International Journal of Research in Biosciences
Vol. 3 Issue 3, pp. (57-62), July 2014
Available online at http://www.ijrbs.in
ISSN 2319-2844

Research Paper

Specific detection of human pathogenic *Vibrio species* in water bodies of north Indian region by using multiplex polymerase chain reaction

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(Received May 12, 2014, Accepted June 30, 2014)

Abstract

The purpose of this study was to investigate the prevalence of Human pathogenic Vibrio species in water bodies of north Indian region using the traditional culture and multiplex PCR methods. Vibrio species are very much prevalent in water bodies of tropical region.The study was conducted on 30 fresh water bodies' samples collected from rivers and ponds of north Indian zones. For this species specific primers were designed targeting the tox gene of the five pathogenic species specific and confirmation was done using multiplex PCR technique for rapid detection of the five selected pathogenic species including *V. cholerae*, *V. alginolyticus, V. parahaemolyticus, V. mimicus* and *V. vulnificus*. Out of the five targeted species three were present in the samples. The major rivers of north India shows high rate of contamination and *Vibrio cholera* was most abundant among all.

Keyword: Multiplex-PCR, TSA, TCBS, tox gene, *V. cholerae, V. alginolyticus, V. parahaemolyticus, V. mimicus and V. vulnificu.*

Introduction

Vibrio species are Gram-negative rod-shaped bacteria and are pathogenic to vertebrate as well as invertebrate animals [1]. In the Asian region, *Vibrio species* have been recognized as the leading cause of foodborne outbreaks in many countries including Japan, India, China, Taiwan, Korea and Malaysia [2]. As food safety is a major global concern that affects the consumer and those in the food service sector, serious attention has to be given to the aquaculture industry as fish can act as a vector for human pathogenic bacteria [3]. The importance of *Vibrio species* as a contaminant of raw or undercooked seafood has been well established and may lead to acute gastroenteritis including diarrhea, headache, vomiting, nausea and fever [4]. Therefore, it is important to have data on the prevalence of *Vibrio species* in freshwater fish. Freshwater fish are easily available in the markets in India and are in high demand by local consumers [5].Humans are the prime reservoir of the organism *V. cholera* and cholera is typically associated with reduced hygiene and contaminated water supplies, although it can also be food borne [6]. Polymerase chain reaction (PCR) quickly detects pathogenic microorganisms without the need of the cultivation process through the oligonucleotide primer-direction method by way of DNA amplification [7]. Primers synthesized from 16S r-DNA gene can be used for the species-specific detection by using PCR for the purpose of phylogenetic classification of microorganisms [8].This technique represented a vigorous tool for the specific and rapid detection of major human pathogenic *Vibrio species.*

Materials and Methods

Collection of Water Sample: A total 30 water samples were collected from various water bodies like rivers, lakes and ponds of north Indian zone.

Isolation of *Vibrio species:* Isolation of different *Vibrio species* from 30 different water samples was done on Thiosulfate Citrate Bile salts Sucrose (TCBS) media (specific for isolation of *Vibrio species*). 200µl of water sample was taken for spreading on TCBS agar plates and left for 24 hours at 37^{o}C for development of *Vibrio* colonies. Single colonies were taken and streaked onto Trypto Soya Agar (TSA) plates for obtaining discrete colonies. Thereafter, the broth was prepared for inoculation of the colony and liquid culture was prepared for isolation of DNA.

Isolation of Genomic DNA: Genomic DNA isolation was done using Phenol Chloroform method. Extraction of DNA was done from broth culture. 2 ml of culture broth was taken in an eppendorf tube and centrifuged at 10000 rpm for 10 mins. After centrifugation supernatant was discarded and pellet was dissolved in 500 µl T.E. buffer.1/20 volume of 10% SDS was added and tubes were kept in waterbath for cell lysis to occur.ater 1-2 hours the cell extract containing eppendorf are again centrifuged at 10000 rpm for 10 mins and supernatant was collected. Phenol chloroform Isoamyl alcohol mix was added in the ratio 25:24:1, and mixed well by inverting the tubes, the samples were again centrifuged .the upper transparent layer was collected which contained DNA and 50 µl of freshly prepared 3 M Sodium Acetate solution was added for precipitation of DNA and kept in ice cold conditions for 10 mins. After that double volume of ethanol was added to the tubes and centrifuged at 10000 rpm for 10 mins. Discarding the supernatant ,the tubes were air dried and 50-100 µl of T.E was added for dissolving the pellet for loading in agarose gel electrophoresis. Similar method was applied for DNA extraction from direct scraping of colonies from TSA plates. in an eppendorf tube 500 µl of T.E. buffer was taken and large amount of Vibrio colonies were scrapped from the plates into the eppendorf tubes containing T.E. buffer. Same steps were repeated after that as done in isolation of DNA from culture broth.

Primer Designing: Oligonucleotides primers, ranging from 20- to 24-mers, were selected. The primer sets used in this study for multiplex-PCR, their corresponding gene targets and size of expected amplification products were shown in Table (1).

Table 1: List of Primers

Name of Primers	Primer Abbreviation	Primer sequence
Universal Forward Species	VM-F	CAGGTTTGYTGCACGGCGAAGA
5 Reverse primer :		
V.cholera	VC- Rmm	AGCAGCTTATGACCAATACGCC
V. parehaemolyticus	VP- MmR	TGCGAAGAAAGGCTCATCAGAG
V. Vunificus	VV- Rmm	GTACGAAATTCTGACCGATCAA
V. mimicus	VM- Rmm	YCTTGAAGAAGCGGTTCGTGCA
V. alginolyticus	V.al2- MmR	GATCGAAGTRCCRACACTMGGA

PCR Amplification and Gel Electrophoresis: The isolated genomic DNA were amplified by PCR using species specific primers. The PCR mix was made of total 20µl. Multiplex PCR was performed on a thermocycler and the total number of cycles set was 35.Further the PCR products were run on agarose gel electrophoresis for checkingamplification. The amplification products were visualized after electrophoresis at 50 V for 45 mins ona 1.2% agrose gel by using 3µlethidium bromide.

Results and Discussion

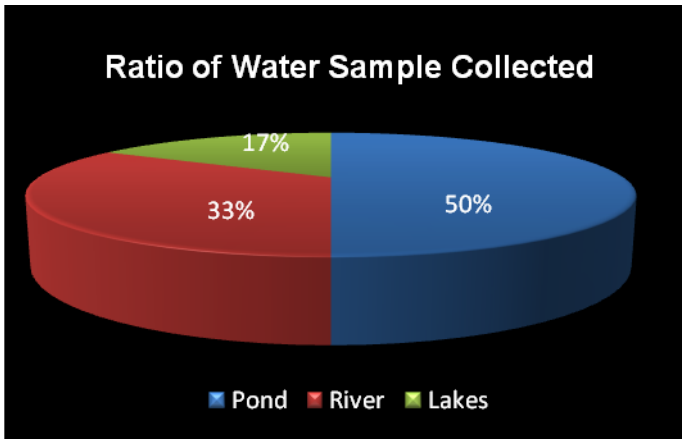

Figure 1: Ratio of Water Reservoir from where sample was collected (30)

Isolation of *Vibrio* species: The yellow and green colonies are grown, when water sample was spread onto the TCBS media, and kept in incubation for 24 hrs. The yellow colonies show the sucrose positive. The green colonies show the sucrose negative as shown in figure 2.

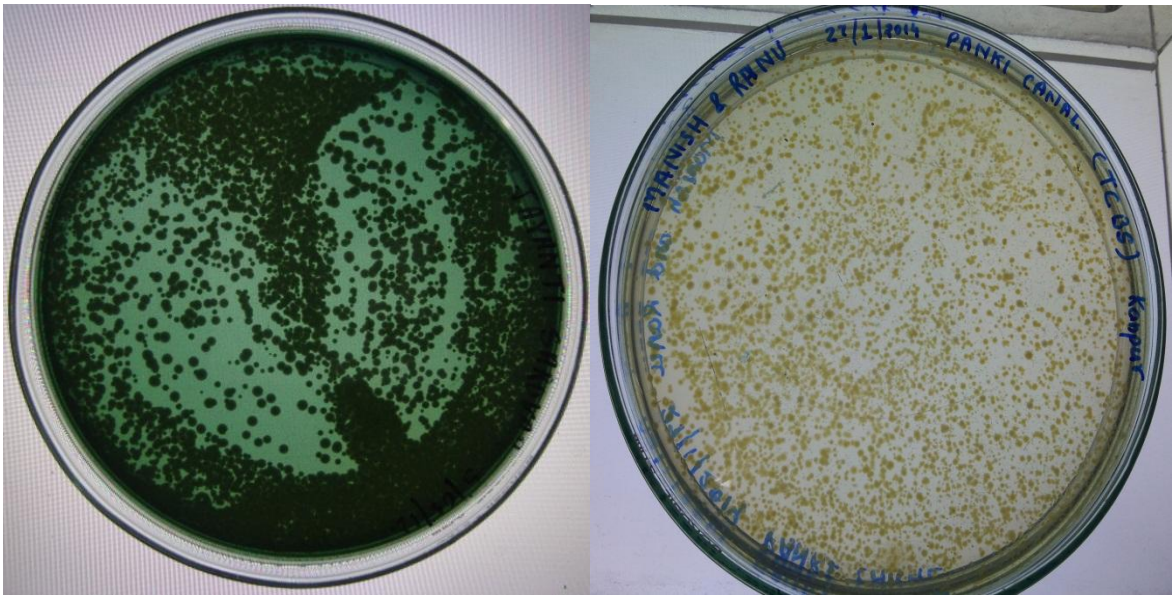

Figure 2: Isolated Green and Yellow *Vibrio* colony on TCBS media

Isolation of Genomic DNA from isolated *Vibrio* species

After isolation of *Vibrio* species on TCBS media from different water samples, the colonies were inoculated on TSA broth for isolation of genomic DNA from *Vibrio* species. The Phenol Chloroform isoamyl method was used for isolation of DNA. The precipitated DNA was reconstituted using 1X TE

buffer.Quality and quantity of DNAextracts were also verified on 1.2% agarose gels stained with ethidium bromide.

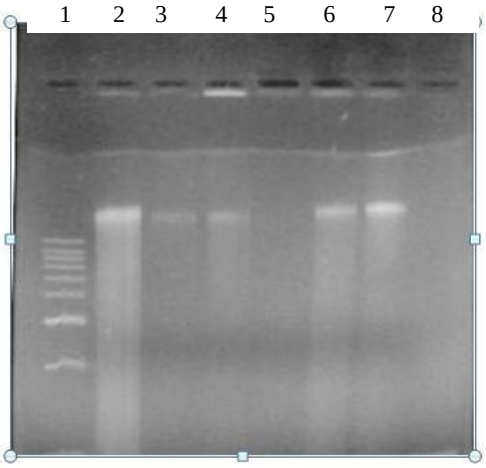

Figure 3: Electrophoretic analysis of Genomic DNA isolated fron *Vibrio species*
(a) Lane 1- 1kb marker

Amplification by Multiplex PCR: Amplification by Multiplex PCR was performed and out of the five targeted species three of them were present in the samples and were used for identification. The amplified products were run in agarose gel electrophoresis for visualization of the amplified products. The major rivers of north India shows high rate of contamination and *Vibrio cholera* was most abundant among all.

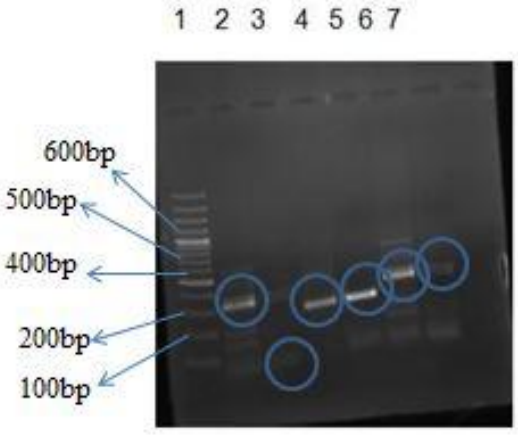

Figure 4: Electrophoretic analysis of PCR amplified target Genes from five different *Vibrio species*

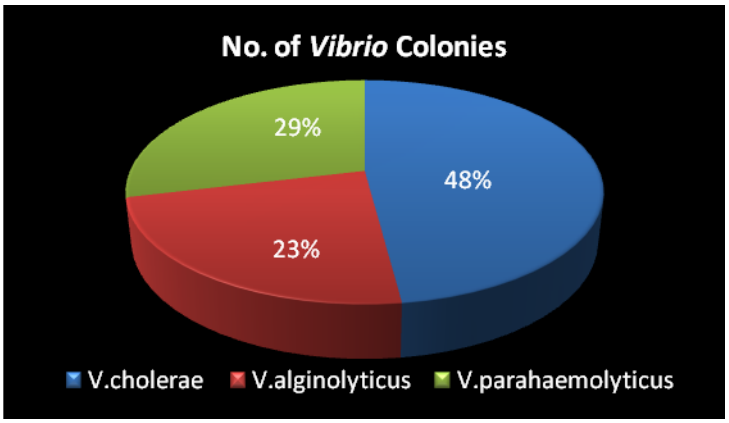

Figure 5: Ratio of three major *Vibrio* species found in 30 water samples

Conclusion

Identification of Human pathogenic Vibrio species in water bodies of north Indian region was done by using the traditional culture and multiplex PCR methods. The study was conducted on 30 fresh water bodies' samples collected from rivers , lakes and ponds of north Indian zones. For this species specific primers were designed targeting the tox gene of the five pathogenic species specific and confirmation was done using multiplex PCR technique for rapid detection of the five selected pathogenic species including *V.cholerae, V.alginolyticus, V.parahaemolyticus, V.mimicus and V.vulnificus.* Out of the five targeted species three were present in the samples. The major rivers of north India shows high rate of contamination and *Vibrio cholera* was most abundant among all.

Acknowledgement

We are thankful to Saroj Institute of Technology & Management, Lucknow for giving us permission to do our project work from CytoGene and we are extremely thankful to CytoGene Research & Development, Lucknow for providing us with necessary facilities for the work. We are also thankful to almighty without whose consent anything is possible**.**

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