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PCR Cloning and Partial Sequencing of *rtx*A Gene of non-O1 / non-O139 *Vibrio cholerae* Isolated from Gold Fish *Carassius auratus* in India.

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Abstract

A total of 16 non-O1/ non-O139 *Vibrio cholerae* isolates were obtained from ornamental fish collected from aquarium traders located in four metro cities of India. All the isolates were confirmed by amplifying the 300 bp fragment in the 16S-23S rRNA intergenic spacer regions. Amplification of 431 bp fragment of gene, which resulted in detectable levels of PCR product, was achieved with a minimum of 8 CFU/ml of *V. cholerae*. The detection limit for *rtxA* gene by PCR amplification of genomic DNA was 20 picogram. A 431 bp fragment of the *rtxA* gene of *V. cholerae* was cloned and sequenced (NCBI accession number EU714289). Nucleotide sequence analysis data of the *rtxA* gene showed that about 96% identical to those of *V. cholerae* RTX toxin gene cluster.

Introduction

Vibrio cholerae is an important cause of diarrhea in many parts of Asia and Africa. *V. cholerae* is an autochthonous inhabitant of brackishwater and estuarine systems (Colwell et al. 1977). Toxigenic *V. cholerae* O1 and *V. cholerae* O139 are etiological agents of epidemic cholera. However, both *V. cholerae* O1 strains that do not produce cholera toxin, i.e., that are nontoxigenic, and non-O1/non-O139 strains have also been associated with cholera, gastroenteritis, septicemia, and/or extraintestinal infections (Mukhopadhyay et al. 1995). Conventional methods used to detect and classify cholera-causing vibrios isolated from clinical and environmental samples require several days to complete and involve culture in alkaline peptone water, thiosulfate citrate bile sucrose agar, slide agglutination with specific antisera, and assay for production of cholera toxin (Sakazaki 1992). Molecular methods, including PCR and DNA-DNA hybridization performed with probes specific for *V. cholerae*, provide more reliable identification (Chun et al. 1999) but have limitations because of cost and the facilities required for analysis; these limitations are particularly significant for field studies involving large numbers of samples.

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Although cholera toxin (CT) is clearly the most important causative factor for cholera, CT deficient isolates of *V. cholerae* also elicit mild to severe diarrhea and other reactogenic symptoms in human, indicating that other toxins are likely to contribute to the pathogenesis of the disease (Coster et al. 1995). A novel toxin in *V. cholerae* that belongs to the RTX (repeat in toxin) family of toxins, which are generally produced by several pathogenic gram-negative bacteria, was recently discovered (Chow et al. 2001). Lin et al. (1999) proposed that the *V. cholerae RTXA* (VcRTX-A) toxin, might play a role in the gastrointestinal virulence property of *V. cholerae*. A total of 16 non-O1 and non-O139 *V. cholerae* were isolated from aquarium fish, koi carp and gold fish in India during the previous study (Swaminathan et al. 2007).

In this study, rapid detection and confirmation of *V. cholerae* non-O1 and non-O139 isolated from koi carp and goldfish by amplification and sequencing of a 431 bp fragment of the *rtxA* gene of *Vibrio cholerae* was carried out.

Materials and methods

Source of the isolates

A total of 16 *V. cholerae* non-O1 and non-O139 serogroups isolated from different regions of India were included in our study. All the isolates were obtained from koi *Cyprinus carpio* koi, goldfish *Carassius auratus* (L.) during 2004-05. All the isolates were subsequently examined and characterized. The identity of the isolates was confirmed by serotyping at the National Institute for Cholera and Enteric Diseases , Kolkata, India.

Identification of V. cholerae by PCR

Identification and confirmation of isolated *V. cholerae* was done by amplification of a fragment of 16S-23S rRNA intergenic spacer region (ISR) of *V. cholerae* as described by Chun et al. (1999).

Construction of specific PCR primers

The *rtxA* sequences of these *V. cholerae* was obtained by accessing the nucleotide sequence database (GenBank accession # AF119150). The data were examined with multiple alignment analysis (CLUSTAL W), the specific region for bacteria was identified, and the specificity was confirmed by FASTA. Specific PCR primers for identification of *V. cholerae* were designed from the sequence of *rtxA* using Laser gene 6 software. Primers were tested for specificity among different bacterial isolates. The cross-reactivity of *V. cholerae rtxA* gene primers were checked by NCBI–BLAST and by genomic DNA amplification of other bacteria, i.e. *Escherichia coli, Salmonella arizonae, Pseudomonas alcaligenes, Aeromomas hydrophila, Edwardsiella tarda, Staphylococcus aureus* and *Flavobacterium* sp.

Preparation of PCR samples

Bacterial pellets were diluted in sterile saline prior to lysis by 10 min of boiling in a water bath. Bacterial genomic DNA isolation of all *V. cholerae* was according to Hiney et al. (1992). The nucleic acid preparation was finally suspended in 50 µl of TE buffer. The isolated nucleic acid was qualified and quantified at 260 nm and 260/280 ratios, respectively.

PCR amplification

The PCR amplification was performed using the primers designed in this study. We used 10 ng of genomic DNA, 50 pmoles of primers, 100 µ moles of each dNTP's and 2 mM of $MgCl_2$. Samples were subjected to 35 cycles of amplification (94^0C for 2 min, 64^0C for 1 min and 72^0C for 3 min on a Master cycler (Eppendorf). 10 µl of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.2% agarose.

Cloning and Sequencing of 431 bp fragment of V. cholerae rtx A gene

A fragment of 431 bp of *V. cholerae rtxA* gene was amplified using designed primer. The DNA band of interest was excised and purified from the gel at position 431 bp and was ligated to pCR 2.1 TOPO cloning vector and transformed into DH5 α *E. coli* strain (Invitrogen). The recombinant clones were confirmed by PCR using designed primer. The two terminal sequences of the cloned genes fragment was determined with the ABI PRISM Dye primer cycle sequencing ready reaction kit and ABI 377 DNA auto sequencing machine by using M13sequence primers.

Sensitivity of the PCR

For determining the sensitivity of the PCR 10-fold dilutions (8 X 10^{-5} to 8 cells) were tested. When nucleic acids were used, the sensitivity of the PCR was determined by amplifying 5µl of 10-fold serial dilutions (20ng to 2pg). PCR amplification was performed with a DNA thermal cycler as described before.

Results

Primer pairs was designed on the basis of the nucleotide sequence of the *rtxA* downloaded from NCBI and used to amplify target sequences in genomic DNA from 16 isolates of *V. cholerae* and yielded a product of the expected 431 bp size for *V. cholerae* (Fig 1). Similar amplification of the expected product size was not observed for the other bacteria *viz., Escherichia coli, Salmonella arizonae, Pseudomonas alcaligenes, Aeromomas hydrophila, Edwardsiella tarda, Staphylococcus aureus* and *Flavobacterium* sp when the primer pairs were used. All strains of *V. cholerae* isolated in our laboratory were confirmed and a PCR amplification product of the expected length (431 bp) was obtained (Fig.1).

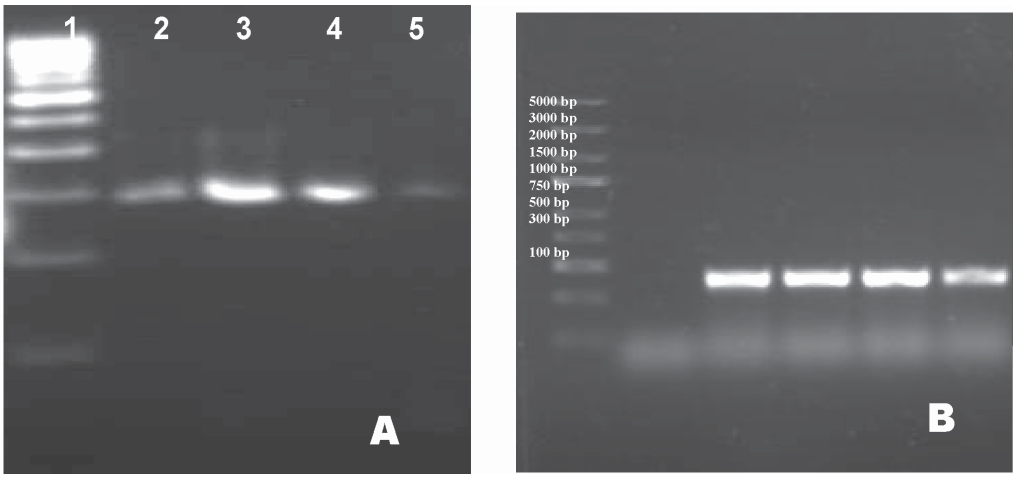

Figure1. Detection of non -O1 and non - O139 *Vibrio cholerae* by PCR

A- Confirmation of *V. cholerae* by amplification of the fragment of 16S-23S ISR by PCR. Lane 1 - 100bp ladder, lane 2- 5 - 16S-23S ISR (300bp).

B-Detection of *V. cholerae* by amplification of the fragment (417bp). of *rtx*A gene by PCR. Lane 1 – Generuler DNA Ladder (Fermentas), Lane 2- Negative control Lane 3 – Positive control lane 4-6 – Positive test samples

The 431 bp fragment of *rtxA* of *V. cholerae* was cloned in PCR 2.1 TOPO cloning vector for sequencing. The cloned 431 bp fragment of the *rtxA* was sequenced and comparison of this sequence with other sequences from DDBJ/EMBL/Genbank was made. Comparing the 431 bp fragment of the *V. cholerae* isolates 96% homology with *rtxA* sequences of *V. cholerae* strains *viz.*, AE003852.1, AF119150.1, was found. The sequence of the 431 bp fragment of *rtxA* of *V. cholerae* was deposited in the NCBI GenBank (EU714289).

Amplification which resulted in detectable levels of PCR product was achieved when a minimum of 8 CFU/ml of *V. cholerae* were lysed, on the basis of an average of five repeated testing of viable cells and PCR assays. The minimum amount of purified DNA in the reaction mixture needed to obtain a detectable PCR product was 20 pg. The lower limit of detection of *V. cholerae* bacterial cells or isolated DNA by PCR was examined for all the strains of *V. cholerae*. A suspension of cells of the isolates was diluted and processed. A PCR amplification product could not be obtained when a sample with more than 10^6 CFU was used in the assay, probably because of accumulation of soluble cell products inhibitory to PCR. Isolated DNA from *V. cholerae* was serially diluted in saline and used as a template. The minimum amount of purified DNA in the reaction mixture needed to obtain a detectable PCR product was 20 pg.

In the study, we used the PCR technique for the identification of the *rtxA* genes of *V. cholerae* and the specificity of the primers used here is noteworthy, and can reach high sensitivity. The detection limit for *rtxA* gene from *V. cholerae* was 20 pg.

Discussion

This study was undertaken to explore the possibility of contamination of *V. cholerae* serogroups O1 and O139, the most important causative organisms for cholera and also potential public health importance, by isolating these organisms from body surface, gill and intestine of common ornamental fishes. *V. cholerae* is often transmitted by water but fish or fish products that have been in contact with contaminated water or faeces from infected persons also frequently serve as a source of infection (Colwell et al. 1977). *V. cholerae* O1 or O139 were not isolated from body surface swabs, gills and intestine of these common table fishes. Dalsgaard et al. (1995) isolated 143 *V. cholerae* non-O1 strains from shrimp farms in Thailand, and characterized and grouped by ribotyping. All the 16 isolates of *V. cholerae* isolated in our laboratory were confirmed and a PCR amplification product of the expected length (431 bp) of *rtxA* gene was obtained. Nonepidemic *V. cholerae* non-O1 serogroup strains, which cause only sporadic, milder cases of diarrhea, do secrete the RTX cytotoxins but do not secrete CT (Chow et al. 2001). The sequence of the 431bp fragment of the *rtxA* gene had showed 96% homology with the other *rtxA* gene sequences of the *V. cholerae*, *viz.*, AE003852, AF119150.

Non-O1/ non-O139 *V. cholerae* strains can no longer be ignored. The rationale for continuous monitoring is based on the emergence of serogroup O139 (Bengal) in Bangladesh (CT positive) and Argentina (CT negative), each of which clearly evolved independently. The sixth pandemic, the seventh pandemic, and U.S. Gulf Coast isolates represent three different clones, each independently evolved from environmental non-O1 *V. cholerae* isolates (Karaolis et al. 1995). Finally, the emergence of a new clone of the *V. cholerae* O1 El Tor in Calcutta, India (Sharma et al. 1997) has been reported. The possibility exists that those additional new strains of toxigenic *V. cholerae* with epidemic potential may emerge in the future. While CT is a principal virulence factor for *V. cholerae*, the contribution of the RTX toxins to its pathogenesis requires further investigation.

In previous study it was described that the rtx gene was absent only from the *V. cholerae* classical O1 serogroup strain, which has greater epidemic potential than strains of the other serogroups, despite its displacement by the El Tor biotype since the seventh pandemic (Chow et al. 2001). Therefore, nonepidemic *V. cholerae* non-O1 serogroup strains, which cause only sporadic, milder cases of diarrhea, does secrete the RTX cytotoxins but do not secrete CT. Further investigation is required to determine the role of RTX in the pathogenicity of non-O1 /non-O139 *V. cholerae*.

Conclusion

The 431 bp fragment of *rtxA* of *V. cholerae* was amplified by the primer designed and the PCR product was cloned in pCR 2.1 TOPO cloning vector for sequencing. The cloned 431 bp fragment of the *rtxA* was sequenced and comparison of this sequence with other sequences from DDBJ/EMBL/Genbank was made. The sequence of the

431 bp fragment of *rtxA* of *V. cholerae* was deposited in the NCBI GenBank (EU714289). The sensitivity and specificity of the primer designed to detect the rtxA gene fragment of *V. cholerae* were checked. The primer could identify 8 CFU/mL *V. cholerae* and 20 pg of purified DNA of *V. cholerae*.

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