

Detection and confirmation of toxigenic *Vibrio cholerae* O1 in environmental and clinical samples by a direct cell multiplex PCR

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ABSTRACT

Epidemic cholera caused by toxigenic *Vibrio cholerae* O1 is a major health problem in several developing countries. Traditional methods for identifying *V. cholerae* involve cultural, biochemical and immunological assays which are cumbersome and often take several days to complete. In the present study, a direct cell multiplex PCR was developed targeting the *ompW*, *ctxB* and *rfbO1* genes for confirmation of *V. cholerae*, its toxigenicity and serogroup O1, respectively from clinical and environmental samples. The detection sensitivity of the multiplex PCR was 1.9×10^3 *V. cholerae* per PCR reaction. A total of 31 environmental samples and 45 clinical *V. cholerae* isolates from different outbreaks were examined by the PCR. The assay was simple and specific, as there was no requirement for DNA extraction and no amplification was observed with other homologous bacteria used. The assay can be very useful for rapid surveillance of toxigenic *V. cholerae* O1 in environmental water samples, as well as for confirmation of clinical isolates.

Keywords: cholera, *Vibrio cholerae*, PCR, environmental samples

INTRODUCTION

Cholera is an epidemic diarrheal disease that continues to devastate many countries where primary health and sanitation is still a challenge. Several cholera episodes are reported from developing countries due to contamination of water supplies. In 2010, a total of 48 countries reported 317 534 cases of cholera to the WHO, of which 36% were reported from Africa and 56.6% from the Americas, where a large outbreak was reported in Haiti (WHO, 2011). A total of 5 155 cases including 9 deaths were reported from India (WHO, 2011). Globally, the true number of cholera cases may be much higher because many cholera cases remain unreported. Cholera is caused by *Vibrio cholerae*, which is primarily an inhabitant of aquatic environments (Faruque et al., 1998, Islam et al., 1994). Therefore, water plays a significant role in the transmission and epidemiology of cholera. Monitoring the presence of *V. cholerae* in drinking water sources prior to disease outbreak is important to enable effective resource management and public health protection (Choopun et al., 2002).

V. cholerae is classified into more than 200 serogroups, but only *V. cholerae* O1 and O139 that produce cholera toxin (CT) have been associated with epidemics and pandemics (Rivera et al., 2001). The other non-O1, non-O139 serogroups are usually associated with some cases of mild gastroenteritis (Kaper et al., 1995). The ability to produce CT has been used to distinguish between cholerae and non-cholerae strains. CT non-producer O1 and O139 (non-cholerae) strains do not cause epidemic cholera (Levine et al., 1983); and CT-producing non-O1/non-O139 strains, although pathogenic, lack epidemic potential. Hence, detection and monitoring of toxigenic *V. cholerae* O1 or O139 is important during surveillance.

Various conventional cultural-, biochemical- and immunological-based assays are used for detection of *V. cholerae*. However, many of these methods are time-consuming and laborious. Moreover, biochemical identification systems may not always be accurate as several *Vibrio* species display similar biochemical characteristics (Nishibuchi, 2006; O'Hara et al., 2003). Several investigators have employed a direct immunofluorescence technique using polyclonal or monoclonal antibodies for detection of various bacteria and spores (Goel et al., 2005a; Huq et al., 1990). In contrast to culturing and immunological techniques, molecular methods have several advantages: they are rapid, sensitive, highly selective and do not require extensive hands-on time. In this study, we have developed a simple, sensitive and specific multiplex PCR that confirms the presence of *V. cholerae* in environmental and clinical samples, and exhibits its epidemic potential.

MATERIALS AND METHODS

Bacterial strains

The reference strain of *Vibrio cholerae* 20 O1 Ogawa was procured from the National Institute of Cholera and Enteric Disease (NICED), Kolkata, India, and was maintained on Brain Heart Infusion (BHI) agar (Difco, USA) at 22 °C. The other bacterial strains used in this study were *V. cholerae* O1 El Tor N16961 (ATCC 39315), *V. cholerae* O1 El Tor (ATCC 14033), *V. cholerae* O1 Classical 569B (ATCC 25870), *V. cholerae* O1 Classical (ATCC 11623), *V. cholerae* O139 (ATCC 51394), *V. fischeri* (MTCC 1738), *V. parahaemolyticus* (MTCC 451), *Salmonella typhimurium* (MTCC 98), *S. paratyphi* A (MTCC 735), *S. virchow* (MTCC 1163), *S. infantis* (MTCC 1167), *S. bovis* (MTCC 1162), *Shigella sonnei* (MTCC 2957), *Shigella flexneri* (MTCC 1457), *Shigella dysenteriae* (NICED isolate), *Escherichia coli* (ATCC 11775) and *Bacillus cereus* (ATCC 10876).

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Received 18 June 2012; accepted in revised form 20 August 2013.

Determination of sensitivity of direct cell PCR

Vibrio cholerae 20 O1 was grown in Brain Heart Infusion (BHI) broth for 16–18 h at 37°C and 10-fold serial dilutions were made in PBS, pH 7.4. Bacterial enumeration to determine CFU/ml was done by plating 100 µl of each dilution on BHI agar plates, followed by incubation at 37°C for 18 h. A 1-ml culture from each dilution was washed twice with de-ionised water by centrifugation at 10 000 x g for 10 min and finally re-suspended in 1 ml de-ionised water. Five microlitres (5 µl) of each dilution was used directly as template in PCR.

Processing of clinical samples

Clinical samples were processed for isolation of *V. cholerae* as described previously (Pourshafie et al., 2007). *V. cholerae* isolates from different outbreaks were grown on BHI agar plate for 16–18 h at 37°C and a loopful of bacterial culture was suspended in 500 µl de-ionised water. After mixing well, 5 µl of the solution was used directly in PCR. A total of 45 isolates from 3 different cholera outbreaks (15 isolates from each outbreak) in India were used in this study.

Processing of environmental water samples

A total of 31 water samples were collected from surface water (10), groundwater (10) and sewage water (11) from in and around Gwalior, Madhya Pradesh, India (26°13' N; 78°13' E). A total of 100 ml of water sample was filtered through a polycarbonate membrane filter of 0.45 µm pore size using vacuum pressure of 103–137 kPa. Sewage samples (100 ml) were mixed with an equal volume of distilled water, mixed well and allowed to settle. Slurry from the upper surface was processed for filtration. After this, the membranes were incubated in 5 ml of alkaline peptone water (APW, 1% peptone (w/v), 1% NaCl (w/v), pH 8.6) for 6 h at 37 ± 1°C under shaking conditions (100 r/min). One millilitre (1 ml) of enriched culture broth, taken from the top layer of APW was centrifuged at 10 000 x g for 10 min to collect the bacterial cells. The pellet was washed twice with de-ionised water and finally suspended in 20 µl of de-ionised water; 5 µl of the suspension was used for PCR. Negative, un-inoculated water samples were included as controls in each of the experiments. Simultaneously, all of the APW enriched samples were also grown on TCBS agar plates for confirmation of *V. cholerae*. Two loopfuls of the APW culture broth were streaked on to thiosulphate bile salt sucrose (TCBS) agar plates (Difco, USA) and incubated overnight at 37 ± 1°C. The yellow, flat colonies of 1–3 mm diameter were considered as *V. cholerae* (Tamrakar et al., 2006)

PCR

Three sets of primers for genes encoding the outer-membrane protein *OmpW* (*ompW*), cholera toxin (*ctxB*) and O1 somatic antigen (*rfbO1*) were used in this study. The sequences of primers (5'–3') used were:

ompW (For: CACCAAGAAGGTGACTTTATTGTG;

Rev: GAACTTATAACCCCGCG);

ctxB (For: GCACATGGAACACCTCAA;

Rev: GGCAAAACGGTTGCTTCT) and

rfbO1 (For: TCTATGTGCTGCGATTGGTG;

Rev: CCCCAGAAAACCTAATGTGAG).

The primers targeting the genes encoding *ompW*, *ctxB* and *rfbO1* amplify fragments of 588 bp, 386 bp and 638 bp,

respectively. Primers were selected from previous studies (Nandi et al., 2000) or were designed on the basis of GenBank gene sequences of *V. cholerae* using Oligo Explorer version 1.2 (Gene Link). In the PCR assay, bacterial cells suspended in de-ionised water were used as template for amplification. To determine the specificity of the PCR assay, a single colony of other bacteria strains mentioned above were suspended in 500 µl de-ionised water and used in the PCR. *Vibrio cholerae* cells were subjected to triplex PCR assay by simultaneous addition of the primer pairs for *ompW*, *ctxB* and *rfbO1* in the same reaction mixture. PCR amplification of the target DNA was carried out in a 200-µl PCR tube with a reaction mixture of 25 µl. Each of the reaction mixtures contained 1 x master mix (Fermentas, USA), 10 pmol each of the *ompW* and *rfbO1* primers, 20 pmol of the *ctxB* primers, 5 µl of template (direct cells), and distilled water. The master mix was supplied as a 2 x concentrated solution containing *Taq* DNA polymerase, reaction buffer, MgCl₂ and dNTPs. Amplifications were carried out in a thermal cycler (Corbett Life Sciences, Australia) programmed for 30 three-step cycles starting with denaturation of the template DNA at 95°C for 45 s; annealing at 57°C for 45 s and extension of the primers at 72°C for 1 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. After the last cycle, the reaction mixture was subjected to 72°C for 10 min to ensure final extension. In a control PCR reaction, deionised water was added to the reaction mixture instead of bacterial cells. PCR products thus obtained were electrophoresed on a 1.8% (w/v) agarose gel containing ethidium bromide (0.5 µg·ml⁻¹) to resolve the amplified products. A 100 bp DNA ladder (Fermentas, USA) was loaded onto the agarose gel as a molecular size standard.

RESULTS AND DISCUSSION

Multiplex PCR enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. The isolates were screened for the presence of 3 different genes for identification, toxigenicity and serogroup of *V. cholerae*. PCR sensitivity was determined by using 10-fold serially diluted *V. cholerae* cells for amplification of the *ompW*, *ctxAB* and *rfbO1* genes. Amplified fragments of 588, 386 and 638 bp could be visualised on 1.8% agarose with ethidium bromide in a reaction mixture that contained 1.9 x 10³ CFU/ reaction i.e 3.8 x 10⁵ CFU/ml (Fig. 1). The results showed that direct bacterial cells of a toxigenic *V. cholerae* culture could readily serve as template for PCR and there is no need for DNA extraction. Cell lysis during the initial denaturation at 94°C for 10 min during PCR is sufficient to provide a suitable template.

In the multiplex PCR, all the reference isolates of *V. cholerae*, except *V. cholerae* O1 El Tor (ATCC 14033) and *V. cholerae* O139 (ATCC 51394), showed all three genes (Table 1, Fig. 2). *V. cholerae* O1 El Tor ATCC 14033 does not possess the *ctxB* gene and hence was PCR positive only for the *ompW* and *rfbO1* genes. Likewise, *V. cholerae* O139 (ATCC 51394) does not possess the *rfbO1* gene and hence was PCR positive only for *ompW* and *ctxB* genes (Fig. 2). The multiplex PCR was specific as no amplification was observed with other bacteria used in the study (Table 1).

The *ompW* gene is species-specific for *V. cholerae* and its presence confirmed that the isolates were *V. cholerae*. Nucleotide sequence data has shown that the *ompW* sequence is highly conserved among *V. cholerae* strains belonging to different biotypes or serotypes (Nandi et al., 2000). The presence

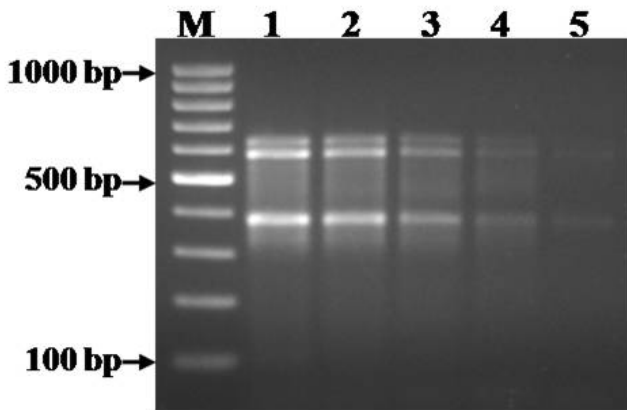


Figure 1

Sensitivity of direct cell multiplex PCR assay for the detection of *V. cholerae*. Lane M: 100 bp ladder (Fermentas); Lane 1: 1.9×10^5 cells/reaction; Lane 2: 1.9×10^4 cells/reaction; Lane 3: 1.9×10^3 cells/reaction; Lane 4: 1.9×10^2 cells/reaction; Lane 5: 1.9×10^1 cells/reaction

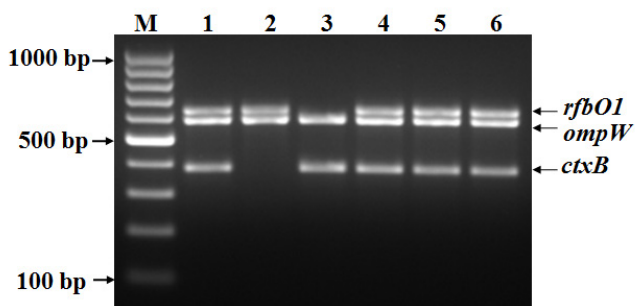


Figure 2

Direct cell multiplex PCR assay for the detection of *V. cholerae*. Lane M: 100 bp ladder (Fermentas); Lane 1: *V. cholerae* 20 O1; Lane 2: *V. cholerae* O1 ATCC 14033; Lane 3: *V. cholerae* O139 ATCC 51394; Lane 4: *V. cholerae* O1 ATCC 11623; Lane 5: *V. cholerae* O1 ATCC 39315; Lane 6: *V. cholerae* O1 569B ATCC 25870

of the *ctxB* and *rfbO1* genes confirmed the toxigenicity and O1 serogroup of the isolates, respectively. The cholera toxin is considered the most important epidemic marker among various toxins produced by *V. cholerae* (Kaper et al., 1995). The presence of the somatic O-antigen biosynthesis gene *rfbO1* provided molecular evidence for O1 serogroups, which has epidemic and pandemic potential.

A total of 31 water samples collected from different locations and 45 *V. cholerae* isolates from 3 different cholera outbreaks in India were subjected to multiplex PCR. Out of 31 environmental samples, only 14 samples tested positive for bacteria containing *ompW* gene (Table 1). One sewage water sample tested positive for *ompW* and *ctxB* gene, indicating the presence of toxigenic *V. cholerae* (Table 1). However, the *rfbO1* gene was not present in any of the samples, indicating the presence of non-O1 *V. cholerae* in the water samples. Prior to PCR, environmental water samples were filtered and enriched in APW, which specifically promotes the growth of *Vibrio* spp. due to its alkaline pH. The enriched samples were centrifuged and washed with deionised water and directly used in PCR. Enrichment of *V. cholerae* in APW for 6 h was sufficient to multiply the bacteria to within the limits of sensitivity of PCR. In earlier studies, it was found that about a single CFU/ml of *V. cholerae* could be detected in water after 6 h enrichment

Bacteria/sample	<i>ompW</i>	<i>ctxB</i>	<i>rfbO1</i>
<i>V. cholerae</i> 20 O1 Ogawa	+	+	+
<i>V. cholerae</i> O139 ATCC 51394	+	+	-
<i>V. cholerae</i> O1 El Tor N16961 ATCC 39315	+	+	+
<i>V. cholerae</i> O1 Classical ATCC 11623	+	+	+
<i>V. cholerae</i> O1 Classical 569B ATCC 25870	+	+	+
<i>V. cholerae</i> O1 El Tor ATCC 14033	+	-	+
<i>V. fischeri</i> (MTCC 1738)	-	-	-
<i>V. parahaemolyticus</i> (MTCC 451)	-	-	-
<i>Salmonella typhimurium</i> (MTCC 98)	-	-	-
<i>S. paratyphi</i> A (MTCC 735)	-	-	-
<i>S. virchow</i> (MTCC 1163)	-	-	-
<i>S. infantis</i> (MTCC 1167)	-	-	-
<i>S. bovis/morficans</i> (MTCC 1162)	-	-	-
<i>Shigella flexneri</i> (MTCC 1457)	-	-	-
<i>Shigella sonnei</i> (MTCC 2957)	-	-	-
<i>Shigella dysenteriae</i> (NICED isolate)	-	-	-
<i>Escherichia coli</i> (ATCC 11775)	-	-	-
<i>Bacillus cereus</i> (ATCC 10876)	-	-	-
Hand-pump water samples (2/10)	+	-	-
Hand-pump water samples (8/10)	-	-	-
Surface water samples (4/10)	+	-	-
Surface water samples (6/10)	-	-	-
Sewage water samples (7/11)	+	-	-
Sewage water samples (1/11)	+	+	-
Sewage water samples (3/11)	-	-	-
Clinical isolates from Orissa outbreak (15)	+	+	+
Clinical isolates from Chennai outbreak (15)	+	+	+
Clinical isolates from Solapur outbreak (15)	+	+	+

in APW (Goel et al., 2005b). All 14 samples tested positive on TCBS plates and using other biochemical tests, which indicated that environmental strains contained non-O1 *V. cholerae*. The 45 clinical *V. cholerae* isolates from different cholera outbreaks all harboured the three genes i.e. *ompW*, *ctxB* and *rfbO1* (Table 1). The study thus indicated that the clinical *V. cholerae* isolates possess the cholera toxin gene and generally belong to the O1 serogroup. In contrast, most of the environmental samples contained non-toxicogenic, non-O1 *V. cholerae*.

Traditional methods for cholera diagnosis are time consuming and further do not confirm the toxigenicity of strains. PCR has become an important and powerful tool for the detection of pathogenic micro-organisms. However, DNA isolation is a cumbersome process in PCR. Hence, the present PCR assay obviates the extraction of DNA and bacteria are rather used as PCR template. Therefore, the multiplex PCR assay described herein is a quick, sensitive and effective method for monitoring of *V. cholerae* in the environment as well as for confirmation of its toxigenicity and epidemic potential.

CONCLUSION

Toxigenic *Vibrio cholerae* O1 is the major causative agent of cholera. In this study, a simple, sensitive and specific multiplex PCR has been designed that confirms *V. cholerae*, its toxigenicity and serogroup O1. The assay obviates the need for extraction

of DNA and the enriched environmental samples or clinical isolates can be used directly as PCR templates. Thus, the present assay can be a useful tool for monitoring the presence *V. cholerae* in environmental water samples.

ACKNOWLEDGEMENTS

Authors are thankful to Director, Defence Research & Development Establishment (DRDE), Gwalior for providing necessary facilities and funds for the work.

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