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Simple and Accurate Detection of *Vibrio Cholera* Using Triplex Dot Blotting Assay

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ABSTRACT

Cholera outbreak is more common in developing countries. The causative agent of the disease is Vibrio cholerae strains O1 and O139. Traditional diagnostic testing for Vibrio is not always reliable, because Vibrio can enter a viable but non cultivable state. Therefore, nucleic acid-based tests have emerged as a useful alternative to traditional enrichment testing.

In this investigation, a triplex dot blotting assay has been developed for accurate and simple detection of V. cholerae using cholera toxin (ctxA, ctxB) and outer membrane protein (ompW) genes. The target genes were amplified using specific primers during monoplex polymerase chain reaction (PCR) and the amplicons were blotted on a nylon membrane. DIG-labeled PCR products in size of 219 (ctxA), 317 (ctxB) and 498 (ompW) bp, were amplified by a triplex PCR and used in a hybridization step as a probe. The positive signal was detected by applying anti-DIG HRP conjugate and chromogenic substrate. The results showed that the assay is sensitive enough to detect 10 cfu of V. cholerae O1. Also, the assay is specific enough to differentiate V. cholera from enterotoxigenic E. coli. The triplex dot blotting on clinical samples showed that it is more sensitive than monoplex and triplex PCR. In conclusion, we introduce a new rapid, sensitive and specific method for diagnosis of V. cholera in clinical specimens.

Keywords: Vibrio, Blotting, Detection, DIG

INTRODUCTION

Vibrio cholerae is an important agent of diarrheal disease in many parts of Asia and Africa. It is an enteric pathogen which can produce a global pandemic of the disease. Therefore, it is very important disease from point of public health. In spite of the appearance of more than 100 serogroups of *V. cholera*, the disease is only induced by two serogroups, O_1 and O_{139} which can produce cholera enterotoxin (CT) [1-4]. The pathogenesis of cholera is a complex process, and the major virulence factors of *V. cholerae* are CT encoded by the ctxAB genes and the toxin-coregulated pilus (TCP) encoded by the tcpA gene [5-7]. From a diagnostic point of view, toxigenic and non-toxigenic strains of *V. cholerae* can be differentiated by the presence of the cholera toxin and toxin-coregulated pilus genes [8].

Detection and identification of CT-producing *V. cholerae* by conventional culture, biochemical and immunological based assays is time consuming and laborious, which requiring more than three days [2]. On the other hand, commercially available kits cannot distinguish between the heat-labile enterotoxin (LT) of Escherichia coli and CT [9]. Due to nutrient starvation and physical stress, *Vibrio* cells may also enter a viable but non-culturable (VBNC) state. This may explain the failure of traditional culture techniques to isolate this organism from contaminated water and food samples implicated in food-borne outbreaks [10,11]. Therefore a rapid, reliable and practical assay for the detection of CT-producing *V. cholerae* is very important. Several rapid diagnostic tests for cholera have been described by detecting cholera toxin

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and lipopolysaccharide (LPS) antigen [12-14]. Polymerase chain reaction (PCR) and DNA probe techniques have been developed for rapid and sensitive detecting pathogenic *Vibrio* species. Several PCR protocols have been developed for *V. cholera*. PCR followed by hybridization techniques increase the sensitivity and specificity of the detection. Also, PCR detection confirms the presence of specific genetic regions in a target organism [15-20]. In this study a novel PCR-based method, named as triplex dot blotting assay, was developed for rapid, sensitive and specific detection of *V. cholerae* in clinical and environmental samples.

MATERIALS AND METHODS

DNA Extraction

V. cholerae serogroup O_1, enterotoxigenic E. coli (ETEC) and clinical samples were obtained from Bou-Ali Hospital in Tehran. All Strains were grown aerobically in LB broth for 24 h. The cultures were centrifuged at 9000 rpm for 3 min and genomic DNA was extracted by DNA isolation kit (Bioneer, South Korea) according to manufacturer's protocol.

Primer Design and PCR for ctxA, ctxB and ompW

Six pairs of primers were designed for specific amplification of ctxA, ctxB and ompW gene by Generunner software (Table 1). The primer Blast was performed using NCBI tool to study the specificity of the primers. PCR was carried out in two steps. In first step, the target genes, ctxA, ctxB and ompW, were amplified using M3-90, M5-100 and M8-90 primer pairs, respectively. In second step, three pairs of primers (M3-219, M5-317 and M8-498) were used in triplex PCR with DIG-labeled nucleotide. The amplification reaction was prepared in 25 µ1 containing 170 ng of genomic DNA, 2.5 µ1 of 10X PCR buffer, 10 pmol of each primers, 2 mM of each dNTP or DIG-dUTP, 1.5 mM of $Mgcl_2$ and 2.5 U of Taq DNA polymerase. The PCR was performed by initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 57.5 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. All PCR assays were performed using an automated thermal cycler (Techgene, Germany) and all PCR products were analyzed by 1% agarose gel electrophoresis.

Triplex Dot Blotting

The PCR products of the first step was denatured by 100 °C heating for 10 min and the blotted on three position of nylon membrane (Roche, Germany) using slot blot system (Amersham pharmacia biotech, Germany). UV crosslinking was performed by 40 joule for 10 min. Pre-hybridization and hybridization was carried out with DIG easy

Table 1. Primer Sequences of Used in this Investigation

Primer name	Target Gene	Oligonucleotide	Sequence	PCR product size (bp)
M3-90	ctxA	ctxA F1	5'- GGTCTTATGCCAAGAGGACAG - 3'	90
		ctxA R1	5'- CGTCTGAGTTCCTCTTGCATG - 3'	
M3-219	ctxA	ctxA F2	5'- GGTCTTATGCCAAGAGGACAG - 3'	219
		ctxA R2	5'- GTTGGGTGCAGTGGCTATAAC - 3'	
M5-100	ctxB	ctxB F1	5'- GAAGGATACCCTGAGGATTGC - 3'	100
		ctxB R1	5'- CATACTAATTGCGGCAATCG - 3'	
M5-317	ctxB	ctxB F2	5'- CATATGCACATGGAACACCTC - 3'	317
		ctxB R2	5'- CATACTAATTGCGGCAATCG - 3'	
M8-90	ompW	ompW F1	5'- CTGTATTTGCTCACCAAGAAGG - 3'	90
		ompW R1	5'- TGTTTAACACTTTATCGCTACTGTC - 3'	
M8-498	ompW	ompW F2	5'- CTGTATTTGCTCACCAAGAAGG - 3'	498
		ompW R2	5'- TTGGCATACCACACAGAAGC - 3'	

hybridization kit (Roche, Germany) using the denatured triplex PCR products with 219, 317 and 498-bp size fragment. In the next step, blots were incubated for 1 h at 1:1000 dilution of anti-DIG antibody conjugated to horseradish peroxidase (Roche, Germany). The enzymatic reaction was developed using H_2O_2 (0.1%) and diaminobenzidine (DAB, 400 µg ml^{-1}) in PBS as a chromogenic substrate.

The specificity of triplex dot blotting was determined by compared of *Vibrio* with ETEC genome in this test. Also, a known concentration (4.0×10^5 CFU/ml) of *V.cholerae* O1 was used to determine the sensitivity of the test. The known concentration of *V.cholerae* O1 was determined using adjusting the concentration to a level equivalent to a MacFarland standard and confirmed the concentration by plate count on tryptic soy agar.

RESULTS

Gene Amplification

DNA was extracted from bacterial strains *V. cholera* O1 and ETEC. The target genes were amplified by monoplex and triplex PCR using designed primers (Table 1) and then analyzed on 1% (w/v) agarose gel electrophoresis. The monoplex PCR was performed using pairs of primers M3-90, M5-100 and M8-90 which amplified 90 and 100 bp DNA fragments (Fig. 1A). In triplex PCR, three DNA fragments in size of 219, 317 and 498 bp were amplified using pairs of primers M3-219, M5-317 and M8-498 (Fig. 1B).

Triplex Dot Blot

For triplex dot blotting assay, amplified fragments of the monoplex PCR were denatured and blotted onto a nylon membrane. Hybridization was carried out using denatured DIG-labeled PCR products from triplex PCR as a probe. The blots were detected by anti-DIG antibody conjugated to horseradish peroxidase (HRP) and DAB chromogene. As shown in Fig. 2, amplicons of ctxA (M3), ctxB (M5) and ompW (M8) genes produced positive signals.

Specificity and Sensitivity of Triplex Dot Blot

Specificity of the triplex dot blotting assay was studied

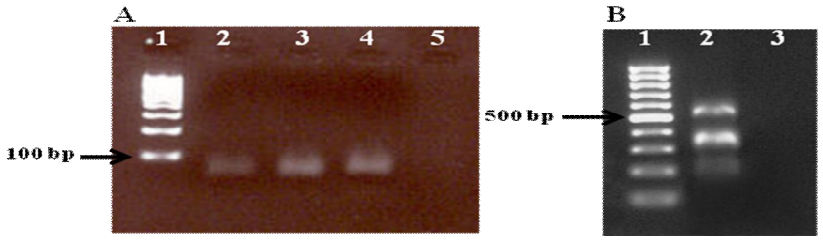

Fig. 1. (A) Monoplex PCR, lane 1: 100 bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp), lane 2: PCR products using M3-90 primer, lane 3: PCR products using M5-100 primer, lane 4: PCR products using M8-90 primer, lane 5: negative control. (B) Triplex PCR, lane 1: 100 bp DNA ladder, lane 2: PCR products using M3-219, M5-317, M8-498 primer, lane 3: negative control.

Fig. 2. (A) Triplex dot blot analysis of ctxA (M3), ctxB (M5) and ompW (M8) from *V.cholerae* O1. Monoplex PCR products with 90 or 100 bp fragment size was blotted, respectively and then hybrid into triplex PCR products that contend 219, 317 and 498 bp fragment size.

with ETEC. There was no positive signal when genomic DNA of ETEC was used as a template in triplex dot blot assay and triplex PCR (Fig. 3).

The sensitivity of triplex dot blot for DNA template at triplex PCR was estimated about 10 cfu (data was not shown). Also, triplex dot blotting using 10 clinical isolates of *V.cholerae* causing epidemics and sporadic cases of cholera in various parts of Iran confirmed that this detection assay is more sensitivity than mono and triplex PCR (Table 2).

DISCUSSIONS

Rapid, sensitive and specific measurement of *V. cholerae* is interest of many clinical laboratories. Application of traditional bacterial culture and microscopic approach with fluorescent-labeled antibodies is limited by direct detection of cholera toxin in clinical samples [1,2]. In this area, DNA-based amplification methods offer a useful alternative to conventional techniques, especially in clinic [15-20]. Although PCR assays provide more rapid

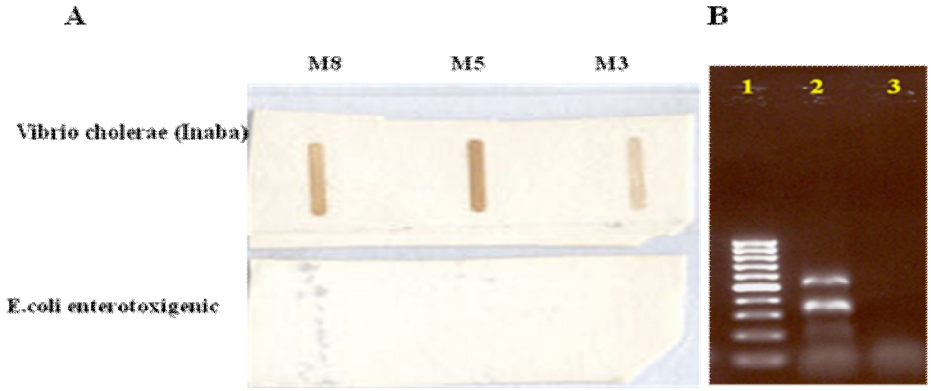

Fig. 3. (A) Specificity of triplex dot blot analysis of ctxA (M3), ctxB (M5) and ompW (M8) from *V.cholerae* and *E.coli* enterotoxigenic. (B) Specificity of triplex PCR of ctxA (M3-219), ctxB (M5-317) and ompW (M8-498) from *V.cholerae* (2) and *E.coli* enterotoxigenic(3) using ethidium bromide-stained agarose gel electrophoresis. Lane 1, 100 bp DNA ladder.

Table 2. Summarized Results of Mono/Triplex PCR and Triplex Dot Blot Analysis of 10 Clinical Isolates of *V.cholerae*. Genomic DNA in All Experiments was 8 Picogram (10 cfu/ml)

Sample	Monoplex PCR			Triplex PCR	Triplex dot blot
	ctx A	ctx B	ompW		
1	+	+	+	-	+
2	+	-	+	-	+
3	-	+	+	-	+
4	+	+	+	-	+
5	+	+	+	-	+
6	-	+	+	-	+
7	+	-	+	-	+
8	+	+	-	-	+
9	-	+	+	-	+
10	-	+	+	-	+

identification of *V. cholera* than conventional assays, they require the use of electrophoresis to detect amplified products, which is time-consuming, tedious and may be suffers from lack of specificity and sensitivity. In this case, PCR combined with a hybridization assay may be improves experiment specificity and sensitivity. Specific oligonucleotide probe has been successfully applied in detection and differentiation of *V. cholerae* strains using nucleic acid hybridization assay [21, 22]. In the present study, a new triplex dot blotting assay was developed to identify *V. cholerae*. The preferred targets for pathogen detection are pathogen virulence genes. Virulence genes have been successfully applied in PCR detection assays for *V. cholera*.

We selected three genes of ctxA, ctxB and ompW for detection of all *V. cholerae* species. It is because of, for example, the *ctx* gene is expressed in some of the serotypes of *V. cholerae* O1/O139 and some strains of non-O1/O139, but not at all in other non-O1/O139 strains [23-25]. The results showed that triplex dot blotting assay was more sensitive than PCR. Kapley and Purohit reported the sensitivity levels of 100 CFU for *V. cholerae* using conventional PCR assay [19] while 10-fold increase in sensitivity can be achieved using triplex dot blotting assay.

CT-producing *V. cholera* is closely related to LT-producing *E. coli* at the immunological and genetic levels [26], therefore their discernment is critical. A commercial reversed passive latex agglutination assay kit for the detection of CT/LT is available. However, this kit is unable to discern between CT and LT. PCR assays have been shown suitable for the specific detection of the *ctx* gene without confusing the *lt* gene [9,26]. Here, we developed a new specific triplex dot blotting assay for detecting of CT-producing *V. cholera.*

In conclusion, the triplex dot blotting assay provided sensitive, simple and rapid detection of *V. cholerae* which can be used as an alternative to conventional assays.

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