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Rapid Identification of Human Pathogenic Vibrio Species in Fresh Water using Multiplex PCR

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***Abstract*— In the present investigation we focus on identification of Vibrio species in different rivers and ponds of Indian northern plains. This study targets five species of Vibrio such as Vibrio alginolyticus, Vibrio cholerae, Vibrio mimicus, Vibrio parahaemolyticus, Vibrio vulnificus which are the major human disease causing Vibrio species. Primarily identification of Vibrio species done through gram's staining and further by color of colonies developed on TCBS agar plates. A multiplex PCR assay had been developed for the detection of targeted five species of Vibrio for the DNA isolated from various samples of water using specific primers targeting tox gene. The assay was specific as no amplification occurred for other bacterial DNA.**

Keywords—Multiplex PCR, Vibrio sp.,amplification, TCBS,tox gene

I.INTRODUCTION

Water is a vital natural resource because of its basic role to life, quality of life, the environment, food production, hygiene, industry, and power generation (Meays et al., 2004). With the rapid increase in world population and increased urbanization, THERE IS A MASSIVE STRAIN ON THE EXISTING WATER SUPPLY AND sanitation facilities (UNDPI, 2005). In the developing world, poor access to safe water and inadequate sanitation continues to be a danger to human health (World Health Organisation [WHO], 2004).India's 14 major, 55 minor and several hundred small rivers receive millions of litres of sewage, industrial and agricultural wastes. The most polluting source for rivers is the city sewage and industrial waste discharge. Presently, only about 10 per cent of the waste water generated is treated; the rest is discharged as it is into our water bodies. Due to this, pollutants enter rivers, lakes and groundwater (Ministry of Environment and Forests; 2011-12).

The paucity of clean water for domestic use has led to the increase in the number of deaths in both the urban and rural parts of developing economies. Deaths due to water related diseases in India are in the range of nearly 80%.Lack of water, sanitation, and hygiene results in the loss of 0.4 million lives while air pollution contributes to the death of 0.52 million people annually in India (WHO 2007). Environmental factors contribute to 60 years of ill-health per 1,000 population in India compared to 54 in Russia, 37 in Brazil, and 34 in China. The socio-economic costs of water pollution are extremely high: 1.5 million children less than 5 years die each year due to water related diseases; 200 million person days. Water related diseases plague many Indians. The availability of fresh and good quality drinking water to all Indians remains a concern.

Pathogenic microbes have been implicated in human diseases linked with the use of contaminated water and food. Adequate sanitation and clean water, being two critical factors in ensuring human health, protects against a wide range of water-related diseases. These include diarrhoea, cholera, trachoma, dysentery, typhoid, hepatitis, polio, malaria, and filariasis (United Nations Department of Public Information (UNDPI, 2005).

Vibrio-species is widely acknowledged as one of the most important waterborne pathogen causing gastrointestinal disorders. Cholera is one of the five most deadly water related diseases that occur in India .In India cholera related deaths are most common in places with shortage of good quality water. In 2010, nearly 140 people died of cholera in Odisha (formerly known as Orissa). Vibrio species bacteria are ubiquitous in aquatic environments including fresh, coastal and marine habitats. They are also found as commensals on the surfaces and in the digestive tracts of fish and in zooplanktons (Drake et al., 2007; Montanari et al., 1999). They are transmitted to humans via raw or improperly cooked fish or contaminated water.

The importance of Vibrio-spp.asa contaminant of raw or undercooked aqua-culture food has been well established (Gopal et al., 2005; Di Pinto et al., 2008; Luan et al., 2008) and may lead to acute gastroenteritis including diarrhea, headache, vomiting, nausea and fever (Apun et al., 1999; Vongxay et al., 2008; Yang et al., 2008). As food safety is a major global concern that affects the consumer and those in the food service sector (Badrie et al., 2006; Jacxsens et al., 2009), serious attention has to be given to the aquaculture industry as fish can act as a vector for human pathogenic bacteria. Therefore, it is important to have data on the prevalence of Vibrio spp. in freshwater. Freshwater fish are easily available in local market and these fishes are highly consumed by customers.

Multiplex PCR-based detection is a popular and effective method to distinguish closely related bacterial species such as Vibrio-species (Edwards & Gibbs 1994; Haldar et al., 2010). This is carried out either through the use of different gene-specific primers to detect various strains of a particular species of Vibrio (e.g. Rodkhum et al., 2006) or through the use of a single gene-specific primer set to differentiate Vibrios (e.g. Haldar et al., 2010).

II.MATERIALS & METHODOLOGY

A. Collection of Water Samples

For the present study water samples were collected from different rivers and ponds of Indian northern plains where Vibrio related waterborne diseases are major concern. In this study pond water samples were collected from various regions of Bundelkhand where people highly rely on these sources as major water source. For each site five water samples were collected.

TABLE I. LIST OF SAMPLES COLLECTED

S.N.	Sample Name	Collection Place	Type
1.	Bakshi ka Talab	Lucknow	Pound water
2.	MahirkaTalab	Orai	Pound water
3.	Ram kund	Orai	Pound water
4.	KeemathJheel	Agara	Pound water
5.	PalahariTalab	Banda	Pound water
6.	BadokharTalab	Banda	Pound water
7.	Fun Pound	Lucknow	Pound water
8.	Mama kaTalab	Allahabad	Pound water
9.	PragiTalab	Banda	Pound water
10.	Sarada River	Lakhimpurkhiri	River water
11	Ramganga River	Barelli	River water
12	Betawa River	Hamirpur	River water
13.	Cane River	Hamirpur	River water
14	Son River	Sasaram	River water
15.	Punpun River	Aurangabad	River water
16	Adari River	Aurangabad	River water
17	Pandu River	Panka Village	River water
18.	Pandu River	Kanpur city	River water
19.	PankiNahar	Kanpur	River water
20.	Gomti River	Chandrika , Lucknow	River water
21.	Gomti River	Hanumansetu , Lucknow	River water
22.	Gomti River	Laxaman Park , Lucknow	River water
23	Gomti River	Jaunpur	River water
23	Tonse River	Azamgarh	River water
22.	Tonse River	Allahabad	River water
23	Yamuna River	New Delhi	River water
24.	Yamuna River	Agara	River water
25.	Yamuna River	Hamirpur	River water
26.	Yamuna River	Kaushambi	River water
27.	Yamuna River	Gaughat , Allahabad	River water
28.	Yamuna River	Baluaghat , Allahabad	River water
29.	Ganga River	Kanpur	River water
30.	Ganga River	Kaushambi	River water
31.	Ganga River	Araighat , Allahabad	River water
32.	Ganga River	Ramghat , Allahabad	River water
33.	Ganga River	Haridawar	River water
34.	Mandakani River	Chitrakut	River water
35.	Rapti River	Gorakhpur	River water
37	Ghaghara River	Bashti	River water
38	Ghaghara River	Ambedkarnagar	River water

B. Analysis of water samples

For each site

Spreading had been done with 200µl of water sample on the TCBS media, and kept in incubation for 24 hrs at 37°C for colony growth. Streaking of yellow and green colonies obtained on TCBS media had been done separately on TSA (Trypto Soya Agar) Media for isolation of pure colonies. Bacterial DNA isolation was done with colonies obtained on TSA plates.

C. Isolation of Bacterial Genomic DNA

A chemical method was used for the isolation of bacterial DNA from TSA plates. Bacterial colonies were first dissolved in TE buffer. This mixture had been centrifuged at 10,000rpm for 10 minutes, supernatant was discarded and the pellet was dissolved in mixture of 467µl TE buffer, 30µl 10% SDS and 3 µl Proteinase K. This mixture incubated for one hour at 45°C. After one hour equal volume of phenol: chloroform (1:1) is added to mixture. After 10 minutes of invert mix centrifuged at 10,000 rpm. Upper aqueous layer separated by denatured protein transferred in to new eppendrop and 1/10 volume of sodium acetate and remaining volume of ice chilled isopropanol added. This mixture is incubated at 0°C for overnight. After incubation the mixture is centrifuged at 10,000rpm that's formed a pellet that is DNA. This pellet is washed with 70% ethanol and after washing this pellet is stored in 50 to 100µl TE buffer. This isolated DNA is stored and further used as raw material for PCR amplification.

D. Primer Designing

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 3 lists the primers used for the amplification of these genes and the predicted sizes of the amplification products. To facilitate PCR product detection, the primers were designed such that the predicted sizes of the amplification products of each target gene would be different to permit size discrimination by gel electrophoresis.

TABLE II. OPTIMIZATION OF MULTIPLEX PCR

Universal Forward	VM-F	CAGGTTTGYTGCACGGCGAAGA
5' Reverse primer :		
V. cholera	VC-Rmm	AGCAGCTTATGACCAATAACGCC
V. parahaemolyticus	VP-MmR	TGCGAAGAAAGGCTCATCAGAG
V. vulnificus	VV-Rmm	GTACGAAATTCTGACCGATCAA
V.mimicus	VM-Rmm	YCTTGAAGAAGCGGTTCGTGCA
V.algicusinolyt	V.a12MmR	GATCGAAGTRCCRACACTMGGA

E. OPTIMIZATION OF MULTIPLEX PCR

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 (HenegariuO et al.,1997). A systematic study was, therefore, performed to optimize the m-PCR conditions to obtain similar and maximal band intensities for each of the gene amplicons.

TABLE III. PCR COMPONENTS

Chemical	**Stock**	**Working**
PCR buffer	10 x	2µl (1 x)
DNTP	2.5 mM	1.6 µl (0.2 mM/L)
Primer	100 ppm	
Universal forward primer		1µl (8 ppm)
Reverse primer *Vibrio cholera*		1µl (8 ppm)
Reverse primer *Vibrio vulnificius*		1µl (8 ppm)
Reverse primer *Vibrio parahaemolyticus*		1µl (8 ppm)
Reverse primer *Vibrio mimicus*		1µl (8 ppm)
Reverse primer *Vibrio alginolyticus*		1µl (8 ppm)
Taq Polymerase	5 U	0.2µl (5 Unit)
Distilled Water		10.2µl
Total		20µl

TABLE IV.PCR CONDITIONS

Initial Denaturation	94°C for 3 min
Denaturation	94°C for 30 sec
Annealing	60°C for 30 sec
Extension	72°C for 60 sec
Final extension	72°C for 7 min
Number of cycles	35

The amplification products were visualized after electrophoresis at 100 V for 45 mins on a 1% agrose gel by ethidium bromide staining.

III.RESULT & DISCUSSION

The isolated Vibrio species were primarily confirmed by Gram staining and colony morphology on TCBS agar. Gram negative, rods, characteristically curved or comma-shaped. After 18-24 hours incubation colonies on TCBS are at least 2 mm in diameter and yellow in the case of sucrose fermenters and green non-sucrose fermenters.

TABLE V.COLONY COLOUR

Organism	Color of Colonies on TCBS
V. alginolyticus	Yellow
V. cholera	Yellow
V. parehaemolyticus	Green
V. vulnificus	Green
V. mimicus	Green

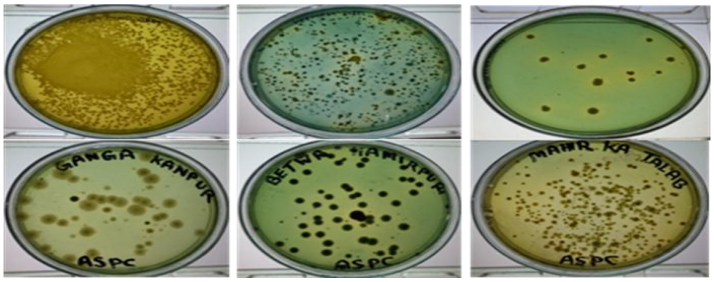

Fig 1: Colony of Vibrio on TCBS media

Water sample collected from Ganga river, Haridwar and from Mandakani river, chitrakut, Banda did not shown any colony growth on TCBS media. Water sample from Ramkund (Orai), Mahirkatalab (Orai), Tonse River (Allahabad), Ganga River (Arailghat, Allahabad), Yamuna River(New Delhi), Gomti River (Chandrika, Lucknow), Yamuna River (Gaughat, Allahabad), Kukrail River (Lucknow), Rapti River (Gorakhpur) shown maximum growth of yellow colonies. Water sample from Badokhar Talab (Banda), Bakshi ka Talab (Lucknow), Mama ka Talab (Allahabad), keemath Jheel (Agra), Pragi Talab (Banda), Ganga River(Kanpur), Yamuna River (Kaushambi), Cane River(Banda), Ramganga, Barelli, Betwa River (Hamirpur), Sharda River(Lakhimpur khiri), Tonse River , Azamgarh, Ganga River (Kaushambi), Ganga River (Ramghat , Allahabad) shows maximum growth of green colonies.

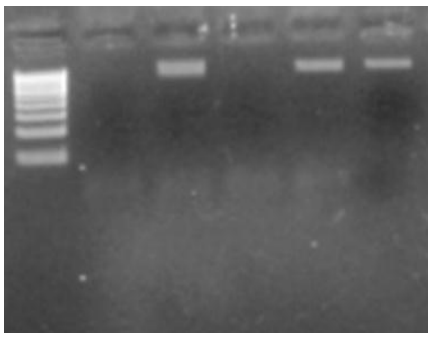

Fig 2: Electrophoretic analysis of isolated DNA on a 0.8% agarose gel

0.8% Agarose Gel was prepared.

Loading dye – 3µl + DNA sample – 5µl

TABLE VI.0.8% AGAROSE GEL

Lane 1	DNA ladder
Lane 2	Sample 1
Lane 3	Sample 2
Lane 4	Sample 3
Lane 5	Sample 4
Lane 6	Sample 5

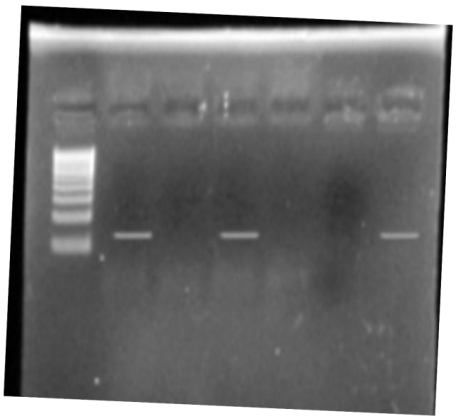

Fig 3: Electrophoretic analysis of PCR – amplified target Genes from different Vibrio species.

1% Agarose Gel

Loading Dye – 3µl + Sample – 5µl

TABLE VII.1.2% AGAROSE GEL

Lane 1	DNA ladder
Lane 2	*Vibrio vulnificus*
Lane 3	*Vibrio parehaemolyticus*
Lane 4	*Vibrio cholerae*
Lane 5	*Vibrio mimicus*
Lane 6	*Vibrio alginolyticus*

TABLE VIII. SHOWING AMPLIFIED SAMPLES BY MULTIPLEX – PCR

Samle Name	Vp	Vv	Vm	Vc	Va
Bakshi ka Talab, Lucknow (G)	+	+	+	_	_
Mahir Ka Talab, Orai(G)	+	_	+	_	_
Mahir Ka Talab, Orai(Y)	_	_	_	+	+
Palaharitalab,Banda(G)	_	_	+	_	_
Palaharitalab,Banda(Y)	_	_	_	+	_
Ram Kund, Orai(Y)	_	_	_	+	+
Pragi Talab,Banda(G)	+	_	_	_	_
Keemath Jheel, Agara(G)	_	+	+	_	_
Keemath Jheel, Agara(Y)	_	_	_	+	+
Mama Ka Talab, Allahabad(G)	_	+	+	_	_
Mama Ka Talab, Allahabad(Y)	_	_	_	+	+
Fun Pond, Lucknow(G)	_	+	_	_	_
Fun Pond, Lucknow (Y)	_	_	_	+	_
Sarada River, Lakhimpurkhiri(G)	+	+	+	_	_
Sarada River, Lakhimpurkhiri(Y)	_	_	_	_	+
Ram Ganga River, Barelli(G)	_	_	+	_	_
Ram Ganga River, Barelli(Y)	_	_	_	+	+

VP: Vibrio parahaemolyticus; VV: Vibriovulnificius; VM:Vibriomimicus; VC: Vibrio cholera; VA: Vibrio alginolyticus;+ indicates the presence of species; – indicates the absence of species in the sample.(g) represents green Vibrio colonies; (y) represent yellow colonies.

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