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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF *VIBRIO* SPP. FORM WATER RESOURCES OF NORTHERN INDIA USING MULTIPLEX PCR

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ABSTRACT

Vibrio is a diverse group of aquatic bacteria, currently include 72 species, 12 of which occur in human clinical samples. Some species are pathogens of fish, eel and frogs as well as other vertebrate and invertebrates. 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. The aim of the present work is to screen different water bodies for the presence of *Vibrio* species and determine their pathogenicity, which will help to control *Vibrio* –associated gastroenteritis in India.

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INTRODUCTION

Vibrio is gram negative curved, rod-shaped, non motile bacteria, inhabits aquatic environment worldwide, which do not form spores, and usually bears single polar flagellum. *Vibrio* species are among the most common surface water organism across the world. They occur in both marine and freshwater habitats in association with aquatic animals. Some species of *Vibrio* are pathogens of fish, eel and frogs as well as other vertebrate and invertebrates (Todar, 2005). 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 (Baron *et al .,* 1994; Bhaskar et. al., 1998; Byoid *et. al.,* 2000). There are at least 12 pathogenic vibrio species recognize to cause human illness. Vibrio infections are largely classified into two distinct groups: Vibrio cholera infections and noncholera Vibrio infections. Because most Vibrio infections are associated with the consumption of contaminated food, these infections are often considered a food borne disease. The prevalence of noncholera Vibrio infections in India appears to have increased in recent years.

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The aim of the present work is to screen different water bodies for the presence of *Vibrio* species and determine their pathogenicity, which will help to control *Vibrio* –associated gastroenteritis in India. Gugliandolo (2009) used molecular methods that utilize the PCR and nucleotide sequence determinations overcome many of the limitations of phenotypic methods. Most importantly, molecular methods are comparatively easy, efficient, laborsaving, of high discriminative and reproducible abilities, accurate, less time consuming, identify multiple species in one assay which would be a useful for clinical laboratories and would enhance *Vibrio surveillance* and diagnosis. Daniels and Shafaie (2000) reviewed the pathogenic vibrio infections. The acute, voluminous diarrhoea caused by *Vibrio cholerae* represents a dramatic example of enteropathogen invasion and gut microbial community disruption (Ansel *et al.,* 2014).

MATERIAL AND METHODS

Sample collection

Total 13 samples were collected from different areas of Northern India to perform this experiment. The samples were collected from river, pond and tap water at Haridwar, Rishikesh, Pilibheet, Pantnagar, Ambala, Lucknow, Gonda, Pratapgarh, Allahabad, Varanasi, Azamgarh, Banda and Chitrakoot.

Spreading of Water Sample on TCBS Media

Water samples were poured on 25ml autoclaved media in each petriplate and leave it to solidify. Spread all inoculums over the surface of TCBS media evenly. Wrapped plates with clean film, then incubated at 37ºC for 2 days and plates were examined for bacterial growth. After incubation, petriplates were taken out from the incubator and bacterial colonies were examined over the surface of media. Picked colonies from TCBS media. Streak the inoculums over the surface of TSA media in a zig zag manner.

Bacterial DNA Isolation by Phenol Chloroform Method

Inoculate single bacterial colony from TSA media to the TS broth and incubated for 1 day. From the liquid culture 1.5 ml of culture was taken in microcentrifuge tube and centrifuge it at 8000 rpm for 5 min and pellet the bacterial cells. Resuspended the pellet into 900µl TE buffer. Added 1/10 volume of 10% SDS solution. Water bathed the tubes at 50-60ºC and incubated it for approximately 2 hours. After 2 hours the solution become thicker then it was in the starting, now mix 600µl of Phenol: chloroform isoamyl alcohol (25:24:1) into it and shake gently by repeatedly inverting the tube. White precipitate appeared when the mixture was added to the tube and shaked, the solution becomes creamy orange. Kept for 5 min and then centrifuged at 10000 rpm for 10 min. Upon centrifugation 3 layers appear in the tube. Upper aqueous layer in transparent like water which contain DNA below this is a thin layer of white colour, which is protein precipitate and the lower dark orange layer is phenol chloroform mixture. Collected upper transparent aqueous layer in fresh new eppendorf tube and discard the lower layer. Kept the eppendorf tube in refrigerator to cool it for 10-15 min. Added double volume of chilled propanol in to the aqueous DNA solution drop by drop from its wall. After adding DNA precipitate out from the solution and kept it again on 0ºC for 15 min, so that DNA is precipitated easily. After precipitation centrifuged the tube at 10000 rpm for 10 min and discarded the supernatant, before discarding, mark the DNA pellet with marker on wall of tube. Dried the DNA pellet in air or by keeping the tube inverted position on filter paper. Dissolved the dry DNA pellet in 50µl TE buffer. Checked the quality of DNA isolated by agarose gel electrophoresis.

Bacterial Genomic DNA by Agarose Gel Electrophoresis (0.8%)

Filled the tank with TAE buffer. Placed the agarose gel in the electrophoresis tank. The well will be placed toward the cathode. Loaded the sample into the gel carefully using pipettes. Run the sample till it travels the half area of the gel. Removed the gel from the electrophoresis tank and observed it on UV transilluminator or DOC system.

Spectrophotometric quantification of DNA

Switched on the UV Double beam Spectrophotometer and put the type of DNA to be quantified. Prepared the dilution 10 µl ds DNA sample and 990µl ultrapure distilled water. Then kept the sample in spectrophotometer and take the reading of sample at 260/280 nm. Prepared a known dilution of DNA sample in TE buffer which was used to dissolve the DNA sample. Recorded the OD of the sample at 260 and 280 nm.

Polymerase Chain Reaction

Mixed the reagent 0.5 ml tubes or the 0.2 ml PCR tubes. The reagent was kept on ice. Vortex the tube so that all the composition mix properly. Placed all tube inside the thermo cycler. Until completion of PCR, hold sample at 4ºC. Prepared the DNA for loading by addition 1/10 volume stop-loading buffer (containing EDTA, glycerol and bromophenol blue). Analysed by agarose gel electrophoresis and include size marker in at least one well on the same gel. The amplification products were visualized after electrophoresis at 50V for 45 min on a 1.2% gel. A multiplex PCR specially designed for application in routine diagnostic laboratories, was developed for identifying human pathogen *Vibrio* species (Nhung et al., 2007).

RESULT AND DISSCUSTION

Large pointed yellow and green colonies are grown on TCBS spreads petriplates after incubation of 48 hours. The yellow colonies show the sucrose positive and green colonies show sucrose negative.

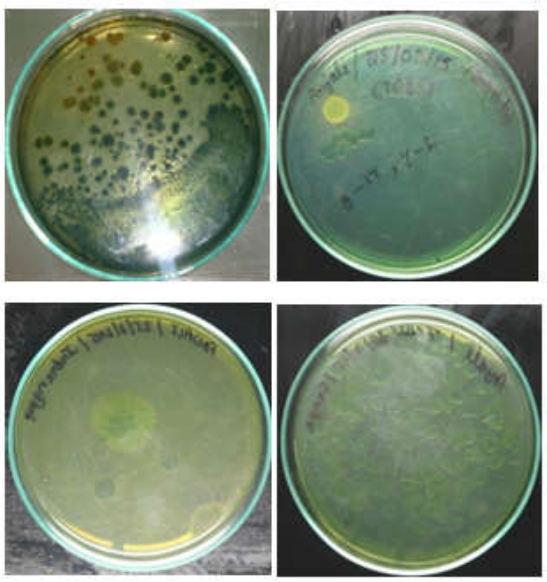

Fig. 1. Vibrio colonies TCBS Medium

Isolation of DNA

DNA was isolated from culture (nutrient broth) grown overnight at 37^0C for 24 hours. The isolated DNA was run on a 0.8% agrose gel at 90v. The gel was stained with ethidium bromide and then visualized in gel documentation systems. The figures shows ladder in lane 1: and sample 2,3,4,5,6,7.

Spectrophotometric quantification of DNA using UV double beam spectrophotometer

The quality of DNA was estimated by measuring the 260:280 UV absorbance ratios which varied between 1.6 and 1.8. The results are presented in the following table.

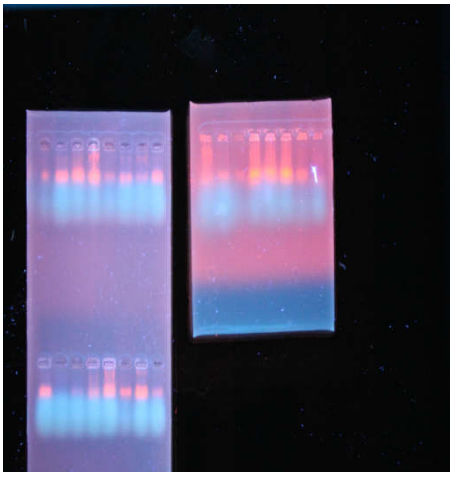

Fig. 2. DNA bands visualized in gel DOC system

Table 1. Spectrophotometric analysis of DNA (tap water)

Sl No.	Tap water samples	M factor	Ratio	DNA Conc.	Protein Conc.
1.	Allahabad (Y)	1.00	1.90	1.13	0.49
2.	Ambala (Y)	1.00	1.02	2.20	5.01
3.	Azamgarh (G)	1.00	1.02	5.56	12.70
4.	Azamgarh (Y)	1.00	0.96	1.35	3.41
5.	Banda (Y)	1.00	1.12	3.07	5.82
6.	Chitrakoot (G)	1.00	1.02	4.25	9.66
7.	Gonda (G)	1.00	1.06	1.46	1.09
8.	Haridwar (G)	1.00	1.09	6.61	13.22
9.	Lucknow (G)	1.00	1.01	5.91	13.74
10.	Lucknow (Y)	1.00	1.01	4.07	9.47
11.	Pant Nagar (Y)	1.00	1.81	0.84	0.44
12.	Pilibhit (Y)	1.00	1.02	5.42	12.34
13.	Rishikesh (Y)	1.00	1.04	5.35	11.69
14.	Varanasi (Y)	1.00	1.04	5.87	12.80
15.	Pratapgarh	1.00	1.67	1.48	1.07

Vibrio alginolyticus - yellow colony, *Vibrio vulnificus* -green 85% or yellow 15% colony, *Vibrio cholera* -yellow colony, *Vibrio mimicus* - green colony, *Vibrio parahaemolyticus* - green centered colony

Table 2. *Vibrio* spp in water samples

S. No.	Water Samples	Colony	V.A	V.V (y)	V.V (g)	V.C	V.M	V.P
1.	Varanasi tap water	Y						
2.	Azamgarh tap water	Y						
3.	Lucknow tap water	G						
4	Gonda	G,Y						
5.	Pilibheet tap water	Y						
6.	Ambala tap water	G,Y						
7.	Chirtrakoot tap water	G,Y						
8.	Pratapgarh	Y,G						
9.	Rishikesh Ganga river	G						
10.	Pantnager tap water	Y						
11.	Haridwar Tap water	G						
12.	Gomti river, Lucknow	G,Y						
13.	Ganga river, Allahabad	G						
14.	Banda	Y						

Multiplex PCR

For the confirmation of bacteria, responsible for contaminating water sample, 3 primers were used with each bacterium. The bacteria give yellow colour on TCBS media with DNA amplified with *vibrio alginolyticus, vibrio vulnificus, Vibrio cholera*. Those give green colour on TCBS the DNA amplified with *vibrio vulnificus, vibrio mimicus* and *vibrio parahaemolyticus*.

Conclusion

In the present study *Vibrio* species were isolated from 13 different water sample of Haridwar, Rishikesh, Pilibheet, Pantnagar, Ambala, Lucknow, Gonda, Pratapgarh, Allahabad,

Varanasi, Azamgarh, Banda and Chitrakoot. *Vibrio vulnificus* is a lactose-fermenting, halophilic vibrio causing severe disease in immunocompromised patients, but causing a mild type of gastroenteritis in healthy people, usually associated with consumption of seafood (De and Mathur, 2011). *V. vulnificus* is the most important pathogenic vibrio because of its invasiveness and the high fatality rate associated with infections. It can also cause infection of the skin, open wounds are expose to warm seawater, primary septicemia and gastroenteritis etc. *Vibrio cholerae* is the primary causative of cholera. Infection with this organism can cause profuse watery diarrhea, vomiting and muscle cramps. *Vibrio parahaemolyticus* has been implicated as a common cause of sea food associated gastroenteritis, acute watery diarrhea, abdominal cramps and nausea usually characterize the illness. *Vibrio alginolyticus* is first recognizing as being pathogenic in humans. Gastroenteritis is a major disease caused by this species. The chronic diarrhea, conjunctivitis and post traumatic intracranial infection etc. are other diseases associated with it. *Vibrio mimicus* is a non halophilic species, causes sporadic episode of acute gastroenteritis and ear infection.

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