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Single Multiplex Polymerase Chain Reaction for Environmental Surveillance of Toxigenic–Pathogenic O1 and Non-O1 *Vibrio cholerae*

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ABSTRACT. A multiplex PCR assay was developed for the detection of toxigenic and pathogenic *V. cholerae* from direct water sources using specific primers targeting diverse genes, *viz*. outer membrane protein (*ompW*), cholera toxin (*ctxB*), ORF specific for O1 (*rfbG*), zonula occludens (*zot*) and toxin co-regulated pilus (*tcpB*); among these genes, *ompW* acts as internal control for *V. cholerae*, the *ctx* gene as a marker for toxigenicity and *tcp* for pathogenicity. The sensitivity of multiplex PCR was 5×10^4 *V. cholerae* cells per reaction. The procedure was simplified as direct bacterial cells were used as template and there was no need for DNA extraction. The assay was specific as no amplification occurred with the other bacteria used. Toxigenic *V. cholerae* were artificially spiked in different water samples, filtered through a 0.45 μm membrane, and the filters containing bacteria were enriched in APW for 6 h. PCR following filtration and enrichment could detect as little as 8 *V. cholerae* cells per mL in different spiked water samples. Various environmental potable water samples were screened for the presence of *V. cholerae* using this assay procedure. The proposed method is rapid, sensitive and specific for environmental surveillance for the presence of toxigenic–pathogenic and nonpathogenic *V. cholerae*.

Abbreviations

APW	alkaline peptone water	PBS	phosphate-buffered saline
BHI	brain heart infusion (agar)	PCR	polymerase chain reaction
CFU	colony-forming unit	TCBS	thiocitrate–bile salt–sucrose (agar)

Cholera is an epidemic and life-threatening diarrhea, which is transmitted generally by the fecal–oral route. The disease is caused by *Vibrio cholerae* which primarily is an inhabitant of the aquatic environment. Therefore, water plays an important role in transmission and epidemiology of cholera (Faruque *et al.* 1998). The organism has the ability to cause outbreaks of epidemic and pandemic proportions. Cholera has been a consistent public health problem for most of the developing countries, which severely affects the economy too. Although *V. cholerae* is sensitive to ordinary disinfectants and chlorination, yet periodically occurring outbreaks of enteric diseases by contaminated water supplies prove the lapses of our system.

V. cholerae is classified into more than 200 serogroups. Of these, mainly O1 and O139 serogroups are capable of causing epidemic cholera. The other non-O1 and non-O139 serogroups are associated only with the sporadic cases of diarrhea (Morris 1990).

Early monitoring of *V. cholerae* in drinking-water sources is important to enable effective resource management and public health protection (Madico *et al*. 1996; Choopun *et al.* 2002). The conventional culture methods used to detect and classify *V. cholerae* are time-consuming and laborious. They require prolonged incubation and growth on selective media to reduce the number of other nonspecific organisms (Tamrakar *et al.* 2006). Moreover, these tests do not explain the serotype, toxigenicity and pathogenicity of the isolates. Many other techniques, such as ELISA, agglutination, immunofluorescence and immunosensors have also been employed for identification of *V. cholerae* (Goel *et al*. 2005*a*; Rao *et al*. 2006). However, these techniques have certain limitations and it is difficult to ascertain the toxigenicity and pathogenicity of isolates by using these techniques. Nucleic-acid-based techniques have recently emerged as powerful alternatives for rapid and reliable identification. Toxigenic *V. cholerae* can be identified by the presence of *ompW* and cholera toxin genes (Goel *et al*. 2005). However, from the epidemiological point of view, information about the serotype and the presence of other virulence genes in environmental strains can help in taking timely preventive measures. The cholera toxin (*ctx*) gene is essential for causing cholera. However, only *V. cholerae* O1 and O139 strains having the *ctx* gene possess the potential to cause epidemics while other, non-O1/non-

O139, strains possessing this gene cause only sporadic cases of gastroenteritis (Morris 1990). The toxin-coregulated pilus (*tcp*) gene is supposed to be important to acquire the *ctx* gene (Waldor and Mekalanos 1996). Therefore, any strain possessing this gene is considered as pathogenic and it can become toxigenic after acquiring the *ctx* gene. Zonula occludens toxin is another toxin which sometimes is involved in cholera.

Therefore, this study was aimed to design a quick and reliable method for identification of toxigenic *V. cholerae* in environmental water samples and determination of other important genes involved in epidemics by PCR.

MATERIALS AND METHODS

Bacterial cultures. Standard strain of *V. cholerae* 20 O1 Ogawa was obtained from *National Institute of Cholera and Enteric Disease*s (NICED; Kolkata, India) and was maintained on BHI agar (*Difco*, USA) at 22 °C. Other bacterial strains used: *Vibrio fischeri* (MTCC 1738), *V. parahaemolyticus* (MTCC 451), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) (MTCC 98), *Salmonella enterica* subsp. *enterica* sv. Paratyphi A (*Salmonella* Paratyphi) (MTCC 735), *Salmonella enterica* subsp. *enterica* sv. Virchow (*Salmonella* Virchow) (MTCC 1163), *Salmonella enterica* subsp. *enterica* sv. Infantis (*Salmonella* Infantis) (MTCC 1167), *Salmonella enterica* subsp. *enterica* sv. Bovismorbificans (*Salmonella* Bovismorbificans) (MTCC 1162), *Shigella flexneri* (MTCC 1457), *S. dysenteriae* (NICED isolate), *S. sonnei* (MTCC 2957). These cultures were obtained from the *Microbial Type Culture Collection and Gene Bank* (MTCC), *Institute of Microbial Technology* (Chandigarh, India).

Sensitivity of multiplex PCR. *V. cholerae* 20 O1 was grown overnight in a BHI broth and 10-fold serial dilutions were made in PBS (pH 7.4). Bacterial concentration (CFU/mL) was estimated by plating 100 μL of each dilution on BHI agar plates followed by incubation for 18 h at 37 °C. One-mL culture from each dilution was washed twice with deionized water by centrifugation (10 000 ***g***, 10 min) and finally re-suspended in 1 mL of deionized water. Ten-μL of each dilution was used directly as template in PCR.

Preparation of seeded environmental water samples. Water samples (ground water, tap water, pond water and sewer water) were collected locally from different places of Gwalior (longitude 78°13′E, latitude 26°13′N). Sea water samples were collected from Marina Beach, Chennai (longitude 80°17′E, latitude 13°04′N). Ten-fold serial dilutions of *V. cholerae* were made in respective non-sterilized water samples. One mL of each dilution was used to seed 99 mL of respective environmental water samples and left for 1 h at room temperature. Each spiked water sample was filtered through a polycarbonate membrane filter (0.45 μm pore size, 47 mm diameter; *Millipore*) using a hand-operated vacuum pump (vacuum pressure of 103–137 kPa). Each membrane was added to tubes containing 5 mL of APW (1 % peptone, 1 % NaCl, both *W*/*V*; pH 8.6), vortexed well to release the bacteria from the membrane and then incubated for 6 h at 37 °C. Three mL of enriched culture broth was taken from the upper surface and centrifuged (10 000 ***g***, 10 min) to collect the bacterial cells. The pellet was washed twice with deionized water and finally suspended in 100 μL of deionized water. Ten μL of suspension was used in PCR. Negative, non-inoculated water samples were included as controls in each of the experiments.

Processing of naturally contaminated environmental water samples. Water samples were collected from freshwater wells, hand pumps and sewers from different locations of Gwalior, Bhind and Datia (different districts of Madhya Pradesh, India) and processed (*see above*) for filtration and enrichment. Sewer samples (100 mL) were mixed with equal volume of distilled water, mixed well and allowed to settle. Slurry from the upper surface was processed as described *above*. Simultaneously, all the enriched samples were also grown on TCBS agar plates for confirmation of *V. cholerae*.

Multiplex PCR was developed to detect diverse traits of *V. cholerae* using specific oligonucleotide primers (Table I), 5 sets being used. Primers for the *ompW* gene were reported by Nandi *et al*. (2000); it amplifies a 304-bp fragment. Primers for *ctxAB* were designed on the basis of *ctx*-gene sequence of *V. cholerae* (*EMBL* and *GenBank* accession no. AF390572); it amplifies a 536-bp fragment of *V. cholerae*. Primers for *rfbO1*, *zot* and *tcp* gene were also designed from the available gene sequences. The primers were custom-synthesized from *Operon Biotechnologies* (Germany). In the PCR assay, direct bacterial cells suspended in deionized water were used as template for amplification. The specificity of multiplex PCR was determined by using the other homologous bacterial strains mentioned *above*. Different bacterial cells were subjected to multiplex PCR assay by simultaneous addition of primer pair for different genes in the same reaction mixture. PCR amplification of the target DNA was carried out using 200 μL PCR tube with a reaction mixture of 25 μL. Each of the reaction mixtures contained 1× reaction buffer, 200 μmol/L each of dATP, dCTP, dGTP and dTTP (*Promega*), 1.5 mmol/L $MgCl_2$, 1 U of Taq polymerase (*Promega*), 10× reaction buffer, varied concentrations of primers specific for each gene (Table I), 10 μL of template (direct bacterial

cells) and milli-Q water up to 25 μL. A gradient PCR was carried out in a thermal cycler (*BioRad*, USA) with the annealing temperature in the range of 52–62 °C to find out the appropriate annealing temperature

Table I. Details of primers used

Target	Nucleotide sequence	Amplicon size, bp	Primer concentration per reaction, pmol	Reference
ompW (F)	CAC CAA GAA GGT GAC TTT ATT GTG	304	12	Nandi *et al*. 2000
(R)	GGT TTG TCG AAT TAG CTT CAC C		12	
ctxAB (F)	GCC GGG TTG TGG GAA TGC TCC AAG	536	9	*this paper*
(R)	GCC ATA CTA ATT GCG GCA ATC GCA TG		9	
rfbO1 (F)	TCT ATG TGC TGC GAT TGG TG	638	10	*ditto*
(R)	CCC CGA AAA CCT AAT GTG AG		10	
tcp (F)	CGT TGG CGG TCA GTC TTG	805	12	*ditto*
(R)	CGG GCT TTC TTC TTG TTC G		12	
zot (F)	TCG CTT AAC GAT GGC GCG TTT T	947	7	Singh *et al*. 2001
(R)	AAC CCC GTT TCA CTT CTA CCC A		7	

that did not interfere with annealing of any of the primers. The optimum annealing temperature for the multiplex reaction was found to be 59 °C. The thermal cycler was programmed for 30 cycles starting with denaturation of the template DNA for 1 min at 94 °C, annealing of primers with template DNA for 1 min at 59 °C, and extension of the primers for 2 min at 72 °C. Before initiation of the first cycle, the reaction mixture was heated (10 min, 94 °C) to allow complete denaturation of the template and extraction of DNA from bacterial cells. After the last cycle, the reaction mixture was subjected for 10 min to 72 °C to ensure final extension. In a control reaction, deionized water was added to the reaction mixture instead of bacterial cells. PCR products were separated by electrophoresis on a 1.5 % (*W*/*V*) agarose gel with ethidium bromide (0.5 μg/mL) to resolve the amplified products. A 100-bp DNA ladder (*Sigma*) was used as a molecular size standard.

RESULTS AND DISCUSSION

Sensitivity of the multiplex PCR reaction was determined by using serially diluted *V. cholerae* cells. Amplicons of different genes could be visualized clearly on a 1.5 % agarose gel with ethidium bromide in a reaction mixture that contained 5/nL (*i.e.* 5×10^6 CFU per mL which corresponds to 5×10^4 CFU per reaction) (Fig. 1). The *ompW* primer yielded an amplicon of 304 bp. Primers of other genes yielded amplicons

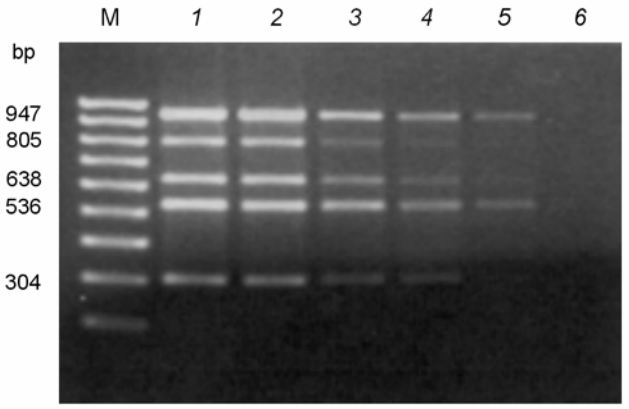

Fig. 1. Detection sensitivity of multiplex PCR assay with five primer sets of *V. cholerae* genes; *1* – 5×10^6 CFU per reaction, *2* – 5×10^5, *3* – 5×10^4, *4* – 5×10^3, *5* – 5×10^2, *6* – negative control; M – 100-bp DNA ladder (*Sigma*).

of 536 bp (*ctx*), 638 bp (*rfbO1*), 805 bp (*tcp*) and 947 bp (*zot*). These amplicons were considerably different in size from one another. The desired amplification of various genes showed that the whole bacterial cells could readily serve as template for PCR. The cell lysis during the initial 10-min denaturation at 94 °C during PCR was sufficient to provide suitable template. No amplification was observed with bacterial cells other than *V. cholerae*.

Environmental water samples, collected from different sources were spiked artificially with a known number of *cells* and, after filtration and enrichment in specific media, bacterial cells were harvested and directly used for PCR. The detection limit of multiplex PCR was 8 CFU/mL in ground water and sea water; in the case of tap water and pond water 80 CFU/mL could be detected (Table II). However, the detection sensitivity in spiked sewer water was 800 CFU/mL. No amplification was observed in any of the non-seeded water samples. The detection limit was higher in seeded tap water, pond water and sewer water samples probably due to the presence of disinfectants and other microflora.

Table II. Detection sensitivity[a] of multiplex PCR for *V. cholerae* in artificially spiked water samples[b]

Sample	8 CFU/mL	80 CFU/mL
Ground water	+	+
Tap water	–	+
Pond water	–	+
Sea water	+	+
Sewer water	–	–

[a](+) – positive, (–) – negative.
[b]Cell concentration 0.8–8000 CFU/mL; in samples with concentration of 800 and 8000 CFU/mL all results positive, at 0.8 CFU/mL all samples negative; control – unseeded, all negative.

A total of 56 water samples were collected from different locations and multiplex PCR was after filtration and enrichment employed. Of these enriched samples, 12 contained the *ompW* gene; by contrast, the *ctx* and *rfbO1* genes were present in none of the samples, indicating that these samples contained non-O1 *V. cholerae*. Both the *tcp* and *zot* genes simultaneously were found in only 3 samples (2× in a ground well, 1× in a sewer). All 12 samples were found to be positive on TCBS plates and by other biochemical tests (*data not shown*) which indicates that environmental samples contained non-O1 *V. cholerae*.

Prior to PCR, environmental water samples were filtered and enriched in APW which specifically promotes the growth of vibrios due to its alkaline pH. The enriched samples were centrifuged, washed with deionized water and directly used in PCR. Enrichment of *V. cholerae* in APW for 6 h was sufficient to multiply the bacteria to the sensitivity level of PCR. Our earlier studies showed that >1.2 CFU/mL of *V. cholerae* could be detected in water after a 6-h enrichment in APW by duplex PCR (Goel *et al*. 2005). It was also shown that undesired microorganisms and other PCR-interfering factors do not greatly influence the PCR reaction.

Monitoring the presence of *V. cholerae* in the water sources is helpful in early assessment of the public health threat posed by these bacteria, which needs a rapid and reliable method of *V. cholerae* detection in a heterogeneous population of bacteria. PCR has become an important and powerful tool for the detection of pathogenic microorganisms in the environmental as well as clinical samples (Bej and Mahbubani 1992; Koenraad *et al*. 1995; Catalan *et al*. 1997; Čermáková *et al*. 2005; Rudenko *et al*. 2005; Růžičková *et al*. 2005). Selective enrichment followed by PCR is a specific, sensitive and viable method for screening of a large number of bacteria. Primers used in this study were specific and no amplification was observed with other bacterial strains except *V. cholerae*. Nucleotide sequence data has proved that the *ompW* sequence is highly conserved among *V. cholerae* strains belonging to different biotypes or serotypes (Nandi *et al*. 2000). Therefore, the *ompW* gene acts as internal control for *V. cholerae*.

Among various toxins produced by *V. cholerae*, cholera toxin is the most potent and serves as a marker of epidemic potential (Kaper *et al*. 1995) but a number of other genes are involved in cholera pathogenesis. The *tcp* gene is required for cholera pathogenesis. Cholera toxin gene (*ctxAB*) is acquired from the genome of a filamentous CTX bacteriophage. The pilus colonization factor TCP acts as a receptor for CTXφ, which can infect non-toxigenic *V. cholerae*, leading to the emergence of new toxigenic strains (Waldor and Mekalanos 1996). Zonula occludens (*zot*) toxin is another toxin involved in cholera. The serotype of isolates is also very important because mainly *V. cholerae* O1 and O139 strains possess epidemic potential. In this PCR assay, all important genes could be detected simultaneously and we can determine in a short time that *V. cholerae* O1 or non-O1 is cholera toxin producer and pathogenic.

In environmental water samples, all isolates were *ctx*-gene-negative. We showed that most of the environmental *V. cholerae* strains are non-cholera toxin producers (Tamrakar *et al*. 2006). Therefore, the presence of non-toxigenic *V. cholerae* in potable water does not mean that the water is safe as the intestine is supposed to be the site where these non-toxigenic strains can be converted into toxigenic ones (Faruque *et al*. 1998).

PCR assay coupled with enrichment is a rapid and effective method for environmental surveillance and monitoring of *V. cholerae* in drinking-water sources. The method does not require DNA extraction and it can differentiate between toxigenic pathogenic and non-pathogenic *V. cholerae* strains.

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