Short Communication

Amplification of *ctxA* gene in *Vibrio cholerae* 0139

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Abstract

The direct detection of cholera enterotoxin (*ctxA*) gene in *Vibrio cholerae* 0139 was examined using polymerase chain reaction (PCR). A pair of synthetic primers, each of which were 24 bases in length, were used with Taq polymerase to amplify the cholera enterotoxin. *Vibrio cholerae* 0139 strains isolated from clinical samples during the 1993 cholera outbreak were tested by PCR to detect the presence of the *ctxA* gene. The amplified PCR product of 290-bp was detected by either gel electrophoresis or hybridization to a 25-base synthetic oligonucleotide probe labeled with biotin. The fragment was produced following 35 cycles of amplification plus an additional cycle for the extension step. The PCR method did not detect *ctxA* gene in diarrhoeagenic *E.coli, Shigella* sp., *Aeromonas* sp. or *Plesiomonas shigelloides*. This amplification of *ctxA* gene by PCR provides a highly sensitive and specific tool for detection of toxigenic *Vibrio cholerae* directly from clinical samples.

Key words: *Vibrio cholerae* 0139; polymerase chain reaction (PCR); cholera enterotoxin endonuclease restriction.

A major virulence determinant of *Vibrio cholerae* 01 is the cholera enterotoxin, a protein composed of one A subunit and five B subunits. The genes for A and B subunits of cholera toxin (CT) are arranged in a single transcriptional unit with the A cistron (*ctxA*) preceding the B cistron (*ctxB*) (Moseley & Falkow, 1980). The complete nucleotide sequence of the cholera enterotoxin in *V. cholerae* 01 was shown as nucleotides 516 to 1,292 and 1,289 to 1,663 from the coding sequences for *ctx A* and *ctx B*, respectively (Mekalanos *et al.*, 1983). Although the non-01 *V.cholerae* CT or CT-like genes have not been sequenced, data available suggest that *ctxA* is well conserved within the species (Wright *et al.*, 1992).

The detection of cholera toxin is an important step in the diagnosis of cholera and there are reports describing a similar PCR assay for the cholera toxin gene. We examined our *V.cholerae* 0139 isolates for their ability to produce cholera enterotoxin as a significant virulence factor using PCR method to amplify the *ctxA* operon encoding the enterotoxin.

Amplification of *ctxA* gene was performed on *V. cholerae* 0139 isolated from clinical cases during the cholera outbreak in Klang Valley, Malaysia, in August/ September 1993, *V. cholerae* 01 serotype Inaba, biotype El Tor isolated from cholera patients in 1987, diarrhoeagenic *E. coli*, *Shigella* sp., *Plesiomonas shigelloides*, *Aeromonas sobria* and *A. hydrophila* isolated from patients with diarrhoea. Chromosomal DNA of the bacterial isolates was extracted by the mini preparation method described by Ausubel *et al.* (1987). For amplification, 5.0 µl of template DNA solution (purified chromosomal DNA dissolved in sterile double distilled water), 5.0 µl of buffer solution ($MgCl_2$), 6.4 µl of dNTPs, (1 mM each of dATP, dGTP, dTTP, and dCTP), 1 µl of each primer stock (10 µM each) and water to a final volume of 50 µl were used. The reaction mixtures were overlaid with mineral oil and heated to 95°C for 10 min to denature the DNA and 1.25 U of Taq polymerase (BRL, USA) was added. The samples were subjected to rounds of amplification steps consisting of a 1-min denaturing at 94°C, a 1-min annealing at 54°C, and a 1-min extension at 72°C, repeated for 35 cycles in a programmable temperature cycler (Perkin-Elmer Corp., USA). An additional cycle for extension step at 72°C, for 5 min was added. The amplified mixture and 100 bp molecular weight marker (BRL, USA) were subjected to electrophoresis through 2% agarose gel and the DNA fragment of specific sizes were visualized by UV fluorescence. The amplified DNA were confirmed by Southern membrane hybridization using PhotoGene™ nucleic acid detection system (BRL, USA). Oligoribonucleotides used as primers for amplification and hybridization probes were purchased from ImmunoGen International Ltd., (USA). The probes were labeled with biotin at the 5' end.

The various parameters of the amplification method were evaluated with the chromosomal DNA extracted from *V. cholerae* 0139 strains. The selected primer set; designated CTXA 1(24-bp; 5'-CTCAGACGGGA-TTTGTTAGGCACG-3'; located at nucleotide 712 to 735) and primer CTXA2 (24-bp; 3'-GCATTAT-CCCCGATGTCTCTATCT-5'; located at nucleotide 990 to 1013), most effectively amplified the 290-bp fragment (expected size for the primers). This was determined by the intensity of the amplicons after horizontal gel electrophoresis and hybridization. Experiments with primers and the reaction conditions described above revealed that 290-bp fragment was a *ctx*-specific sequence.

Screening of isolates of diarrhoeagenic *E. coli, Shigella* sp., *Aeromonas* sp. and *Plasiomonas shigelloides* was performed to assess the specificity of this amplification method. The target sequence amplified the *V. cholerae* 01 and *V. cholerae* 0139 but not that of other isolates (Fig. 1). *Aeromonas hydrophila* which was reported to produce protein similar to cholera toxin, was negative for *ctx*A gene by PCR in this study.

Isolates of *V. cholerae* 0139 were tested for amplification and showed a single 290-bp fragment on gel electrophoresis (Fig.2). All *V. cholerae* 0139 isolates tested were positive and hybridized with the biotin labeled probe (Fig.3). The probe used was a 5'-ACTATATTGTCTGGT-CATTCTACT-3' inner sequence (corresponding to 792 to 815) as reported by Mekalanos *et al.* (1983).

Strains of *V. cholerae* 0139 have been recognized as the causative agents of the August/September 1993 cholera outbreak in Klang Valley, Malaysia. The clinical spectrum ranged from mild, self-limited infection to severe dehydrating diarrhoea and dysentery as described by Nair *et al.* (1996). The mechanisms responsible for the pathogenesis of *V. cholerae* non-01 particularly in diarrhoea disease, are only now being elucidated. The proposed virulence factors include the El Tor and Kanagawa hemolysins (Ichinose *et al.*, 1987), cell associated hemagglutinins (Datta-Roy *et al.*, 1986), and a 17-amino-acid heat-stable enterotoxin, NAG (Hoge *et al.*, 1990)

In this report, we used primers and probe previously reported by Shirai *et al.*, (1991) to detect cholera enterotoxin operon of *V. cholerae* 01. We felt it was necessary to ascertain the specificity and sensitivity of these primers and probe for cholera enterotoxin genes in *V. cholerae* 0139. A single 290-bp amplified fragment was observed on gel electrophoresis. The specificity and sensitivity of the method were enhanced by hybridization with an inner sequence probe. We have used the chemilluminescent method to remove the need of handling and disposing radioisotopes. The result was in agreement with other reports since this method enables as few as 10^2 CFU to be detected in a sample (Wright *et al.*, 1992). The choice of primers and probes is critical since the sensitivity of this method is affected by the nature of the target sequence.

This *in vitro* amplification allows rapid analysis of isolates and is applicable to nonviable cells. This is important for outbreak investigations in which the original isolates fail to grow because the samples were not inoculated into enrichment medium or because of an insufficient period of incubation.

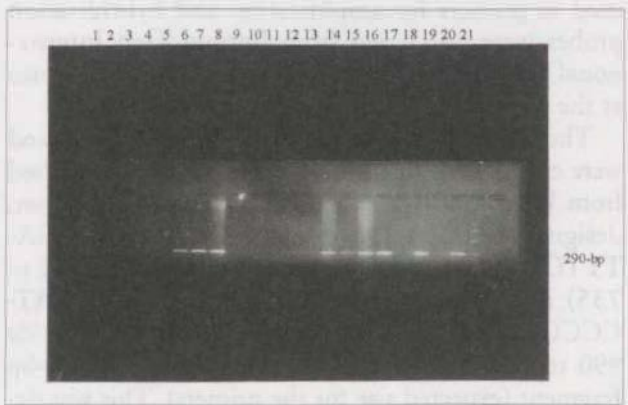

Fig.1. Analysis of PCR products for the ctxA genes. Lanes 1& 2, *Aeromonas hydrophila*; lanes 3 & 4, *A. sobria*; lanes 5 - 7, *V. cholerae 01*; lanes 8 - 10, *Plesiomonas shigelloides*; lanes 11 & 12, *Shigella sp.*; lanes 13 - 16, *V. cholerae 0139*; lane 17 diarrhoeagenic *E. coli*; lanes 18 & 20, *V. cholerae 01* as positive controls; lane 19, negative control; lane 21, PCR marker (Promega).

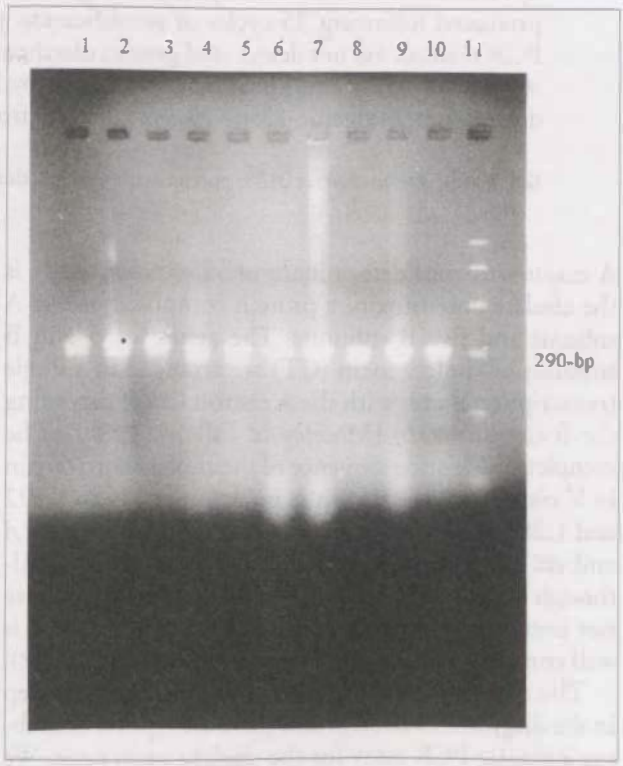

Fig. 2. Analysis of PCR products for the ctxA genes. Lanes 1-10, *V.cholerae 0139*; lane 11, PCR marker (Promega).

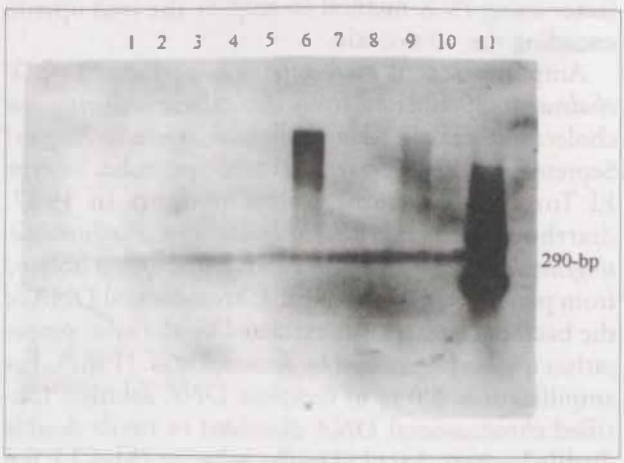

Fig. 3. Hybridization of PCR products for the ctxA genes. Lanes 1-10. *V.cholerae 0139*; lane 11, 147 *Hae*III biotinylated marker (Promega).

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