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RAPID IDENTIFICATION OF HUMAN PATHOGENIC VIBRIO SPECIES USING MULTIPLEX PCR - A CASE STUDY

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ABSTRACT

There are several water-borne diseases which are highly pathogenic for humans. Amongst all, genus *Vibrio* plays a major role. In this case study of “RAPID IDENTIFICATION OF HUMAN PATHOGENIC VIBRIO SPECIES USING MULTIPLEX PCR” We have chosen salt water bodies such as sea and estuaries for sampling. To facilitate the identification of human-pathogenic species, we have used multiplex PCR to amplify gene regions in five species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus and V. alginolyticus*). The assay was tested on a sample of 82 *Vibrio* isolates for identification of five common human pathogenic vibrio species. Multiplex PCR was performed targeting the tox gene present in the five most common pathogenic vibrio species. After analysis of all the results we have found that salt water is having a higher concentration of vibrio species than freshwater & *V.vulnificus* was most abundant among all.

KEYWORDS:Multiplex PCR, Halophilic, tox gene, Human pathogenic.

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INTRODUCTION

The increasing population of the coastal zone introduces a high percentage of untreated sewage Into the sea, and this form of pollution is probably responsible for the greatest number of human morbidities and mortality worldwide [1]. In particular, waterborne infections like typhoid fever, cholera, dysentery and traveller's diarrhea, caused by different types of bacterial pathogens pose a major public health hazard [2], especially in developing countries. The spectrum of waterborne infections is also expanding, and many infectious diseases once believed to be conquered are on the rise [3]. In order to protect public health, regular monitoring of waterborne pathogens is required. However, the deficiency of precise and cost-effective diagnostic methods is a major obstacle in the prevention and control of infections and outbreaks transmitted by waterborne pathogens. The aim of this study is to screen different water bodies (sea, rivers, estuarine, lakes and ponds) for the presence of *Vibrio* species and determine their pathogenicity. The knowledge of this will help in the control of *Vibrio* – associated gastroenteritis in India as the awareness of the dangers associated with the consumption of raw or undercooked seafood will be created for the masses. Shrimp accounts for about 20% of the value of exported fishery products over the past 20 years [4]. Imports into developed countries accounted for about 40% of intra-developed countries trade, while about 60% comes from developing countries; out of the exports from developing countries 80% goes for developed countries and only 20% stays in the group [5]. Also, shrimps are one of the major aquaculture products of export importance from the tropics [6]. Eighty percent of the world's farms raised shrimps is contributed through aquaculture in Asia [7]. The number of detention and rejection cases due to the detection *Vibrio bacteria* from Asia is increasing.

Vibrios are gram-negative and largely halophilic. Although, depending on their sodium chloride requirements a few species are nonhalophilic also. Most of the species are oxidase-positive too. Mostly sensitive to acidic pH, while tolerant to alkaline pH. As pathogenic organisms, the CDC estimates that there are 8,000 infections and 60 deaths each year that are the result of Vibrio infections (Centre for disease control). Traditionally, detection and enumeration of bacterial pathogens have been largely based on the use of selective culture and standard biochemical methods [8]. But these methods suffer from a number of drawbacks. First, pathogenic bacteria which normally occur in low numbers tend to incur large errors in sampling and enumeration [9]. Second, culture-based methods are time-consuming, tedious, invariably monospecific (i.e. detecting low output). Third, many pathogenic organisms in the environment, although viable, are either difficult to culture or non-culturable [10], but can still cause illnesses [11]. Due to these difficulties, examination of water samples for pathogens like Vibrio cholerae, Shigella dysenteriae, Aeromonas spp. and Campylobacter spp., etc. is normally not performed during routine microbiological assessment of water quality [8].

Vibrio, a diverse genus of aquatic bacteria, currently includes 72 species, 12 of which occur in human clinical samples. Of these 12 some account for the majority of *Vibrio* infections in humans. Members of the genus *Vibrio* are all Gram-negative straight or curved rods, which do not form spores and are motile, usually by a single polar flagellum. Most are oxidase and catalase positive and ferment glucose without gas production. Vibrio species are among the most common surface water organisms across the world. They occur in both marine and freshwater habitats and in associations with aquatic animals. Some species are pathogens of fish, eels and frogs as well as other vertebrates and invertebrates [12]. Species such as *V. cholerae, V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. mimicus, V. fluvialis, V. furnissii, V. metschnikovii, V. hollisae* and *V. damsela* are

human pathogens [13]. They account for a significant proportion of human infections such as gastroenteritis, usually associated with consumption of raw or undercooked seafood, wound infections, septicemia and ear infections [14]. Most of these vibrios secrete enterotoxins in foods, water or in the gastrointestinal tract [15]. Identification of *Vibrio* pathogen in water bodies or the aquatic life farming system is very important because these are the main cause of human infection. If we could identify these contaminated water bodies then reclamation of those contaminated water bodies can make them free from pathogens so that it would not cause any water borne disease again. Not only this if we could identify the source of contamination of water sampling at various places, then prevention and treatment of those source of pollution would provide us contamination free water and that contamination free water can be used without any risk.

A multiplex polymerase chain reaction (PCR) method, specifically designed for application in routine diagnostic laboratories, was developed for identifying 5 human pathogenic *Vibrio* species: *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio mimicus*, and *Vibrio alginolyticus.* [16] This assay directed toward the *dnaJ* gene(a housekeeping gene that encodes heat shock protein 40, for the identification of vibrio species) was tested on a total of 38 strains representing 23 *Vibrio* species .Specific PCR fragments were formed in isolates which belonged to the 5 target species and were absent from all strains other than these 5 species, indicating high specificity of this multiplex PCR. This technique represented a vigorous tool for the specific and rapid detection of the 5 major pathogenic *Vibrio* species. Till date not much work have been done in context of identification of *Vibrio species* from aquatic water bodies in India, and whatever work is done is based on marine water samples. So the present study was based on fresh water sample along with marine water sample for identification.

MATERIALS AND METHODS

Analysis of water samples

Total 82 Water samples collected from various sea beaches, rivers, estaurines, lakes and shrimp culture ponds of south, north and east India. Plating of media i.e., TCBS agar supplied by Titan Biotech Ltd, Rajasthan, was done for spreading of water samples.100 µl of seawater and 200 µl of fresh water was taken for spreading onto TCBS agar plates and left for the 24 hours incubation period for development of the colonies. Over development of green and yellow colonies on TCBS plates, single colonies were taken and streaked onto Trypto Soya Agar supplied by Titan Biotech Ltd, Rajasthan, 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

Isolation of genomic DNA was performed using the Phenol chloroform method. Extraction of bacterial DNA was done by broth. 2 ml broth was taken in an eppendorf tube and centrifuged at 10000 rpm for 10 mins. There after supernatant was discarded and the pellet was dissolved in 500 µl T.E. buffer.1/20 of 10% SDS was added and tubes were kept in waterbath for cell lysis. After 1-2 hours tubes were again centrifuged and the supernatant was collected. Phenol chloroform Isoamyl mix was added in the ratio 25:24:1 and the samples were again centrifuged .An upper transparent layer containing DNA was collected and 50 µl of 3 M sodium acetate was added for precipitation of DNA and kept in ice cold conditions for 10 mins. Again add double volume of ethanol to the tubes and centrifuge at 10000 rpm for 10 mins. Discard the supernatant and air dry the tubes and add 50-100 µl of T.E. buffer to the tubes for loading in agarose gel electrophoresis. The same method was used for DNA extraction from direct scraping of colonies from TSA plates.500 µl of T.E. the buffer was taken in an eppendorf tube and large amount of vibrio colonies were scrapped from the plates into the eppendorf

tubes containing T.E. buffer. After that same steps were repeated as done in DNA isolated from broth.

Designing of Primer

In this identification method, five pairs of oligonucleotide primers were designed to simultaneously detect five different types of vibrio species by m-PCR. They are targeted at a species-specific tox gene region of the *Vibrio*. Table 1 lists the primers used for the amplification of these genes and the predicted sizes of the amplification products. To assist PCR product detection, the primers were designed in such a way that the predicted sizes of the amplification products of each target gene would be different from each other to permit size discrimination using gel electrophoresis technique.

Table 1

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

After performing gel electrophoresis of the isolated DNA, we performed PCR on the isolated samples using species specific primers supplied by Bangalore Genei. The PCR mix was made of total 20μl.PCR was performed on a thermocycler supplied by Bangalore genei and the total number of cycles set was 35.Further the PCR products were run on agarose gel electrophoresis for checking amplification. The amplification products were visualized after electrophoresis at 50 V for 45 mins on a 1.2% agrose gel by ethidium bromide staining.

RESULT AND DISCUSSION

Figure 1
Ratio of water Reservoire from where sample was collected (82)

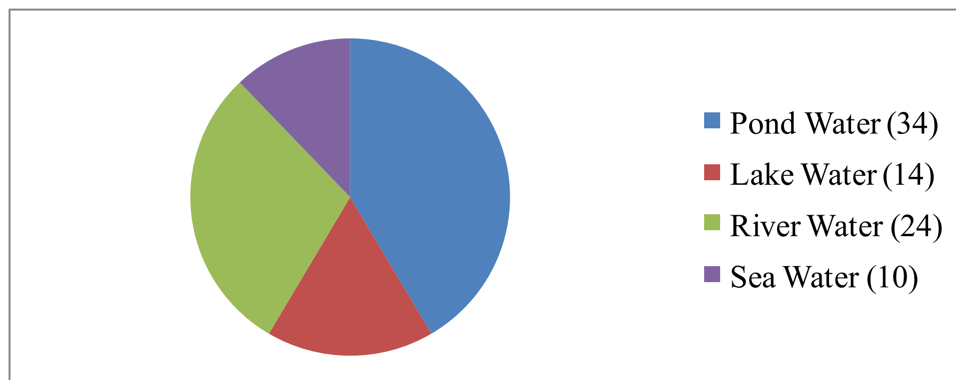


Figure 2: Chilka site 1

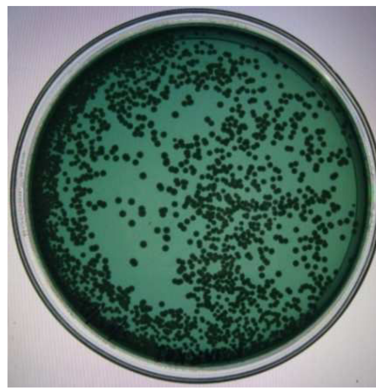


Figure 3: Kharkai River(Jamshedpur)

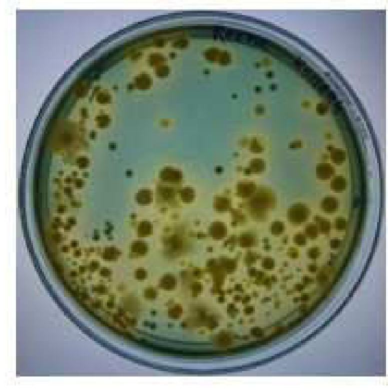


Figure 4: Konark (Orissa)

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. Chilka site 1 (Orissa) shows maximum no of yellow colonies. Kharkai river (Jamshedpur) shows mostly green colonies while Konark (Orissa) shows maximum no of yellow colonies

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 [17]. Therefore,a methodical study was prepared to optimize the m-PCR conditions in order to get similar and maximum band intensities for each of the gene amplicons.

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. Concentration is found to be high in saline water in comparison to fresh water. The amplied products were run in agarose gel electrophoresis for visualization of the amplified products. The first pcr reaction was set for 16 samples taking 100 bp ladder.lane 1 and 18 contains the ladder while lane 2,3,4,6,7 and 10 respectively shows the presence of *Vibrio* species. The second PCR cycle was set for 6 *Vibrio species.* Lane 1 contains the ladder of 100 bp and the rest 6 lanes contain the amplified samples.Out of which lane 4 conatins *Vibrio* species.

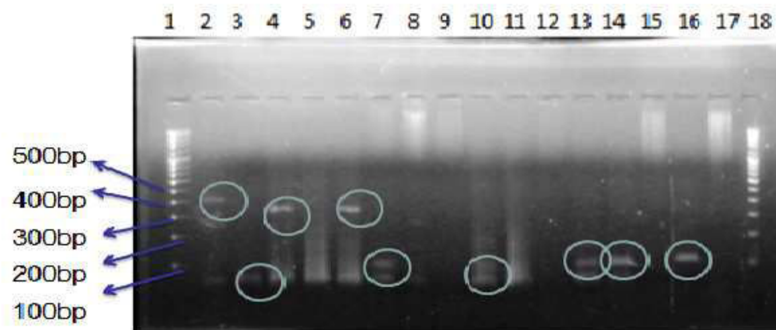


Figure 5

Electrophoretic 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 2: V.vulnificus, Lane 3:V.parahaemolyticus, Lane 4: V.vulnificus, Lane 6: V.vulnificus, Lane 7: V.parahaemolyticus, Lane 10: V.vulnificus, Lane 13: V.vulnificus, Lane 14: V.vulnificus, Lane 16: V.vulnificus .

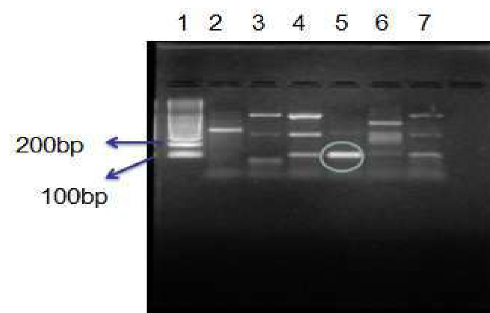
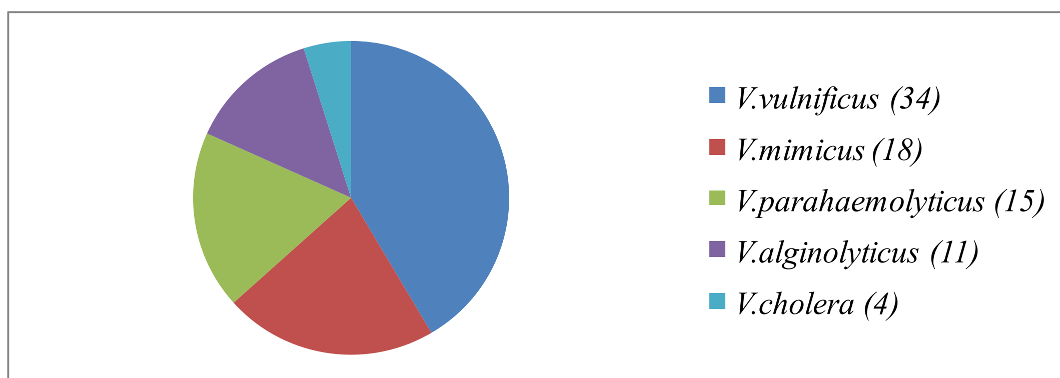


Figure 6
Electrophoretic analysis of target gene showing presence of V.alginolyticus in lane 5. Lane 1: 100 bp marker.

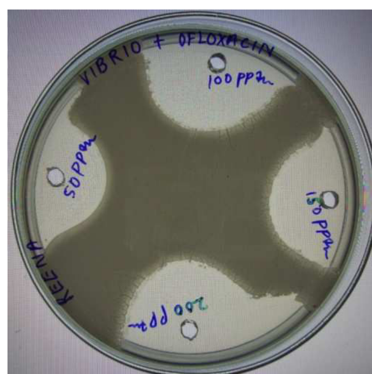
Figure 7
Ratio of the Specieses found after analysis, out of 82 sample



Antibiotic test by Well Diffusion Method

To check whether *Vibrio* is resistance or sensitive to antibiotics, an antibiotic test was performed by well diffusion method. TSA agar plate was used for spreading of *Vibrio* culture and different concentration (50 ppm,100 ppm,150 ppm,200 ppm) of ofloxacin drug was prepared from the stock solution of 1000 ppm.all the concentrations shows maximum inhibition zone .

Figure 8
TSA Plate With Vibrio And Ofloxacin



Diameter of inhibition zone = 1 cm (50 ppm)
Diameter of inhibition zone = 1.3 cm (100 ppm)
Diameter of inhibition zone = 1.4 cm (150 ppm)
Diameter of inhibition zone = 1.5 cm (200ppm)

The water samples were collected from different water bodies such as saline water bodies(sea and estuarine) as well as from fresh water bodies (rivers, lakes and ponds) India .Till now not much 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. Very less of work has been done on the biosafety 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 23 samples from five types of water bodies i.e. sea, rivers, lakes, estuarine and pond. Sampling was done on north ,south and east India's water systems. More samples can be included from major water sources of rest parts of India for the detection of *Vibrio* species. The conclusion on the biosafety evaluation of *Vibrio species* in freshwater system as well as marine system indicates another promising source of food safety issues to consumers. Many other methods other than multiplex PCR can also be used such as RT-PCR, NASBA (Nucleic Acids Sequence Based Amplification) for the identification of pathogenic *Vibrio* species. But we have used multiplex PCR technique for identification of Vibrio species as it is a rapid technique for detection of pathogens.

CONCLUSION

Identification of pathogenic *Vibrio* species through mPCR is a cost effective method providing high output and less time consuming. It can provide a powerful supplement to the conventional methods for more accurate monitoring of pathogenic bacteria in fresh water system as well as marine system. 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.

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