CHAPTER FOUR

DETECTION OF VIABLE TOXIGENIC *VIBRIO CHOLERAE* AND VIRULENT *SHIGELLA* SPP. IN NATURALLY CONTAMINATED ENVIRONMENTAL WATERS BY PIT-STOP SEMINESTED PCR ASSAYS AND COMPARISON TO THE CULTURE TECHNIQUE

4.1 INTRODUCTION

Most pathogens occur in relatively low numbers in environmental waters and because adequate protection of public health requires their rapid detection, the range of available detection methods for pathogenic microorganisms is rapidly increasing, both with regard to the types of pathogens that can be detected as well as the methodology involved. Some form of concentration procedure will usually be the first step in a method for pathogen detection (Oyofo and Rollins, 1993; Gerhardt and Drew, 1994; Shepard and Wyn-Jones, 1995; Graczyk *et al.*, 1997; Vilaginès *et al.*, 1997). Following concentration of the sample, bacteria retained on the filter can be detected by culturing in or on selective media (Toro *et al.*, 1995; Høi *et al.*, 1998; Cerdà-Cuéllar *et al.*, 2000). Some methods may give reliable results in one single step, but usually additional steps are necessary such as resuscitation to allow detection of sublethally injured cells, selective incubation, and confirmatory tests (Kang and Siragusa,1999; Reissbrodt *et al.*, 2000). The sensitivity and specificity of these methods depend strongly on the number of confirmatory tests performed and such tests may take several days to complete.

Given these drawbacks of culture techniques, molecular biology methods appear as a primary alternative for detecting pathogenic bacteria in water samples. Unlike traditional techniques, these methods are based on the detection of a fraction of the genetic material of the targeted bacteria. By using such techniques, the selectivity and sensitivity problems associated with culture techniques can be overcome. Various detection protocols have thus been developed based on cell filtration and PCR amplification of target sequences. Detection limits of 5×10^3 spiked cells/ml in water samples were demonstrated by Pepper *et al.* (1991) using a two-stage filtration and

concentration process, PCR amplification of the target gene, and ethidium bromide staining of gels. Bej *et al.* (1990; 1991a) used a single stage filtration method combined with PCR and Southern hybridization and observed a detection limit of 1 cell/100 ml.

A disadvantage of the above direct detection assays is that inhibitors may hamper the PCR reaction. Such inhibition phenomena have often been described for different types of matrixes, including water concentrates (Kreader, 1996; Wilson, 1997). In addition, it is not possible to assess the viability of the detected bacteria (Josephson *et al.*, 1993). Yet, the viability concept is fundamental for interpreting the result in terms of public health when dealing with water samples. The PCR technique must consequently be associated with a viability test. In the preceding two chapters, the development of protocols consisting of an enrichment step followed by a novel pit-stop seminested PCR for the detection of viable toxigenic *V. cholerae* and entero-invasive *Shigella* spp. in environmental water has been described. Although these methods were developed using 1-ml volumes of seeded water samples, the optimized PCR methods nevertheless showed excellent specificity with a high level of sensitivity and the results could be obtained rapidly. However, the physiological state of the pure cultures used to artificially seed the water samples does not reflect the physiological state of naturally occurring cells, which may have been exposed to a variety of unfavourable conditions or suffered some degree of sublethal injury.

Thus, to establish the seminested pit-stop PCR as a practical tool for routine laboratory use, its performance with regards to the detection of toxigenic *V. cholerae* and enteroinvasive *Shigella* spp. in large volumes of naturally contaminated environmental waters would have to be evaluated. Since conventional culture-based detection methods require the analysis of 100-ml volumes of the water samples, analysis of similar volumes of water would align the pit-stop seminested PCR method with these conventional methods and standards for water quality monitoring. The aims of this part of the research were thus (i) to determine the sensitivity of the pit-stop seminested PCR assays using large volumes (100 ml) of seeded environmental water samples, (ii) to compare the sensitivity of PCR assays with that of conventional culture methods, and (iii) to use the developed detection protocols to analyse naturally contaminated

environmental water samples for the presence of toxigenic *V. cholerae* and enteroinvasive *Shigella* spp.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

Vibrio cholerae strain NCTC 5941, obtained from the National Collection of Type Cultures, London, UK, and *Shigella flexneri* strain CCRC 1077, obtained from the Taiwanese Culture Collection, were used for seeding of environmental water samples. The bacterial strains were cultivated and maintained on nutrient agar or in nutrient broth at 37˚C.

4.2.2 Preparation of bacterial lysates for PCR

DNA was extracted from the bacterial cells by a rapid boiled-lysate method, as previously described (Sections 2.2.2 and 3.2.2). Bacterial cells were concentrated by centrifugation, the pellets suspended in 20 µl sterile Milli-Q water by vortexing and then lysed by boiling. Following removal of the bacterial debris by centrifugation, 10 µl of the supernatant was immediately used as template in the PCR assays.

4.2.3 Sensitivity of the pit-stop seminested PCR assays

The bacterial strains were streaked onto nutrient agar and after overnight incubation of the plates, a single colony was suspended in test tubes containing sterile saline (0.8% (w/v) NaCl in distilled water). For sensitivity determinations, serial 10-fold dilutions of the *V. cholerae* and *S. flexneri* suspensions were prepared in 9 ml sterile saline as diluent. Enumeration of the bacteria to determine the cfu/ml were performed in triplicate by plating each of the 10-fold dilutions onto nutrient agar plates and incubating at 37˚C for 18 h before counting colonies. One ml of the prepared 10-fold dilution series' was subsequently used to seed 99-ml volumes of different environmental water samples (surface water, tap water and treated effluent). The serially-diluted *V. cholerae* and *S. flexneri* cells in the 100-ml water samples were then recovered by filtering through 50-mm cellulose nitrate filters with a pore size of 0.45 µm (Millipore Corp.) using a vacuum

pump (APHA, 1998). The filter membranes and trapped cells were rolled and aseptically transferred to 50-ml Schott bottles containing 4 ml of either CDC or GN broth for enrichment of *V. cholerae* and *S. flexneri* cells, respectively. The filters were vortexed for 5-10 s to release the cells from the filter surface to the liquid phase. Following incubation at 37˚C for 6 h with shaking, 1 ml of the broth was removed and used to prepare template DNA for pit-stop seminested PCR analysis. Negative, uninoculated water samples were included as controls in each of the experiments.

4.2.4 Analysis of water samples by comparison of culturing and PCR methods

To compare the sensitivity of the seminested PCR assays to culturing, 30 naturally contaminated environmental water samples, as indicated in Table 4.2, were analyzed. The water samples were collected in KwaZulu-Natal and were supplied by the CSIR, Durban after the following had been performed. The microorganisms from 50 lt of the environmental water samples were concentrated by making use of Moore swabs, prepared by folding a 100 mm × 8 ply gauze swab into three. The Moore swabs were placed in funnels and after the water samples had been filtered through the swabs, they were removed and transferred into 100 ml CDC broth and incubated at room temperature for 12 h. These samples were subsequently transported to the CSIR, Pretoria by airfreight in cooler bags with ice-packs. The respective samples were immediately analysed on arrival, which was within 20 h after collection of the water samples. The presence of *V. cholerae* and *S. flexneri* in the samples was determined by bacteriological culturing methods as well as by pit-stop seminested PCR assays. In addition, 10 of the original water samples collected, were included for analysis by seminested PCR assays following membrane filtration and enrichment.

4.2.4.1 Isolation of *V. cholerae* by culture methods

(i) Isolation media

For the isolation of *Vibrio* spp., a loopful of growth was obtained from the area just below the culture surface (pellicle), inoculated into *Vibrio* enrichment broth (Biolab) and incubated at 37˚C for 18 h. Freshly prepared thiosulfate-citrate-bile salt-sucrose agar plates (TCBS; Difco) were streaked with bacterial growth before and after culturing in

the *Vibrio* enrichment broth. The agar plates were incubated at 37°C for 18 h and presumptive *Vibrio* isolates were selected based on a yellow (sucrose-fermenting) colony phenotype.

(ii) Biochemical identification

The yellow sucrose-fermenting colonies were streaked on the slant and stabbed into the butt of triple sugar iron agar (TSI; Oxoid) slants and the tubes incubated at 37°C for 18 h. Isolates of which the reactions in TSI agar medium were acid butt and acid slope without production of gas and hydrogen sulphide (H_2S) were selected for further analysis. A colony of each isolate was subsequently inoculated into tryptone water after which a loopful of the cell suspension was streaked onto MacConkey agar (Oxoid) and brain heart infusion (BHI) agar (Oxoid), while SIM agar (Oxoid) slants were stab-inoculated to within a few centimetres from the bottom of the tube. The agar plates and tubes were incubated at 37°C for 18 h. The individual colonies growing on BHI agar plates were analysed for oxidase activity by using strips of filter paper impregnated with a 1% (w/v) tetramethyl-*p*-phenylenediamine solution and 0.1% (w/v) ascorbic acid, to retard auto-oxidation. The strips were moistened with sterile water just prior to use. A colour change to purple in less than 10 s was regarded as a positive reaction, but if it took longer than 60 s it was considered to be negative. To determine indole production, 0.5 ml of Kovac's reagent (Merck) was added to the cell suspensions prepared in tryptone water. Indole production was indicated by the development of a red colour. Only isolates which displayed motility, grew on MacConkey agar and were postive for oxidase and indole, but negative for H_2S production, were retained. These isolates were confirmed as belonging to the genus *Vibrio* by the API 20E system (bioMérieux) according to the manufacturer's instructions.

(iii) Serological identification

Toxigenic *V. cholerae* isolates were identified by agglutination assays using O1-polyvalent antiserum (Murex). For each isolate, a single colony was emulsified in duplicate in a drop of sterile saline to uniform turbidity. A drop of the polyvalent antiserum was added to one of the emulsions, while sterile saline was added to the duplicate emulsion which served as a negative control. Mixing of the reagents were

performed by tilting the slide back and forth for 60 s. Agglutination was investigated microscopically by viewing under indirect light against a dark background.

4.2.4.2 Isolation of *S. flexneri* by culture methods

(i) Isolation media

For the isolation of *Shigella* isolates, a loopful of growth was streaked onto xylose-lysine-deoxycholate (XLD; Biolab) agar and the plates incubated at 37°C for 24 h. Presumptive *Shigella* isolates were identified as colourless (non-lactose fermenting) colonies on the XLD agar plates.

(ii) Biochemical identification

Non-lactose fermenting colonies were streaked on the slant and stabbed into the butt of triple sugar iron agar (TSI; Oxoid) and lysine iron agar (LIA; Oxoid). The colonies were also stabbed into the butt of urea agar (Biolab) slants. All of the tubes were incubated at 37°C for 18 h. Isolates which were urease negative (urea agar medium remained yellow) and the reactions in TSI and LIA agar media were acid butt and alkaline slant without production of gas, and without production of H_2S in TSI agar medium, were retained. These presumptive *Shigella* isolates were then investigated for catalase activity as follow. Following overnight incubation of inoculated nutrient agar slants, a drop of 3% (v/v) hydrogen peroxide was added to the culture on the agar slant. The formation of oxygen bubbles was regarded as a positive test. Oxidase activity was determined as described in Section 4.2.4.1. Only the oxidase-negative, but catalase-positive isolates were retained and characterized serologically.

(iii) Serological identification

Presumptive *Shigella* isolates were identified to the species level by latex agglutination assays using a commercial kit, Wellcolex Colour Shigella (Murex), according to the manufacturer's instructions. Briefly, one drop of each latex reagent was dispensed onto a disposable reaction card supplied by the manufacturer. One drop (40 µl) of the bacterial suspension, prepared in 200 µl sterile saline, was added to the respective latex reagents and mixed thoroughly. The card was placed on a flat-bed orbital shaker and

incubated for 2 min at 150±5 rpm. The cards were observed for agglutination without removing from the shaker. A positive and negative control provided by the manufacturer were included in each test. A summary of the results that can be obtained, is indicated in Table 4.1.

Table 4.1 Wellcolex Colour Shigella identification of *Shigella* spp.

Latex reagent*	Particle colour	Species	Serotype covered
1	Red Blue	*S sonnei* *S flexneri*	Forms I and II 1 to 6, X, Y
2	Red Blue	*S. dysentarieae* *S. boydii*	1 to 12 1 to 15

*Wellcolex Colour Shigella uses the principle of coloured latex particle mixtures. It is a two colour system: Red and blue latex particles coated with antibodies to the four different *Shigella* spp. are mixed together to make two different (purple) test reagents. In the presence of homologous antigen, one of the colours will agglutinate. The identity of the antigen is determined by the colour of the agglutination and the resulting change in the background colour.

4.2.5 Pit-stop seminested PCR

For detection of *V. cholerae*, the pit-stop seminested PCR consisted of 10 cycles with primers CTX2 and CTX3, and 1 µl of product thus obtained was subjected to 20 cycles of amplification with primers CTX2 and CTX15, as described in Section 2.2.5.2. In the case of *S. flexneri*, pit-stop seminested PCR was similarly performed with H8 and H15 as the outer primer pair and H8 and H10 as the seminested primer pair, as described in Section 3.2.5.2. Following PCR amplification, the amplicons were analyzed with 2% (w/v) agarose gels (Section 2.2.6), and the sizes of the amplicons were estimated by comparison with a molecular weight marker (pBR 328 DNA cleaved with *Bgl* I and *Hinf* I, Roche Diagnostics).

4.2.6 Analysis of naturally contaminated environmental water samples

A total of 84 water samples were analyzed for the presence of *V. cholerae*, while 48 water samples were analyzed for the presence of *S. flexneri*. Aliquots of the samples (100 ml) were concentrated by membrane filtration after which the membranes were transferred to Schott bottles containing 4 ml of either CDC or GN broth for enrichment of *V. cholerae* and *Shigella* spp., respectively, and then incubated at 37°C for 6 h.

Positive control samples were prepared by seeding duplicate water samples with *V. cholerae* and *S. flexneri* cell suspensions at approximately 10^2-10^3 cells/100 ml and 10^3-10^4 cells/100 ml, respectively. The control samples were processed by the same procedures used for the test samples. Following incubation, 1 ml of the broth was removed from both the test and control samples and used to prepare bacterial lysates for pit-stop seminested PCR analysis.

4.3 RESULTS

4.3.1 Sensitivity of the pit-stop seminested PCR assays following concentration of large volumes of seeded environmental water samples

For monitoring purposes, PCR-based detection of indicator and pathogenic organisms requires not only specificity, but also sufficient sensitivity to ensure the safety of the various water users. For monitoring of potable water quality, 100-ml volumes are typically tested for the presence of indicator and/or pathogenic microorganisms. Protocols based on PCR have previously been developed in this study for the detection of viable *V. cholerae* and *Shigella* spp. In order to assess the feasibility of the overall protocol and to evaluate its sensitivity and robustness with regards to water samples from diverse sources, 100-ml aliquots of different water samples were analyzed after seeding with serially-diluted *V. cholerae* and *Shigella flexneri*, respectively. Following concentration of the samples by membrane filtration and incubation of the membranes in the appropriate enrichment broths for 6 h at 37˚C, the cells from 1 ml of the enrichment broth of each dilution were recovered, lysed and subjected to the pit-stop seminested PCR protocols. Water samples without seeding were analyzed as controls.

The results obtained for toxigenic *V. cholerae* indicated that the minimum number of cells that could be detected in seeded drinking water and seeded treated effluent, were 15 cfu/100 ml and 3 cfu/100 ml, respectively. However, in the case of seeded surface water, 1 cfu/100 ml toxigenic *V. cholerae* could be detected. In contrast, the detection limits obtained for virulent *S. flexneri* in the same types of water samples were slightly lower. While 8 cfu/100 ml of virulent *S. flexneri* could be detected in seeded treated

effluent, 21 cfu/100 ml could be detected in seeded drinking water. A detection limit of 28 cfu/100 ml was obtained in seeded surface water. No amplification products were observed in unseeded water samples, indicating an absence of *V. cholerae* and *Shigella* spp. from the water samples prior to spiking.

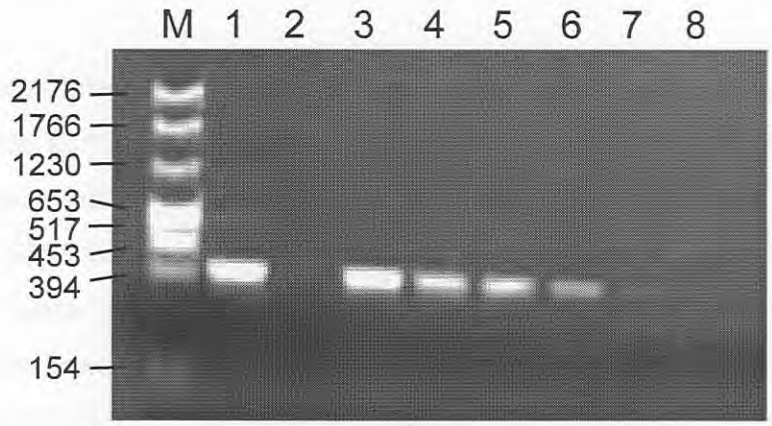

Fig: 4.1 Analysis of the limit of detection upon seeding a surface water sample with serially diluted toxigenic *V. cholerae* following enrichment in CDC broth using the pit-stop seminested PCR protocol for large volume samples. Lane 1, positive toxigenic *V. cholerae* control; Lane 2, negative control. Lanes 3 through 8, dam water seeded with 3.9×10^5 (lane 3), 3.9×10^4 (lane 4), 3.9×10^3 (lane 5), 3.9×10^2 (lane 6), 3.9×10^1 (lane 7), 3.9×10^0 (lane 8) cfu/100 ml. The sizes of the molecular weight marker (lane M) are indicated to the left of the figure.

4.3.2 Examination of environmental samples by culture-based methods and pit-stop seminested PCR assay

A total of 30 water samples were tested in parallel for the presence of toxigenic *V. cholerae* and virulent *Shigella* spp., and the results obtained by bacteriological culturing were compared to those obtained by pit-stop seminested PCR. Although many different conventional culture media and enrichment regimes have been proposed for isolating *V. cholerae* and *Shigella* spp., with some having been reported to be superior to others, no single standardized method exists as yet (Gonzalez, 1995; Donovan and van Netten, 1995; Shears, 1996; APHA, 1998; Lindqvist, 1999). The bacteriological culturing procedures used in this investigation for the isolation of *V. cholerae* and *Shigella* spp. were identical to those routinely used by laboratories in South Africa. Prior to analysis, the water samples were filtered through Moore swabs which were then recovered and incubated in CDC enrichment broth for 12 h at room temperature. These samples were

transported from the CSIR laboratories in Durban to those in Pretoria for subsequent analysis. For 10 of the above samples, the original water samples collected were also supplied. These samples were investigated by pit-stop seminested PCR following concentration of 100 ml of the samples by membrane filtration and enrichment in CDC broth for 6 h.

All of the samples tested negative for *Shigella* spp. in both the bacteriological culturing as well as pit-stop seminested PCR assays (data not shown). In contrast, toxigenic *V. cholerae* could be detected in several of the water samples (Table 4.2). Analysis of the water samples supplied as enrichment cultures, indicated that only 2 of the 30 (7%) water samples were positive for toxigenic *V. cholerae* in culture, as confirmed by biochemical and serological analysis. However, 4 of the water samples tested positive by pit-stop seminested PCR. Analysis of 10 water samples concentrated by membrane filtration prior to enrichment and PCR (Table 4.3) revealed 3 positive samples. Two of these samples were positive in culture, but PCR-negative in instances where the water samples had been concentrated by making use of Moore swabs (Table 4.2). This may have been due to the ineffective concentration capability of the Moore swabs which consisted merely of folded surgical gauze through which samples were poured. In contrast, filtration of 100 ml water samples through 0.45 µm membrane is considerably more effective in trapping the bacterial cells present in the sample. Thus, of the 30 water samples analyzed, using PCR and culturing, a total of 7 samples (23%), tested positive for *V. cholerae*. Of the 7 positive samples, 5 were negative for *V. cholerae* in culture and only one sample was positive for both culture and PCR. Conversely, 1 of the 24 PCR-negative samples was found to be positive for *V. cholerae* in culture. No amplification products could be obtained from two control, seeded water samples. The lack of PCR amplification in these control samples may be due to substances that inhibited the PCR. However, both samples tested negative for *V. cholerae* in culture.

Table 4.2 **Comparison of culture based and pit-stop seminested PCR methods for detection of *V. cholerae* from environmental water samples following filtration through Moore swabs and enrichment**

No	Sample name	Culturing	Seminested PCR	Seeded control samples
1	Gezinsila effluent	-	-	+
2	Gezinsila pond effluent	-	-	-
3	Nyezane river	-	-	+
4	Mnanda river	-	-	+
5	Bumba river	-	-	+
6	Judea river	-	-	+
7	Mpushin river	-	-	+
8	Mabhokweni river	-	-	+
9	Kwamfana river	-	-	+
10	Ithole river	-	-	+
11	Ngwelezana effluent	-	-	-
12	Vondlo river	-	+	+
13	Ofasimba river	-	-	+
14	Mpangeni river	-	-	+
15	Nseleni river	-	-	+
16	Ndabayake river	-	-	+
17	Mhlatuze river	-	+	+
18	Mhlatuze estuary	-	+	+
19	Makwensi foutain	-	-	+
20	Ntuthunga foutain	-	-	+
21	Guncu river	-	-	+
22	Mduki river	-	+	+
23	Mkhiwana river	-	-	+
24	Mvuzana river	-	-	+
25	Mzumbi river	+	-	+
26	Mnambithi river	+	-	+
27	Dikwe river	-	-	+
28	Kwakhomo fountain	-	-	+
29	Mgolomi river	-	-	+
30	Umgeni river	-	-	+

4.3.3 Examination of naturally contaminated environmental water samples

From the above results, it was concluded that the pit-stop seminested PCR assays may be sufficiently sensitive for monitoring purposes. Additionally, results were obtained more rapidly with the PCR assays than with culture and subsequent biochemical and serological assays. Thus, to test the efficacy of the pit-stop seminested PCR assays for monitoring environmental water samples, samples from various sources in South Africa were examined. Aliquots (100 ml) of the samples were filtered through cellulose nitrate filters and following enrichment in CDC broth for 6 h, cell lysates were prepared and subjected to PCR analysis. Control, seeded samples were performed to confirm that chemicals and particulates from the water did not interfere with PCR amplification.

Analysis of 84 environmental water samples for *V. cholerae* by the enrichment broth pit-stop seminested PCR detection protocol, resulted in amplification of amplicons of the expected size from only 5 samples (6%) (Table 4.4). Of the 84 duplicate control seeded water samples, nine samples did not result in an amplification product. These samples consisted of six highly contaminated sewage effluents and three drinking water samples, six showed no amplification product. Three samples consisted of chlorinated tap water, while the other samples were highly contaminated sewage effluents. For all of the test samples that yielded positive results, the duplicate seeded control samples lso yielded amplification products of the expected sizes. The lack of PCR amplification in some of the control samples therefore indicated that these water samples probably contained substances that inhibited the PCR and proves the necessity of including internal controls in order to interpret the negative results.

Table 4.3 Detection of *V. cholerae* in selected water samples by pit-stop seminested PCR following filtration through cellulose nitrate filters and enrichment

No	Sample	Seminested PCR	Seeded control sample
21	Guncu river	-	+
22	Mduki river	+	+
23	Mkhiwana river	-	+
24	Mvuzana river	-	+
25	Mzumbi river	-	+
26	Mnambithi river	+	+
27	Dikwe river	-	+
28	Kwakhomo fountain	-	+
29	Mgolomi river	-	+
30	Umgeni river	+	+

Table 4.4 Detection of *V. cholerae* and *Shigella* spp. in naturally contaminated samples by pit-stop seminested PCR assays

	Tap		River		Dam		Treated sewage effluents	
V. cholerae	No of samples	PCR results	No of samples	PCR results	No of samples	PCR results	No of samples	PCR result
	9	0/9	6	2/6	20	1/20	49	2/49
Shigella spp.	4	0/4	4	0/4	9	2/9	31	1/31

4.4. DISCUSSION

Health risks associated with the waterborne transmission of disease make the detection of pathogenic organisms critical for water quality monitoring. Since many pathogens are present in the natural environment only at low cell densities, a technique to detect low

levels of cells is essential. Filtration methods are typically used to concentrate microorganisms for analysis requiring low detection levels. The analysis is often completed by placing the filter directly on a selective medium and performing a series of biochemical and/or serological tests for specific identification of an indicator microbe or microbial pathogen (Bobb *et al.*, 1981; Lewis and Mak, 1989; De Ryck *et al.*, 1994; Barrett and Feeley, 1994; Lewis, 1997). Alternatively, bacteria can be washed off the filter surface and reconcentrated in a small volume of suspension medium, which is either plated on agar media or prepared for DNA extraction. This procedure allows pre-treatment of samples in order to enhance suppression of background flora and PCR inhibitory substances (Knight *et al.* 1991; Rafii and Lunsford, 1997). However, there can be problems associated with these methods. Enrichment involves competition with other, possibly faster growing organisms in a mixed population, and may result in overgrowth of unwanted bacteria (Arroyo and Arroyo, 1995; Huang *et al.*, 1999). The use of selective media containing inhibitory compounds to eliminate background bacteria may also be inhibitory to environmentally stressed isolates (Arroyo and Arroyo,1996). Biochemical tests and/or serological assays require tedious subculture of numerous individual isolates, and the strain variability for many biochemical assays makes identification questionable, unless a sufficient number of assays are done. In addition, the ability of microorganisms to enter a viable but nonculturable state due to starvation and physical stress may result in failure to isolate these organisms from contaminated water samples by culture techniques, thus usually leading to an underestimation of their numbers (Colwell *et al.*, 1985; Morinigo *et al.*, 1989; Colwell *et al.*, 1990; Byrd et al., 1991; Nilsson *et al.*, 1991; Warner and Oliver, 1998; Dixon,1998).

Since the cost and labour involved in this type of assessment can be prohibitive for many laboratories, methods which could directly detect pathogenic microorganisms in environmental water samples would be preferable. Molecular-based methods, particularly the PCR, are able to achieve the desired sensitivity and reliability without the need to first culture the organism (Bej *et al.*, 1990; 1991a; Oyofo and Rollins, 1993; Toranzos *et al.*, 1993; Juck *et al.*, 1996). By combining filtration and PCR methods, samples contaminated with targeted pathogens and especially viable but nonculturable organisms can be detected within hours, instead of the days required for traditional

biochemical methods (Knight *et al.*, 1991; Palmer *et al.*, 1993; Way *et al.*, 1993; McDonald *et al.*, 1995; Sinigalliano *et al.*, 1995). The most commonly used filters for the filtration of water samples are cellulose nitrate, cellulose acetate and polycarbonate filters (APHA, 1998). However, such filters have been reported to be inhibitory to PCR DNA amplification as DNA amplification is inconsistent in the presence of these filters (Bej *et al.*, 1991a; Oyofo and Rollins, 1993; Juck *et al.*, 1996). The combination of filtration, enrichment and PCR offers the advantage of reducing the negative influence of the membrane filters and complex water matrix on DNA amplification by dilution of the potential inhibitory substances present in the water sample. Also, with this procedure, nonviable cells will not be able to grow during the enrichment step, thereby reducing the risk of false-positives (Maiwald *et al.*, 1994; Dupray *et al.*, 1997; Waage *et al.*, 1999).

The development and evaluation of methods for pathogen detection have focused largely on the use of serially diluted pure culture cells or lysates. Although amplification from pure culture is relatively simple, the true test of a PCR-based method for detection of waterborne pathogens is its robustness and sensitivity in terms of its application to water samples from diverse sources. The sensitivity of the pit-stop seminested PCR was determined with 100-ml volumes of seeded environmental water samples. Depending on the type of water sample, detection limits of 1 cfu/100 ml and 8 cfu/100 ml for toxigenic *V. cholerae* and virulent S. *flexneri*, respectively, could be obtained. The results proved that the protocol developed allows for sensitive detection of the targeted bacteria in different types of water matrixes.

Comparative studies regarding detection of *V. cholerae* and *Shigella* spp. in 30 environmental water samples by bacteriological culturing methods and by pit-stop seminested PCR was undertaken. Analysis of the samples for the presence of *Shigella* spp. yielded no positive results for either of the methods. Since the corresponding control seeded samples yielded amplification products, the lack of these products in the test samples was thought not to be the result of failures in the reactions due to the presence of inhibitors and/or the unavailibility of the DNA, e.g. no bacterial lysis. In contrast, analysis of the samples for *V. cholerae* yielded 6 positive samples by pit-stop

seminested PCR and only 2 positive samples by culturing methods. Thus, culturing appears to be less sensitive than PCR for detection of *V. cholerae* in environmental samples. This may be due to large numbers of other organisms that can outcompete the toxigenic *V. cholerae* on the media, the inability of injured or nonculturable *V. cholerae* to form colonies on the media, low concentrations of viable cells that were below the level of detection by culture, or inhibition owing to bactericidal products produced by other microorganisms. The higher sensitivity of PCR-based methods, in comparison to culture-based methods, has also been reported for other pathogenic microorganisms, including *Legionella* spp. (Frahm and Obst, 1995) and *Shigella* spp. (Islam *et al.*, 2001).

Application of the pit-stop seminested PCR to environmental water samples for the detection of *V. cholerae* and *Shigellae* spp. indicated that these pathogens could be detected in 5 of 84 and 3 of 48 samples, respectively. The low incidence of *Shigella* spp. is in agreement with the current low prevalence of *Shigella* infections (Personal communication, L. Taylor, Addington Hospital, Durban). Some of the water samples exhibited inhibition of the PCR, most notably drinking water and sewage effluent. The inhibition observed for the tap water samples may have been due to the presence of residual chlorine which was not neutralized by sodium thiosulphate prior to analysis. The inhibition observed for the heavily contaminated effluent may have been due to volatile acid by-products derived from the growth of a large number of antagonistic and competing bacteria present in these samples. However, the sensitivity of the PCR for the detection of pathogens in these types water samples may be enhanced by further dilution of sample prior to PCR analysis. This may result in dilution of the PCR-inhibitory substances to a point where they no longer interfere with the assay (DePaola and Hwang, 1995; Weaver and Rowe, 1997).

Rapid tests for identification of *V. cholerae* and *Shigella* spp., such as those described here, will probably complement, not replace, bacterial culture techniques as there will always be a need to culture the organism for serotyping and epidemiological purposes. However, when combined with a cultivation procedure, these pit-stop seminested PCR assays may significantly increase the number of positive results, while reducing the

number of false-negative results. In addition, the methodology may also allow for the processing of a large number of samples in a relatively short period of time.

CHAPTER FIVE

CONCLUDING REMARKS

Despite drinking water being a precious resource and necessary to human health, well-being and socio-economic development, nearly one-half of the world's population suffers from diseases contracted by drinking water of inadequate quality (WHO, 1997). Due to the large number of infectious agents, reservoirs and asymptomatic infected individuals, it may not be possible to eradicate waterborne diseases. However, it may be possible to control these agents as long as they can be detected and monitored. Monitoring of microbial contaminants typically involves filtration and cultivation of indicator bacteria on selective media, followed by colony counting. These traditional methods are laborious, time-consuming and not always sensitive enough to exclude risk of non-specific contamination. In addition, coliforms, the traditional indicators of pathogens, are beginning to fail in some cases by giving misleading information. There is thus a great need for more appropriate methodologies, both for routine monitoring and for investigating disease outbreaks. Due to the rapid advances in biotechnological research, a wide range of new molecular methods have become available. The use of PCR, especially, as a means whereby specific microbial strains in a mixed microbial population can be detected, has become a primary alternative for the detection of pathogens in different types of environmental samples. In this study, PCR technology was used to develop novel approaches aimed at detecting toxigenic *Vibrio cholerae* and *Shigella* spp. with a high degree of sensitivity and specificity. Furthermore, the newly developed detection protocols were modified and their applicability for the detection of toxigenic *Vibrio cholerae* and *Shigella* spp. in environmental water samples was investigated.

Rapid sensitive pit-stop seminested PCR assays for the detection of toxigenic *V. cholerae* (Chapter 2) and virulent *Shigella* spp. (Chapter 3) in pure culture were developed. The performance of these assays were subsequently evaluated using small volumes (1 ml) of environmental water samples seeded with the respective bacterial

pathogens. The *V. cholerae*-specific PCR amplifies sequences within the cholera toxin operon. The PCR procedure coupled with an enrichment culture procedure 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 one *V. cholerae* cfu/ml was obtained with amplification reactions from crude bacterial lysates. Similarly, a set of primers specific for the invasion plasmid antigen gene (*ipaH*) of virulent *Shigella* spp. and enteroinvasive *E. coli* produced a 620 bp fragment that was used as template for the seminested primer pair delineating a 401 bp fragment. By using agarose gel electrophoresis for detection of the seminested PCR-amplified products, a detection limit of 1.6×10^3 cfu *S. flexneri* was obtained with amplification reactions from crude bacterial lysates. The PCR procedure coupled with an enrichment culture incubated for 6 h detected as few as 1.6 organisms in pure culture. Small volumes (1 ml) treated sewage, ground, surface and drinking water samples collected from various sources were seeded with *S. flexneri* and incubated in GN broth for 6 h before detection by seminested pit-stop PCR. A detection limit lower than 14 cfu/ml was achieved in some water samples.

To establish the seminested pit-stop PCR assay as a practical tool for routine laboratory use, its performance with regards to the detection of toxigenic *V. cholerae* and entero-invasive *Shigella* spp. in large volumes (100 ml) of seeded and environmental waters was evaluated (Chapter 4). The basic PCR detection protocol was modified to incorporate a membrane filtration step in order to concentrate large volumes of water samples. By making use of seeded environmental water samples, detection limits of as few as 1 cfu/100 ml and 8 cfu/100 ml of toxigenic *V. cholerae* and *S. flexneri*, respectively, were obtained. Comparative analysis of environmental samples using bacteriological culturing methods and PCR indicated that the pit-stop seminested PCR assay was more sensitive for the detection *V. cholerae*. The higher sensitivity of the PCR-based detection methods may be due to its ability to amplify a fraction of the genetic material of the targeted bacteria, even in the presence of mixed microbial populations. In contrast culture-based methods usually rely on the use of selective media which may inhibit growth of the targeted bacteria and these methods may also

yield false-negative results due to the inability of injured or viable, but non-culturable organisms, to form colonies on the media. In the case of *Shigella* spp. the results obtained by seminested PCR and culturing were in agreement, despite the fact that no bacterial cells could be detected in the tested water samples. Inhibiting substances hampered PCR detection only in a very limited number of samples. These consisted mainly of drinking water and heavily contaminated effluents. These problems may be overcome by extracting the DNA prior to analysis or by further dilution of the sample prior to analysis. Application of the pit-stop seminested PCR for the detection of *V. cholerae* and *Shigella* spp. in environmental water samples yielded positive results for 6% of the samples analyzed, indicating that the methods may be suitable for use in the analysis of environmental water samples. In instances where the invasive plasmid antigen gene, *ipa*H, are used as the targeted gene in seminested pit-stop PCR, a positive amplification result maybe due to the presence of shigellae and/or EIEC bacteria as *ipa*H, occurs in both virulent shigellae and EIEC. However, both organisms cause bacillary dysentery and their presence in water is highly undesirable.

Although molecular technologies, such as those developed in this study, present potential new tools for assessing microbial quality of water, their widespread application to water may depend on several factors. For example, the detection costs must be low and the benefits must outweigh the continued use of conventional methods; the molecular methods must be specific for the microorganisms of concern, which means specifically being able to detect live organisms capable of causing disease; and the sensitivity must be adequate to provide protection against waterborne disease, which means being able to concentrate targets for detection from large volumes of water and to overcome interfering factors that may be present so as to detect very low numbers of microorganisms. The results obtained during the course of this investigation have shown that it is possible to detect pathogenic bacteria from the water samples within 12 hours. The combination of membrane filtration, an enrichment procedure and the pit-stop seminested PCR provided a sensitive, specific and easy method for the detection of vaible *V. cholerae* and *Shigella* spp. in environmental water samples.