CHAPTER TWO*

DEVELOPMENT OF AN ENRICHMENT BROTH CULTIVATION-PIT-STOP SEMINESTED PCR PROCEDURE FOR THE DETECTION OF TOXIGENIC *VIBRIO CHOLERAE* IN VARIOUS TYPES OF WATERS

2.1 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 access to safe drinking water is limited. In 1992 to 1994, 700 000 cholera cases were reported by 21 countries in the Western Hemisphere, mostly in coastal areas, with more than 5 000 cases resulting in death (Tauxe *et al.*, 1994; Popovic *et al.*, 1995). 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 1 521 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). During a recent outbreak in South Africa, a 100 000 cholera cases were reported and the outbreak resulted in 228 fatalities (National Cholera Status Report, 2001). 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 to 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

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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; Kay *et al.*, 1994).

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 for visualization 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* diagnostic 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 aims of this part of the research were (i) to develop a rapid and specific PCR-based detection protocol for low levels of toxigenic *V. cholerae* in water, and (ii) to determine the specificity and sensitivity of the newly developed PCR procedure using small volumes of seeded environmental water samples collected from natural sources.

2.2 MATERIALS AND METHODS

2.2.1 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 2.1. The strains were cultivated and maintained in nutrient broth (Difco) at 37˚C, unless the Culture Collection instructions specified otherwise.

2.2.2 Preparation of DNA for PCR

For specificity determination, DNA was extracted from bacterial strains (Table 2.1) by a rapid boiled-lysate technique. Briefly, bacterial colonies from an overnight cultivation on nutrient agar plates were suspended in 1 ml of sterile distilled water to a concentration of approximately 10^6 organisms per ml. 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 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 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.

2.2.3 Enrichment of *Vibrio cholerae* in samples

Serially diluted bacterial cells were recovered from each dilution tube 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 per milliter 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 was used in the pit-stop seminested PCR method as described below.

Table 2.1 Bacterial strains examined

Microorganism	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

2.2.4 Preparation of seeded environmental water samples for PCR

Treated sewage, surface, ground as well as drinking water samples were collected from various localities in and near Pretoria, South Africa. The water samples were pretested 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 (autoclaving at 121°C for 15 min at a pressure of 15 psi) and then used to seed two dilution series' consisting of sterile and nonsterile treated sewage, lake, river, ground and tap water as diluent. The bacterial cells from 1 ml of each dilution were recovered by centrifugation at 10 000 × *g* for 5 min and then resuspended in 1 ml of CDC enrichment medium. Following incubation at 37°C for 6 h, template DNA for pit-stop seminested PCR analysis was prepared as described above for enrichment samples. Positive as well as negative, uninoculated controls were included in each experiment.

2.2.5 DNA amplification

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

2.2.5.1 Selection and synthesis of primers

Oligonucleotide primers CTX2, CTX3 and CTX15 from *V. cholerae ctxA* sequences were used in a pit-stop seminested PCR assay. Primers CTX2 (5' - CGGGCAGATTCTAGACCTCCTG - 3') and CTX3 (5' - CGATGATCTTGGAGCATTCCCAC - 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 (Germany) with automatic DNA synthesizers.

2.2.5.2 Pit-stop seminested PCR

The reaction mixtures used for both PCR steps contained 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5% (vol/vol) Triton X-100), $MgCl_2$ at 1.5 mM, each deoxynucleoside triphosphate at a concentration of 0.1 mM, and 1 U of Taq DNA polymerase (all these were purchased from Promega) per 50 µl of reaction mixture. For the first PCR step, 22 pmol of primer CTX2, 32 pmol of primer CTX3, and a sample volume of 10 µl was used. The reaction tubes were placed in a GeneAmp 2400 thermal cycler (Perkin-Elmer). 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 by using a total volume of 50 µl. A 1 µl aliquot of the first PCR product was used as the template. Primer concentrations of 22 pmol of primer CTX2 and 32 pmol 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.

2.2.5.3 Single-step PCR reactions

PCR reactions were performed by using 10 µl of 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 as described above.

2.2.6 Electrophoretic detection of amplicons

The amplification products were analysed by agarose gel electrophoresis and sized according to their migration in the gel as compared to that of a standard molecular weight marker (100 bp DNA ladder; Gibco BRL, Life Technologies). For this purpose,

horizontal 2% (w/v) agarose slab gels were cast and electrophoresed at 100 V in 1 × TAE buffer (40 mM Tris-HCl, 20 mM Na.acetate, 1 mM EDTA; pH 8.5) using a BioRad Mini Sub™ electrophoresis unit. The agarose gels were stained with ethidium bromide (0.5 µg/ml) and the DNA fragments visualised by UV fluorescence.

2.2.7 Nucleic acid sequencing of amplicons

2.2.7.1 DNA purification

The amplicons were concentrated and purified by ethanol precipitation. Briefly, the DNA was precipitated by the addition of 2 volumes 96% ethanol and Na. acetate (pH 7.0), to a final concentration of 0.3 M. Following incubation for 30 min at -70˚C, the DNA was recovered by centrifugation at 10 000 × *g* for 15 min, washed twice with 70% ethanol and dried under vacuum before being suspended in 20 µl UHQ water.

2.2.7.2 Nucleotide sequencing and sequence analysis

Sequencing of the amplified DNA was performed using an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Perkin-Elmer). Each reaction mixture contained 100 ng of template DNA, 3.2 pmol sequencing primer (CTX2 or CTX3), 2 µl Terminator Ready Reaction Mix, 2 µl of 5 × buffer and UHQ water in a final reaction volume of 10 µl. Cycle sequencing was performed using the following program for 25 cycles: denaturation at 96˚C for 30 s, annealing at 50˚C for 15 s and extension at 60˚C for 4 min. Following brief centrifugation, the extension products were precipitated by the addition of 8 µl UHQ water and 32 µl 95% ethanol (final concentration 60%). The tubes were incubated at room temperature for 15 min, centrifuged at 10 000 × *g* for 15 min and the supernatant carefully aspirated. The pellets were washed twice with 50 µl 70% ethanol, vacuum-dried for 10 min and then stored at -20˚C. Prior to electrophoresis the purified extension products were resuspended in 3.5 µl Blue dextran/EDTA loading buffer (PE Applied Biosystems), denatured for 2 min at 90˚C and loaded onto the ABI PRISM model 377 DNA sequencer. The obtained nucleotide sequences were edited with the ABI PRISM Sequencing Analysis 3.1 and the ABI PRISM Sequencing Navigator 1.0.1 software programs (PE Applied Biosystems). Sequence comparison was subsequently carried

out using the BLAST 2.1 program and the GenBank non-redundant database.

2.3 RESULTS

2.3.1 Specificity of PCR

A novel protocol for detection of low numbers of *V. cholerae* in water samples was developed based on the technique of seminested PCR. Any nonspecific 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 due to modifications of the amplification conditions, compared to those described by Fields *et al.* (1992). Thus, to confirm and to validate amplicon integrity in the present study, sequencing reactions were performed on aliquots of the purified amplicon generated from toxigenic *Vibrio cholerae* culture and primers CTX2-CTX3. The sequence was identical to nucleotide sequences of the targeted area of the *ctxA* gene (GenBank, accession no. AF 175708). In order to study the specificity of the seminested CTX15 primer, crude lysates of a panel of different bacterial strains (Table 2.1) 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 seminested PCR on these bacterial extracts also resulted in the amplification of a 347 bp amplicon from only toxigenic *Vibrio* strains.

2.3.2 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 in Materials and Methods (Section 2.2.2). The bacteria present in the 10-fold dilutions were enumerated by dilutional plating. Lysate supernatants were subjected to PCR amplification, and the products were visualised 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 1.3×10^7 cfu/ml of *V. cholerae*, which corresponds to 1.3×10^5 cfu per PCR reaction. The sensitivity was improved by the pit-stop seminested 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 1.3×10^5 cfu/ml without loss of specificity (1 300 cfu per reaction). The results of gel electrophoresis analysis following CTX2-CTX3 PCR, and after pit-stop seminested PCR are presented in Fig. 2.1.

The sensitivity following enrichment of pure cultures was also investigated. Serially-diluted and previously enumerated *V. cholerae* cells were recovered by centrifugation, resuspended in CDC enrichment broth and incubated at 37°C. Pit-stop seminested 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 detection limit was greatly enhanced after an 6 h enrichment. The obtained results demonstrated that 390 cfu/ml (3.9 cfu per reaction) could be detected after 6 h of incubation.

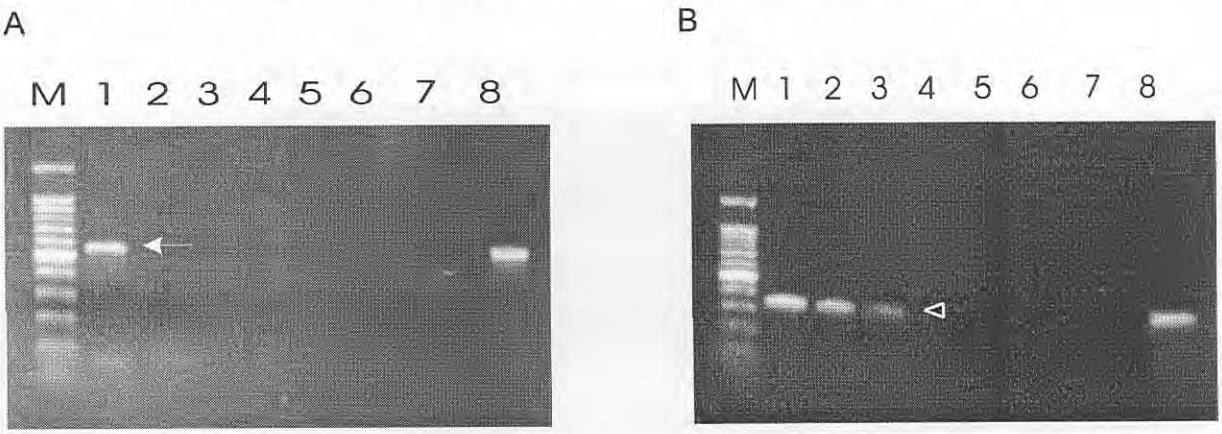

Fig. 2.1 Sensitivity of the PCR assay for the detection of toxigenic *V. cholerae* by a single (A) and with a pit-stop seminested (B) PCR, evaluated with serially diluted whole cells from toxigenic *Vibrio cholerae*. Lanes: 1, 1.3×10^5 cfu; 2, 1.3×10^4 cfu; 3, 1.3×10^3 cfu; 4, 1.3×10^2 cfu; 5, 1.3×10^1 cfu; 6, 1.3×10^0 cfu; 7, DNA negative control; lane 8, positive toxigenic *V. cholerae* control. Lanes 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.

2.3.4 Examination of small volumes of seeded environmental water samples

To test the applicability of the proposed detection method to different types of water samples, treated sewage as well as surface, ground and drinking water samples were artificially inoculated with toxigenic *V. cholerae*. Following enrichment for 6 h in CDC broth, bacterial lysates were prepared and subjected to the pit-stop seminested PCR.

Application of this PCR detection assay to these diverse environmental water samples (Fig. 2.2) yielded the following detection limits. While as few as 1 cfu/ml (0.01 cfu) of toxigenic *V. cholerae* organisms could be detected in seeded ground water, 92 cfu/ml (0.92 cfu) and 510 cfu/ml (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 (9.6 cfu) and 13 cfu/ml (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 resulted in positive amplification, indicating that humic acids, microorganisms 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 nonsterile samples, or showed a 10-fold increase.

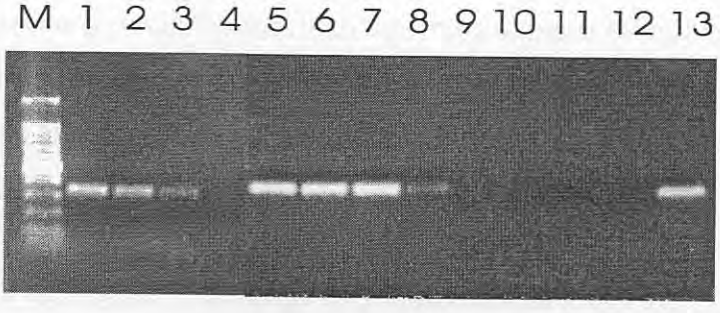

Fig. 2.2 Analysis of the limit of detection upon seeding environmental water samples with toxigenic *V. cholerae* following enrichment in CDC broth using the pit-stop seminested PCR protocol. Lanes: 1 through 4, well water seeded with 1×10^2 (lane 1), 1×10^1 (lane 2), 1×10^0 (lane 3), 1×10^{-1} (lane 4) cfu/ml; 5 through 10, tap water seeded with 9.6×10^5 (lane 5), 9.6×10^4 (lane 6), 9.6×10^3 (lane 7), 9.6×10^2 (lane 8), 9.6×10^1 (lane 9), 9.6×10^0 (lane 10) cfu/ml; 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.

2.4 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 nonculturable 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). Amplified DNA produced using these methods are, however, 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; Bej and Mahbubani, 1992; Pillai *et al.,* 1991; Koenraad *et al.,* 1995; Juck *et al.,* 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, fecal and food samples.

In this part of the investigation, a modified seminested PCR assay (pit-stop seminested PCR) for the detection of toxigenic *V. cholerae* in small volumes of environmental water was developed and evaluated. The pit-stop seminested 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 seminested 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 seminested PCR is performed in a total of 30 cycles. Not only did the pit-stop seminested PCR assay 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 seminested PCR system yielded a positive signal from 1 300 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 nonspecific amplification products that result from mispriming within nontarget 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 occurring. To overcome these problems, methods to separate DNA from extracts containing humic acid substances (Tsai and Olssen, 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.,* 1993b). 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, however, is that they may detect dead bacteria as well as viable bacteria.

To minimize these problems, the present study utilized 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 to use 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 seminested PCR assays (Starnbach *et al.,* 1989; Lindqvest, 1999; Waage *et al.,* 1999), is thus minimized. In this investigation, enrichment in CDC broth for as short as 6 h of incubation before amplification enhances 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 seminested PCR assay was also determined using small volumes of 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, as few as 1 cfu/ml could be detected by this method. The obtained results 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 cultured. 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 sublethally 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 newly developed method which includes a combination of enrichment, rapid sample preparation and pit-stop seminested PCR, is specific for detection of toxigenic *V. cholerae* and can be used for detection of these pathogens in small volumes of 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 seminested 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.

CHAPTER THREE*

A SENSITIVE SEMINESTED PCR METHOD FOR THE DETECTION OF *SHIGELLA* IN SPIKED ENVIRONMENTAL WATER SAMPLES

3.1 INTRODUCTION

The genus *Shigella* is composed of Gram-negative facultative anaerobes of four species: *Shigella dysenteriae*, *S. boydii*, *S. sonnei* and *S. flexneri*. All are pathogens of humans and are usually transmitted from person to person as well as by ingestion of contaminated water and foods. The infective dose is very low, varying from 10^1 to 10^4 organisms (Rowe and Gross, 1984; DuPont, 1990). Virulent *Shigella* organisms cause the human illness known as bacillary dysentery, as do enteroinvasive *Escherichia coli* (EIEC) strains. Clinical features of bacillary dysentery (shigellosis) include diarrhoea, fever, dysentery, and even death in some cases if effective intervention strategies are not used. Epidemiological studies of shigellosis in Bangladesh have shown that various water sources, e.g. ponds, lakes, wells, and rivers, can act as sources of infection (Islam *et al.*, 1993a). In the United States, outbreaks of shigellosis have also been attributed to swimming in contaminated water (Rosenberg *et al.*, 1976; Blostein, 1991; Fleming *et al.*, 2000). In South Africa, children under five years of age living in settlements with rudimentary access to water supply and sanitation are the most susceptible to diarrhoea whereas adults often become symptomless carriers (Pergram *et al.*, 1998). The difficulty in detecting carriers by culture techniques makes them a potential source of environmental contamination.

All of the virulent *Shigella* spp. and EIEC strains harbor a 120 to 230 kb plasmid named the virulence plasmid, which was first described for *S. flexneri* 2a. It was established that the invasion plasmid antigen gene (*ipaH*), unique to shigellae and EIEC and

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implicated in virulent functions, is present in multiple copies on the invasion plasmid and the chromosome (Venkatesan *et al.*, 1989; Hartman *et al.*, 1990; Hale, 1991; Venkatesan *et al.*, 1991).

The standard procedure for *Shigella* spp. detection is based on isolation of *Shigella* by selective culture media followed by identification by biochemical tests and agglutination assays (Frankel *et al.*, 1989; June *et al.*, 1993). This process may take 48 to 72 h or even longer to obtain results. Since shigellae are very fastidious organisms, appropriate collection, rapid transport to the laboratory and rapid plating of the sample are important for isolation. Such conditions are often difficult to attain in developing countries. Thus, rapid, highly sensitive and specific techniques based on genetic characteristics have been developed. DNA hybridization (Venkatesan *et al.*, 1988) and PCR are the best known of these techniques and are often used as tests for the detection and identification of pathogenic microorganisms. Several PCR protocols for detection of *Shigella* in faeces (Frankel *et al.*, 1989; Sethabutr *et al.*, 1993; Yavzori *et al.*, 1994), food (Rafii *et al.*, 1995; Lindqvist, 1999), and some in water (Bej *et al.*, 1991a; Bej *et al.*, 1991b) have been published. These protocols use primers directed at sequences located on the invasion plasmid of *Shigella* spp. and EIEC (Frankel *et al.*, 1989; Yavzori *et al.*, 1994; Lindqvist, 1999), or on both the plasmid and the chromosome (Sethabutr *et al.*, 1993). The major obstacle to using PCR for the detection and identification of pathogenic organisms from clinical samples or environmental water samples is the presence of substances that are inhibitory to PCR (Wilde *et al.*, 1990; Rossen *et al.*, 1992). This obstacle can be minimized by direct chemical extraction of nucleic acids (Frankel *et al.*, 1989; Rafii *et al.*, 1995), immunomagnetic separation (IMS) of bacteria with antibodies (Islam and Lindberg 1992), or buoyant density gradient centrifugation (Lindqvist, 1999), but these procedures are laborious and expensive.

The aims of this part of the research were (i) to develop a highly sensitive and specific detection method for virulent *Shigella* organisms and EIEC by seminested PCR combined with a short enrichment step, and (ii) to determine the sensitivity of the procedure by using artificially seeded ground and surface water samples collected from different natural sources.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains

Shigella flexneri, strain CCRC 10772, obtained from the Taiwanese Culture Collection, Taiwan was used as the test organism in this study. This strain was reconfirmed by cultural, morphological and biochemical tests according to standard procedures (June *et al.*, 1993). Numerous bacterial strains were tested to determine the specificity of the detection protocol (Table 3.1). The organisms were cultivated on MacConkey agar plates and maintained in LB broth (10 % (w/v) peptone, 5 % (w/v) yeast extract, 10 % (w/v) NaCl, 1 % (w/v) glucose) at 37˚C, unless the Culture Collection instructions specified otherwise.

3.2.2 Preparation of lysates for PCR

For specificity determination, a direct lysis method was used for isolation of DNA from bacteria. Bacterial colonies were suspended in 1 ml of sterile water to a concentration of 10^6 organisms per ml. The bacteria were lysed by heating for 10 min at 100˚C and then immediately placed on ice for 5 min. Particulate material present after processing was removed by centrifugation at 10 000 × *g* for 5 min. The lysate supernatant was removed and 10 µl used as the template in the PCR assays immediately or following storage at -20˚C.

3.2.3 Enrichment and enumeration of *Shigella*

S. flexneri CCRC 10772, which was used to seed water samples and to determine the sensitivity of the seminested PCR assay, was grown in LB medium to mid-exponential phase (A_{600} = 0.35), corresponding to 10^8 cfu/ml. Appropriate bacterial concentrations were obtained by preparing serial 10-fold dilutions in sterile distilled water. To enumerate the bacteria, aliquots were spread onto nutrient agar plates and incubated at 37˚C overnight and the bacterial concentration was estimated by calculating the average colony count on plates containing between 30 and 300 colonies. For enrichment of the bacterial cultures, serially diluted bacterial cells were recovered by centrifugation at 10 000 × *g* for 5 min. The cells were then resuspended in 1 ml of Gram-negative broth (GN broth; Difco) and the tubes were incubated at 37°C with

shaking. At time 0, 2, 4 and 6 h after seeding, the numbers of cells per milliter were assessed by viable plate counts and template DNA was prepared from each tube as described above, except that the collected bacterial cells were washed twice in distilled water prior to heating in a water bath.

3.2.4 Preparation of seeded environmental samples prior to PCR

Mid-exponential phase *S. flexneri* cells were serially diluted (10^{-1} to 10^{-9}); 100 µl of the serial dilutions were seeded into 900 µl of both sterile and nonsterilized environmental water samples (treated sewage, lake, river, well and tap water) and enumerated by plate counting. The respective water samples were pretested for the presence of amplifiable *Shigella* DNA by PCR and found to be negative. The bacterial cells from each dilution were recovered by centrifugation at 10 000 × *g* for 5 min and then resuspended in 1 ml of GN broth. Following incubation at 37°C for 6 h, template DNA for seminested PCR analysis was prepared as described above for enrichment samples. Positive as well as negative, uninoculated controls were included in each experiment.

3.2.5 DNA amplification

3.2.5.1 Selection of primers

Oligonucleotide primers H8, H15 and H10 from the multicopy invasion plasmid antigen gene, *ipaH*, were used in a seminested PCR assay. Primers H8 (5' - GTTCCTTGACCGCCTTTCCGATAC - 3') and H15 (5' - GCCGGTCAGCCACCCTC - 3') have been described previously by Islam *et al.* (1993a). An internal primer, H10 (5' - CATTTCCTTCACGGCAGTGGA - 3'), was designed based on the *ipaH* gene sequence reported by Hartman *et al.* (1990). The first PCR step, performed with primers H8 and H15, amplified a 620 bp region of the *ipaH* gene, while the size of the final PCR product obtained with primers H8 and H10 was 401 bp.

3.2.5.2 Seminested PCR

The reaction mixtures used in the PCR steps contained 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5% (vol/vol) Triton X-100), 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.1 mM, 24 pmol of primer H8, 34

Table 3.1 Bacterial strains examined

Microorganism	Amplification (Seminested PCR)	*Source or strain
Aeromonas hydrophila	-	RW
Bacillus cereus	-	CSIR
Citrobacter freundii	-	CSIR
Escherichia coli HB101	-	DSM
Escherichia coli	-	ATCC 25922
Escherichia coli	-	CCRC 13086
Escherichia coli	-	CCRC 14824
Escherichia coli O:112	+	OVI
Escherichia coli O:102	+	OVI
Enterobacter aerogenes	-	ATCC 25922
Klebsiella pneumoniae	-	ATCC 49472
Legionella pneumophila	-	ATCC 33153
Proteus mirabilis	-	ATCC 49469
Pseudomonas diminuta	-	CSIR
Pseudomonas aeruginosa	-	ATCC 27853
Salmonella enteritidis	-	ATCC 13076
Salmonella typhi	-	ATCC 49469
Salmonella dublin	-	CCRC 13852
Salmonella heidelberg	-	CCRC 123437
Shigella dysenteriae	+	NCTC 1311
Shigella flexneri	+	CCRC 10772
Shigella sonnei	+	ATCC 8574
Shigella boydii	+	SAIMR
Vibrio cholerae	-	ATCC 25870

*CTC = National Collection of Type Cultures, Public Health Laboratory Service, London, UK
ATCC = American Type Culture Collection, Rockville, MD, USA
DSM = Deutsche Sammlung von Mikroorganism und Zelkulturen, Braunschweig, Germany
CCRC = Culture Collection and Research Center, Hsinchu, Taiwan
OVI = Culture Collection, Onderstepoort Veterinary Research Institute, Onderstepoort, South Africa
RW = Rand Water, Johannesburg, South Africa
CSIR = Council for Scientific and Industrial Research, Pretoria, South Africa

pmol of primer H15, and 1 U of *Taq* DNA polymerase (Promega) per 50 µl of reaction mixture. The sample volume was 10 µl. The reaction tubes were placed in a Model 2400 GeneAmp thermal cycler (Perkin-Elmer). 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 60°C for 1 min, and DNA extension at 72°C for 1 min. The second PCR step was performed by using a total volume of 50 µl. A 1 µl aliquot of the first PCR was used as the template and 24 pmol H8 primer and 31 pmol H10, as the seminested primer, 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.

3.2.5.3 Single-step PCR reactions

PCR reactions were performed by using 10 µl of bacterial lysate as template DNA, buffer, deoxynucleoside triphosphate mixture, primers H8 and H15 or primers H8 and H10, 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 as described above. Control reaction mixtures containing distilled water and all other reagents but no template were amplified along with the test samples throughout the amplification reaction. A positive control consisting of a *S. flexneri* suspension was included.

3.2.6 Electrophoretic detection

The amplicons were resolved on a 2% (w/v) agarose gel in 1 × TAE (40 mM Tris-HCl, 20 mM Na.acetate, 1 mM EDTA, pH 8.5), as described in Section 2.2.6, and visualized by UV-induced fluorescence after staining with 0.5 µg of ethidium bromide per ml. A 100 bp DNA ladder (Gibco BRL, Life Technologies) was included on each gel as a molecular size standard.

3.2.7 Restriction enzyme digestion and polyacrylamide gel electrophoresis

3.2.7.1 Purification of the amplified DNA

The 620 bp H8-H15 amplified DNA fragment was purified by phenol-chloroform

extraction. The volume of the sample was adjusted to 400 µl by the addition of 1 × TE (1 mM Tris.HCl, 0.1 mM EDTA; pH 7.6). An equal volume of a phenol:chloroform:isoamyl alcohol mixture (25:24:1) was added to the samples, mixed and the organic and aqueous phases separated by centrifugation at 10 000 × *g* for 5 min. The upper aqueous phase was recovered and extracted twice with an equal volume of chloroform. The DNA was precipitated from the aqueous phase by the addition of 2.5 volumes 96% ethanol and Na.acetate (pH 7.0), to a final concentration of 0.3 M. The DNA was recovered by centrifugation at 10 000 × *g* for 10 min, washed with 70% ethanol and dried under vacuum before being suspended in a suitable volume of 1 × TE.

3.2.7.2 Restriction endonuclease digestion of DNA

Samples (10 µl) of the purified amplicon were subsequently digested with 5 U *Hae* III (Roche Diagnostics) in the appropriate restriction endonuclease buffer. Following incubation at 37°C for 1.5 h, the fragments were separated on a polyacrylamide gel. *Hae* III was predicted to digest the 620 bp *ipaH* amplicon into 273, 215 and 135 bp fragments. The sizes of the restriction fragments were estimated by comparison with a molecular weight marker (pBR 328 DNA cleaved with *Bgl* I and *Hinf* I, Roche Diagnostics).

3.2.7.3 Polyacrylamide gel electrophoresis

An aliquot of the restriction enzyme digestion reaction mixture was separated by vertical 8% polyacrylamide gel electrophoresis at 9.0 V/cm for 2-3 h in 1 × TAE buffer (Sambrook *et al.*, 1989). Following electrophoresis, the gel was stained in ethidium bromide solution for 5 to 10 min and visualized with a UV transilluminator.

3.3 RESULTS

3.3.1 Specificity of PCR

The oligonucleotide primer pair H8 and H15 has previously been described by Islam *et al.* (1993a) and was used in a PCR to detect viable but nonculturable *S. dysenteriae* in

laboratory microcosms. However, no primer specificity data were provided. In the absence of such data, specificity testing of four strains of *Shigella* and 21 strains belonging to other genera was performed. To investigate the specificity of the H8-H15 PCR, samples of all strains listed in Table 3.1 were subjected to 30 cycles of amplification. All *Shigella* and EIEC strains produced an intense band of 620 bp. With all other strains tested, no PCR product was detectable. To confirm and to validate amplicon integrity, restriction enzyme digestions were performed with *Hae* III on aliquots of the purified 620 bp amplicons generated in PCR using crude cell lysates from the *Shigella* spp. The polymorphism patterns of the restriction fragments obtained experimentally were identical to those predicted from published nucleotide sequences of the targeted area of the *ipaH* gene (Fig. 3.1).

To investigate the specificity of the seminested PCR, cell lysates prepared of all strains listed in Table 3.1 were subjected to a 10-cycle PCR amplification with primers H8 and H15, and 1 µl of this PCR was subjected to another 20 cycles of amplification with oligonucleotides H8 and the nested primer, H10. The PCR amplified not only DNA from *S. flexneri*, but also from all the other *Shigella* and EIEC strains tested. All amplification-generated products were of the expected size (approximately 401 bp) on agarose gel electrophoresis. No amplification product was observed from microorganisms other than

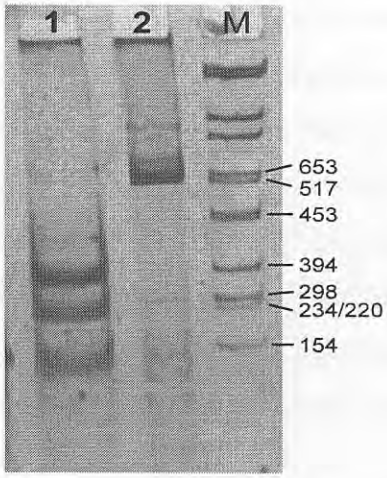

Fig 3.1. Characterisation of the amplicon generated by the H8-H10 single step PCR by resctriction enzyme digestion. The identity of the amplicon (lane 2) was confirmed by restriction with *Hae* III (lane 1), prior to poylacrylamide gel electrophoresis. The sizes of the molecular weight marker (lane M) are indicated to the right of the figure.

Shigella and EIEC, even though there was sufficient DNA to detect a single copy sequence of *ipaH*. In addition to the 401 bp fragment, amplification of the DNA of the *Shigella* and EIEC strains occasionally did produce larger amplicons. The size of these amplified fragments was identical to those obtained in the single step PCR using primers H8 and H15 (620 bp). These amplicons may be the amplified products of the first PCR step, which is used as template for the second round PCR amplification, resulting in amplification of the expected 401 bp DNA fragment. Raising the primer annealing temperature to 61°C during the first PCR step decreased this problem to a minimum and did not influence the sensitivity of the assay. Since no amplification was observed when DNAs from other bacterial strains were used as targets for PCR, we concluded that the seminested PCR assay can be used to specifically detect virulent *Shigella* spp. and
EIEC strains.

3.3.2 Sensitivity of PCR

In order to evaluate the minimal detectable number of *S. flexneri* organisms, viable CCRC 10772 cells were diluted 10-fold in sterile distilled water, enumerated by dilutional plating and template DNA was prepared by the boiling method described in Materials and Methods. Aliquots of the lysate supernatants were first assayed for sensitivity with the single primer pair H8 and H15. The detection limit was at least 1.6×10^4 cfu of *S. flexneri* per assay, corresponding to 1.6×10^6 cfu/ml of lysate. To reach a lower detection limit with simultaneous confirmation of the reaction product, the samples were subjected to the seminested PCR assay. After seminested PCR, the last dilution step amplified corresponded to approximately 1.6×10^3 cfu per assay (1.6×10^5 cfu/ml of lysate). Thus, detection levels were increased 10-fold in seminested PCR assays. The sensitivity of the PCR assays following enrichment of pure cultures in GN broth was also investigated. PCR reactions were performed on crude lysates after 0, 2, 4, and 6 h of enrichment. The obtained results demonstrated that 1.6 cfu (160 cfu/ml) *S. flexneri* organisms could be detected in the seminested PCR assay after 6 h of incubation. In contrast, the limit of detection in the single-step PCR reaction using primers H8 and H10 was 16 cfu (1.6×10^3 cfu/ml), which gave a faintly stained band (Fig. 3.2).

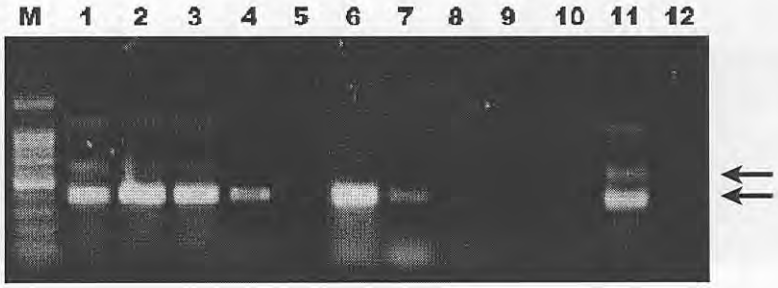

Fig. 3.2 Sensitivity of the PCR assay by a seminested (lanes 1 - 5) and single-step H8-H10 (lanes 6 - 10) PCR following enrichment in GN broth for 6 h. Lane 11, positive control; lane 12, negative virulent *S. flexneri* control. Lanes 1 and 6, 1.6×10^5; 2 and 7, 1.6×10^4; 3 and 8, 1.6×10^3; 4 and 9, 1.6×10^2; and 5 and 10, 16 cfu/ml. Lane M, 100-base pair ladder as a molecular size standard. The bottom arrow indicates the expected seminested PCR-amplified fragment length of 401 bp. The top arrow indicates the first round 620 bp *S. flexneri* fragment occasionally observed during seminested PCR amplification.

3.3.3 Examination of seeded environmental water samples

To test the efficacy of the seminested PCR assay for monitoring small volumes of environmental water samples, serial dilutions of artificially contaminated treated sewage, lake, river, well and tap water samples were prepared for seminested PCR as described in Materials and Methods, and 1/100 of the lysed cells were amplified by the seminested PCR system. Application of this PCR detection assay to these diverse environmental water samples (Fig. 3.3) yielded the following detection limits. While 2×10^3 cfu/ml (20 cfu per reaction) of virulent *S. flexneri* organisms could be detected in seeded well water, 14 cfu/ml (0.14 cfu per reaction) and 580 cfu/ml (5.8 cfu per reaction) could be detected in seeded lake and river water samples, respectively. In the case of seeded sewage and tap water samples, 610 cfu/ml (6.1 cfu per reaction) and 11 cfu/ml (0.11 cfu per reaction) virulent *S. flexneri* organisms could be detected, respectively. No amplified products were detected in sterile and nonsterile unseeded water samples. All seeded control environmental water samples did give positive amplification, indicating that humic acids, microorganisms and other interfering substances which may be present in the water samples did not greatly influence the seminested 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 nonsterile samples, or showed a 10-fold increase in sensitivity.

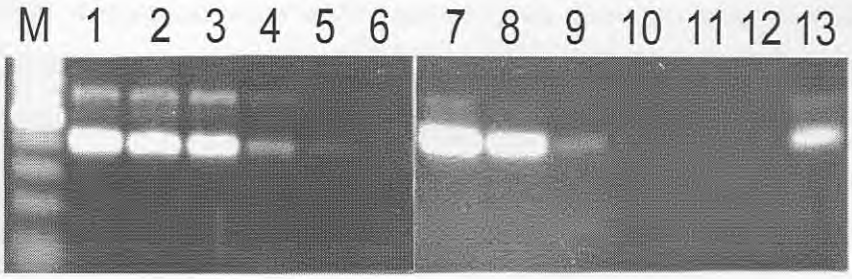

Fig. 3.3. Analysis of the limit of detection upon seeding environmental water samples with virulent *S. flexneri* following enrichment in GN broth using the seminested PCR protocol. Lanes 1 through 6, lake water seeded with 1.4×10^5 (lane 1), 1.4×10^4 (lane 2), 1.4×10^3 (lane 3), 1.4×10^2 (lane 4), 1.4×10^1 (lane 5), 1.4×10^0 (lane 6) cfu/ml; lanes 7 through 11, river water seeded with 5.8×10^4 (lane 7), 5.8×10^3 (lane 8), 5.8×10^2 (lane 9), 5.8×10^1 (lane 10), 5.8×10^0 (lane 11) cfu/ml; lane 12, negative control; lane 13, positive virulent *S. flexneri* control. Lane M, 100-base pair ladder as a molecular size standard.

3.4 DISCUSSION

Detection and identification of *Shigella* from clinical samples has traditionally involved microbiological cultures, biochemical analyses and in some cases, serological methods. The same methods are used to identify suspected *Shigella* colonies isolated from water, food and other environmental samples. However, these methods are not well-suited to the unique situations associated with environmental water samples, where many of the organisms present are stressed and do not perform as expected in clinical testing methods. In the case of *Shigella*, testing problems arise due to the instability of some biochemical characteristics. Studies have also shown that shigellae fail to grow in conventional culture media but remain viable when grown in laboratory microcosms (Islam *et al.*, 1993a). The potential health hazard presented by such *Shigella* species existing in the nonculturable state may therefore be significant. One difficulty in elucidating the potential hazard of viable but nonculturable pathogenic bacteria is the inability to detect such cells in the natural environment by employing routine bacteriological methods. Any detection method that is employed must therefore be capable of detecting low numbers of shigellae against a large background of other cells and of organic material which may be present in the sample. Previous studies have

described PCR-hybridization approaches for the detection of *Shigella* spp. (Islam and Lindberg, 1992). However, most testing procedures describe laborious DNA extraction procedures which are necessary to eliminate substances in samples that can inhibit PCR. To minimize these problems, the present study utilized seminested PCR technology coupled with an enrichment procedure that not only diluted PCR inhibitors, but also resulted in increased numbers of *S. flexneri* organisms in reaction mixtures. This combined procedure requires minimal sample manipulation, but is still applicable to most diagnostic laboratories for detection of small numbers of *Shigella* spp. and EIEC cells in environmental water samples.

The primers selected for this study were based on the sequences of the *Shigella ipaH* gene whose protein product is necessary for invasion of colonic epithelial cells (June *et al.*, 1993). All virulent *Shigella* strains as well as enteroinvasive *E. coli* (EIEC) screened by the seminested PCR assay resulted in visualization of the predicted 401 bp amplified product in ethidium bromide-stained gels. There was no amplification of DNA from samples not inoculated with either the *Shigella* or EIEC. However, amplification of a 620 bp fragment from *Shigella* spp. and EIEC was occasionally observed. Because this fragment was amplified only in the presence of *Shigella* spp. and EIEC, but was not observed when other bacterial strains were used, we attribute its presence to amplification of this fragment from these organisms rather than amplification of DNA from any other bacteria. This may be the result of further amplification of the 620 bp first round amplification product, together with the expected 401 bp product, during the second round of amplification in the seminested PCR assay. Detection of *Shigella flexneri* by a single-step PCR assay was possible with 1.6×10^4 bacteria per reaction. Under the tested parameters, 1.6×10^3 bacteria per reaction were detectable with the seminested PCR system. A low detection limit is, however, indispensable for analysis of environmental water samples, with the infectious dose of shigellae being very low. Enrichment in GN broth for as short as 6 h of incubation before seminested PCR amplification enhanced the limit of detection considerably (at least 1000-fold), and 1.6 cfu of *Shigella flexneri* organisms were detectable in this assay. These results indicate that a high level of sensitivity can be obtained by including an enrichment process.

Methods which could directly detect *Shigella* cells in environmental water samples without an enrichment step would be preferable, especially when viable but nonculturable cells are present. The major obstacle to the development of such methods is the presence of PCR inhibitors, such as humic substances. Insoluble fractions of these substances are concentrated along with bacteria on membrane filters, and extensive extraction procedures may be required to eliminate the inhibitors prior to PCR. Extraction of total DNA by a proteinase K and phenol-chloroform treatment (Frankel, 1989; Bej *et al.*, 1991b; Rafii *et al.*, 1995) or by use of immunomagnetic beads attached to specific antibodies to assist in capturing and concentrating organisms prior to DNA extraction have also been attempted (Islam and Lindberg, 1992; Islam *et al.*, 1993b). These procedures greatly increase the cost and time required to identify pathogenic organisms and certain chemicals used for extraction of nucleic acids inhibit PCR (Rossen *et al.*, 1992).

In this study, use of an enrichment procedure prior to the seminested PCR analysis sufficiently diluted PCR inhibitory substances, while presumably increasing the sensitivity caused by multiplication of the organisms. Furthermore, collection of bacterial cells from the enrichment broth by centrifugation followed by subsequent boiling to lyse the bacteria is a simple and rapid method for preparing DNA for PCR and does not involve any of the aforementioned costly or laborious extraction or purification steps. Minimizing the number of manipulations reduces the risk of contamination and loss of target DNA in each purification step. The sensitivity obtained for boiled *S. flexneri* lysate was 1.6 cfu, which corresponds to the detection sensitivity of 10 cfu obtained by Islam and Lindberg (1992) using an IMS-PCR assay. In addition, small volumes of a variety of different environmental samples were inoculated with *S. flexneri* and analysed using the seminested PCR assay to determine the sensitivity of the assay. The detectable numbers of cells, to some degree, depended on the type of water sample that was seeded. As few as 11 to 14 cfu of *Shigella flexneri* per ml could be detected in some samples.

In conclusion, the PCR system described can permit a rapid and reliable means of assessing the bacteriological safety of water and should provide an effective alternative

methodology to the conventional viable culture methods. Cultures as well as environmental samples can be tested for the occurrence of virulent *Shigella* spp. and EIEC by a seminested PCR assay and ethidium bromide visualization of the PCR products in agarose gels following an enrichment step. The method is easy to perform, sensitive, requires little specialized equipment or training, and provides same day results necessary for rapid action in the case of potential disease outbreaks.