et al.* 1989; Koch *et al.* 1993; Juck *et al.* 1996). In addition, the presence of various interfering substances, such as humic acids, metal ions and organic matter (Rossen *et al.* 1992), may lead to false-negative results. To overcome these problems, methods to separate DNA from extracts containing humic acid substances (Tsai and Olson 1992), and filtration through chelating ion exchange resins to eliminate metal ions (Abbaszadegan *et al.* 1993), have been developed. Immunomagnetic beads attached to specific antibodies to assist in capturing and concentrating organisms prior to DNA extraction have also been used (Islam *et al.* 1993). However, such extraction methods greatly increase the cost and time required to identify pathogenic organisms. Furthermore, there is a risk of losing target DNA in each purification step and certain chemicals used for extraction of nucleic acids inhibit PCR (Rossen *et al.* 1992). Assays based on direct detection of bacterial cells in environmental water samples by filtration and without an enrichment procedure have been developed (Starnbach *et al.* 1989; Bej and Mahbubani 1992; McDonald *et al.* 1995; Sinigalliano *et al.* 1995), but a disadvantage of such methods is that they may detect dead bacteria as well as viable bacteria.

To minimize these problems, the present study used PCR technology coupled with an enrichment procedure that not only diluted PCR inhibitors but also resulted in increased numbers of *V. cholerae* organisms in reaction mixtures. Not only does an enrichment procedure dilute any inhibitors present, but dead bacteria are diluted as well, thus reducing the probability of detecting them by the subsequent PCR assay. By allowing exponential bacterial growth to amplify target copy number rather than using increased numbers of amplification cycles to detect less target, the chance that false-positives might be generated during extended PCR cycling, as in the case of conventional nested and semi-nested PCR assays (Starnbach *et al.* 1989; Lindqvest 1999; Waage *et al.* 1999), is thus minimized. In this study, enrichment in CDC broth for as little as 6 h of incubation before amplification enhanced the limit of detection considerably (at least 300-fold) and as few as 4 cfu of *Vibrio* organisms were detectable in the assay. Furthermore, this combined procedure requires minimal sample manipulation, but is still applicable to most diagnostic laboratories for rapid detection of toxigenic *Vibrio* species.

The sensitivity of the pit-stop semi-nested PCR assay was also determined with environmental water samples from various sources inoculated with *V. cholerae*. In these seeding experiments, different numbers of toxigenic *V. cholerae* could be detected and, depending on the water sample examined, the method detected as few as 1 cfu ml^{-1}. The results obtained indicated that inhibitory substances did not interfere significantly with the PCR when the protocol described above was used. The sensitivity of detection of *V. cholerae* organisms in prepared environmental samples was, in some instances, lower than that in pure cultures. Others have reported similar results of sensitivities with clinical and environmental samples (Hermans *et al.* 1990; Shawar *et al.* 1993; Stone *et al.* 1994). It should be noted, however, that the bacteria used to seed the samples were freshly grown. The apparent lower sensitivity observed for the tap water may be due to initial stress on cells inoculated

into this environment of decreased osmotic pressure, and subsequent slower recovery and growth in the enrichment broth (Farmer and Hickman-Brenner 1992). Detection of sub-lethally damaged *V. cholerae* cells in naturally-contaminated water with this assay should depend initially on the ability of the bacteria to recover from injury and enter the growth phase, and subsequently, on their capacity to compete with the background flora.

In conclusion, the method described in this paper, which includes a combination of enrichment, rapid sample preparation and pit-stop semi-nested PCR, is specific for detection of toxigenic *V. cholerae* and can be used for environmental water samples. The assay can detect low numbers of *V. cholerae* cells in contaminated samples when preparations are incubated in an enrichment medium prior to bacterial lysis and pit-stop semi-nested PCR. The analysis can be completed in 10 h, which is a considerably shorter period of time than is needed for traditional culturing and subsequent bacterial identification. The method described here should be a significant tool in monitoring environmental water and drinking water sources, including sources suspected to be involved in outbreaks of cholera, for the presence of toxigenic *V. cholerae* cells.

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