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Rapid Screening of Toxigenic *Vibrio cholerae* O1 Strains from South Iran by PCR-ELISA

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ABSTRACT

Background: The ability to sensitively detect *Vibrio cholera* with PCR-ELISA method represents a considerable advancement over alternative more time-consuming methods for detection of this pathogen. The aim of this research is to evaluate the suitability of a PCR-enzyme-linked immunosorbent assay for sensitive and rapid detection of *V. cholera* O1. **Methods:** The 398-bp sequence of a gene that codes for the cholera toxin B subunit was amplified by PCR. The digoxigenin-labeled amplified products were coated on microplates and detected by ELISA. The PCR product was also hybridized with biotin labelled probe and detected by ELISA using streptavidin. **Results and Conclusion:** The specificity of the PCR was determined using 10 bacterial strains and 50 samples from south Iran. The detection limit was 0.5 pg of the genomic DNA and five bacterial cells. Adaptation of PCR into PCR-ELISA assay format facilitates specific and sensitive detection and diagnosis of human cholera disease. We conclude that this PCR-ELISA is a diagnostic method that specifically detects toxin genes in *V. cholera* O1 strains. It is more rapid and less cumbersome than other diagnostic methods for detection of toxicity in these strains. *Iran. Biomed. J. 12 (1): 15-21, 2008*

Keywords: *Vibrio cholera*, PCR- ELISA, Immunosorbent assay

INTRODUCTION

Cholera disease caused by toxigenic *Vibrio cholera* is of extraordinarily rapid onset and potentially high lethality [1-3]. Although clinical management of Cholera has advanced over the past 40 years, it remains a serious threat in developing countries [4]. The standard routine method for *Vibrio* detection involves isolation on a selective thiosulfate citrate bile salts sucrose medium followed by biochemical and physiological tests. Several problems are encountered with culturing methods such as time consuming [4, 5]. To avoid these problems, different methods based on molecular biology techniques have been developed, such as DNA amplification technique that is rapid and very sensitive [4, 6-8]. Only strains of *V. cholera* O1 that produce cholera toxin have been associated with epidemics and pandemics; therefore, production of cholera toxin has become an important marker for identifying isolates with the potential to cause epidemics [9, 10]. The methods used to detect cholera toxin in clinical microbiological laboratories have so far been based on the immunological property of cholera toxin or a specific DNA sequence encoding cholera toxin in the bacterial chromosome [11-15]. Both methods thus have limited use in practice when dealing with large numbers of clinical or environmental isolates within a relatively short time [15, 16]. Rivera *et al.* [10-17] reported that the use of a PCR technique with water samples provides better results than conventional microbiological techniques in the diagnosis of the cholera disease. PCR method based on amplification of target sequence of the ctxB gene of *V. cholera* O1 has been reported by several workers [6, 7]. The approaches in conventional PCR assays are often time-consuming and poorly suited for use in general diagnostic laboratories and large scale screening [18-20]. In an attempt to avoid all of those difficulties, we have applied PCR-enzyme-linked immunosorbent assay for large scale screening of *V.*

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cholera O1 based on ctxB gene. PCR-ELISA involves incorporation of chemically tagged nucleotides into the PCR amplicon and subsequent detection of the PCR product with antibody-enzyme conjugate that recognizes the unique chemical label present in the incorporated nucleotides [19, 21, 22].

MATERIALS AND METHODS

Bacterial strains and stool samples. Bacterial strains employed as negative and positive control were toxigenic *V. cholera* O1 ATCC 9458, *Salmonella paratyphi C* ATCC 9068-14035-39315, *Klebsiella pneumoniae* ATCC 13883, *Shigella dysenteriae* ATCC 13313, *Enterobacter aerogenes* ATCC 49469, *Pseudomonas aeruginosa* ATCC 35554 and non-toxigenic *V. cholera* ATCC 25872-25874, collected from Reference Laboratory, Boo-Ali Hospital (Tehran, Iran). A total of 50 stool samples from *V. cholera* infected patients were collected from Sarbaz city villages of Sistan and Baluchistan province (Iran).

Culture-based detection and confirmation of V. cholera. The stool specimens from diarrheal patients collected in sterile containers were transferred into 100 ml enrichment broth and incubated at room temperature for 12 h. A loopful of growth was inoculated into alkaline peptone water (APW) and incubated at 37°C for 18 h. A yellow colony phenotype from thiosulfate citrate-bile salt-sucrose agar plates was inoculated into triple sugar iron agar (Oxoid,United Kingdom) slants. Following incubation at 37°C/18 h, a colony of each isolate ($acid^+$, H_2S^-) was suspended in tryptone water and then streaked onto Mac Conkey agar (Oxoid, United Kingdom) and sulfur-indole-motility agar (Oxoid, United Kingdom). Only isolates which displayed motility grew on Mac Conkey agar and were positive for oxidase and indole, but negative for H_2S production, were retained. Toxigenic *V. cholera* isolates were tested for cholera toxin by agglutination assays using O1-polyvalent antiserum (Sigma,USA) [17].

Preparation of samples for direct PCR:

Pure culture suspension. Pure cultures of *V. cholera* were used to determine the sensitivity and specificity of the method. Serial dilutions of this strain were prepared in sterile PBS for colony counting and for heat extraction at 95ºC for 10 min to provide DNA templates.

Diarrheal stool samples. In order to evaluate the usefulness of the PCR DIG-ELISA method for routine application in a diagnostic bacteriology laboratory, stool specimens from patients with diarrhea were inoculated into enrichment and were incubated overnight at 37ºC. After low-speed centrifugation to remove stool particles, the bacteria were pelleted by high-speed centrifugation and washed twice with PBS.

Preparation of genomic DNA. A modified method of Muarry and Thompson [23] was used for DNA extraction. Briefly, cells harvested from 1.5 ml of an Broth culture were resuspended in 56 µl Tris-EDTA buffer, treated with 30 µl of 10% SDS and 3µl freshly prepared proteinase K (100 µg ml^{-1}) followed by incubation at 37ºC for 1 h. A dose of 100 µl cholera CTAB/NaCl (10% cetyl trimethyl ammonium bromide in 0.7 M NaCl) was added and the mixture was again incubated at 65ºC for 10 min. The aqueous phase was obtained from centrifugation at 4000 ×g at 4ºC, treated with an equal volume of phenol-chloroform and the DNA pellet was washed with 1 ml ethanol 70% suspended in Tris-EDTA, and treated with 5 µl RNase at 37ºC for 30 min. The concentration and purity of the DNA was then determined spectrophotometrically at A_{260} and A_{280}.

PCR primers and DNA amplification. A 29-bp forward primer (5'-GGATGAATTATGATTAAATT AAAATTTGG-3') and reverse primer (5'-GGCTT TTTTATATCTTAATTTGCCATACT-3') targeting cholera toxin B subunit gene of *V. cholera* were used in PCR to obtain a 398-bp product. The PCR assay was performed in a final reaction volume of 50 µl. Each reaction mixture consisted of 0.3 µM of each primer, 200 µM of each dNTP, 190 µM dTTP, 10 µM digoxigenin-11-dUTP (Roche Diagnostics, Germany); 0.5 U of *Taq* DNA polymerase (Fermentas, Russia), 5 µl of 10X PCR buffer, 1.5 mM $MgCl_2$ in the presence of genomic DNA and lysed cells. The PCR program was carried out at 94ºC for 5 minutes followed by 5 and 30 cycles at 94ºC, 60ºC and 72ºC each for 45 s and the final extension at 72ºC for 5 minutes. Each PCR product (5 µl) was loaded into a well of a 2% agarose gel containing 0.5 µg ml^{-1} ethidium bromide. A 100-bp ladder plus (Fermentas, Russia) was used as the molecular weight marker. PCR products were electrophoresed and visualized under UV light, and gel images were stored using a gel documentation system (Gel Doc 1000; Bio-Rad, Hercules, CA).

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Detection of PCR products with DIG-ELISA. PCR amplified product (10 µl) was added to 90 µl of PBS (pH 7.2) and was then serially diluted in the wells of the micro titer plates. The plates were dried at 65°C without shaking for 1.5 h. Plates were washed five times with PBS (pH 7.2) containing 0.05% Tween 20 (PBST). Antidigoxigenin fab-peroxidase conjugate (100 µl, Roche Diagnostics, Germany), labeled with horse radish peroxidase and diluted 1:2500 in PBST, was added to each well and incubated at 37°C and shook for 30 min. After washing, 100 µl of substrate solution (O-Phenylene Diamine 0.2mg ml^{-1} of citrate phosphate buffer-pH 5 containing 2.5 µl 30% H_2O_2) was added to each well of microplate and incubated in the dark at room temperature without shaking for 15 min. A dose of 100 µl of 1 M H_2SO_4 was then added to stop the reaction. The optical density was measured at 490 nm using an ELISA reader (Dynex Technologies, Guornesey, Channel Islands and Great Britain) [21, 22].

Hybridization:

Development of the probe. In order to confirm the result obtained, we designed a biotin labelled probe (5′-biotin- AGC TGG AAA AAG AGA GAT GGC-3′) for the middle part of the ctxB gene. For the hybridization reaction, 80 µl of hybridization solution (1X saline-sodium citrate with 0.6 pmol biotinylated probe) was added to 20 µl of the PCR product of primer pairs and denatured by heating at 95°C for 15 min. The hybridization reaction (100 µl) was incubated on ice for 5 min and then transferred into the microplate wells sensitized with streptavidin as described previously by Luck *et al.* [19]. The hybridization was carried out f at 55°C or 3 h. Anti-DIG antibody-peroxidase conjugate (diluted 1:2500 in PBST) was added to each well and incubated at 37°C for 1 h before the wells were washed five times with the wash buffer. Finally, the peroxidase substrate was added to wells and optical density values were recorded with an ELISA plate reader at 490 nm [22-24].

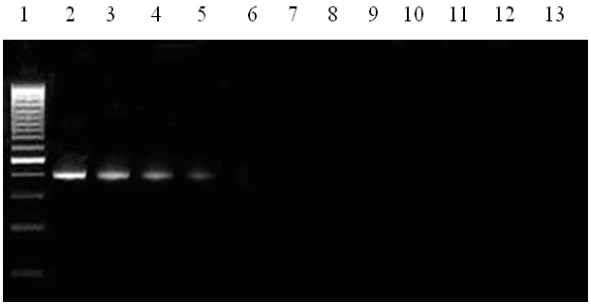

Fig. 1. Sensitivity of PCR product (398 bp) using genomic DNA, followed by agarose gel electrophoresis. Lane 1, DNA marker (100 bp DNA ladder plus); Lanes 2-7, the serial dilution (50, 5, 5×10^{-1}, 5×10^{-2}, 5×10^{-3}, 5×10^{-4} µg) of genomic DNA of *V. chorea* O1 with 30 cycles; Lanes 8-13, The serial dilution of genomic DNA of *Vibrio chorea* O1 with 5 cycles.

Specificity and sensitivity of PCR-ELISA technique. The specificity of the PCR-ELISA was determined using 50 pg of genomic DNA of 50 samples collected from Sistan province and 10 strains of other bacteria (non-toxigenic *Vibrio cholera , Vibrio cholera* O1*, Salmonella paratyphi C, Klebsiella pneumoniae, Shigella dysenteriae, Enterobacter aerogenes and Pseudomonas aeruginosa*). To determine the detection limit for *V. cholera*, genomic DNA was extracted and ten fold serial dilutions were made from the extracted DNA (50µg $µl^{-1}$) and tested with PCR-ELISA. This method was also applied to the cell lystes as DNA source (1 to 1500 cells in each reaction).

RESULTS

Sensitivity of PCR product. The results obtained from PCR with serial dilutions of genomic DNA and bacterial cell samples of *V. cholera* O1 with 5 and 30 cycles are shown in Figures 1 and 2.

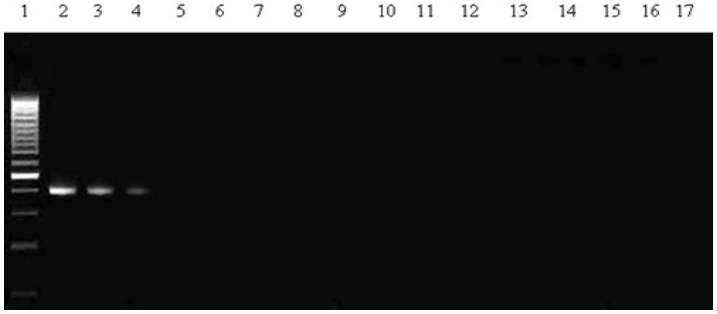

Fig. 2. Sensitivity of PCR using cell lysate, followed by agarose gel electrophoresis. Lane 1, DNA marker (100 bp DNA ladder plus); Lane 2, positive control; Lanes 3-9, the serial dilution of bacterial sample (1000, 750, 500, 250, 100, 20, 5 and 1) cells with 30 cycles; lanes 10-16, the serial dilution of bacterial sample with 5 cycles; Lane 17, negative control.

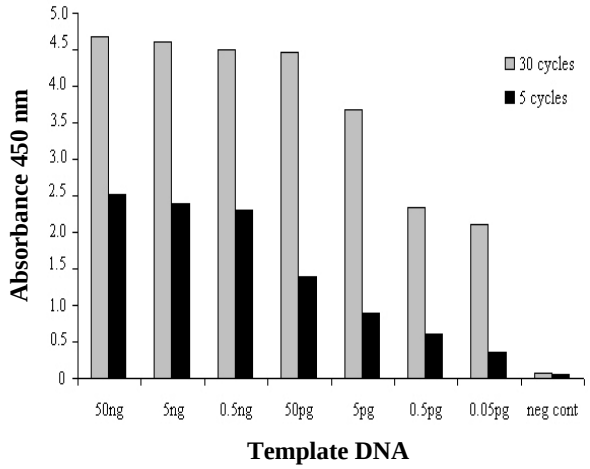

Fig. 3. The sensitivity of the PCR-ELISA technique against samples derived from serial dilution of DNA extraction of *V. cholera* O1.

The minimum amount of genomic DNA and bacterial cell of *V. cholera* O1 that produced an observable band on ethidium bromide-stained agarose gel electrophoresis (AGE) was 50 pg of DNA and 750 bacteria with 30 cycles. No band was observed from low dilutions with 5 and 30 cycles.

PCR-ELISA sensitivity assessment. In order to determine the minimum detectable concentration of DNA and bacteria cell, serial dilutions were subjected to the PCR-ELISA technique. The results (Figs. 3 and 4) demonstrate possibility of detecting 0.5 pg of DNA or 5 bacteria from *V. cholera* O1. In Figures 1-4, the sensitivity between gel electrophoresis and ELISA were compared. Our Elisa method could readily detect the PCR products appearing weakly or as invisible bands on electrophoretic gels.

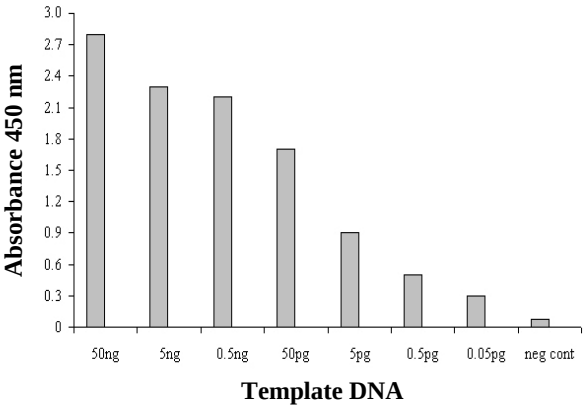

Fig. 4. The sensitivity of PCR-ELISA technique in culture sample *V. cholera* O1.

Specificity of PCR-ELISA technique. The specificity of the PCR-ELISA was assessed on 50 strains of *V. cholera* O1 found in stool samples, one standard strain of the two strains of *V. cholera* O1, two strains of the non-toxigenic *V. cholera*, and five strains of other bacteria including *S. paratyphi* C, *K. pneumoniae*, *Sh. dysenteriae*, *E. aerogenes* and *P. aeruginosa* (Table 1). The absorbance readings obtained with nine strains of non-toxigenic *V. cholera* and other bacteria were lower than 0.072 and considered negative in PCR-ELISA whereas the absorbance readings obtained with the 50 samples of *V. cholera* were higher than the cut-off, indicating that the PCR-ELISA test was fully reliable.

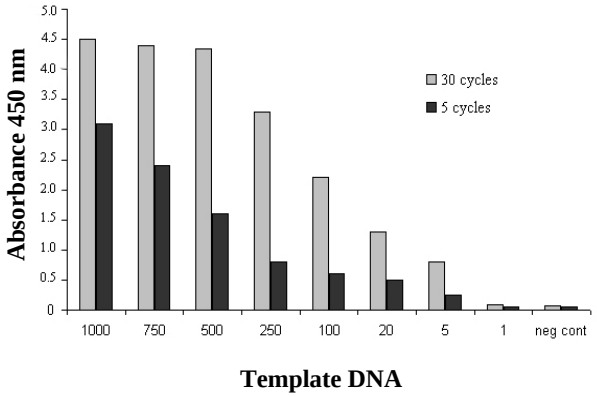

Fig. 5. The sensitivity of detection of hybridization against samples derived from serial dilution of DNA extraction of *Vibrio cholera* O1.

Comparison of PCR- ELISA with culture-based methods assays. A total of 50 stool samples from patients were screened for the presence of *V. cholera* and the results obtained by bacteriological culturing were compared to those obtained by PCR-ELISA. The results obtained from PCR-ELISA assay and from selective culture are compared in Table 2. Out of 50 samples tested, only one sample was culture positive but ELISA negative. The Chi-square test indicated a significant correlation between PCR-ELISA and culture methods for detecting and screening *V. cholera* ($P<0.001$).

DISCUSSION

PCR assays based on amplification of target DNA sequences in the ctx gene of *V. cholera* have been reported [5, 25-27]. These investigators used PCR to identify toxigenic *V. cholera* in stool samples of

Table 1. The Specificity of PCR-ELISA technique.

Sample	PCR 30 cycles		ELISA	
	Positive	Negative	OD 30 cycles	OD 5 cycles
Standard sample	+		>4.255	1.115
1	+		>4.221	1.121
2	+		>4.125	1.145
3	+		>4.123	1.230
4	+		>4.324	1.008
5	+		>4.214	0.050
6	+		>4.250	1.025
7	+		>4.321	1.258
8	+		>4.021	1.155
9	+		>4.301	1.220
10	+		>4.121	١,٢٤٢
11	+		>4.391	1.130
12	+		>4.329	1.100
13	+		>4.321	1.161
14	+		>4.320	1.104
15	+		>4.121	1.030
16	+		>4.401	1.125
17	+		>4.523	1.181
18	+		>4.321	1.102
19	+		>4.321	1.201
20	+		>4.300	1.115
21	+		>4.101	1.171
22	+		>4.221	1.165
23	+		>4.021	1.301
24	+		>4.451	1.205
25	+		>4.191	1.129
26	+		>4.129	1.105
27	+		>4.391	1.280
28	+		>4.281	1.010
29	+		>4.124	1.021
30	+		>4.395	1.365

the patients with cholera, or as an alternative to other *V. cholera* diagnostic methods such as enzyme-linked immunosorbent assay. The present paper reports a PCR-ELISA method for detection of the cholera toxin B subunit in *V. cholera* O1 strains. The PCR-ELISA method offers several advantages over PCR-AGE technique. It combines PCR with a hybridization step that ensures correct identity of amplified product. In the present study, products from 5 and 30 PCR cycles were screened on agarose gel followed by ELISA analysis. The 5 cycles of PCR product, undetectable on agarose gel, were successfully detected by ELISA. The method has the advantages of reduced reaction time by at least one hour in addition to contentment in chemicals such as dNTP and primers while retaining the sensitivity of the method at its precise specificity. Our method shows 150 folds sensitivity over visualization of the amplicons in EtBr-stained agarose gels. Govea *et al.* [28] identified *V. cholera* by ELISA and dot-ELISA with outer membrane protein antisera. In dot-ELISA and ELISA, 106 and 108 CFU/ml were positive. Jesudason *et al.* [29] used coagglutination test for screening of *V. cholera* in food samples. Theron *et al.* [4] reported detection of 1300 cholera bacteria by semi-nested PCR whereas our method facilitates detection of 5 bacteria from each reaction.

Table 2. Comparison of the results of PCR-ELISA and selective culture for the detection of *V. cholera* in stool samples.

Sp detected	No of samples with result/total No				Overall agreement
	PCR- ELISA$^+$ Culture$^-$	PCR-ELISA$^-$ Culture$^+$	PCR-ELISA$^+$ Culture$^+$	PCR- ELISA$^-$ Culture$^-$	
Vibrio cholera	0/50	1/50	49/50	0/50	49/50 (0/97)

Sp, species of bacteria; +, positive result; -, negative result.

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The most common problem when using PCR or PCR-ELISA assay for direct detection of an organism in faeces is the presence of PCR inhibitory substances in faeces. This can be overcome by enrichment of the faecal samples in a suitable broth prior to PCR. The APW broth was used in the present study in order to eliminate inhibitors as well as to increase the sensitivity of the PCR-ELISA assay. Koch *et al.* [8] indicated that selective enrichment in APW for 6 to 8 h before amplification enhances the limit of detection of viable enterotoxigenic *V. cholera*.

The specificity of the PCR was determined using 50 samples collected from south Iran and 10 standard bacterial strains from Boali reference center other bacteria. Among the strains tested, only *V. cholera* O1 was positive by PCR-ELISA. The false negative reported in one case may be due to the presence of PCR inhibitors.

In conclusion, a rapid and accurate method is essential for the detection and screening of the public health threat posed by toxigenic *V. cholera*. Hence, this method represents an assay format for the detection of *V. cholera* O1. Compared to culture and PCR methods, particularly while large scale screening, the technique developed here is faster and less laborious and hence it is a practical and reliable tool for the diagnosis of human Cholera disease in its massive outbreaks.

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