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Design of a multiplex PCR method for detection of toxigenic-pathogenic in *Vibrio cholerae*

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ABSTRACT

Objective: To study virulence and regulatory genes (*hly*A, *ctx*B, *tcp*I) in clinical strains of *Vibrio cholerae* (*V. cholerae*), simultaneously. **Methods:** Three important genes, *tcp*I, *hly*A and *ctx*B were used for detection of toxigenic and pathogenic *V. cholera* by chain reaction assay method. **Results:** According to the results of the PCR, the incidence of *hly*A, *tcp*I, and *ctx*B genes in clinical isolates was obtained as 94.7% (72 sample), 90.8% (69 sample), and 92.1% (70 sample), respectively. Five strains possessed all genes except *ctx*B, six strains possessed all genes except *tcp*I, four strains possessed all genes except *hly*A, one strain possessed only *hly*A and 60 strains contained a combination of three genes, Including *hly*A, *ctx*B and *tcp*I. **Conclusions:** Result show that this method could be reliable to detect toxigenic-pathogenic strains of *V. cholerae* in Iran.

1. Introduction

Cholera is a severe diarrhea that is caused by *Vibrio cholerae* (*V. cholerae*)[1-3]. affects in an important way on the health and economy of the people[1,3,4]. Information about presence of this bacterium in clinical samples can help in properly preventive actions[1].

Additional, there are a high amounts of non molecular microbiological methods that has been used for detection of *V. cholerae*[1] which have the disadvantages of laborious and boring[1,3,4]. Since cholera detection requires urgent testing[5], therefore applied molecular methods that are confident and rapid are needed[1,4]. Among methods based DNA, monoplex PCR is imperfect because it don't give concurrent detection of the toxigenic-pathogenic potential and regulating factors[3,6]. All these reasons have been encouraging us to explore rapid and affordable detection of toxigenic and nontoxigenic *V. cholerae* in clinical samples and, consequently, estuarine environments and food products.

This organism enters in small intestine and produces cholera toxin (CT)[7-9]. Toxin co-regulated toxin (TCP), related to *Vibrio* pathogenicity island (VPI)[9], encode the *tcp*A gene[2,10]. *tcp*A acts as a receptor for CTXΦ[1,9] and colonization of the gut is facilitated due to presence of *tcp*A on the surface of the bacterium[2,7,8,10]. Therefore, any strain possessing *tcp*A gene is regarded as pathogenic and it can become toxigenic after obtaining the CTX cassette gene[1]. On the other hands, *tcp*I-other member in VPI- is an integral inner membrane protein, engaged as environmental sensing and signal transduction and reversely regulates the synthesis of the *tcp*A[7]. Hemolysin (*hly*A) too is another virulence factor that with high frequency found in toxigenic and non-toxigenic strains[8] and it causes diarrhea[8,11].

In this research, we studied virulence and regulatory genes (*hly*A, *ctx*B, *tcp*I) in clinical strains of *V. cholerae*, simultaneously.

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2. Material and methods

2.1. Sampling

A total of 76 stool samples were collected from different patients in diverse provinces of Iran such as Sistan and Baluchestan, Ghom, Tehran, Ghazvin, Kurdestan and Golestan during epidemics, between 2005 and 2010.

2.2. Identification and serogrouping of V. cholerae

First, samples were enriched in alkaline peptone water (APW). The tubes were then shaken (100 rpm) at 37 ℃ for 6 to 8 h. Approximately 5 μL of enriched APW broth was streaked onto TCBS agar (Merck, Germany) and incubated at 37 ℃ for 18-24 h. All the yellow colonies on TCBS plates were selected and assayed by biochemical tests including oxidase, sucrose, mannose and arabinose fermentation, motility, indol, methyl red Voges-Proskauer & resistant to poly-myxin B. Clinical isolates of the *V. cholerae* were serogrouped by using growth from TSI agar slants and slide agglutination test with polyvalent O_1, O_{139} Inaba and Ogawa antisera (Mast Diagnostics Ltd., Bootle, Mersey side, UK).

Afterward, all strains were suspended in BHI broth (Pronadisa, Spain) supplemented with 30% glycerol and stored at -70 ℃[12].

2.3. DNA samples were prepared as follow

Before gene extraction, frozen store strains were subcultured streaked onto LB agar (Pronadisa, Spain) and then onto TCBS agar to confirm purity. For DNA extraction Bidinost *et al* method[13] was modified and used.

2.4. Primers design

The oligonucleotide primers for *ctx*B and *tcp*I were designed based on existing GenBank sequences for all strains of *V. cholerae* by allele ID 6 software and oligonucleotide primer for *hly*A was obtained of Rivera *et al* study[7].

2.5. Chain reaction assay (PCR)

PCR methods were carried out using *V. cholerae* 62013 as standard strain and clinical samples were tested subsequently. *V. cholerae* 62013 is a toxigenic and pathogenic *V. cholerae* strain[16,17].

In the first stage, primers were evaluated by uniplex PCR, When verified the correct functioning of each set, all primers were assayed together by multiplex PCR. One isolate of *V. cholerae* 62013 was used as the PCR positive control for *ctx*B, *hly*A, *tcp*I, and *Salmonella typhi, Shigella dysenteriae, Escherichia coli* O_{157}:H_7 were used as negative controls.

PCR was carried out in 25 μL containing 2.5 μL of 10× PCR amplification buffer (500 mM KCl; 100 mM Tris-HCl pH 9.0; 1% Triton X-100), 1 μL dNTPs (2.5 mM each), 1 μL (10 pmol) of each of the primers, 1 μL of $MgCl_2$ 50 mM, 0.20 μL of *Taq* DNA polymerase(1U) (Cinagen. Iran) and 5 μL of DNA template and double distilled water to a final volume of 25 μL. The PCR circumstances for all the reactions consisted of a first step of 5 min at 94 ℃ and a final step of 5 min at 72 ℃. The middle step included 35 cycles of denaturation, annealing and extension times for: a) *ctx*B: 1 min at 94 ℃, 1 min at 60 ℃ and 1 min at 72 ℃; b) *hly*A: 1 min at 94 ℃, 1 min at 62 ℃ and 1 min at 72 ℃. c) *tcp*I: 1 min at 94 ℃, 1 min at 60 ℃ and 1 min at 72 ℃ respectively.

Amplified products (5 μL) were analyzed based on the appoint of their sizes by 1.5% agarose gel electrophoresis in TAE buffer. A 100 base pair ladder (Fermentas, Germany) was used as molecular size standard, Stained with ethidium bromide, and visualized under a Bio-Rad UV transilluminator (Hercules, CA, USA).

Relative bands from three different PCRs were extracted and purified from agarose gel (Cinagen. Iran) using the DNA extraction kit (Ferrmentas, Germany) and sequenced (Macrogen Research, Seoul, Korea).

2.6. Multiplex PCR assay

Briefly, all three primer pairs were added to the PCR mixture of a standard strain. The following reagents were added to achieve each 25 of PCR mixture: 2.5 μL of 10 × PCR amplification buffer (500 mM KCl; 100 mM Tris-HCl pH 9.0; 1% Triton X-100), 1 μL of $MgCl_2$ 50 mM, 1 μL dNTPs, 0.3 μL (10 pmol) of each of forward and reverse primers, 0.2 μL (1U) of *Taq* DNA polymerase and 5 μL of DNA template and double distilled water to a final volume of 25 μL. The automated thermal cycler (Master cycler gradient, Eppendorf, Germany) was programmed as follows: initial denaturation at 94 ℃ for 5 min, followed by 35 cycles consisting of 94 ℃ for 1 min, annealing at 58 ℃ for 1 min, and extension at 72 ℃ for 1 min and a final extension phase of 72 ℃ for 5 min. The PCR products were separated by 1.5% agarose gel and stained with ethidium bromide. The agarose gel was imagined with a Bio-Rad UV transilluminator.

2.7. Specificity of multiplex PCR technique for ctxB, tcpI and hlyA genes

To confirm the specificity of this study primers, we used 50 pg of genomic DNA of standard strain of *V. cholera* and 3 strains of other digestive system pathogenic bacteria's (*Salmonella typhi, Shigella dysenteriae*, and *Escherichia coli* O_{157}:H_7).

Primers applied in this research were specific and no amplification was seen with other strains of *Salmonella typhi, Shigella dysenteriae* and *Escherichia coli* O_{157}:H_7 and nonspecific bands were not seen .

2.8. Sensitivity of multiplex PCR technique for ctxB, tcpI and hlyA genes

Performing sensitivity test and determine the detection

Table 1
Primers characterization applied for this study.

Target genes	Nucleotide sequences 5'-3'	Accession no.	Tm [a] (℃)	Amplicon size(bp)	Source or reference
*ctx*B-F	ATG AGG CGT TTT ATT ATT CCA TAC AC	HQ599508.1	57.5	128	Original
*ctx*B-R	TAC CAG GTA GTC AAC ATA TAG ATT CA				
*tcp*I-F	TGC GTG ATG CTA ATT GGA CT	L25659.1	60.4	444	Original
*tcp*I-R	TTC GGT TTG TTT GCT TGA TG	-	-	-	-
*hly*A-F	GGC AAA CAG CGA AAC AAA TAC C	M36855.1	59.0	738/727 (ET/Clas)	15
*hly*A-R	CTC AGC GGG CTA ATA CGG TTT A	-	-	-	-

Tm [a], melting temperature.

limit for *V. cholerae* can define the strength of this test. The sensitivity of multiplex PCR assay was tested with standard strain of *V. cholerae* O_1. Standard strain was grown overnight in a BHI broth. Genomic DNA was extracted by the method described above and tenfold serial dilutions were prepared from 100 ng to 10 pg, and multiplex-PCR was carried out at each concentration.

3. Results

Biochemical and serological assays defined that all isolates belongs to the *V. cholerae* O_1 category. Extracting gene process was confirmed through observation of appropriate bands in gel agarose 1%. The simple and multiplex PCR products gained for each gene studied were shown in Figure 1 and size of each amplicon was confirmed. Primers applied in this research were specific and no amplification was seen with other strains of *Salmonella typhi*, *Shigella dysenteria*e and *Salmonella coli* O_{157}:H_7 and nonspecific bands were not seen (Figure 1).The sensitivity or minimum concentration of detection of *ctx*B, *tcp*I, and *hly*A genes for O_1 strain were calculated 5, 50, and 50 pg, respectively.

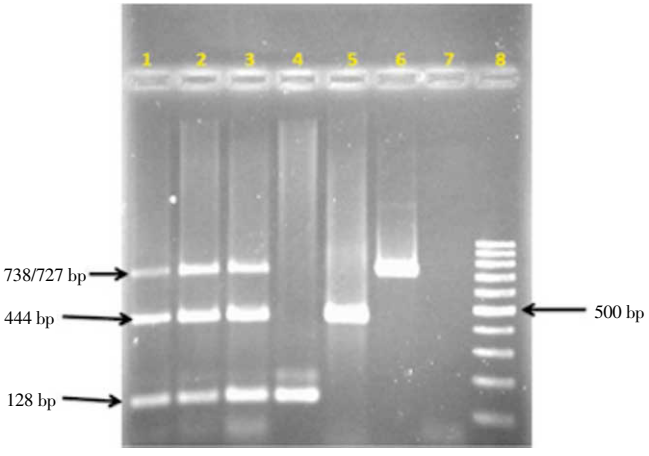

Figure 1. Uniplex and Multiplex PCR for detection of virulence-associated genes in clinical strains of *V. cholerae* O_1 biotype El Tor. Lane 1, 2, 3, 7 multiplex PCR for the two clinical samples, positive and negative controls strains respectively, lane 4, 5, 6 uniplex PCR for *ctx*B, *tcp*I and *hly*A, respectively, lane 8 molecular weight marker 100-bp DNA.

According to the results of the PCR, the incidence of *hly*A, *tcp*I, and *ctx*B genes in clinical isolates was obtained as 94.7% (72 strains), 90.8% (69 strains), and 92.1% (70 strains), respectively. Five strains possessed all genes except *ctx*B, six strains possessed all genes except *tcp*I, four strains possessed all genes except *hly*A, one strain possessed only *hly*A and 60 strains contained a combination of three genes, Including *hly*A, *ctx*B and *tcp*I (Table 2). The *tcp*I and *hly*A gene were frequently detected in toxigenic *V. cholera* O_1 serogroup (84.21%) and (86.84%) respectively. The majority genotype observed in these samples was *ctx*B *hly*A ET *tcp*I (78.95%).

4. Discussion

Formerly, many studies has been carried out with multiplex PCR method for study of virulence factors in *V. cholera*, but many of those studies were detecting virulence genes that are not frequently detected[6,8] or many of them were not searching toxicity and pathogenicity simultaneously[18]. Moreover, many of these methods are time consuming and unspecified.

Among diverse toxins generated by *V. cholerae*, cholera toxin is the most strong but a number of other genes such as *hly*A and *tcp*I are concerned with cholera pathogenesis[1]. The sequence of *hly*A gene in the classical biotype has an 11-bp deletion, contrasted to the El Tor biotype[7,11]. Despite exist differences in the nucleotide sequences of *hly*A in El Tor and classical biotypes, the oligonucleotide primer applied in this study had been chosen from the fragment of the gene possessing the consensus sequence, make possible the detection of both biotypes[15]. Recently in Iran, Fallah and his group[5] applied multiplex PCR to detect *V. cholera* O_1 and non O_1 using the *ctx*A, *tcp*A and *omp*W genes on standard strains. All target genes were detected in *V. cholera* O_1 but only *omp*W gene was detected in *V. cholera* non O_1. Moreover, non-clinical samples were analyzed.

In other PCR multiplex studies that had been done on a variety of serogroups of *V. cholerae* by other researchers, these results were reported. In many studies, *hly*A gene was found in all toxigenic and non toxigenic *V. cholerae* O_1 and O_{139}[7,8,11,14]. The presence of *hly*A (100%) gene in non toxigenic isolates of our study with high frequency in above

studies, prove these genes can be used for detection of non toxigenic serogroups. The *tcp*I gene was reported in studies of Singh *et al*[11] and Rivera *et al*[7] with frequency of 100% and 84.2% for *V. cholerae* O_1 serogroup respectively.

Also, *ctx* gene frequency of our study (92.1%) was approximately equal acquired results of similar studies with O_1 and O_{139} serogroups, for example, frequency of this gene in studies has been reported equally, 89.7%, 94.7% and 96.7%, respectively[7,11,19].

In the present study the results of acquired with Multiplex PCR were compared to monoplex PCR in 76 clinical isolates of the *V. cholerae* O_1. This analysis determined that there was perfect homology between these methods. Although six strains were belonging to the O_1 serogroup but don't containing the *ctx* gene cluster. Although, the *hly*A gene used in this study has a common sequence in *V. cholerae* O_1, O_{139}, non-O_1 and non-O_{139} serogroups[15], but since all isolates studied in this research had belonged to O_1 serogroup, and as regards classical biotype to be extinct nowadays[14, 20] therefore it can be concluded that strains possessor these gene (72 strains) are belonging to biotype Eltor. In addition, all these strains were Voges-Proskauer positive and resistant to polymixin B and these can confirm our hypothesis. We believe that our study is the first report for rapid detection of these genes by multiplex PCR from clinical isolates of *V. cholerae* in Iran.

Conflict of interest statement

We declare that we have no conflict of interest.

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