Evaluation of new primers for detecting toxigenic *Vibrio cholerae* by multiplex PCR

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**Abstract**

*Vibrio cholerae* is the etiological agent of cholera that has emerged as an endemic disease in different regions of the world in recent years. Traditional microbial culture and microscopy methods are considered to be the best standard for diagnosing *V. cholerae* infection. These methods, however, delay any available confirmatory answer by days. Molecular methods have the potential to provide sensitive, accurate, and rapid analysis of *V. cholerae* infection. We have developed a multiplex PCR assay to detect virulence and toxigenic-associated (VTA) genes (*ctxA, tcpA*, and *ompW*). To evaluate PCR specificity, additional bacteria from the enterobacteriaceae family (*Salmonella typhi*, *Shigella dysenteriae*, and *entrotoxigenic E. coli*) and *Aeromonas hydrophila* were examined in this study. Specificity tests were evaluated using the genome dilution method. Importantly, the results show that our PCR specificity method represents the best tool for the rapid detection of *VTA* genes because of its simplicity, cost effectiveness, and accuracy. This multiplex PCR method can be used for examining the existence of *VTA* genes in patient samples, and therefore will distinguish *V. cholerae* from other vibrios and bacteria. This method is able to detect 10-100 colony forming units (CFUs) of *V. cholerae* and 8.5-85 picograms (pg) of genomic DNA. The multiplex PCR method is also more specific and sensitive than other methods, validating it as an appropriate and sensitive tool for detecting the presence of toxigenic and pathogenic *V. cholerae*.

**Introduction**

Diarrheal diseases are among the greatest world healthcare problems. Cholera is one of the most serious diarrheal diseases, causing an epidemic with *Vibrio cholerae* O1 or O139 serogroups.1 Primary virulence factors of *V.cholerae* are cholera toxin and toxin-co-regulated pili, which are encoded by the ctxAB and tcpA genes, respectively.2,3 CtxA and tcpA are uncommon in environmental strains,4 yet are responsible for the pathogenesis in *V.cholerae* serogroups O1 and O139.5-7 Biochemical methods for the detection of *V.cholerae* are time-consuming and can require 2 to 7 days for assessment. Cholera detection requires urgent testing, and efficiency is a critical factor for determining the usefulness of any detection method. Moreover, special expertise is required to process these tests, but such expertise is not available in all laboratories.8 Thus, rapid detection tests are functional and necessary for creating a better detection method for cholera infection. These tests are fast and sensitive techniques for the primary diagnosis and control of pathogenicity, and employ several bio-type-specific genes to diagnose the presence of *V.cholerae*, such as *ctxA* and *tcpA*. The aim of this study was to develop such an assay for the rapid detection of pathogenic and toxogenic *V. cholerae* bacteria. To develop a standard, accurate, and rapid method for the detection of *V. cholerae*, we designed the Multiplex PCR method for genes *ctxA, tcpA,* and *ompW*.

**Materials and Methods**

**Bacterial strains**

*V. cholerae* O1 and non-O1 stains, *Salmonella typhi*, *Shigella dysenteriae*, *Aeromonas hydrophila*, and *Entero-toxigenic Escherichia coli* (ETEC) were all included in this study and were provided by the Bu-Ali reference laboratory in Iran.

**DNA extraction**

All strains were cultured in pepton soy broth and incubated at 37°C overnight. Genomic DNA was extracted using the DNA Pure Extraction Kit (Bioneer, South Korea) according to the manufacturer’s protocol.

**PCR amplification for uniplex and multiplex assays**

Three uniplex PCR assays were carried out in 50 μL reaction volumes consisting of 20 mM Tris-HCl (pH 8.4), 1 unit of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, California, USA), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen, Carlsbad, California, USA), 2 mM MgCl₂, 25 pmol of each primer, and 50 ng of DNA template. Multiplex PCR reactions were performed with the simultaneous addition of primers for the three genes in the same mixture. Thermocycling conditions consisted of an initial denaturation at 94°C for 4 min, followed by 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 1 min of extension at 72°C. The final extension was carried out at 72°C for 5 min.

**Table 1. Primer sequences used in this survey.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Target gene</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctxF</td>
<td>gttCTTATgCCCAGAggACAg</td>
<td>ctxA</td>
<td>219 bp</td>
</tr>
<tr>
<td>ctxR</td>
<td>ATgTgTTgCAgTgCTATAAC</td>
<td>ctxA</td>
<td>219 bp</td>
</tr>
<tr>
<td>tcpF</td>
<td>ATCTTCgTgATCTCATgATAAgg</td>
<td>tcpA</td>
<td>285 bp</td>
</tr>
<tr>
<td>tcpR</td>
<td>TTAATgTgCCACAAATATgTgccc</td>
<td>tcpA</td>
<td>285 bp</td>
</tr>
<tr>
<td>ompF</td>
<td>CACAgAggTgACTTTATgTg</td>
<td>ompW</td>
<td>588 bp</td>
</tr>
<tr>
<td>ompR</td>
<td>gACTTTATgCCACACCgG</td>
<td>ompW</td>
<td>588 bp</td>
</tr>
</tbody>
</table>
Specificity test
For assessment and specificity determination of the designed primers, genomic DNA of *V. cholerae* O1 and non-O1, *ETEC, S. typhi, Sh. dysenteriae,* and *A. hydrophila* was used as templates in uni- and multiplex PCR reactions.

Sensitivity test
For detection of *V. cholerae* genomic DNA, serial dilutions of DNA were made ranging from 100 ng to 10 pg. PCR was performed at each dilution value.

Results

**PCR of ctxA, tcpA, and ompW genes**

ctxA, tcpA, and ompW genes were amplified by PCR using genomic DNA of both *V. cholerae* O1 and non-O1 serogroups. The amplicons related to *V. cholerae* O1 ctxA, tcpA and ompW genes were 219 bp, 295 bp, and 588 bp, respectively. The PCR reaction with the *V. cholerae* non-O1 ompW gene was the only reaction that resulted in an amplicon of the same size (Figure 1).

**Specificity test for ctxA, tcpA, and ompW genes**

To confirm the specificity of the designed primers, genomic DNA of *S. typhi, Sh. dysenteriae, A. hydrophila,* and *ETEC* was used as a template. The results showed that the designed primers were specific for these genes, since no additional amplicons were detected on gel electrophoresis.

**Sensitivity test for ctxA, tcpA, and ompW genes**

Although the infective dose of *V. cholerae* is approximately 10^6 bacteria, performing a sensitivity test by PCR is imperative for determining the robustness of the test. Genomic DNA of *V. cholerae* O1 was extracted and diluted from 100 ng to 10 pg, and PCR was performed at each concentration. The minimum concentration of genomic DNA at which ctxA, tcpA, and ompW could be detected were 8.5, 85, and 85 pg, respectively. Moreover, the ompW gene was detected when the DNA was diluted to 14 pg in the in the *V. cholerae* non-O1 sample (Figure 2).

**Triplex PCR of *V. cholerae* O1 and non-O1 serogroups**

Using all three sets of primers and *V. cholerae* O1 genomic DNA in a single PCR assay, the ctxA, tcpA, and ompW genes were amplified. The amplicon sizes were 219 bp, 295 bp, and 588 bp for the ctxA, tcpA, and ompW genes, respectively (Figure 1). The same triplex PCR reaction was carried out for detection of three genes in the *V. cholerae* non-O1 strain, but only showed a 588 bp amplicon, which corresponded to ompW.

**Discussion**

The standard methods in microbiology that are traditionally used to detect *V. cholerae* infection have several disadvantages. Therefore, a rapid and more efficient method is needed. We employed an updated nucleic acid based approach based on PCR assays, which simplified bacteria detection. It has been shown that toxinogen-regulated pilis are used by *V. cholerae* for intestinal colonization, and the main mediator is the regulatory ctxA gene. Therefore, an important standard gene for the detection of toxigenic *V. cholerae* is ctxA.10,11

Nandi et al. designed rapid detection methods using ompW and toxR and analyzed 254 *V. cholerae* samples.15 Samples 229 and 239 were shown to have the ompW and toxR genes, respectively. This was reported for the O1, O139, non-O1, and non-O139 serogroups, but was undetectable in other Vibrio serogroups.12

In that study, similar to our research, the ompW gene was 588 bp in length. In our particular test, ctxA was used for *V. cholerae* detection.

Panikar et al. applied DNA microarrays and Multiplex PCR to detect pathogenic *V. cholerae*.13 In that particular study, 10 genes were used for diagnosing the Vibrio family. For *V. cholerae*, *rhh* and *vihA* were detected with specific primers and probes. Moreover, for *V. parahaemolyticus*, the *thl*, *tdh*, *trh*, and *ORF8* genes were used for bacterial detection; for *V. cholerae*, the *ompU*, *taxR*, and *tcpI* genes were used for detection; and for El Tor and the classical biotype, the *hhA* gene was used for bacterial detection. The specificity for this method was 100%. Diagnosis in non-broth culture was 100-1000 CFU/mL, while it was 1 CFU/mL in broth culture. The genes ompW, ctxA, and tcpA were used to detect *V. cholerae* stains.13

Gubala et al. applied Multiplex Real Time PCR to amplify *rtpA, epsM, tcpC*, and *mshA* genes as a method for detecting toxigenic *V. cholerae*.14 In this study, the tcpC gene was not detected in the non-O1 and O1 serogroups. Non-Vibrio was not detected in the specificity analysis of primers, but the tcpC gene was detected in *V. cholerae* serogroups O1 and non-O1.14

Katsuki Hoshino and his group applied Multiplex PCR to detect toxigenic Vibrio O1 and O139 using the ctxA and *rhh* genes.15 Of 121 patient waste samples, 38 samples tested positive by this method, and importantly, the method sensitivity was 100% and specificity was 95.5% compared to the bacteriologic culture method. Detection in this study was reported for *V. cholerae* O1 at 65 CFUs and for O139 at 200 CFUs.15

Dalsgaard et al. analyzed the virulence of *V. cholerae* non-O1 and non-O139 by amplifying genes ctxA and tcpA via PCR.16 In this study, ctxA and tcpA genes were detected in the *V. cholerae* serogroup O1.

In our research study, the ompW gene was used to detect all Vibrio species. Multiplex PCR was applied for rapid and specific detection of Vibrio containing the toxigenic gene as well as other Vibrio of gram-negative bacteria. In addition to using the cholerae toxin gene to specifically identify *V. cholerae* pathogenic strains, we applied the tcpA and ompW genes for detection of other Vibrio species. Method sensitivity was analyzed by diluting *V. cholerae* bac-

![Figure 1. PCR (uni, duplex and triplex) of *V. cholerae* O1. Lane 1(Ladder 100bp), lane 2(ctxA amplicon, 219 bp), lane 3 tcpA amplicon, 295 bp), lane 4(ompW amplicon, 588 bp), lane 5(1/100 dilution), lane 6(1/1000 dilution), lane7(1/10000 dilution), lane 8(1/100000 dilution).](image1)

![Figure 2. Sensitivity test of triplex PCR of *V. cholerae* O1. Lane 1(ladder100bp),lane 2(ctxA & tcpA & ompW amplicons), lane 3(tcpA & ompW amplicons), lane 4(ompW amplicons), lane 5(1/10dilution), lane 6(1/100 dilution), lane7(1/1000 dilution), lane 8(1/10000 dilution), lane9(1/100000 dilution).](image2)
terial genomic DNA, and specificity was determined by using bacteria genomes of *S. typhi*, *A. hydrophila*, *Sh. dysantri*, and ETEC. Multiplex PCR distinguished the ctxA gene at a template concentration of 85 pg, while uniplex PCR distinguished the gene at a template concentration of 8.0 pg.

The method described in this study was applied for the rapid detection of *V. cholerae* from water and food sources. Based on the results of our study and others, the multiplex PCR method is the best approach for the rapid detection of cholera infection and provides a useful method of detection even when the bacterial count is low.

**References**