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Original Article

Comparison of toxicities between *Vibrio fluvialis* and *Vibrio furnissii* strains isolated from environmental samples

Tahsina Jainab[1], Sayeema Hasan[1], Mohammed Abul Manchur[2], Mahmuda Yasmin[1], Jamalun Nessa[1] and Chowdhury Rafiqul Ahsan[1]*

[1]*Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh;* [2]*Department of Microbiology, University of Chittagong, Chittagong-4331, Bangladesh.*

Gastrointestinal episodes associated with *Vibrio* species have been rising worldwide in the last few years. In this study, toxicities of two very well characterized *V. fluvialis* and *V. furnissii strains*, isolated from environmental samples, were used to investigate the comparative pathogenicity of these strains. The results showed that there is a positive relationship between toxin production as well as a high interspecies correlation. In the analysis of toxicity, both species showed positive results with close similarity in cell death on BHK-21 cell line and destruction of RBC, which were indicative of cytotoxic and hemolytic activity, respectively. Besides, molecular analysis showed the absence of *ctx, tdh, trh, stx1* and *stx2* genes. On the other hand, reduced fluid accumulation ratio in rabbit ileal loop assay and paralysis of hind legs of Swiss Albino mice were observed for *V. furnissi* strain, which indicates the differences in pathogenicity between the two species.

Keywords: *V. fluvialis*, *V. furnissii*, enterotoxicity, cytotoxicity, neurotoxicity, hemolytic activity.

Introduction

Gastrointestinal pathogens invade or disrupt the intestinal barrier by the action of secreted toxins. They can modify cell physiology by multiple mechanisms, being directly responsible for the pathology of the disease or favoring other processes such as penetration of host barriers, escape from the intracellular environment and manipulation of the immune response, among others[1]. The Vibrionaceae family includes several species of major importance in the clinical field that can cause gastroenteritis. Therefore, Group F vibrios should perhaps form a new genus within the family Vibrionaceae[2]. This group of vibrios has been isolated from cases of diarrhea in Bahrain, Jordan, Bangladesh, and marine and estuarine environments around Britain[3,4]. In this group, the halophilic *V. fluvialis* phenotypically resembles *Aeromonas* species[5] and taxonomically lies between *Aeromonas* and *Vibrio* species[6]. Among the halophilic vibrios, it has a close similarity to *V. furnissii*, but, unlike *V. fluvialis*, *V. furnissii* is aerogenic in nature[7]. Strains isolated from diarrheal patients were found to be anaerogenic, whereas those from the environment included both aerogenic and anaerogenic strains[4]. Both the *V. fluvialis*[8] and *V. furnissii*[9] have been associated with outbreaks and sporadic cases of acute diarrhea and are resistant to many antimicrobial agents[8]. The largest outbreak of *V. fluvialis* infection was reported in Bangladesh between October 1976 and November 1977, with more than 500 patients[10].

Several toxins that may be important in pathogenesis have been reported in *V. fluvialis* including a Chinese hamster ovary (CHO) cell elongation factor, CHO cell killing factor, enterotoxin-like substance, lipase, protease cytotoxin and hemolysin[11,12]. None of these factors, however, has been correlated with the diarrheal activity, and similarity and dissimilarity between the related substances and the factors are also unclear. The epidemiological importance of *V. fluvialis* was reported in several recent publications[13,14]. *V. furnissii* is one of the 11 non-cholera *Vibrio* species pathogenic in humans[15], which can lead to human gastroenteritis and extra-intestinal infection and is also ubiquitously present in the marine environment. The pathology of *V. furnissii* in gastroenteritis is potentially related to cytolysin and hemolysin production[16]. However, the question regarding the microbiological characteristics, mechanism of pathogenicity, and ecology of these organisms remain mostly unanswered. More studies are necessary to define risk factors and to determine the pathogenesis of these organisms. Therefore, this study addresses comparisons of toxicities between the environmental *V. fluvialis* and *V. furnissii* strains for a better understanding of the pathogenicity of these organisms.

Methods

Bacterial strains

Samples were collected from Buriganga and Turag rivers surrounding the Dhaka city, shrimp field near Nalta area of Satkhira and ponds near Tala area of Khulna in Bangladesh. After processing, the collected samples were enriched in alkaline peptone water. The characteristic yellow colonies on the Thiosulfate-citrate-bile salt-sucrose (TCBS) agar plate were subcultured on *Vibrio* Chromogenic agar for white colonies, which was indicative of desired organisms. Salt tolerance was

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

determined by growing the strains in Luria broth overnight with shaking at 37æ%C with 0%, 3%, 5%, 7% 8% and 10%. And API 20E identification strip was used to characterize the biochemical features.

Molecular Identification and characterization by Polymerase chain reaction (PCR)

PCR was used to confirm for species-specific identification to target the *toxR* gene of *V. fluvialis* and *V. furnissii* strains. PCR technique was also done to screen the virulence genes like *ctxA, tdh, trh, stx1 and stx2,* which encode the enterotoxic activity. Template DNA was prepared by boiling DNA method using fresh culture for PCR assay.

Preparation of culture filtrate

Fresh cultures of *V. fluvialis* and *V. furnissii* were inoculated into a synthetic medium containing different kinds of amino acids at an incubation temperature of 37æ%C for 48 hours with shaking at 120 rpm, followed by centrifugation at 8,000 rpm for 10 minutes. The supernatant containing extracellular proteins was used as culture filtrates.

Rabbit Ileal Loop (RIL) assay for determination of enterotoxicity

Enterotoxicity of live cells and culture filtrates of the *V. fluvialis* and *V. furnissii* strains were determined following previous methods[17,18]. Albino rabbits (New Zealand strain) weighing 2-3 kg were used in this study. The animals were fasted for overnight with allowing water prior to testing. A total of 6-8 loops were made per rabbit maintaining proper anesthesia. One ml of live cells and culture filtrates were injected into each loop. *V. cholerae* 569B was used as positive control and cell free culture media was used as a negative control. Each test was done in three rabbits and separate rabbits were used for live cells and culture filtrates.

Mouse lethality assay

An indirect method for neurotoxin detection was carried out by mouse lethality assay. Four to six weeks old Swiss albino mice were intraperitonealy injected with 0.1ml of culture filtrates of the *V. fluvialis* and *V. furnissii* strains and observed for 2-5 days for any physical change *viz*: paralysis of muscles. *E. coli* O157:H7 culture filtrate was used as positive control and culture media to prepare the culture filtrate was used the negative control.

Hemolysin assay

Hemolytic activity of the *V. fluvialis* and *V. furnissii* culture filtrates were investigated by tube hemolysis method, where, sheep blood was centrifuged at 2,000 rpm for 5 min and the erythrocyte was diluted to about 1.0% with phosphate buffer saline (PBS). A reaction mixture was prepared with 1 ml of culture filtrate and 0.5 ml of erythrocyte and was incubated at 37æ%C for 1 hour followed by centrifugation at 2,000 rpm for 5 minutes. The optical density (OD) of the supernatant was measured for released hemoglobin with a spectrophotometer at 450 nm.

Cytotoxicity assay

BHK-21 cell line was used to determine the cytotoxicity of the *V. fluvialis* and *V. furnissii* culture filtrates. A monolayer of BHK-21 was grown in a tissue culture flask using Dulbecco Modified Eagle's medium with 10% Fetal Bovine Serum and antibiotics (Penicillin, Gentamycin and Streptomycin). After getting confluent growth, 3.5×10^4 cells/450µL were distributed into each well of 24 well cell culture plates and incubated with the proper cultural condition. Then culture filtrates were added to the wells in duplicate and mixed properly. The plate was then incubated for 18-20 hours at 37æ%C in a humid atmosphere with 5% CO_2. The cells were then examined under an inverted microscope for necrosis, elongation, or rounding. *E. coli* O157:H7 culture filtrate was used as positive control and only culture medium was used as the negative control.

Results

Primary isolation of V. fluvialis and V. furnissii strains *from environmental samples*

For the isolation of *V. fluvialis* and *V. furnissii* organisms, samples were enriched in alkaline peptone water (APW) and plated on TCBS medium. All the strains of *V. fluvialis* and *V. furnissii* showed growth in 7% and 10% NaCl but no growth in 0% NaCl containing media. The isolates were confirmed by using the API 20E kit (Table 1). Among these isolates, *V. fluvialis* strain S-10 was isolated from sediment beside the shrimp fields of Satkhira and the *V. furnissii* strain S-18 was isolated from the sediment of a pond in Khulna.

Molecular identification and characterization of toxic genes

Both the isolates also gave positive results for *toxR* gene, using its specific primers (Table 1). PCR analysis followed by agarose gel electrophoresis failed to provide the appearance of any desired band for *ctxA*, *tdh*, *trh*, *stx1* and *stx2* from both the *V. fluvialis* and *V. furnissii* strains except in positive control.

Rabbit ileal loop assay

Both the live cells of *V. fluvialis* and *V. furnissii* strains caused fluid accumulation in the rabbit ileal loop (Figure 1). However, the fluid accumulation ratios varied between these two strains and from rabbit loop to rabbit loop. The fluid accumulation activity of *V. fluvialis* was comparable to the positive control *V. cholerae* 569B strain. Culture filtrates were prepared from both the strains of *V. fluvialis* and *V. furnissii* gave positive ileal loop reactions (Figure 2).

Table 1. *Identification of V. fluvialis and V. furnissii strains*

Isolate no.	TCBS	CV	TSI	Gas	Citrate	MR	VP	Oxidase	Growth in NaCl Conc.					VF-	API
									0%	3%	5%	7%	8%	10%	*toxR*	20E
Ref. strain	Y	W	+/+	-	+	+		+	-	+	+	+	+	+	+	*V. fluvialis*
S-10	Y	W	+/+	-	+	+		+	-	+	+	+	+	+	+	*V. fluvialis*
S-18	Y	W	+/+	+	+	+		+	-	+	+	+	+	+	+	*V. furnissii*

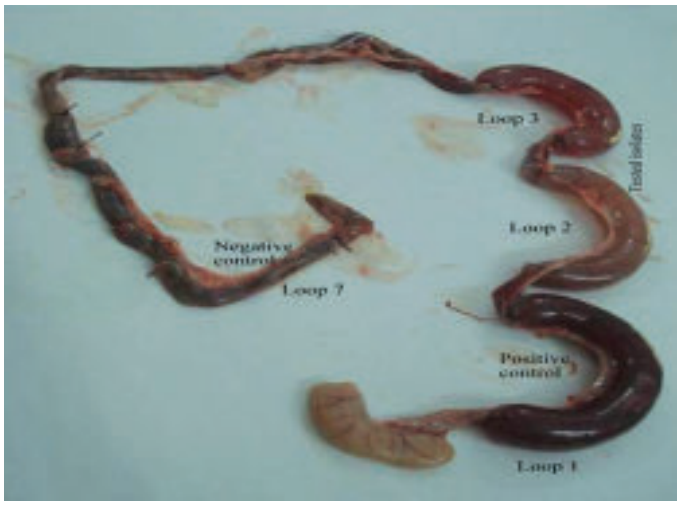

Fig. 1. *Fluid accumulation in rabbit ileal loop (Loop 1 and Loop 7 contains live cells of positive control and negative control respectively. Loop 2 and 3 contains live cells of V. fluvialis and V. furnissii).*

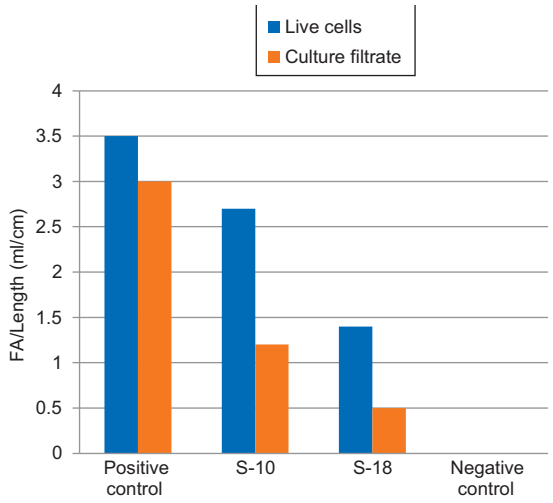

Fig. 2. *Graphical representation of mean ratio of fluid accumulation (FA) per length by live cells and culture filtrates of V. fluvialis and V. furnissii.*

Table 2: Comparative results of mouse lethality assay, hemolysis assay and cytotoxicity assay

Toxicity assay			Control		*V. fluvialis*	*V. furnissii*
			Positive	Negative	S-10	S-18
Mouse lethality assay (Positive/ Tested)			4/4	0/4	4/4	2/4
Hemolysis assay	Tube method (OD@450 nm)	Live cells	1.113	0.00	0.774	0.841
		Culture filtrate	0.297	0.00	0.246	0.232
	Blood agar plate		Beta hemolysis	No hemolysis	Beta hemolysis	Beta hemolysis
Cytotoxicity assay	BHK-21cell line (% of cell death)		70%	0%	25%	30%

Mouse lethality assay

Hind limb paralysis and some sort of dizziness were observed within 2-4 days upon intraperitoneal injection of 0.1 ml of culture filtrate. All those mice died within five days after injection (Table 2).

Hemolysis assay

Both the *V. fluvialis* and *V. furnissii* strains showed positive results, that is the lysis of RBC indicating the production of hemolysin. In the tube method, the development of reddish color throughout the suspension indicated the production of hemolysin by the organisms. The results of hemolytic activity were measured at 450 nm. Live cells of both the isolates also showed a zone of beta hemolysis around their growth (Table 2). *E. coli* O157:H7 was used as positive control and culture media as a negative control for the detection of hemolytic activity in this study.

Cytotoxicity assay

BHK-21 cell line was used to determine cytotoxic effect of *V. fluvialis* and *V. furnissii* Culture filtrates of both the isolates showed 25-30% cell death on BHK-21 cell line (Table 2) when compared with the positive controls.

Discussion

The members of the Vibrionaceae family namely *V. mimicus*, *V. fluvialis* and *V. furnissii* were also frequently found to be associated with diarrheal outbreaks[15,19,20]. The clinical symptoms of *V. fluvialis* include mild to moderate dehydration, vomiting, fever, abdominal pain and diarrhea[5]. According to *Vibrio* Reference Laboratory at the CDC, 16 *V. furnissii* were isolated among 1230 *Vibrio* and all the *V. furnissii* isolates were associated with intestinal infections[21]. There are very little information available on the virulence factors associated with infection and much less information on the mechanism of pathogenicity of this organism. Besides, very limited information has been published on the toxicity of *V. furnissii*. So, this study is taken to compare between the toxicities of *V. fluvialis* and *V. furnissii*. In this study, environmental samples were collected from rivers around Dhaka city and southern parts of Bangladesh in the month of November, when water temperature lies between 20-25°C. Among all

isolates, *V. fluvialis* organisms were isolated from Dhaka, Satkhira and Khulna, and *V. furnissii* organisms solely from Dhaka.

To determine the toxic activities of these organisms, we prepared both live cell preparations and culture filtrates of the *V. fluvialis* and *V. furnissii* strains and carried out *in vivo* and *in vitro* experiments. Both the isolates produced different ranges of fluid accumulation in rabbit ileal loop assay and *V. fluvialis* fluid accumulation ratio is almost comparable to the positive control, *V. cholerae* 569B. Fluid accumulation in the rabbit loop indicated that both *V. fluvialis* and *V. furnissii* strains liberated an enterotoxic-like substance(s) during multiplication in the intestine as well as in the synthetic medium. The differences in fluid accumulation between strains may have been due to the variation in the release of toxins. Cell free culture filtrates of all *V. fluvialis* strains gave positive ileal loop reactions indicated that enterotoxic substance(s) were liberated during *in vitro* multiplication in the medium. Similar observations were made with enterotoxic *A. hydrophila*, *V. cholerae* serotypes other than O1[19]. Strain to strain variations in the amount of fluid accumulation was probably due to differences in the quantitative release of the toxin from cells into the medium[22] and to biological variations in rabbits[23].

In this study, the fluid accumulating activity by culture filtrates was found to be less than the live cells for both *V. fluvialis* and *V. furnissii* strains. It may occur due to enterotoxin production which is medium dependent and also depends on other factors. Previous report also showed that the enterotoxin production from *V. fluvialis* strains was culture medium dependent and found that clinical strains grown in BHI broth supplemented with 0.5% NaCl induced large amounts of fluid accumulation in mouse intestines[24]. BHI media may contain any protein that is toxin destroying or may inhibit the toxin secretion.

The clinical symptoms of gastroenteritis caused by *V. fluvialis* are quite similar to those caused by *V. cholerae*, except for the frequent occurrence of blood in stools[25]. For that reason PCR analysis of *ctxA* gene was done to determine the possible reason for fluid accumulation in the rabbit ileal loop. However, no band for the *ctxA* gene was obtained in the PCR analysis, when compared with the positive control *V. cholerae* 569B. So the fluid accumulation factors may be distinct from CT-like enterotoxin. A similar result of *ctxA* negative in PCR analysis was also obtained for the *V. furnissii* strain. Subsequently we moved further to determine whether other known toxic genes accountable for enterotoxicities were responsible for fluid accumulation and PCR analysis was done for *tdh, trh, stx1* and *stx2* gene responsible for CT, TDH, TRH and STX. However, both *V. fluvialis* and *V. furnissii* strains gave negative results for all genes. More investigation is required to find out the possible reason behind the fluid accumulation of both of the species.

Besides enterotoxicity, the toxin of *V. fluvialis and V. furnisssii* showed hind limb paralysis in Swiss Albino mice within 2-3 days and all the mice died within five days. These results suggested that both the species produced neurotoxin like substances and in the case of *V. furnissii* it is less severe than *V. fluvialis*. We also found both live cells and culture filtrate of two species were capable of causing the comparable amount of lysis of sheep red blood cells. Production of hemolysin may correlate with the blood in the stool of *V. fluvialis* diarrhea. These results are coherent with the study which reported that *V. fluvialis* hemolysin lyses a wide variety of erythrocytes and is immunologically related to the El Tor hemolysin[11]. Cytotoxicity assay was done on BHK-21 cell lines showed positive results as compared to the positive control *E.coli* O157:H7. In this study, both the isolates showed the comparable amount of cell death (about 25-30%). All these data clearly showed differences in toxicities between the environmental *V. fluvialis* and *V. furnissii* strains for a better understanding of the pathogenicity of these organisms. More studies are needed on the purification of the toxins to confirm the findings that can evaluate the role of the toxin(s) in the pathogenesis of the enteric disease and to determine their mechanisms of actions on host target cells.

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