Detection of viable toxigenic *Vibrio cholerae* and virulent *Shigella* spp. in environmental waters by pit-stop seminested polymerase chain reaction assays

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Abstract

A rapid and sensitive assay was developed for the detection of low numbers of viable *Vibrio cholerae* and *Shigella* spp. cells in environmental and drinking water samples. Water samples were filtered, and the filters were enriched in a non-selective medium. The enrichment cultures were prepared for polymerase chain reactions (PCR) by a rapid and simple DNA extraction procedure consisting of boiling. Seminested PCR, based on specific amplification of the cholera toxin operon of *V. cholerae* and the invasion plasmid antigen gene (*ipaH*) of virulent *Shigella* spp., was performed and the PCR products were visualised by agarose gel electrophoresis. The assay allowed the detection of as few as 1 cfu/100 mℓ of *V. cholerae* and 8 cfu/100 mℓ of *Shigella* cells. A comparison of the PCR method and culturing methods by using environmental water samples showed that the PCR method has a higher level of sensitivity than culturing methods. As an application of the PCR detection protocol, environmental water samples were screened for the presence of *V. cholerae* and *Shigella* spp. Positive amplifications resulted from *V. cholerae* and *Shigella* species in environmental samples. The results obtained indicate that the described seminested PCR has the advantage of a rapid turn-around time and fulfils the requirements of sensitivity for use in an environmental laboratory.

Key words: *Vibrio cholerae*, *Shigella*, water-borne pathogens, polymerase chain reaction, environmental waters, drinking water, detection

Introduction

Protection of public health requires the rapid detection of water-borne pathogens which often occur in relatively low levels in environmental waters. Consequently, some form of concentration procedure will usually be the first step in traditional, culture-based methods for pathogen detection (Oyofo and Rollins, 1993; Graczyk et al., 1997). Following concentration of the sample, bacteria retained on the filters 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 culturing techniques, molecular biology methods have emerged as a primary alternative for detecting pathogenic bacteria in water samples. Unlike traditional culturing 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. The polymerase chain reaction (PCR) is one such molecular technique. It relies on the *in vitro* amplification of a DNA fragment and due to its simplicity and rapidity, a result is obtained in a short period of time after receiving the sample. Various detection protocols have thus been developed based on cell filtration and PCR amplification of target sequences (Bej et al., 1991; Juck et al., 1996). A disadvantage of these direct detection assays is that inhibitors may hamper the PCR reaction. Such inhibition phenomena have often been described for different types of matrices, 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 results in terms of public health when dealing with water samples. The PCR technique must consequently be associated with a viability test.

South Africa has recently experienced outbreaks of *V. cholerae* and *S. dysenteriae* that have resulted in 288 and 13 fatalities, respectively (Pegram et al., 1998; Dept. of Provincial and Local Government, 2001). Both these pathogenic bacteria are usually transmitted to humans by ingestion of contaminated water and foods. The genus *Shigella* is composed of four species, *Shigella dysenteriae*, *S. boydii*, *S. sonnei* and *S. flexneri*. The infective dose of *Shigella* spp. is very low, varying from 10^1 to 10^4 organisms (Rowe and Gross, 1984). Virulent *Shigella* organisms cause bacillary dysentery (shigellosis) which may lead to death in some cases if effective intervention strategies are not used. Toxigenic *V. cholerae* is responsible for causing cholera, a highly epidemic diarrhoeal disease which continues to devastate many developing countries where socio-economic conditions are poor, sanitary systems and public hygiene are rudimentary, and safe drinking water is not available. The ingestion of approximately 10^4 to 10^6 *V. cholerae* O1 organisms is likely to produce clinical cholera (Cash et al., 1974). The detection of these microbial contaminants in drinking water supplies and source waters should thus be viewed as a high priority.

In this paper, protocols have been developed for the detection of viable *Shigella* spp. and toxigenic *Vibrio cholerae*. The detection protocol described here, consists of a non-selective enrich-

ment process followed by a rapid and simple DNA preparation step and a pit-stop seminested PCR assay. The sensitivity of the procedure was determined by using artificially seeded water samples collected from natural sources. We furthermore demonstrate the successful application of the newly developed detection protocols for the detection of toxigenic *V. cholerae* and entero-invasive *Shigella* spp. in naturally contaminated environmental water samples.

Materials and methods

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.

Sensitivity of the pit-stop seminested PCR detection protocol using seeded environmental water samples

Single bacterial colonies from an overnight cultivation on nutrient agar plates were suspended in sterile saline (0.8% w/v NaCl in distilled water) to a concentration of approximately 10^6 organisms per ml. 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 was 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 water samples (surface water, tap water and treated effluent). The bacterial cells from each dilution were recovered by filtering through 50 mm cellulose nitrate filters with a pore size of 0.45 µm (Millipore Corp.) using a vacuum pump (*Standard Methods*, 1998). The filter membranes containing the trapped cells were rolled and aseptically transferred to 50 ml Schott bottles containing 4 ml of enrichment broth. In the case of *V. cholerae*, CDC broth (1% w/v peptone, 0.5% w/v NaCl, pH 8.4) (Farmer and Hickmann-Brenner, 1992) was used, whilst Gram-negative broth (GN broth, Difco) was used for enrichment of S*higella* spp. The filters were vortexed for 5 to 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.

Preparation of bacterial lysates for PCR

A direct lysis method was used for isolation of DNA from bacteria. Briefly, bacterial cells were concentrated by centrifugation at 10 000 x *g* for 5 min and then suspended in 20 µl sterile Milli-Q water. The bacteria were lysed by heating for 10 min at 100°C. Following removal of the bacterial debris by centrifugation, 10 µl of the supernatant was immediately used as template in the PCR assays.

Pit-stop seminested PCR and electrophoretic detection of amplicons

The reaction mixtures used for both PCR steps contained 1 X PCR buffer, 0.15 mM MgCl$_2$, 100 µM of each deoxynucleoside triphos-phate (dNTP), 1 U of Taq DNA polymerase (all of these were purchased from Promega) and 30 µmol of the required primers. 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 previously described (Theron et al., 2000). The specific fragment amplified (347 bp) is localised within the cholera toxin operon. 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 (Theron et al., 2001). The primer pairs used allow amplification of a specific fragment from the invasion plasmid antigen (*ipaH*) gene with an expected size of 401 bp. The amplicons were resolved on a 2% (w/v) agarose gel in 1 X TAE (40 mM Tris-HCl, 20 mM Na.acetate, 1 mM EDTA, pH 8.5) and visualised by UV-induced fluorescence after staining with 0.5 µg of ethidium bromide per ml. A molecular weight marker was included on each gel as a molecular size standard (Marker VI, Roche).

Comparison of PCR and culturing

To compare the sensitivity of the seminested PCR assays to culture-based detection procedures, 30 environmental water samples, as indicated in Table 1, were analysed. The water samples were collected in sterile containers and the micro-organisms from 50 l of the environmental water samples were concentrated by filtration through Moore swabs. Each swab was transferred into 100 ml enrichment broth and incubated at room temperature for 12 h. These samples were subsequently analysed for the presence of *V. cholerae* and *S. flexneri* by pit-stop seminested PCR assays and by bacteriological culturing methods, as described below. In addition, 10 of the original water samples collected, were included for analysis by semi-nested PCR assays following membrane filtration and enrichment.

Culture-based detection and confirmation of *V. cholerae*

A loopful of growth was obtained from the area just below the enrichment culture surface, 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 and incubated at 37°C for 18 h. Presumptive *Vibrio* isolates were selected based on a yellow (sucrose-fermenting) colony phenotype and inoculated into Triple Sugar Iron agar (TSI; Oxoid) slants. Following incubation (37°C, 18 h), isolates of which the reactions were acid butt and acid slope without production of gas and hydrogen sulphide (H$_2$S), were selected. A colony of each isolate was suspended in Tryptone water and then streaked onto MacConkey agar (Oxoid) and SIM agar (Oxoid). The agar plates were incubated at 37°C for 18 h. The individual colonies were analysed for oxidase activity (*Standard Methods*, 1998) and indole production (*Standard Methods*, 1998). Only isolates which displayed motility, grew on MacConkey agar and were positive for oxidase and indole, but negative for H$_2$S 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. Toxigenic *V. cholerae* isolates were subsequently identified by agglutination assays using O1-polyvalent antiserum (Murex).

Culture-based detection and confirmation of *S. flexneri*

A loopful of growth from enrichment cultures was streaked onto Xylose-Lysine-Deoxycholate (XLD; Biolab) agar plates and incu-

bated at 37°C for 18 h. Presumptive *Shigella* isolates were identified as colourless (non-lactose fermenting) colonies and inoculated into Triple Sugar Iron agar (TSI; Oxoid), Lysine Iron agar (LIA; Oxoid) and urea agar (Biolab). All of the tubes were incubated at 37°C for 18 h. Isolates which were urease negative 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 oxidase (*Standard Methods*, 1998) and catalase activity (Smibert and Krieg, 1994). Only the oxidase-negative, but catalase-positive isolates were retained and characterised serologically. 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. A positive and negative control provided by the manufacturer was included in each test.

Analysis of naturally contaminated environmental water samples by seminested PCR

A total of 84 water samples were analysed for the presence of *V. cholerae*, while 48 water samples were analysed 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 to 10^3 cells/100 ml and 10^3 to 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, as described above.

Results

Sensitivity of the pit-stop seminested PCR assays following concentration and enrichment of seeded environmental water samples

The specificity of the oligonucleotide primer pairs used in this study has been previously demonstrated by Theron et al. (2000; 2001). For monitoring purposes, however, 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 micro-organisms. In order to assess the feasibility of the overall PCR detection protocol and to evaluate its sensitivity and robustness with regards to water samples from diverse sources, 100 ml aliquots of different water samples were thus analysed, as described under **Materials and Methods**, after seeding with serially-diluted *V. cholerae* and *S. flexneri*, respectively. Water samples without seeding were analysed 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 *S. flexneri* in the same types of water samples were slightly lower. While 8 cfu/100 ml of *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
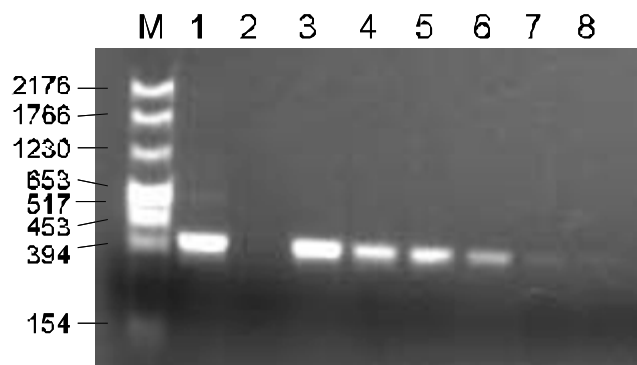

Figure 1

Analysis of the limit of detection upon seeding environmental water samples with toxigenic V. cholerae *following enrichment using the pit-stop seminested PCR protocol. Lane 1, positive toxigenic* V. cholerae *control; lane 2, DNA negative control. Lanes 3 through 8, dam water seeded with 3.9 x 10^5 (lane 3), 3.9 x 10^4 (lane 4), 3.9 x 10^3 (lane 5), 3.9 x 10^2 (lane 6) 3.9 x 10^1 (lane 7), 3.9 x 10^0 (lane 8) cfu/100 ml of* Vibrio cholerae. *The sizes of the molecular weight marker (lane M) are indicated to the left of the figure.*

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. Representative results are shown in Fig.1.

Comparison of culture-based methods to pit-stop seminested PCR assays

A total of 30 environmental water samples were tested in parallel for the presence of *V. cholerae* and *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 standardised method exists as yet (Gonzales et al., 1995; Donovan and Netten, 1995; 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 a number of laboratories in South Africa. Prior to analysis by bacteriological culturing and by seminested PCR analysis, the water samples were filtered through Moore swabs which were then recovered and incubated in enrichment broth for 12 h at room temperature. For 10 of the above samples, the original water samples collected were also analysed. These samples were investigated by pit-stop seminested PCR following concentration of 100 ml of the samples by membrane filtration and enrichment 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 1). Analysis of the water samples supplied as enrichment cultures, indicated that only 2 of the 30 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. No amplification products could be obtained from two control, seeded water samples. Both samples also tested negative for *V. cholerae* in culture. The lack of PCR amplification in these control samples may have been due to substances that inhibited the PCR. Analysis of 10 water samples concentrated by

TABLE 1
Comparison of culture-based and pit-stop semi-nested PCR methods for detection of *V. cholerae* from environmental water samples following filtration through Moore swabs and enrichment

No	Sample	Culturing	Semi-nested PCR	Seeded control sample
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 fountain	-	-	+
20	Ntuthunga fountain	-	-	+
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 2
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	+	+

membrane filtration prior to enrichment and PCR (Table 2), revealed 3 positive samples. Two of these samples were found to be positive in culture, but PCR-negative in instances where the water samples had been concentrated by making use of Moore swabs (Table 1). 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 water samples through a 0.45 μm membrane is considerably more effective in trapping and concentrating the bacterial cells present in the sample. Thus, of the 30 water samples analysed, a total of 7 samples (23%) showed positive amplification for *V. cholerae*. Of the 7 positive samples, 5 were negative for *V. cholerae* in culture. Conversely, 1 of the 24 PCR-negative samples was found to be positive for *V. cholerae* in culture.

Examination of naturally contaminated environmental water samples

From the above results, it was concluded that the developed pit-stop seminested PCR assays may be sufficiently sensitive for monitoring of environmental and drinking water samples. 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 included in the analysis.

Analysis of 84 naturally contaminated 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 5 samples (6%) (Table 3). Of the 84 duplicate control seeded water samples, 9 samples did not result in an amplification product. These samples consisted of 6 highly contaminated sewage effluents and 3 drinking water samples. Application of the *S. flexneri*-specific pit-stop seminested PCR assay to a total of 48 environmental water samples resulted in amplicons of the expected size being obtained for 3 samples (6%) (Table 3). Of the 48 duplicate seeded environmental water samples, 6 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 also 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.

Discussion

Health risks associated with the water-borne transmission of diseases make the detection of pathogenic organisms critical for water quality monitoring. Since many bacteria are present in the natural environment only at low cell densities, filtration methods are typically used to concentrate micro-organisms 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 confirmatory biochemical and/or serological tests for specific identification of an indicator microbe or microbial pathogen (Bobb et al., 1981; De Ryck et al., 1994). However, there can be problems associated with these methods. The use of selective media containing inhibitory compounds to eliminate background bacteria may also be inhibitory to environmentally stressed isolates (Arroyo and Arroyo, 1996). Confirmation 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 micro-organisms to enter a viable but non-culturable 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 (Byrd et al., 1991; Nilsson et al., 1991; Yokomaku et al., 2000).

In contrast to the above, by combining filtration and PCR methods, samples containing the targeted pathogens and especially viable but non-culturable organisms can be detected within hours, instead of the days required for traditional biochemical methods (Palmer et al., 1993; McDonald et al., 1995). The most commonly used filters for the filtration of water samples are cellulose nitrate, cellulose acetate and polycarbonate filters (*Standard Methods*, 1998), but these filters have been reported to be inhibitory to PCR DNA amplification (Bej et al., 1991; Oyofo and Rollins, 1993; Juck et al., 1996). The combination of filtration, enrichment and PCR, as described in this study, offers the advantage of reducing the negative influence of the filters and complex water matrix on DNA amplification by dilution of the inhibitors present in the sample. Also, with this procedure, non-viable cells will not be able to grow during the enrichment step, thereby reducing the risk of false-positives.

The development and evaluation of rapid 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 water-borne pathogens is its robustness and sensitivity in terms of its application to water samples from diverse sources. In this study, the sensitivity of the pit-stop seminested PCR was determined with 100 mł volumes of seeded environmental water samples. Depending on the type of water sample, detection limits of 1 cfu/mł and 8 cfu/mł for *V. cholerae* and 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 matrices.

Comparative studies regarding detection of *V. cholerae* and *Shigella* spp. in 30 naturally contaminated 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 amplified 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 7 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 out-compete the toxigenic *V. cholerae* on the media, the inability of injured or non-culturable *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 micro-organisms. The higher sensitivity of PCR-based methods, in comparison to culture-based methods, has also been reported for other pathogenic micro-organisms, including *Legionella* spp. (Frahm and Obst, 1995).

Application of the pit-stop seminested PCR to environmental water samples for the detection of *V. cholerae* and *Shigella* spp. indicated that these pathogens could be detected in 5 of 84 and 3 of 48 samples, respectively (Table 3). Some of the water samples exhibited inhibition of the PCR in some instances, 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 neutralised by sodium thiosulphate prior to analysis. The inhibition observed for the heavily contaminated effluents 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 (Weaver and Rowe, 1997). However, the sensitivity of the PCR for the detection of pathogens in these types of water samples may be enhanced by further dilution of the 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 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.

Conclusions

Although molecular technologies, such as those developed in this study, present potentially 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 micro-organisms 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 water-borne 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 micro-organisms. The results obtained during the course of this study have shown that it is possible to detect different types of pathogenic bacteria from water samples within 24 h. The combination of membrane filtration, an enrichment procedure and the pit-stop seminested PCR provided a sensitive, specific and easy method for

TABLE 3
Detection of *V. cholerae* and *Shigella* spp. in environmental samples by pit-stop seminested PCR assays

	Tap		River		Dam		Treated sewage effluents	
	No of samples	PCR results	No of samples	PCR results	No of samples	PCR results	No of samples	PCR results
Vibrio cholerae	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

the detection of *V. cholerae* and *Shigella* spp. in environmental water samples. The inclusion of an enrichment period allows for the detection of culturable bacteria which is crucial as PCR detection does not give indications on the viability of the detected material. Inhibiting substances hampered PCR detection only in a very limited number of samples and these consisted mainly of drinking water and heavily contaminated effluents. The inhibitory substances, however, may be removed by extracting the DNA prior to analysis or by further dilution of the samples.

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