Determination of five selected human pathogenic *Vibrio* species in water bodies of north Indian zone using multiplex PCR technique

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Abstract

Vibrio species are prevalent in tropical region water bodies in all seasons throughout the year. A total of 47 fresh water bodies were used for sample collection including rivers & ponds of north Indian zone. 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. Being source of livelihood for a huge population these water bodies are continuously being used as dumping ground for wastes.

Keyword: *m - PCR, TSA, TCBS, Halophillic*

1. Introduction

Members of the genus *Vibrio* are defined as Gram negative, asporogenous rods that are straight or have a single rigid curve and are motile with a single polar flagellum when grown in liquid

Medium (1). In the Asian region, *Vibrio* spp. Have been recognized as the leading cause of foodborne outbreaks in many countries including Japan (2), India (3), China (4), Taiwan (5), Korea (6) and Malaysia (7). As food safety is a major global concern that affects the consumer and those in the food service sector (8), serious attention has to be given to the aquaculture industry as fish can act as a vector for human pathogenic bacteria (9). (10) reported that apart from seafood, *Vibrio* spp. can be found naturally in brackish water and estuarine ecosystems with optimal salinity and temperature conditions. The importance of *Vibrio* spp. as a contaminant of raw or undercooked seafood has been well established (11) and may lead to acute gastroenteritis including diarrhea, headache, vomiting, nausea and fever (9). Therefore, it is important to have data on the prevalence of *Vibrio* spp. in freshwater fish. Freshwater fish are easily available in the markets in India and are in high demand by local consumers.

In the recent years, the use of nucleic acid probes and PCR have provided highly sensitive methods for detection of specific pathogens in environmental samples. More recently, the use of the multiplex polymerase chain reaction (m-PCR) has provided rapid and extremely sensitive methods for the particular detection of pathogenic microorganisms in the aquatic environment. In this study, we report the development of an m-PCR method that permits the simultaneous detection of five different species of *Vibrio* pathogen such as *V.cholerae, V.parahaemolyticus, V.alginolyticus, V.mimicus* and *V.vulnificus.*

2. Materials & Methods

2.1. Water sample analysis

A Total of 47 Water samples were collected from various rivers and lakes and shrimp culture ponds of north India. Media plating was done i.e., TCBS agar supplied by Titan Biotech Ltd, Rajasthan for spreading of water samples. 200 µl of fresh water was taken for spreading on TCBS agar plates and left for 24 hours incubation period for colony developing. After the development of green and yellow colonies on TCBS agar plates, single colonies were taken and streaked on Trypton Soya Agar supplied by Titan Biotech Ltd, Rajasthan, plates for obtaining different colonies. After that

Trypton Soya Broth was prepared for inoculation of colony and liquid culture was prepared for isolation of bacterial DNA

2.2. Genomic DNA isolation

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 eppendorfs 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.

2.3. Designing of Primer

In this detection process, five pairs of oligonucleotides primers were designed to simultaneously detect five different types of *Vibrio* species by multiplex PCR assay targeting the species-specific tox gene region of the *Vibrio*. Table 1 contains the primers used for the amplification of these genes and the predicted sizes of each of the amplification products. In order to assist PCR product detection, the primers were designed to predict sizes of the amplification products of each target gene would be different from each other to permit size discrimination using gel electrophoresis technique.

Universal Forward Species	VM-F	CAGGTTTGYTGCACGG CGAAGA
5 Reverse primer :		
V.cholera	VC-Rmm	AGCAGCTTATGACCAA TACGCC
V. parehaemolyticus	VP-MmR	TGCGAAGAAAGGCTCA TCAGAG
V. Vunificus	VV-Rmm	GTACGAAATTCTGACC GATCAA
V. mimicus	VM-Rmm	YCTTGAAGAAGCGGTT CGTGCA
V. alginolyticus	V.al2-MmR	GATCGAAGTRCCRACA CTMGGA

2.4. PCR Amplification and Agarose Gel Electrophoresis

After running the samples on agarose gel electrophoresis of the isolated DNA, we performed PCR for the same samples using species specific primers supplied by Bangalore Genei. The PCR mix was made of 20µl volume in total for each tube. PCR was performed on a thermocycler instrument supplied by Bangalore Genei and the total number of cycles we set was 30. Further the PCR products were run on an agarose gel electrophoresis for detection of amplification. The amplification products were then visualized after electrophoresis done at 50 V for 45 mins on a 1.2% agrose gel by ethidium bromide staining.

3. Result & Discussion

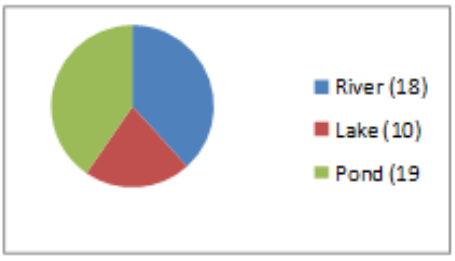

Fig1: Ratio of water Reservoir from where sample was collected (47)

After spreading of the collected samples yellow and green colonies appeared on the TCBS agar plates after 24-48 hours of incubation period given

at 37^0C. The appearance of yellow colonies shows the presence of sucrose fermenting species whereas green colonies shows the non-sucrosefermenting species of the genera *Vibrio*. Ram Ganga (Baliya region) shows maximum no of yellow colonies. On the other hand Ganga (Haridwar region) shows mostly green colonies.

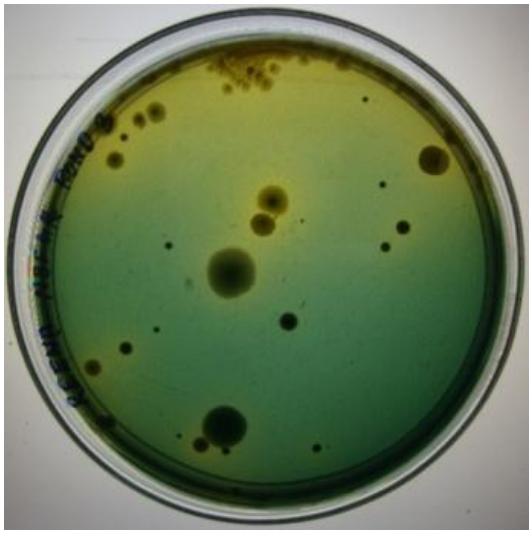

Fig 2: NBFGR Pond3

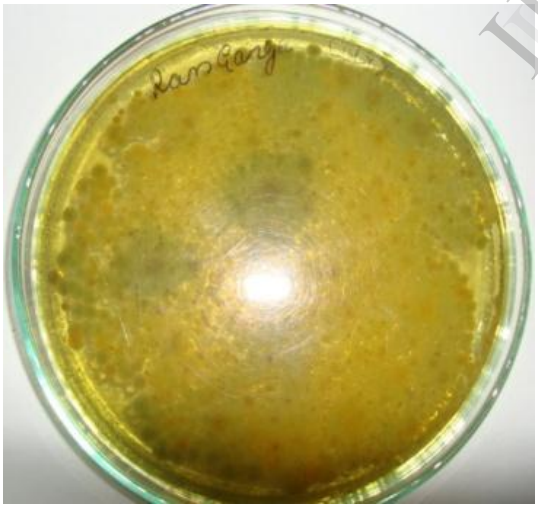

Fig 3: Ram Ganga, Baliya

Fig 4: Kukrail, Lucknow

3.1. Multiplex PCR Optimization

Specific and sensitive amplification of target gene sequences by m-PCR are dependent on a number of key parameters like annealing temperature, primer concentration, Mg2+ concentration, extension time, and the amount and quality of Taq polymerase used(12). Therefore systematic approach was taken into account to optimize the m-PCR conditions in order to get similar and maximum band intensities for each of the gene amplicons.

3.2. Multiplex PCR Amplification

Multiplex PCR was performed and out of the five targeted species three of them were present in the samples we used for analysis(13). Concentration of *Vibrio* pathogens was found to be high in the densely populated and tourist regions in compared to sub- urban areas. The amplied products were run in agarose gel electrophoresis for visualization bands bt ethidium bromide staining. The first PCR was set for 10 samples taking 100 bp ladder. Lane 1 contain the ladder while rest of the lanes were loaded with the PCR amplified samples. Lane 5, 8 and 9 respectively shows the presence of *Vibrio* species. The second PCR cycle was set for 7 isolated samples of DNA. Lane 1 contain the ladder of 100 bp and the rest 7 lanes contain the PCR loaded samples. Out of which lane 2, 4 and 5 containsthe amplified DNA *Vibrio*s.

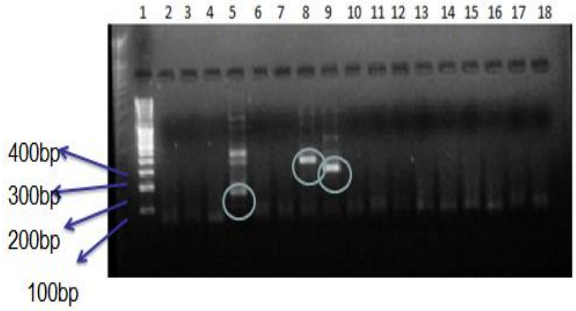

Fig 5: Electrophoresis analysis of PCR-amplified target genes from five different *Vibrio* species. Mobilities of the different target gene amplicons are indicated on the left. Lane 1 :100 bp marker, Lane 5: *V.alginolyticus*, Lane 8: *V.cholerae*, Lane 9: *V.cholerae*

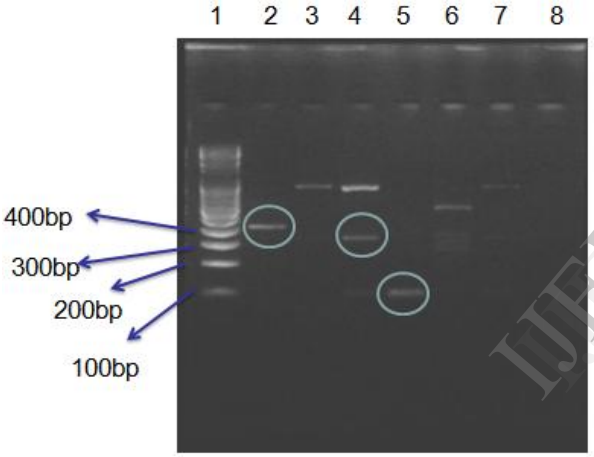

Fig6: Electrophoretic analysis of target gene showing Lane 2: *V.vulnificus*, Lane 4: *V.cholerae*, Lane 5: *V.alginolyticus*. Lane 1: 100 bp marker.

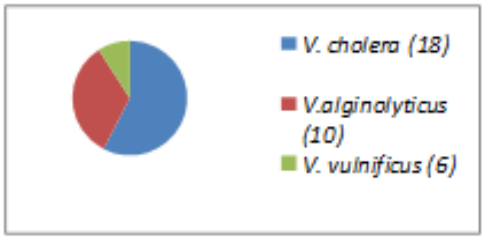

Fig 7: Ratio of the Species found after analysis, out of 47 samples:

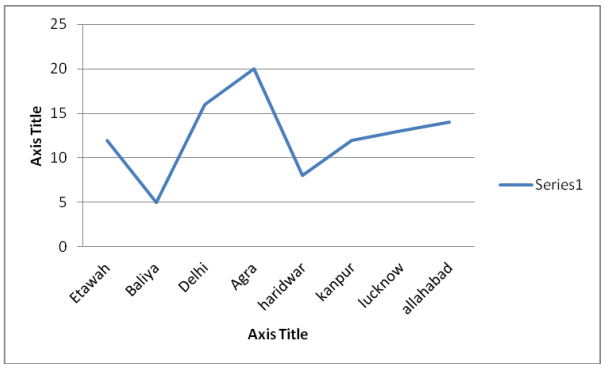

Fig 8: Chart showing area with maximum *Vibrio* concentration water bodies.

3.3. Antibiotic test by Well Diffusion Method

To check whether *Vibrio* is resistance or sensitive to certain specific antibiotics, an antibiotic test was performed using disc diffusion method. TSA agar plate was used for spreading of *Vibrio* culture

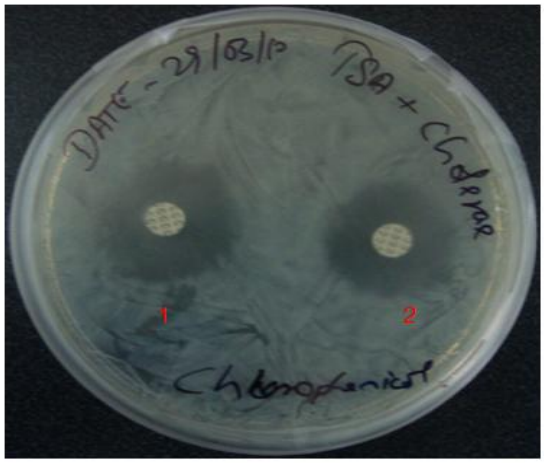

Fig 9: TSA Plate with *Vibrio* And chloramphenicol

Diameter of inhibition zone 1 = 1.5 cm
Diameter of inhibition zone 2= 1.3 cm

Sampling was done from different fresh water bodies such as (rivers, lakes and ponds) of north Indian region. Till date no such relevant work has been done in this prospect in fresh water bodies in India. Dumping of sewage and industrial wastes in rivers causes contamination of these water bodies

with major pathogenic and non pathogenic organisms (13).
Not much work has been done on the bio safety level of *Vibrio* species in freshwater bodies in India. The purpose of this study was to investigate the occurrence and concentration of *Vibrio* species in fresh water as well as sea water using the Multiplex-Polymerase Chain Reaction (m-PCR) method(). The study was conducted on 47 samples from different types of water bodies i.e. rivers, lakes and pond of north India. More samples can be included from major water sources of rest parts of India for the detection of *Vibrio* species (13).

4. Conclusion

Most applied molecular techniques are based on protocols of nucleic acid amplification, of which the polymerase chain reaction (PCR) is the most commonly used. In this we have used the tool m-PCR for microbial identification and surveillance with high sensitivity and specificity. This technique has been successfully applied for the identification and detection of pathogenic bacterial species in clinical and environmental samples, as well as for the analysis of food and waterborne disease outbreaks. Rapid identification of various pathogenic species using mPCR would not only provide a way to routinely screen the water quality to protect and safeguard public health but also allow evaluation of water treatment processes(13).

5. Acknowledgement

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