Semi-nested polymerase chain reaction for detection of toxigenic *Vibrio cholerae* from environmental water samples

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Abstract A rapid and sensitive direct cell semi-nested PCR assay was developed for the detection of viable toxigenic *V. cholerae* in environmental water samples. The semi-nested PCR assay amplified cholera toxin (*ctxA2B*) gene present in the toxigenic *V. cholerae*. The detection sensitivity of direct cell semi-nested PCR was 2×10^3 CFU of *V. cholerae* whereas direct cell single-step PCR could detect 2×10^4 CFU of *V. cholerae*. The performance of the assay was evaluated using environmental water samples after spiking with known number of *Vibrio cholerae* O1. The spiked water samples were filtered through a 0.22 micrometer membrane and the bacteria retained on filters were enriched in alkaline peptone water and then used directly in the PCR assay. The semi-nested PCR procedure coupled with enrichment could detect less than 1 CFU/ml in ground water and sea water whereas 2 CFU/ml and 20 CFU/ml could be detected in pond water and tap water, respectively. The proposed method is simple, faster than the conventional detection assays and can be used for screening of drinking water or environmental water samples for the presence of toxigenic *V. cholerae*.

Keywords Semi-nested PCR · Environmental detection · Toxigenic · Viable · *Vibrio cholerae*

Introduction

Cholera is a severe diarrheal disease, mainly of those countries where access to safe drinking water and adequate sanitation is still a problem. Most of the developing countries are facing either a cholera outbreak or the threat of an epidemic, which along with serious public health problems severely affects the economy too. It is caused by toxigenic *Vibrio cholerae*, a Gram-negative, non-spore forming bacterium, which is primarily an inhabitant of the aquatic environment. Therefore, water plays an important role in the transmission and epidemiology of cholera^{1,2}. *V. cholerae* is a very delicate organism and is highly sensitive to dry heat, steam, boiling, short-term exposure to ordinary disinfectants, and chlorination of water³. Despite of this fact, cholera outbreaks do occur almost every year and that it may therefore be indicative of poor water supplies and water systems in third world countries.

V. cholerae has been reported to produce many toxins viz. cholera enterotoxin (CT), zonula occludens (Zot), accessory cholera enterotoxin (Ace), hemolysin and a new cholera toxin⁴. However, cholera enterotoxin is the major virulence factor responsible for profuse diarrhea, typically known as cholera gravis. Only cholera enterotoxin producer *V. cholerae* harness the potential to cause disease in the epidemic form. The disease manifests with profuse watery diarrhea, vomiting and dehydration, often associated with leg cramps due to electrolyte imbalance leading to severe dehydration and death. Although, cholera can be identified easily from clinical samples, yet rapid and accurate identification of *Vibrio cholerae* in drinking-water sources is important to enable effective resource management and public health protection^{5,6}. Conventional laboratory methods for detection of *V. cholerae* are time-consuming and laborious

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as these require growth on selective media to reduce the number of non-specific organisms⁷. However, these tests are not always reliable because the bacterium can remain in a viable but non-culturable state^{8,9}. Several other methods like ELISA, immunofluorescence and immunosensors also have been developed for detection of *V. cholerae*^{10–12}. But, these tests neither indicate about the presence of cholera enterotoxin gene nor about its expression in the isolates and to ascertain this trait, further immunological tests are required. Nucleic acid based techniques have emerged as powerful alternatives for fast and reliable identification of micro-organisms. In this study, a quick, reliable and sensitive method has been designed employing semi-nested PCR for detection of toxigenic *V. cholerae*. The performance of assay was evaluated using the environmental water samples after seeding with known number of *V. cholerae* followed by enrichment.

Materials and Methods

Bacterial strains: Reference strain of *V. cholerae* 20 O1 Ogawa used in this study was capable of producing cholera enterotoxin and was procured from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. This was maintained on tryptic soy agar (Difco, USA) at 22 °C. Sources and names of various other bacterial strains used in this study are listed in Table 1.

Sensitivity of direct cell PCR: *V. cholerae* was cultured overnight in brain heart infusion (BHI) broth after which ten-fold serial dilutions were made in PBS, pH 7.4 from overnight culture. Bacterial enumeration to determine CFU ml⁻¹ was done by plating 100 µl of each dilution on BHI agar plates followed by incubation at 37 °C for 18 h. One ml culture from each dilution was washed twice with de-ionized water by centrifugation at 10,000 × g for 10 min and finally re-suspended in 1 ml de-ionized water. Ten µl of each dilution was used directly as template in PCR.

Preparation of seeded environmental water samples: Ground water, pond water and tap water samples were collected locally from different places in 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). *V. cholerae* was cultured overnight in BHI broth and then ten-fold serial dilutions were made in respective water sample from overnight broth. Five ml of each dilution was used to seed 495 ml of respective environmental water samples and left for 1 h at room temperature. These inoculated water samples were filtered through a polycarbonate membrane filter of 0.22 µm pore size using vacuum pressure of 15–20 lb in⁻². After this, membranes were incubated in 5 ml of alkaline peptone water (APW, 1% peptone (w/v), 1% NaCl (w/v), pH 8.6) for 6 hrs at 37±1 °C under shaking conditions (100 rev. min⁻¹). One ml of enriched culture broth, taken from the top layer of APW was centrifuged at 10,000 × g for 10 min to collect the

Table 1 Bacterial strains used in this study

Bacterial strain	Source/strain
<i>Bacillus cereus</i>	ATCC 10876
<i>Bacillus subtilis</i>	ATCC 6633
<i>Escherichia coli</i>	ATCC 11775
<i>Salmonella bovis</i> MTCC 1162	Institute of Microbial Technology (IMTech), Chandigarh, India
<i>Salmonella infantis</i> MTCC 1167	IMTech, Chandigarh
<i>Salmonella paratyphi</i> A MTCC 735	IMTech, Chandigarh
<i>Salmonella sonnei</i> MTCC 2957	IMTech, Chandigarh
<i>Salmonella typhimurium</i> MTCC 98	IMTech, Chandigarh
<i>Salmonella virchow</i> MTCC 1163	IMTech, Chandigarh
<i>Shigella dysenteriae</i>	NICED, Kolkatta
<i>Shigella flexneri</i> MTCC 1457	IMTech, Chandigarh
<i>Staphylococcus aureus</i>	ATCC 12600
<i>Vibrio fischeri</i> MTCC 1738	IMTech, Chandigarh
<i>Vibrio parahaemolyticus</i> MTCC 451	IMTech, Chandigarh
<i>Vibrio vulnificus</i>	IMTech, Chandigarh
<i>Vibrio cholerae</i> O139	National Institute of Cholera and Enteric Diseases (NICED) Kolkatta
<i>Vibrio cholerae</i> 20 O1 Ogawa	National Institute of Cholera and Enteric Diseases (NICED) Kolkatta
<i>Vibrio cholerae</i> AVL6 (non-O1)	Environmental isolate

bacterial cells. The pellet was washed twice with de-ionized water, finally suspended in 20 μ l sterilized water and 10 μ l of suspension was used in PCR. Negative, un-inoculated water samples were included as controls in each of the experiments.

Processing of environmental water samples: A total of 17 metro water samples were collected in sterilized containers from cholera-affected areas of Chennai for analysis of the presence of *V. cholerae*. Aliquots of the samples were processed as described earlier for PCR assay.

Semi-nested PCR: Synthetic oligonucleotide primers PCR1, PCR2 and PCR3 were designed on the basis of cholera toxin (*ctxA2B*) gene sequence of *V. cholerae* (GenBank accession number AF390572) and were synthesized by Operon Biotechnologies, Germany. The first PCR step was performed with PCR1 (5'-GCCGGGTTGTGGGAATGCTCCAAG-3') and PCR2 (5'-CATGCGATTGCCGCAATTAGTATGGC-3') primers, which amplified a 536 bp product. An internal forward primer PCR3 (5'-GCACATGGAACACCTCAA-3') was designed from the *ctxA2B* sequence, which along with PCR2 primer amplified a product of 313 bp from the first PCR product.

In the first-step, PCR amplification of the target DNA was carried out using 200 μ l PCR tube with a reaction mixture of 25 μ l. Each of the reaction mixtures contained 1x PCR reaction buffer, 0.4 μ M each of PCR 1 and PCR2 primer, 200 μ M each of dATP, dCTP, dGTP and dTTP (Promega), 1.5 mM $MgCl_2$, 0.625 U of Taq polymerase (Promega) and 10 μ l of template (direct cells). Amplifications were carried out in a thermal cycler (BioRad, USA) programmed for 12 cycles starting with denaturation of the template DNA at 94 °C for 1 min, annealing of primers with template DNA at 59 °C for 1 min and extension of the primers at 72 °C for 2 min. Before initiation of the first cycle, the reaction mixture

was heated at 94 °C for 10 min to allow complete denaturation of the template. In the second step, the reaction mixture was same except that 5 μ l of the first-step PCR product was used as template and primers used were PCR2 and PCR3 (0.4 μ M each). The thermal cycler was programmed for 30 cycles with the same cycling conditions as in first-step PCR. After the last cycle, reaction mixture was subjected to 72 °C for 10 min to ensure final extension. In control reaction, deionized water was added to reaction mixture instead of bacterial cells. To determine the specificity of PCR, different bacterial cells listed in Table 1 were used in PCR. Sensitivity of the nested PCR was determined and compared with a single-step PCR by amplifying a serially diluted culture of *V. cholerae*. In this single-step whole cell PCR, thermal cycler was programmed for 30 cycles with the same cycling conditions as in first-step PCR. PCR products thus obtained were electrophoresed through 1.5 % (wt/vol) agarose gel containing ethidium bromide (0.5 μ g ml⁻¹) to resolve the amplified products. A 100 bp DNA ladder (Sigma) was loaded on gel as a molecular size standard.

Results and discussion

PCR sensitivity was determined by using 10-fold serially diluted *V. cholerae* cells for PCR amplification of *ctxA2B* gene. The detection limit of *V. cholerae* using the single-step direct cell PCR by using PCR1 and PCR2 primers was 2×10^4 CFU/reaction (Fig. 1a). These primers yielded an amplicon of 536 bp and showed that the viable cells of toxigenic *V. cholerae* culture could readily serve as DNA template for PCR. The cell lysis during the initial denaturation at 94 °C for 10 min during PCR is sufficient to provide suitable DNA template. The sensitivity was improved 10-fold by the

Fig. 1 Sensitivity of direct cell PCR assay for the detection of toxigenic *V. cholerae* by single-step (A) and semi-nested (B) PCR. Lane 1: 2×10^6 CFU; lane 2: 2×10^5 CFU; lane 3: 2×10^4 CFU; lane 4: 2×10^3 CFU; lane 5: 2×10^2 CFU; lane 6: 2×10^1 CFU. Lane M: 100 bp ladder (Sigma).

Table 2 Detection sensitivity of semi-nested PCR in artificially seeded water samples after enrichment

Sample	Semi-nested PCR for <i>V. cholerae</i> spiked water samples					Unseeded control
	2×10^{-2} CFU/ml	2×10^{-1} CFU/ml	2CFU/ml	2×10^1 CFU/ml	2×10^2 CFU/ml	
Ground water	–	+	+	+	+	–
Pond water	–	–	+	+	+	–
Tap water	–	–	–	+	+	–
Sea water	–	+	+	+	+	–

second PCR called semi-nested PCR using PCR3 and PCR2 primers which yielded an amplicon of 313 bp (Fig. 1b). To ascertain the specificity of semi-nested PCR, crude lysate of different bacterial strains listed in Table 1 were subjected to PCR. PCR using the two different primer combinations yielded amplification products of the expected sizes in the case of toxigenic *V. cholerae* strains (data not shown). The detection sensitivity of this semi-nested PCR was comparable with real-time PCR, which could detect 10^3 CFU of *V. cholerae* from the pure culture¹³.

Water samples collected from different places were artificially spiked with known number of viable toxigenic *V. cholerae* O1 cells to determine the detection limit of semi-nested PCR. These spiked samples were enriched for 6 h in APW after filtration and the samples were prepared for PCR. The detection limit of *V. cholerae* by semi-nested PCR was 0.2 CFU/ml in ground water and sea water, whereas, in case of pond water, 2 CFU/ml could be detected after enrichment (Table 2). However, the detection sensitivity in seeded tap water was 20 CFU/ml. All unseeded water samples were found negative for the presence of *V. cholerae*. PCR detection of *V. cholerae* in spiked pond and sea water samples indicated that micro-organisms and other PCR interfering factors do not greatly influence the PCR reaction. The detection sensitivity was lower in seeded tap water samples, probably due to the stress posed by chlorinating agents in the potable water. In earlier studies also, sensitivity of PCR was lower in tap water samples^{14, 15}.

Molecular monitoring of microbial ecosystems is a very rapid and reliable tool to detect specific micro-organisms. *V. cholerae* is a heterogenous species based on the heat-stable somatic O-antigen and only two serotypes, *V. cholerae* O1 and O139 have been characterized as toxigenic. Cholera is caused only by the toxigenic *V. cholerae* which is primarily an inhabitant of the aquatic environment. Laboratory methods for diagnosis are time consuming and further it is cumbersome to ascertain the toxigenicity of strains. For rapid clinical diagnosis, a wet mount of liquid stool is examined for typical darting movement of *Vibrio* bacteria. But for environmental detection of *V. cholerae*, several biochemical tests like growth of bacteria on selective TCBS medium, oxidase tests, arginine and esculin hydrolysis tests are performed⁷.

Therefore, PCR is becoming an important and powerful tool for detection of pathogenic micro-organisms in the environment^{16–18}. Selective enrichment followed by PCR is a rapid, sensitive, specific and viable method for screening of a large number of samples. Sometimes, DNA extraction complicates the protocol. But, in this study, there was no need of DNA extraction as live bacteria could serve as template after denaturation at 94 °C in PCR cycle. Primers used here were specific and no cross-reactivity was observed with other bacterial strains included in this study.

When this PCR assay was used to screen natural environmental water samples, out of 17 water samples, only 2 were found positive for toxigenic *V. cholerae*. These environmental water samples were found positive for *V. cholerae* on TCBS agar plates and by other biochemical tests (data not shown). Our earlier study has also shown that most of the environmental *V. cholerae* strains are non-cholera toxin producers^{7, 14}. In this study, sensitivity of PCR was enhanced at least 10 times than the normal PCR. Moreover, semi-nested PCR is highly specific because if there is any wrong amplification in the first cycle, it will not be amplified in the semi-nested cycle.

Therefore, semi-nested PCR assay coupled with enrichment is a rapid and effective method for assessing the microbial quality of water and environmental surveillance and monitoring of *V. cholerae* in water sources. The method is highly sensitive and specific. It does not require DNA extraction and samples can be analyzed quickly.

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