Bangladesh J Microbiol, Volume 36, Number 2, December 2019, pp 91-97

Original Article

Characterization of *ctx* gene Negative *Vibrio fluvialis* Organisms Isolated from the Environment

Juthi Biswas[1], Tahsina Jainab[1], Mahmud Hossain[2], Mahmuda Yasmin[1], Jamalun Nessa[1] and Chowdhury Rafiqul Ahsan[1*]

[1]*Department Microbiology, University of Dhaka, Dhaka-1000, Bangladesh.* [2]*Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka-1000, Bangladesh*

A total of five *Vibrio fluvialis* organisms were isolated from the different environmental samples collected from Dhaka, Satkhira and Khulna. All these isolates were confirmed following API 20NE tests. Molecular analysis showed the absence of *ctx, tox*R, *tdh*, *trh*, *stx1,* and *stx2* genes in these organisms. However, culture filtrates and crude proteins prepared from these organisms showed fluid accumulation in rabbit ileal loop assay, haemolysis of sheep red blood cess, rounding of BHK-21, HeLa and MDCK cells in cell culture assay, hind limb paralysis and death of mice in mice lethality assay and morphological changes in mouse neuronal cell assay. All these results indicated that the environmental *V. fluvialis* organisms, may not contain different virulence genes, including the *ctx* gene. However, the other *in vivo* and *in vitro* assays indicate that the toxins produced by the *V. fluvialis* organisms may contain enterotoxin, haemolysin, cytotoxin and neurotoxin.

Keywords:*V. fluvialis*, cytotoxicity, neurotoxicity, Mouse lethality assay, Rabbit ileal loop assay

Introduction

V. fluvialis is considered to be an emerging food borne pathogen and has been implicated in outbreaks and sporadic cases of acute diarrhea in several countries including Bangladesh[1]. The largest outbreak of *V. fluvialis* infection was reported in Bangladesh between October 1976 and November 1977, with more than 500 patients[2]. The distribution of *V. fluvialis* is worldwide[3] and this organism is not only isolated from human diarrheal cases[4] but also from marine and estuarine environments[5]. There are reports of food poisoning caused by this organism[3], especially due to consumption of raw shellfish[6]. *Vibrio* mediated infections frequently occur in countries where the raw seafood was largely consumed. In many instances, *V. fluvialis* was found to be associated with cholera-like diarrhea[7]. Between 1982 and 1988, 10 gastroenteritis cases of *V. fluvialis* have been reported in Florida due to consumption of contaminated seafood[4]. In the Gulf coast, the majority of the *Vibrio* mediated gastroenteritis has been associated with intake of raw oysters and in about 6% of the cases *V. fluvialis* was the causative pathogen[6]. Foodborne outbreaks were reported in several communities implicating *V. fluvialis* alone or with either *V. parahaemolyticus/Salmonella* spp.[8] Food borne diarrheal outbreaks caused by *V. fluvialis* have been reported during 1981 in Maharashtra[3]. Several toxins that may be important in pathogenesis have been reported including a Chinese hamster ovary (CHO) cell elongation factor, CHO cell killing factor, enterotoxin-like substance, lipase, protease cytotoxin and hemolysin[9-13]. However, most of these previous studies were done using clinical stains of *V. fluvialis* and very little is known about the toxigenic nature of the environmental *V. fluvialis* strains. Therefore, this work was undertaken to characterize the toxins produced by the *V. fluvialis* organisms isolated from different environmental sources of Dhaka and the southern districts like Satkhira and Khulna of Bangladesh.

Materials and Methods

Bacterial strains

Samples were collected from different environmental sources, such as water, hyacinth root and mud samples of Buriganga river near Sadarghat and Turag river near Abdullahpur bridge, Dhaka and shrimp fields of Satkhira and ponds near Tala area of Khulna. Isolated strains were plated on thiosulfate bile salt sucrose (TCBS) and CHROM agar followed by incubation at 37°C overnight. Salt tolerance tests were done by growing the isolates in nutrient broth containing 0, 1, 6, and 8% (wt/vol) NaCl. The medium was incubated at 30 or 37°C for up to 7 days, and positive results were determined by examining the turbidity. All the strains were further tested by API 20NE kit to confirm the identity of the *V. fluvialis* isolates. Five isolates (S-5, S-9, S-10, S-14 and S-17), which showed positive results for *V. fluvialis*, were taken for further studies.

Isolation of genomic DNA and molecular characterization

PCR was done to detect the presence of virulence genes like *tox*R, *tdh*, *trh*, *stx1, stx2* using specific primers. PCR assays were also done to screen the presence of *ctx* gene. Chromosomal DNA was extracted from the isolates of pure culture using boiling DNA method and used as templates for all PCR assays.

Corresponding author:
Chowdhury Rafiqul Ahsan, Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh. Email: crahsan@du.ac.bd

Preparation of culture filtrate

Chemically defined medium containing amino acids as well as glucose and buffering agents was used to prepare the culture filtrates of the *V. fluvialis* isolates. The medium was inoculated with the organisms and incubated at 37^0C for 48 hours, followed by centrifugation at 10,000 g for 10 min. Supernatant containing extracellular proteins of the *V. fluvialis* organisms were used as culture filtrates.

Preparation of crude protein toxins

Ammonium sulfate was added slowly to the culture filtrate (up to the 80% saturation) while pH 7 to 7.2 was maintained by the addition of liquid ammonia. Concentrated supernatant was kept at 4^0C overnight and centrifuged at 8,000 rpm for 10 minutes. The precipitates containing crude protein toxins were re-suspended in 1-1.5 ml of PBS. The protein content was estimated by Bio-Rad protein Assay[14], based on the Bradford method. In parallel, *Escherichia coli* O157:H7was also grown in Brain Heart Infusion broth and crude proteins were prepared from it to serve as positive control.

Effect of toxin on BHK-21, HeLa and MDCK cell lines

The ability of the toxin to alter the morphology or kill the BHK-21, HeLa cells and Madin-Darby Canine Kidney (MDCK) cells was examined in 24- well, flat -bottom tissue culture plate with either BHK-21, HeLa cells or MDCL cells. Cells were firstly grown in tissue culture flask using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, kept at 37ºC in a humidified 5% CO_2 atmosphere. After confluent growth in the flask, cells were treated with 0.025% trypsin, dispersed in 24-well plates and purified toxins were added to the wells. The plates were then incubated for 48 hours in a CO_2 incubator and treated cells were examined under an inverted microscope for cytotoxic and morphological examination. Crude protein toxin of *E. coli* 0157:H7 was used as positive control and DMEM and BHI with no inocula acted as negative controls for *V. fluvialis* and *E. coli* O157:H7, respectively.

Haemolysin assay

Tube haemolysin method: To determine the hemolytic activity, sheep blood was centrifuged at 2,000 rpm for 5 min. The erythrocyte was diluted to about 1.0% with phosphate buffer saline (PBS). Reaction mixture containing 1 ml of culture filtrate and 0.5 ml of erythrocyte solution was incubated at 37^0C for 1 hour followed by centrifugation at 2,000 rpm for 5 min. Optical density of the supernatant was measured at 450 nm.

Blood Agar Plate Method: Blood agar plates were used to demonstrate the alpha and beta hemolytic reaction of the *V. fluvialis* organisms. It was performed by simply streaking a single isolated colony on the media followed by overnight incubation at 37^0 C. Complete clear or partial clear zone around the colony was an indicative of beta or alpha hemolysis production, respectively.

Rabbit ileal loop assay for determination of enterotoxicity of V. fluvialis crude protein toxin

Enteropathogenecity of crude toxin was estimated by following previous methods[15, 16]. New Zealand white rabbits weighing 2.2 kg on average were used in this study. The animals were fasted for 24 hours immediately prior to testing. One ml each of live cells grown in synthetic media or culture filtrates, were injected in each rabbit ileal loop. The secretory response in each loop was determined in terms of the dilatation index. A DI equal to 1.0 or more was taken as positive for ileal loop assay. Each test was done in three rabbits and separate sets of rabbits were used for live cells or culture filtrates as inocula. *V. cholerae* 569B was used as positive control and Alkaline peptone water was used as negative controls, respectively. To determine the heat sensitivity, 1 ml of *V. fluvialis* crude protein was heated at 80^0C or 50^0C for 30 min respectively and the toxin activity of treated samples were assessed in rabbit ileal loop assay and compared to that of untreated control protein samples. For acid treatment, 1ml of crude protein was treated with 250 µl HCl (0.05M) before performing rabbit ileal loop assay. Again, 1 ml of extracellular crude protein was treated with 10 µL of Proteinase K and the mixture was heated at 37^0C for 15 minutes. All these samples were taken for rabbit ileal loop assay.

Mouse lethality assay

Six weeks old Swiss albino mice were each injected with 0.1 ml of crude protein of *V. fluvialis* intraperitoneally and observed for 2-5 days for any physical change *viz:* paralysis of muscles. Crude protein toxin of *E. coli* 0157:H7 was used as positive control and phosphate buffer saline was used as negative controls, respectively.

Effect of crude toxin on mice neuronal cells

Primary cultures of cortical neurons were prepared from the cerebral cortices of 16-day-old mice fetuses, whereas those of cerebellar granule cells are prepared from 7-day-old mice pups[17]. Pregnant mice between 9 and 15 days gestation were cervically dislocated and the embryos removed individually under sterile conditions. Fetuses were prepared one at a time in order to minimize neural cell death in the remaining embryos. The embryos were decapitated and the heads were kept in the L15 medium on ice. The skull was pulled off with the small spatula and the brain and cerebellum were exposed. Meninges were removed and cortices were taken out. Dishes were kept on ice during the dissection. Tissue chunk was put in sterile Eppendorf containing PBS and the medium was removed by washing with PBS. PBS was removed and 1 ml of 0.25% trypsin EDTA with 50 µl DNAse/ml was added to the Eppendorf. Tissue suspension was then added in 4 ml of neurobasal medium supplemented with B27, L-Glutamax, Penicillin/Streptomycin and Hepes (final concentration 10 mM). Cell suspension was passed through 70µ mesh put on a 50 ml falcon tube and the flow through was centrifuged at 1000 rpm for 5 minutes. The supernatant was

removed and the pellet containing the cells was re-suspended in neurobasal medium supplemented with B27, L- Glutamax, penicillin/Streptomycin and incubated at 37ºC in a humidified 5% CO_2 incubator.

After confluent growth in the dishes, cells were treated with crude protein at different concentrations. Treated cells were examined under an inverted microscope to check for cytotoxic and morphological changes.

Results

Primary isolation of V. fluvialis from environment

Water and sediment samples were collected from Dhaka, Satkhira and Khulna area for the isolation of *V. fluvialis* from environmental samples. After collection, samples were enriched in alkaline peptone water (APW) and plated on TCBS medium. All the strains of *V. fluvialis* showed growth in 7% NaCl but no growth in 0% NaCl containing media. The isolates were confirmed as *V. fluvialis* using the API 20E kit. Five isolates (S-5, S-9, S-10, S-14 and S-17) which showed positive results for *V. fluvialis*, were taken for further studies.

Among the *V. fluvialis* isolates, S-5 was obtained from the downstream sediment of Turag river, S-9 and S-10 were from sediment of beside the shrimp fields of Satkhira, S-14 was from sediment of a pond in Khulna and S-17 was from the sediment of Buriganga river.

Molecular analysis of toxic genes

PCR analysis followed by agarose gel electrophoresis failed to provide any desired band from the five isolates of *V. fluvialis*, indicating lack of the *ctxA, tdh, trh, stx1* and *stx2* genes in tested *V. fluvialis* organisms.

Cytotoxic activity

Several cytotoxicity assays were done on BHK-21, HeLa and MDCK cell lines using culture filtrates of *V. fluvialis* organisms. Cytoplasmic vacuolation, cell rounding, and destruction of the monolayer confirmed the crude toxin of *V. fluvialis* as a cytotoxic agent. It was also observed that treated cells had become compact and granular in appearance and they either were necrotic or showed various degenerative morphological changes (Figure 1). Culture filtrate of *E. coli* 0517:H7, which was used as a positive control, also showed similar results. Culture filtrates of *V. fluvialis* organisms showed 25%, 60% and 50% cell death on BHK-21, MDCK cells and HeLa cells, respectively (Table 1). On the other hand, ammonium sulfate precipitated crude protein preparation of *V. fluvialis* gave a complete destruction of monolayer (up to 95% of cytotoxicity) on both MDCK and HeLa cells. Control HeLa and MDCK cells exhibited a normal epithelioid shape and confluent growth. In all cases, the incubation time was 18 to 48 hours.

Detection of Haemolysin

Tube Haemolysin Method: Tube hemolysin method was used for culture filtrates and live cells. All the *V. fluvialis* isolaes showed positive results, that is the lysis of RBC indicating production of hemolysin. Development of reddish color throughout the suspension indicated the production of hemolysin by the organisms. Positive control used was *E. coli* O157:H7culture filtrate (Table 2) and the release of hemolysin of sheep RBC was measured at 450 nm.

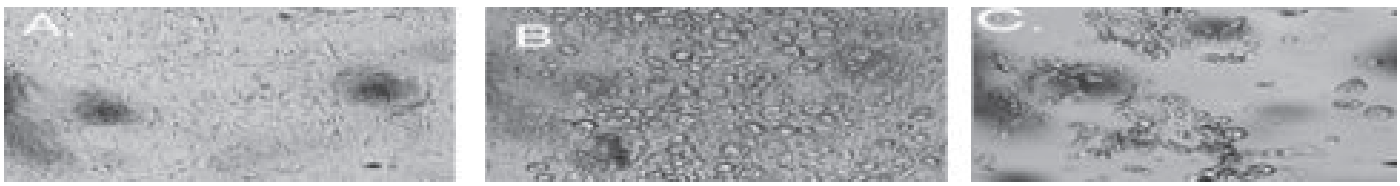
Blood Agar Plate Method: Blood agar plate was used for detection hemolysin. Inoculation of *V. fluvialis* culture on blood agar plate showed lysis of RBC around their growth. This is due to their ability to produce hemolysin that break down RBC. All the five *V. fluvialis* isolates showed zone of beta hemolysis around their growth.

Rabbit ileal loop assay

Live cells of all the five isolates of *V. fluvialis* caused fluid accumulation in rabbit ileal loop assay and the fluid

Figure 1: *Effect of V. fluvialis crude protein on MDCK and HeLa cells.*

Microscopic images of HeLa cell after 18 hours of incubation with *V. fluvialis* crude protein. Here (A) HeLa cells without any treatment (negative control) (B) EHEC 0157:H7 culture filtrate (C) Neat protein



Microscopic images of MDCK and HeLa cell after 18 hours of incubation with *V. fluvialis* crude protein. Here (A) MDCK cells without any treatment (negative control)(B) EHEC 0157:H7 culture filtrate (C) Neat protein

Table 1: *Cytotoxic effect V. fluvialis crude protein on BHK-21, HeLa and MDCK cells at 18 hours and 48 hours after application. Here percentages express the proportion of cell death. Positive control was EHEC 0157:H7 culture filtrate and negative control was DMEM media.*

Cytotoxic effect of crude toxin on different cell line	S-5	S-9	S-10	S-14	S-17	Positive control	Negative control
Cytotoxic effect on BHK-21cells (titre) after 48 hours of incubation with crude protein	20%	25%	35%	30%	25%	30%	0
Cytotoxic effect on HeLa cells (titre) after 48 hours of incubation with crude protein	80%	65%	95%	75%	80%	50%	0
Cytotoxic effect on MDCK cells (titre) after 48 hours of incubation with crude protein	80%	65%	95%	75%	80%	60%	0

accumulating activity of the S-5, S-9 and S-10 were found to be comparable to the positive control *V. cholerae* 569B strain. The others (S-14 and S-17), however, caused relatively smaller amounts of fluid accumulations (Table 2). Culture filtrates were prepared from all the 5 strains of *V. fluvialis* also gave positive ileal loop reactions. The fluid accumulation varied from strain to strain. However, the culture filtrates gave less amount of fluid accumulation than the live cells (Table 2). Crude protein samples isolated from S-10, treated with different physiochemical factors such as acid, enzymes and heat were injected into the loop. Negligible amount of fluid accumulation was observed which indicated that the crude protein was heat labile, sensitive to acid and proteinase K (Table 2).

Mouse lethality assay

Hind legs of all the mice became paralyzed within 2-5 days, when mice were intraperitoneally injected with 100 µl of crude protein. Again all those mice died within seven days after injection (Table 2). This observation indicated the probable presence of neurotoxic agent in the prepared crude protein toxin.

Neurotoxicity assay

To examine the neurotoxic activity of *V. fluvialis* crude protein, mice neuronal cell culture was established and the protein was added at three different concentrations. With the increasing concentration, the proportion of cell death also increased, whereas in case of control, there was no change. These results concluded that *V.fluvialis* crude protein toxin may have cytotoxic effects on mice neuronal cells, and therefore have neurotoxic activity (Figure 2).

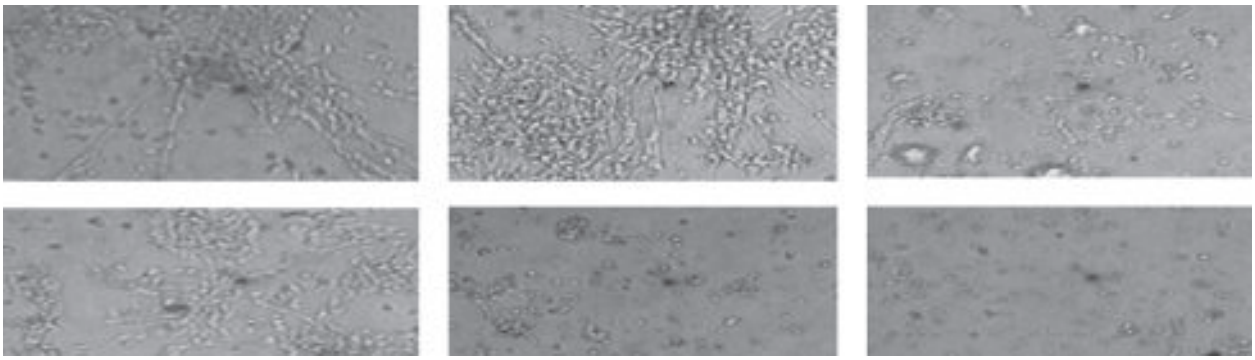
This table represents the results observed from different assays such as fluid accumulation ratio in case of rabbit ileal loop assay, hemolytic characteristic on blood agar, and number of mouse, dead in mouse lethality assay. In rabbit ileal loop assay, positive control was *V. cholera*e 569B and negative control was alkaline peptone water. In case of mouse lethality assay, positive control was EHEC 0157:H7 culture filtrate and phosphate buffer saline was used as negative control.

Hemolysis of RBCs on Sheep Blood (OD at 450 nm). LC = Live Cell, CF= Culture Filtrate

Table 2: *Summary of the biological activities and various functional characteristics of V. fluvialis crude protein.*

		S-5	S-9	S-10	S-14	S-17	Positive control	Negative control
Fluid accumulation ($mLCm^{-1}$)	Live cells	2.9	3.4	2.8	1.3	1.7	3.9	0
	Culture filtrate	0.8	0.6	1.5	0.4	0.5	2.0	0
		S-10						
	Proteinase K	0.05 M HCl		50^0 C-50 minutes			80^0 C-80 minutes	
	0.011	0.22		0.22			0.22	
Blood agar hemolytic characteristics		beta	beta	beta	Beta	beta	beta	No hemolysis
Tube hemolysis	Live cells	0.964	1.11	0.841	0.841	0.849	1.113	0
	Culture filtrate	0.123	0.143	0.232	0.232	0.199	0.297	0
Mouse lethality assay (Positive/ Tested)		2/4	2/4	4/4	2/4	4/4	4/4	0/4

Figure 2: Effect of *V. fluvialis* crude protein on mice neuronal cells. To examine the neurotoxic activity of *V. fluvialis* crude protein, mice neuronal cell culture was established and the protein was added at three different concentrations. With the increasing concentration the proportion of cell death also was increased whereas in case of control (No protein was added) there was no change. Here (A) Control (No protein was added). (B) Protein added at 1.5 µg/ml concentration. (C) Protein added at 3 µg/ml concentration. (D) Protein added at 6µg/ml concentration



Microscopic view of mice neuronal cells (10X)

Discussion

The genus *Vibrio* is an ecologically and metabolically diverse group, autochthonous to the marine, estuarine, and freshwater environment[18]. This genus comprises of nearly 100 species of which, some members are capable of causing severe diarrheal diseases, thus posing a serious threat in the developing world[5,19]. Among these, *V. cholerae* O1/O139 and *V. parahaemolyticus* are considered major diarrheal pathogens and are responsible for several pandemics and epidemics[20, 21].

Previously most of the research focused on *V. cholerae* in water because of the severity of the diseases it causes[22] but over the last decade, several studies have involved relatively minor *Vibrio* species of medical interest[23], some of which are described as emerging pathogens, able to cause mild to severe human diseases[24]. In past thirty years several outbreaks have been occurred which were associated with non O1/O139 vibrios, such as *V. fluvialis* and *V. mimicus*[1,25]. *V. fluvialis* is one of the food borne pathogenic bacteria, which has been implicated in outbreaks and sporadic cases of diarrhea[26]. Between 1976 and 1977, 500 patients (mostly children and young adults) were reported to be infected with *V. fluvialis* in Bangladesh with symptoms marked by vomiting, abdominal pain, moderate to severe dehydration and significant fever[27]. After that, several outbreaks of diarrhea also occurred due to *V. fluvialis* in South Asia, and mostly in Indian subcontinent. The incidence of outbreaks has increased in the past years and that's why *V. fluvialis* is known as emerging food borne pathogen. *V. fluvialis* ranks very high as a human public health hazard amongst bacterial pathogens as well as a contaminant in marine foods and food products, and causing impairment in both freshwater and marine environments. Early reports from the US indicated involvement of *V. fluvialis* with gastroenteritis among infants[28-30]. Since 1979, *V. fluvialis* was isolated as one of the important pathogens in Japan[31]. Investigations carried out after the 1998 floods in Bangladesh showed involvement of *V. fluvialis* in a diarrhea outbreak[32]. Due to complexity in the identification of *V. fluvialis*, information regarding its incidence among diarrheal patients is scanty. In many instances, *V. fluvialis* was found to be associated with cholera-like diarrhea[33]. Studies on the pathogenesis of enteric disease associated with isolation of the bacterium indicated that the clinical features of the disease closely resembled cholera, except that some patients had blood and mucus in their stools and some had abdominal pain and fever[27]. In 2009, an episode of massive diarrhea broke out in coastal regions of India following the cyclone Aila. Further investigation confirmed *V. fluvialis* as the predominant pathogen responsible for this diarrheal outbreak[34]. In many investigations, the detection frequency of *V. fluvialis* is increased both in aquatic environment and in diarrheal cases[35]. Despite of extensive research, the exact mechanism of diseases and virulence factors of *V. fluvialis* are yet to be explored. Though *V. fluvialis* related illness is characterized by gastroenteritis, nausea, vomiting, loss of appetite and watery bloody diarrhea with abdominal cramp, which mostly resemble to cholera or diseases caused by other vibrios, however, all the *V. fluvialis* strains were negative for the virulence genes commonly reported in *V. cholerae* and *V. parahaemolyticus*[36]. An aerogenic strain of *V. fluvialis* elicited fluid accumulation in ligated rabbit ileal loops and it caused cytotoxic and cytotonic responses when growing in Y-1 adrenal cell cultures. Also when the culture filtrates were tested, cytotoxic effect was readily noticed in the Chinese hamster ovary and HeLa cell lines, i.e., cytoplasmic vacuolation, cell rounding, and destruction of the monolayer[37]. The observations cited above stated the possibility that *V. fluvialis* produced products which could contribute to the pathogenesis of the enteric disease attributed to the bacterium. On the other hand, there are very few reports on characterization of *V. fluvialis* from environmental samples in Bangladesh. Therefore, this study was aimed at isolation of *V. fluvialis* from rivers around Dhaka city

and southern parts of Bangladesh and characterization of their toxins.

V. fluvialis is a halophilic organism, which cannot grow in the absence NaCl and in the presence of 12% NaCl in the laboratory; but the interesting thing is that the location from where it was isolated contains 0-0.5% NaCl, indicating that despite of being halophilic it has the tendency to grow without the absolute presence of NaCl. Perhaps the presence of other salts in the sampling area enables them to grow and survive. As *V. fluvialis* causes diseases mostly resemble to *V. cholerae,* we thought weather they contained *ctx* gene. We conducted PCR analysis to detect the *ctx* gene and the PCR analysis showed that the isolates harbor no *ctx* gene. PCR was also conducted for searching some other genes and it was found that the isolates of *V. fluvialis* lacked the *tdh, trh, stx1 and stx2* genes. Extracellular proteins released by the pathogenic organisms are suspected to be associated with virulent factors. Therefore, to characterize the extracellular proteins of *V. fluvialis*, we prepared crude protein from the culture filtrate. For this, a filter sterilized chemically defined medium containing a range of amino acids was prepared for the growth of *V. fluvialis*. The reason for selecting chemically defined medium, instead of BHI (Brain Heart Infusion) broth, is that the BHI broth contains a mixture of proteins of its own. As we tried to obtain those proteins which are only excreted by the bacterium into the medium, so we used the selective medium for this purpose and the *V. fluvialis* grew in liquid medium and produced its own proteins/toxins. After preparation of the crude protein toxin, we went for both in *vivo* and *in vitro* techniques to characterize the crude protein. Several cytotoxicity assays were done on BHK-21, HeLa and MDCK cell lines and cytotoxic activity of the crude protein was demonstrated on these cells. In the rabbit ileal loop assay, both live bacteria and crude protein toxins produced fluid accumulation in the rabbit ileal loops, some of which were comparable to the *V. cholerae* 569B positive control. This assay also indicated the enterotoxigenic nature of the crude protein. Though the *V. fluvialis* organisms showed *ctx* gene negative results in the PCR assay, however, the positive ileal loop assay clearly indicated that the *V. fluvialis* organisms might produce toxins, which are probably different from the known cholera toxin. In further characterization, crude toxin showed positive result in mouse lethality assay and this result suggested the possible presence of neurotoxin in the *V. fluvialis* crude protein. To evaluate the neurotoxic potential of the crude protein, mice neuronal cell culture was established and the effect of crude protein on mice neuronal cells was also observed. All these results suggested that the environmental *V. fluvialis* organisms, which lack the *ctx* gene, are highly pathogenic in nature and may be considered as potential threats for the society.

Reference

1. Chowdhury G, Pazhani GP, Dutta D, Guin S, Dutta S, Ghosh S et al. 2012. *Vibrio ûuvialis* in patients with diarrhea, Kolkata, India. *Emerg. Infect. Dis.***18**: 1868–1871.
2. Huq MI, Alam, AK, Brenner DJ, and Morris and GK. 1980. Isolation of *Vibrio*-like group, EF-6, from patients with diarrhea. *Journal of Clinical Microbiology.* **11**(6): 621-624.
3. Thekdi R, Lakhani AG, Vachha SM, and Chandrakapure MR. 1982. *Vibrio fluvialis* (group F vibrio) in Maharashtra. *The Indian journal of medical research.* **76**: 80-85.
4. Klontz KC and Desenclos JC. 1990. Clinical and epidemiological features of sporadic infections with *Vibrio fluvialis* in Florida, USA. *J. Diarrhoeal Dis. Res*. **8**: 24–26.
5. Morris JG. 2003. Cholera and other types of vibriosis: a story of human pandemics and oysters on the half shell. *Clin. Infect. Dis*.**37**: 272-280.
6. Levine WC, Griffin PM and Gulf Coast *Vibrio* Working Group. 1993. *Vibrio* infections on the Gulf Coast: results of first year of regional surveillance. *Journal of Infectious Diseases*. **167**(2): 479-483.
7. Allton DR, ForgioneJrMA and Gros SP. 2006. Cholera-like presentation in *Vibrio fluvialis* enteritis. *Southern medical journal*. **99**(7): 765-768.
8. Tokoro M, Kato M, Goto K, Watanabe M, Yamada F, Sako T and Niwa S. 1984. Community outbreaks of mixed food-borne infection *with Vibrio parahaemolyticus* and *Vibrio fluvialis*. *Kansenshogakuzasshi. The Journal of the Japanese Association for Infectious Diseases*. **58**(10): 1038.
9. Rahim Z and Aziz KM. 1996. Factors affecting production of haemolysin by strains of *Vibrio fluvialis*. *J Diarrhoeal Dis Res.***14**:113–116.
10. Kothary MH, Lowman H, McCardell BA and Tall BD. 2003. Purification and characterization of enterotoxigenic El Tor-like hemolysin produced by *Viriofluvialis. Infection and immunity*.**71**(6): 3213-3220.
11. Rubin LG, Altman J, Epple LW and Yolken RH. 1981.*Vibrio cholerae* meningitis in a neonate. *J Pediatr.* **98**:940–942.
12. Naidu LS, Bakerman BR, Saubolle MA and Lewis K. 1993. *Vibrio cholerae* non-0:1 meningitis in an infant. *Pediatr Infect Dis J*. **12**(10): 879–881.
13. Kuo-Chin Huang and Robert Wen-Wei Hsu. 2005. Clinical Infectious Diseases, Vol (40): 75–77.
14. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**: 248–254.
15. Singh SJ and Sanyal SC. 1978.Enterotoxicity of the so-called NAG vibrios.*Annales de la SocieteBelge de Medicine Tropicale*. **58**:133–140.
16. De A, Sen PC and Tewari IC. 1993. Enteropathogenic bacteria in river Ganges in Varanasi. *Indian journal of pathology & microbiology*. **36**(4): 425-432.
17. García CV and Antillon F. 1990. Isolation of enteropathogenic Vibrio in bivalves and mud from the Nicoya Gulf, Costa Rica. *Revista de biologia tropical.* **38**(2B): 437-440.
18. Haley BJ, Choi SY, Grim CJ, Onifade TJ, Cinar HN, Tall BD., et al. 2014. Genomic and Phenotypic Characterization of *Vibrio cholerae* Non-O1 Isolates from a US Gulf Coast Cholera Outbreak. *PLoS ONE***9**(4): e86264.
19. Thompson JR, Randa MA, Marcelino LA, TomitaMitchell A, Lim E and Polz MF. 2004. Diversity and dynamics of a north Atlantic Coastal Vibrio community. *Appl Environ Microbiol*. **70**: 4103–4110.
20. Kaper JB, MorrisJGJr and Levine MM. 1995. Cholera, *ClinMicrobiol Rev*.vol(**8**): 48-86.
21. Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y and Sack DA. 2007. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. *ClinMicrobiol Rev.***20**: 39–48.
22. Mishra M, Mohammed F, Akulwar SL, Katkar VJ, Tankhiwale NS and Powar RM. 2004. Re-emergence of El Tor Vibrio in outbreak of cholera in and around Nagpur. *Indian J Med Res*.**120**:478.
23. Daniels NA, MacKinnon L, Bishop R et al. 2000.*Vibrio parahaemolyticus* infections in the United States, 1973–1998. *J Infect Dis*.**181**: 1661–1666.

24. Tantillo GM, Fontanarosa M, Di Pinto A and Musti M. 2004. Updated perspectives on emerging vibrios associated with human infections. *Lett. Appl. Microbiol.* **39**:117–126.

25. Chitov T, Kirikaew P, Yungyune P, Ruengprapan N and Sontikun K. 2009. An incidence of large foodborne outbreak associated with *Vibrio mimicus*. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology.* **28**(4): 421-4.

26. Olivier V, Haines GK, Tan Y and Satchell KJ. 2007. Hemolysin and the multifunctional auto processing RTX toxin are virulence factors during intestinal infection of mice with *Vibrio cholerae* El Tor O1 strains. *Infect Immun*. **75**: 5035–5042.

27. Huq MI, Alam AK, Brenner DJ, and Morris GK. 1980. Isolation of *Vibrio*-like group, EF-6, from patients with diarrhea. *Journal of Clinical Microbiology*.**11**(6): 621-624.

28. Hickman-Brenner FW., et al. 1984. *Vibrio fluvialis* and *Vibrio furnissii* isolated from a stool sample of one patient. *J. Clin. Microbiol*. **20**:125–15

29. Bellet J, Klein B, Altieri M and Ochsenschlager D. 1989. *Vibrio fluvialis*, an unusual pediatric enteric pathogen. *PediatrEmerg Care*. **5**: 27–28.

30. Kolb EA, Eppes SC and Klein JD. 1997. *Vibrio fluvialis*: an underrecognized enteric pathogen in infants.*South Med J*. **90**: 544–545.

31. Aihara M, Sakai M, Iwasaki M, Shimakawa K, Kozaki S, Kubo M and Takahashi, H. (1991). Isolation of enteropathogenic microorganism from patients with infection of the digestive tract during 1976 to 1988 in Tenri Hospital. *Kansenshogakuzasshi. The Journal of the Japanese Association for Infectious Diseases*. **65**(7): 864-874.

32. Tanabe K, Nakamura S and Kunii O. 1999. Bacteriological survey of diarrheal epidemics in the 1998 Bangladesh floods. *KansenshogakuZasshi.***73**: 918–922.

33. Allton DR, ForgioneJrMA and Gros SP. 2006. Cholera-like presentation in *Vibrio fluvialis* enteritis. *Southern medical journal*, **99**(7): 765-768.

34. BhattacharjeeS, Bhattacharjee S and Bal B. 2010. Is *Vibrio fluvialis* emerging as a pathogen with epidemic potential in coastal region of eastern India following cyclone Aila? *J Health PopulNutr*. **28**:311.

35. Ramamurthy T, Chowdhury G, Pazhani GP and Shinoda S. 2014. *Vibrio fluvialis:* an emerging human pathogen. *Front Microbiol*. **5**.

36. Miyoshi SI, Sonoda Y, Wakiyama H, Rahman MM, Tomochika KI, Shinoda S and Tobe, K. (2002). An exocellularthermolysin-like metalloprotease produced by *Vibrio fluvialis*: purification, characterization, and gene cloning. *Microbial pathogenesis*, **33**(3): 127-134.

37. Chakraborty R, Chakraborty S, De K, Sinha S, Mukhopadhyay AK, Khanam J, Ramamurthy T, Takeda Y, Bhattacharya SK and Nair GB. 2005. Cytotoxic and cell vacuolating activity of *Vibrio fluvialis* isolated from paediatric patients with diarrhoea. *J Med Microbiol*. **54**: 707–716.