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 enterotoxigenic *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 pilus, 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 biotype-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 toxigenic *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 strains, *Salmonella typhi*, *Shigella dysenteriae*, *Aeromonas hydrophila*, and Enterotoxigenic *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

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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.

Target sequences and primers

Three target sequences were used that encoded cholera toxin (*ctxAB*), toxin coregulated pilus (*tcpA*), and outer membrane protein (*ompW*). The primers used were *ctxA*, *tcpA*, *ompW* (NC-012583, NC-009457, NC-012583), respectively, and are shown in Table 1.

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.

Primers	Sequence	Target gene	Amplicon size
ctxF	ggTCTTATgCCAgAggACAg	ctxA	219 bp
ctxR	gTTgggTgCAGTggCTATAAC	ctxA	
tcpF	ATTCTTggTgATCTCATgATAAgg	tcpA	295 bp
tcpR	TTAATTCACCACAAATATCTgcc	tcpA	
ompF	CACCAAgAaggTgACTTTATTgTg	ompW	588 bp
ompR	gAACTTATAACCCCGcg	ompW	

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 558 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 10^8 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 *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 toxin-regulated pili are used by *V. cholerae* for intestinal colonization, and the main mediator is the regulatory *ctxA* gene.⁹ 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.¹² 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. vulnificus*, *rrh* and *viuB* were detected with specific primers and probes. Moreover, for *V. parahaemolyticus*, the *tlh*, *tdh*, *trh*, and *ORF8* genes were used for bacterial detection; for *V. cholerae*, the *ompU*, *toxR*, and *tcpI* genes were used for detection; and for El Tor and the classical biotype, the *hlyA* 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 *rtxA*, *epsM*, *tcpA*, and *mshA* genes as a method for detecting toxigenic *V. cholerae*.¹⁴ In this study, the *tcpA* gene was not detected in the non-O1 and O1 serogroups. Non-*Vibrio* was not detected in the specificity analysis of primers, but the *tcpA* gene was detected in *V. cholerae* serogroups O1 and non-O1.¹⁴

Katsaki Hoshino and his group applied Multiplex PCR to detect toxigenic *Vibrio* O1 and O139 using the *ctxA* and *rfb* 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 200CFUs.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 cholera toxin gene to specifically identify *V. cholerae* pathogenic strains, we applied the *tcpA* and *ompW* genes for detection of other *V. cholerae* 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),lane3 *tcpA* amplicon, 295 bp), lane4 (*ompW* amplicon of O1 serotype, 588 bp) lane 5(*ompW* amplicon of non-O1 serotype, 588 bp), lane 6(duplex PCR for *ctxA* & *tcpA*), lane 7(duplex PCR for *ompW* & *tcpA*), lane 8 (duplex PCR for *ctxA* & *ompW*), lane9 (triplex PCR for *tcpA*, *ompW* & *ctxA* of *V. cholerae* O1), lane 10(triplex PCR for *tcpA*, *ompW* & *ctxA* of *V. cholerae* nonO1).

Figure 2. Sensitivity test of triplex PCR of *V. cholerae* O1. Lane1(ladder100bp),lane 2(1/10dilution), lane3 (1/100 dilution), lane 4(1/1000 dilution), lane 5(1/10000 dilution), lane6(1/100000 dilution).

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.

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