Full Length Research Paper

Identification of *Vibrio cholerae* pathogenicity island (ctxA, OmpW and tcpA) in non-O139 and non-O1 *V. cholerae* strains isolated from Karun River in Ahvaz, Iran

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*Vibrio cholerae* is a usual inhabitant of the marine environment and causes severe diarrheal disease contaminating thousands of people each year in developing countries. The virulence of *Vibrio* spp. is regulated by the ctxAB and tcpA genes. These genes are supposed to be exclusively associated with clinical strains of O1 and O139 serogroups. In the present study, we examined the presence of virulence genes that contain OmpW, ctxA and tcpA of *V. cholerae* classical and Eltor variants in environmental strains of non-O1 and non O139 *V. cholerae* that were cultured seasonally from four sampling stations of the Karun River in Ahvaz. One hundred water samples were collected from four stations of Ahvaz Karun River. The colonies that were grown on TCBS confirmed to be *Vibrio* spp. by biochemical, serological and molecular methods. After DNA extraction, a polymeras chian reaction (PCR) assay was performed for detection of ctxA and tcpA (both Classical and Eltor variants) and OmpW was in the strain that was recognized as *V. cholerae* non-O139 and non-O1. Among 100 environmental samples of fresh water in this study, 27 isolate confirmed as *V. cholerae* spp. by PCR assay with ompW gene. Of among them, 4 isolate that were confirmed *V. cholerae* non O1- non O139 had gene for toxin coregulated pilus (Classical and Eltor). *V. cholerae* is one of the major causes of morbidity and mortality in the developing countries. The studies that were performed on *V. cholera* nonO1, nonO139 strains that usually do not carry the ctx genes coding for cholera toxin (CT) or toxin-co regulated pilus which are the most important virulence characteristics of the cholera causing strains.

**Key words:** *Vibrio cholerae* non-O1, non-O139, ctxA, tcpA, OmpW.

INTRODUCTION

Cholera is still a threat to a large number of people in the universe. In 2009, 45 countries reported 221,226 cholera cases and 4,946 cholera deaths (case-fatality ratio, 2.24%) to WHO (WHO, 2010). Haiti is the latest country to be affected by the ongoing cholera pandemic which began 49 years ago in Sulawesi, Indonesia, and has lasted longer and spread farther than any previously known cholera pandemic (Wachsmuth et al., 1994). *Vibrio cholerae*, the causal organism of cholera can be classified into >200 serogroups according to the differences in antigenicity of their heat-stable somatic O antigen (Shimada et al., 1994). Pathogenic and epidemic strains of *V. cholerae* contains two essential genetic elements, CTX element and the vibrio pathogenicity island (VPI) which are concerned in coding for cholera...
toxin and toxin co-regulated pilus (TCP), respectively (Mishra et al., 2011). It is believed that most of the environmental strains do not produce cholera toxin (CT) and are therefore of trifling magnitude in epidemic potential. In spite of copious studies over more than a century, the epidemiology of cholera remains mysterious and challenging to investigators in the field. Until the appearance of *V. cholerae* O139 in 1992, toxigenic strains of *V. cholerae* O1 were considered to be the solitary agents of epidemic and pandemic cholera (Faruque et al., 2000). The knack of pathogenic *Vibrio* spp. to cause disease depends on the expression of virulence factors like a potent enterotoxin (CT), a pilus colonization factor toxin co-regulated pilus; TCP. CT is encoded by a transferable filamentous phage (CTXφ) and reports have implied the acquisition of these CT genes under conditions analogous to those in the aquatic environments (Faruque and Nair, 2002).

The ctxAB operon that encodes cholera toxin resides in filamentous bacteriophage CTXφ genome confer the ctx operon to *V. cholerae* strains as a prophage that carries the ctxA and ctxB genes. Cholera toxin (CT), which is responsible for the life threatening diarrheal disease cholera gravis caused by epidemic cholera (Blackstone et al., 2007). All Vibrio strains that are able of causing cholera continuously carry genes for TCP which is an adhesion that is coordinately regulated with CT production (Sharma and Chaturvedi, 2006). TCP is a single *V. cholerae* pilus that has been demonstrated to date to have a role in colonization of the gut mucosa of humans (Herrington et al., 1998). The nucleotide sequencing of OmpW gene in *V. cholerae* strains has unchanged among different *V. cholerae* strains that has made it a highly proper genetic marker for the organism (Nandy et al., 2000).

**Objective**

In this investigation, we have studied the probability of the presence of genes that codes tcpA and ctxAB in *V. cholerae* non O1 and non O139.

**MATERIALS AND METHODS**

In four stages (April, may, June and July 2010), a total of 100 samples of water from Karun River Ahvaz were collected (500 ml). During the study period, the recorded river temperature was about 25 to 35°C and pH ranged from 7 to 8. Water samples collected were concentrated by centrifuge (6000 rpm) and enriched in alkaline peptone water [APW (1% peptone, 1% NaCl, pH 8.4 to 8.6)] for isolation of *Vibrio* spp (Sharma and Chaturvedi, 2007). After 6 to 8 h incubation, concentrated bacterial colonies were harvested from enrichment cultures cultured on thiosulfate-agar (TCBS) agar. The colonies on TCBS were confirmed to be *Vibrio* spp. By biochemical tests (Sharma and Chaturvedi, 2007), morphological colonies compatible with *V. cholerae* were characterized by oxidase test and agglutinated with antisemur (Difco, Detroit, MI, USA) for serotype determination; then, the isolate that were identified as *V. cholerae* O1 out of this study; but *V. cholerae* O139 was identified by PCR method and out of this study.

**DNA extraction**

The simple boiling method was used for DNA extraction from *V. cholerae* colonies (non O1 and non O139) harvested from surface of TCBS agar medium. In brief, a few colonies were dissolved in TE (Tris-EDTA) buffer and were boiled at 100°C for 10 min with subsequent precipitation in a 1200 g refrigerator centrifuge at 4°C for 3 min. The supernatant containing DNA was used as template for PCR amplification. Extracted DNA was selected for PCR analysis by using genus and species-specific primers (Table 1).

**PCR assay**

PCR analysis was then performed on the extracted DNA from colonies that were identified as *V. cholerae* non O1 and non O139. PCR amplification and cycling conditions: on each of the samples, two sets of PCRs were performed to determine the presence of established virulence genes of *V. cholerae* O1 and O139 in *V. cholerae* non O1 and non O139. The primers used in this study are shown in Table 1. The first step was performed to assess the

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Oligonucleotide</th>
<th>Amplicon size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*R</td>
<td>5-TCTATCTCTGTAGCCCTATTACG-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tcpA (classical)</td>
<td>*F</td>
<td>5-CACGATAAGAAAACCGTCAAGAG-3</td>
<td>617-bp</td>
<td>Keasler and Hall (1993)</td>
</tr>
<tr>
<td></td>
<td>*R</td>
<td>5-ACCAATGCAACGCGGAATGGAGC-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tcpA (El Tor)</td>
<td>*F</td>
<td>5-GAAGAGTTTTGTAAAAGAGAAGACAC-3</td>
<td>471-bp</td>
<td>Keasler and Hall (1993)</td>
</tr>
<tr>
<td></td>
<td>*R</td>
<td>5-GAAAGGACCCTTTTTCACGGTG-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OmpW-</td>
<td>*F</td>
<td>5-CACCAAGAAGGTGACCTTATTTGTG-3</td>
<td>588 bp</td>
<td>Nandy et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>*R</td>
<td>5-GAACTTATAACCCCGC-3</td>
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*F; forward primer and *R; reverse primer.
RESULTS

Among the 100 environmental samples of fresh water in this study, 27 isolates were confirmed as V. cholerae spp. By PCR assay with ompW gene (Figure 1), which showed 100% specificity for all V. cholerae non O1 and non O139 strains tested. Then, presence of the gene for toxin coregulated pili (tcpA of Eltor variants) was proved in 3 isolates and tcpA of classical variants proved in 1 isolates for V. cholerae non O1 and non O139 by PCR detection (Figures 2 and 3); while among 23 V. cholerae, non O1 to non O139, no isolate had gene for ctxA gene. In this study, because we did not have positive control, then sequencing method was used to confirm positive samples and nucleotide sequences were compared with other sequences at the GeneBank using BLAST. The homology of TcpA of classical and eltor biotype were 98 and 97%, respectively.

DISCUSSION

V. cholerae exist as pathogenic (clinical) and nonpathogenic (environmental) variants (Faruque et al., 1998). The ability of the pathogenic strains to cause cholera in humans depends chiefly on two factors: the bacterial ability to colonize the host’s intestinal epithelium

Gene sequencing

The sequences of tcpA and ctxAB were confirmed by sequencing analysis. Then products were collected and sent for sequencing at Bioneer Company, Korea.

Figure 1. PCR analysis for ompW gene of V. cholerae non-O1 used in this study. Agarose gel of PCR products amplified by species-specific primers of ompW gene for detection of V. cholerae spp.; 1: marker 100 bp, 2; control positive, 3 to 8: V. cholerae spp.
Figure 2. PCR analysis for tcpA Eltor gene in *V. cholerae* non-O1 used in this study. Agarose gel of PCR products amplified by species-specific primers of toxin co-regulated pilus (417 bp). 1: marker 100 bp; 2, 3 and 4: TCP positive.

Figure 3. PCR analysis for tcpA classical gene in *V. cholerae* non-O1 used in this study. Agarose gel of PCR products amplified by species-specific primers of toxin co-regulated pilus (617 bp). 1: marker 100 bp; 2 to 4 and 10: TCP positive.
which is mediated by the bacterial adhesion by toxin co-regulated (type-IV) pili (TCP), and the ability of the bacteria to produce toxins, primarily the choleer toxin (CT). The genes for the biosynthesis of TCP are part of the TCP pathogenicity island of V. cholerae chromosome and involve at least 15 open reading frames (Kovach et al., 1996). Among the many TCP subunits, the tcpA encoded by tcpA genes is the main protein. The species of V. cholerae that carry the tcp genes on their genome, they can be infected by lysogenic phage CTXe and produced choleera toxin (CT). Studies have discovered that virulence genes or their homologs are discrete among environmental V. cholerae strains belonging to various serogroups, whereas previously, it was considered that virulence genes are carried only by the clinical isolates. The V. cholerae non O1, non O139 strains classically do not carry the ctx genes coding for choleer toxin (CT) or toxin-co-regulated pilus (TCP) which are the major virulence characteristics of the choleera causing strains. However, recently, the presence of new variants of these genes in some clinical and environmental V. cholerae non O1, non O139 strains has been published (Nandi et al., 2000; Chakra et al., 2000). The basis for this possibility may be due to the fact that the studies do not consider the prospect of genetic variations within the virulent genes to the extent that the variants might escape detection with PCR or probes that were designed strictly based on the sequence of the corresponding genes found in the clinical isolates (Mukhopadhyay et al., 2001).

The result that was obtained from this study confirmed the presence of 4 genes for tcpA in V. cholerae non O1 and non O139, then the species that contains tcpA genes, it can produce pili type IV that are contaminated by lysogenic bacteriophage CTXe and by toxin production can cause choleer disease. In a survey that was performed for the presence of virulence associated genes in non-O1, non-O139 V. cholerae strains, they found tcpA and the putative tcp gene cluster in two distinct non-toxicogenic, non-O1/non-O139 strains. As this was an unexpected result, further investigation was carried out on these two isolates (Novais et al., 1999). In the study that was performed by Singh on Territorial waters of the Baltic Sea as a source of infections caused by V. cholerae non-O1, non-O139, all isolates gave negative agglutination reactions with O1 and O139 antisera. They were also negative for the ctxA gene in PCR. A total of 26 strains of V. cholerae including members of the O1, O139, and non O1, non O139 serogroups from both clinical and environmental sources were examined for the presence of genes encoding cholera toxin (ctxA), zonula occludens toxin (zot), accessory choleera enterotoxin (ace), hemolysin (hlyA), NAG-specific heat-stable toxin (st), toxin coregulated pilus (tcpA); all of the non-O1, non-O139 strains were negative for the ctxA, zot, ace, tcpA and tcpI genes (Singh et al., 2001).

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REFERENCES


