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

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Abstract—A rapid seminested polymerase chain reaction (PCR) method for the specific, sensitive detection of virulent *Shigella* spp. in spiked environmental water samples was developed. A set of primers specific for the invasion plasmid antigen gene (*ipaH*) of virulent *Shigella* spp. and enteroinvasive *Escherichia 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 *S. flexneri* organisms in pure culture. 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 PCR. A detection limit lower than 14 cfu/ml was achieved in some water samples. The results indicate that the described seminested PCR has the advantage of a rapid turnaround time and it fulfills the requirements of sensitivity and specificity for use in an environmental laboratory. © 2001 Elsevier Science Ltd. All rights reserved.

Key words—*Shigella*, enteroinvasive *E. coli*, surface water, ground water, seminested PCR, enrichment

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). 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). In South Africa, children under 5 yr 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 implicated in virulent functions, is present in multiple copies on the invasion plasmid and the chromosome (Venkatesan *et al.*, 1989, 1991; Hartman *et al.*, 1990; Hale, 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 48–72 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

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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, b) 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 (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.

In this paper, we describe the highly sensitive and specific detection of virulent *Shigella* organisms and EIEC by seminested PCR combined with a short enrichment step. The sensitivity of the procedure was determined by using artificially seeded ground and surface water samples collected from different natural sources.

MATERIALS AND METHODS

Bacterial strains

Shigella flexneri; Strain CCRC 10772; obtained from the National Collection of Type Cultures; London, UK 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 1). The organisms were cultivated on MacConkey agar plates and maintained in LB broth (Difco) at 37°C; unless the culture collection instructions specified otherwise.

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/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 $10\,000 \times g$ 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.

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 $10\,000 \times g$ 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 in a shaking incubator. At time 0, 2, 4 and 6 h after seeding, the numbers of cells per milliliter 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.

Table 1. Bacterial strains examined[a]

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

[a] NCTC = 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.

Preparation of seeded environmental samples prior to PCR

Mid-exponential phase *S. flexneri* cells were serially diluted (10^{-1}–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 $10\,000 \times g$ 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.

DNA amplification and electrophoretic detection of amplicons

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.

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 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 GeneAmp thermal cycler (Perkin-Elmer Cetus, Model 2400). 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.

Single 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. The amplicons were resolved on a 2% agarose gel in 1 × TAE (40 mM Tris–HCl, 20 mM Na.acetate, 1 mM EDTA, pH 8.5) 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.

Restriction analysis

Samples (5 µl) of the 620-bp H8-H15 amplified fragment, purified by phenol–chloroform extraction and ethanol precipitation, were digested with 5 U *Hae* III (Roche). Following incubation at 37°C for 1.5 h, the fragments were separated on a 0.8% acrylamide gel and visualized by ethidium bromide staining and UV transillumination. *Hae* III was predicted to digest the 620-bp *ipaH* amplicon into 273-, 215- and 135-bp fragments.

RESULTS

Specificity of the 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 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.

To investigate the specificity of the seminested PCR, cell lysates prepared of all strains listed in Table 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 *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.

Sensitivity of PCR

For monitoring purposes, PCR detection of indicator and pathogenic organisms requires not only specificity, but also sensitivity to ensure the safety of the potable water. 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

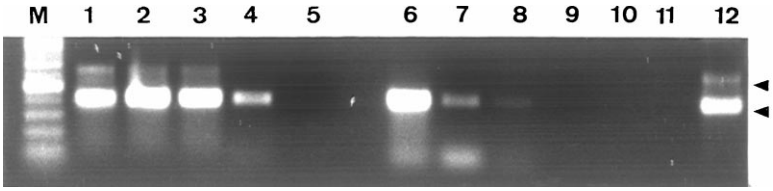

Fig. 1. Sensitivity of the PCR assay by a seminested (lanes 1–5) and single-step H8-H10 (lanes 6–10) PCR following enrichment in GN broth for 6 h. Lane 11, negative control; lane 12, positive virulent *S. flexneri* control. Lanes 1 and 6, 1.6×10^5; 2 and 7, 1.6×10^4; 3 and 8, 1.6×10^3; 4 and 9, 1.6×10^2; 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.

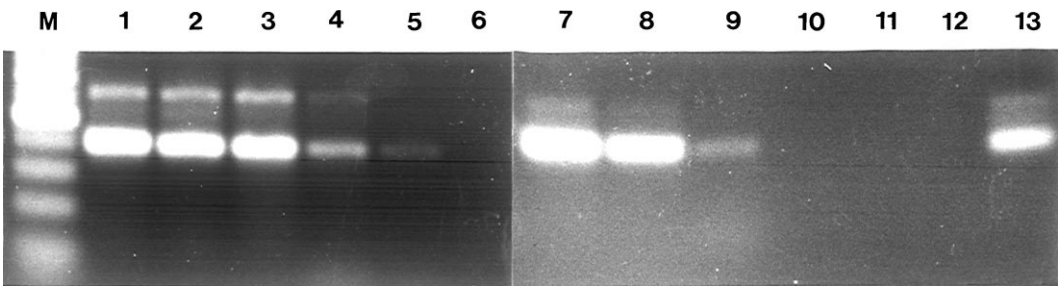

Fig. 2. Analysis of the limit of detection upon seeding environmental water samples with virulent *S. flexneri* following enrichment in GN broth using the seminested PCR protocol. Lanes 1–6, lake water seeded with 1.4×10^5 (lane 1), 1.4×10^4 (lane 2), 1.4×10^3 (lane 3), 1.4×10^2 (lane 4), 1.4×10^1 (lane 5), 1.4×10^0 (lane 6) cfu/ml; lanes 7–11, 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.

plating and template DNA was prepared by the boiling method described herein. 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 (Fig. 1). 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. 1).

Examination of seeded environmental water samples

To test the efficacy of the seminested PCR assay for monitoring 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. 2) yielded the following detection limits. While 2×10^3 cfu/ml (20 cfu/reaction) of virulent *S. flexneri* organisms could be detected in seeded well water, 14 cfu/ml (0.14 cfu/reaction) and 580 cfu/ml (5.8 cfu/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/reaction) and 11 cfu/ml (0.11 cfu/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.

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 (nonculturable) fail to grow in conventional culture media but remain viable when expressed 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/reaction. Under the tested parameters, 1.6×10^3 bacteria/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 with 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). Assays based on direct detection of bacterial cells in environmental water and sewage samples by filtration and PCR without an enrichment procedure have been developed (Bej *et al*., 1991a). A disadvantage of such methods, however, is that they may detect dead as well as viable bacteria. 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.

In the present 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 extraction or purification steps. Minimizing the number of manipulations reduced 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.

The sensitivity of the seminested PCR assay was also determined with environmental water samples from various sources inoculated with *S. flexneri*. In these seeding experiments, different numbers of *S. flexneri* could be detected. Depending on the environmental water sample examined, our method could detect as few as 11–14 cfu of *Shigella flexneri*/ml in some samples. It should be noted, however, that the bacteria used to seed the samples were freshly grown thereby minimizing amplified DNA from nonviable cells. Detection of sublethally damaged *Shigella* 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.

CONCLUSIONS

The results obtained in this work allow the following conclusions to be drawn.

(1) The seminested 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.
(2) 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.
(3) 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.

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REFERENCES

Bej A. K., Mahbubani M. H., DiCesare J. L. and Atlas R. M. (1991a) Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. *Appl. Environ. Microbiol.* **57**(12), 3529–3534.

Bej A. K., Steffan R. J., DiCesare J. L., Haf L. and Atlas R. M. (1991b) Detection of *Escherichia coli* and *Shigella* spp. in water using the polymerase chain reaction and gene probes for *uid*. *Appl. Environ. Microbiol.* **57**(4), 1013–1017.

Frankel G., Giron J. A., Valmassoi J. and Schoolink G. K. (1989) Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. *Mol. Microbiol.* **3**(12), 1729–1734.

Hale T. L. (1991) Genetic basis of virulence in *Shigella* species. *Microbiol. Rev.* **55**(2), 206–224.

Hartman A. B., Venkatesan M. M., Oaks E. V. and van Buysse J. M. (1990) Sequence and molecular characterization of multicopy invasion plasmid antigen gene, *ipaH*, of *Shigella flexneri*. *J. Bacteriol.* **172**(4), 1905–1915.

Islam M. S., Hasan M. K., Miah M. A., Sur G. C., Felsenstein M., Venkatesan M., Sack R. B. and Albert M. J. (1993a) Use of the polymerase chain reaction and fluorescent-antibody methods for detecting viable and nonculturable *Shigella dysenteriae* Type 1 in laboratory microcosms. *Appl. Environ. Microbiol.* **59**(2), 536–540.

Islam D. and Lindberg A. A. (1992) Detection of *Shigella dysenteriae* Type1 and *Shigella flexneri* in faeces by immunomagnetic isolation and polymerase chain reaction. *J. Clin. Microbiol.* **30**(11), 2801–2806.

Islam D., Tzipori S. and Lindberg A. A. (1993b) Rapid detection of *Shigella dysenteriae* and *Shigella flexneri* in faeces by an immunomagnetic assay with monoclonal antibodies. *European J. Clin. Microbiol. Infect. Dis.* **12**(1), 25–32.

June G. A., Sherrod P. S., Imaguana R. M., Andrews W. H. and Hammack T. S. (1993) Effectiveness of the Bacteriological Analytical Manual culture method for the recovery of *Shigella sonnei* from selected foods. *J. AOAC. Int.* **76**(6), 1240–1248.

Lindqvist R. (1999) Detection of *Shigella* spp. in food with a nested PCR method-sensitivity and performance compared with a conventional culture method. *J. Appl. Microbiol.* **86**, 971–978.

Pergram G. C., Rollins N. and Esprey Q. (1998) Estimating the costs of diarrhoea and epidemic dysentery in KwaZulu-Natal and South Africa. *Water SA* **24**(1), 11–20.

Rafii F., Holland M. A., Hill W. E. and Cerniglia C. E. (1995) Survival of *Shigella flexneri* on vegetables and detection by polymerase chain reaction. *J. Food Prot.* **58**(7), 727–732.

Rosenberg M. L., Hazlet K. K., Schaefer J., Wells J. G. and Pruneda R. C. (1976) Shigellosis from swimming. *J. Am. Med. Assoc.* **236**(16), 1849–1852.

Rossen L., Norskov P., Holmstrom K. and Rasmussen O. F. (1992) Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int. J. Food Microbiol.* **17**(1), 37–45.

Rowe B. and Gross R. J. (1984) Facultatively anaerobic gram negative rods. Genus II. Shigella. In *Bergey's Manual of Systematic Bacteriology*, N. R. Krieg and J. G. Holt, eds (pp. 423–427). Williams & Wilkins, Baltimore, MD.

Sethabutr O., Venkatesan M. M., Murphy G. S., Eampokalap B., Hoge C. W. and Echeverria P. (1993) Detection of Shigellae and enteroinvasive *Escherichia coli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. *J. Infect. Dis.* **167**(2), 458–461.

Venkatesan M. M., Buysse J. M. and Hartman A. B. (1991) Sequence variation in two *ipaH* genes of *Shigella flexneri* 5 and homology to the LRG-like family of proteins. *Mol. Microbiol.* **5**(10), 2435–2445.

Venkatesan M. M., Buysse J. M. and Kopecko D. J. (1989) Use of *Shigella flexneri ipaC* and *ipaH* gene sequences for the general identification of *Shigella* spp. and enteroinvasive *Escherichia coli*. *J. Clin. Microbiol.* **27**(12), 2687–2691.

Venkatesan M. M., Buysse J. M., Vandendries E. and Kopecko D. J. (1988) Development and testing of invasion-associated DNA probes for detection of *Shigella* spp. and enteroinvasive *Escherichia coli*. *J. Clin. Microbiol.* **26**(2), 261–266.

Yavzori M., Cohen D., Wasserlauf R., Ambar R., Rechavi G. and Ashkenazi S. (1994) Identification of *Shigella* species by DNA amplification of different loci of the *Shigella* virulence plasmid. *Eur. J. Clin. Microbiol. Infect. Dis.* **13**(3), 232–237.