Enterotoxicity of *Vibrio fluvialis*

M. Phil Thesis

DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF DHAKA
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Enterotoxicity of *Vibrio fluvialis*

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF DHAKA FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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Abbreviations

%	Percentage
et al.	And others
bp	Base pair
mbp	Mega base pair
kbp	Kilo base pair
rpm	Rotation per minute
nm	Nanometer
sec	Second
min	Minute
h	Hour
kDa	Kilodalton
µg	Microgram
kg	Kilogram
mA	Miliampare
Rf	Retention frequency
cm	Centimeter
℃	Degree celsius
L	Liter
µl	Micro liter
ml	Milliliter
mm	Millimeter
M	Molar
N	Normal
mg	Milligram
gm	Gram
Conc.	Concentration
2X	Two times
CDC	Central for disease control and prevention
P^H	Negative logarithm of hydrogen ion concentration
OD	Optical density
RBC	Red blood cell
dH_2O	Distilled water
Cl^-	Chloride
CO_2	Carbon dioxide
HCl	Hydrochloric acid
NaCl	Sodium chloride
KCl	Potassium chloride
NaOH	Sodium hydroxide
KOH	Potassium hydroxide
Na_2HPO_4	Disodium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate

$MgCl_2$	Magnesium chloride
PBS	Phosphate buffer saline
EDTA	Ethylene diamine tetra acetic acid
TE	Tris EDTA
TBE	Tris borate EDTA
ADP	Adinosine diphosphate
NAD	Nicotinamide adenine dinnucleotide
GTP	Guanosine triphosphate
dNTP	Deoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
V.	*Vibrio*
spp.	Species
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
Taq	*Thermus aquaticus*
BHK-21	Baby Hamster Kidney-21
HeLa	Henrietta Lacks
TDH	Thermostable direct haemolysin
TRH	TDH related haemolysin
*tox*R	Toxin regulatory gene
tdh	Thermostable direct haemolysin gene
trh	Thermostable direct haemolysin related haemolysin gene
stx1	Shigella toxin 1 gene
stx2	Shigella toxin 2 gene
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Abstract

Vibrio fluvialis is a halophilic bacterial species which emerged as a diarrhoeal pathogen and has been a human public health hazard all over the world, especially in coastal areas of developing countries and regions with poor sanitation. In this study, we isolated, identified and characterized the enterotoxic and other toxic activities of five *V. fluvialis* organisms isolated from the environmental sources. Though they are halophilic in nature, however, these organisms were isolated from fresh environmental water sources having 0% salinity from rivers around Dhaka city and ponds from Khulna, and higher salinity in Satkhira area. All the *V. fluvialis* strains showed growth in 7% salinity and did not produce gas from glucose. In the toxicity assay, live cells and culture filtrates of all five isolates gave positive results in rabbit ileal loop assay indicating their enterotoxicity. Two strains which gave poor results in enterotoxin production initially, showed enhancement of enterotoxicity as judged by increased fluid outpouring after two consecutive passages in rabbit gut indicating that, the enteric environment probably influences toxin production. On the other hand, PCR analysis showed the absence of *ctxA* gene and other genes like *tdh, trh, stx1* and *stx2*, which are commonly associated with enterotoxicity. The culture filtrate of the isolates showed cytotoxic activities on BHK-21 and HeLa cells lines, gave positive results for haemolysin in blood agar plate and tube methods, and caused paralysis of hind legs of Swiss Albino mice within 2-3 days. The toxic activities shown by culture filtrates of *V. fluvilais* were increased after concentration of culture filtrates by ammonium sulphate precipitation method. All these results suggested that these environmental *V. fluvialis* organisms, which lacked *ctxA* gene, are highly pathogenic. They also lacked other common enterotoxic genes. Although *ctxA* gene negative, these organisms showed rabbit ileal loop positive reactions, which indicated that they might produce a toxin which is different from the known cholera toxin. Apart from the enterotoxic activity, these organisms also produced neurotoxin, cytotoxin and haemolysin. Therefore, the presence of *V. fluvialis* organisms in aquatic environment of Bangladesh constitutes a threat to public and environmental health.

1.0 Introduction and Review of Literature

1.1. General Introduction

Vibrio fluvialis is a halophilic gram-negative bacterium. It has a straight to slightly curved rod cell morphology that is motile by means of polar flagella. It is a sodium chloride-requiring, oxidase-positive, nitrate-positive organism that has 50% guanine-plus-cytosine in its DNA and, ferment D-glucose and other carbohydrates with the production of acid (Lee et al., 1978) but no gas from glucose (Brenner et al., 1983). The halophilic *V. fluvialis* (Lee et al., 1981; Lockwood et al., 1982) phenotypically resembles *Aeromonas* species (Seidler et al., 1980) and taxonomically lies between *Aeromonas* and *Vibrio* species (Thekdi et al., 1990). Among the halophilic vibrios, it has close similarity to *V. furnissii*, but, unlike *V. fluvialis*, *V. funissii* is aerogenic in nature (Brenner et al., 1983).

It is considered to be an emerging food borne pathogen and has been implicated in outbreaks and sporadic cases of acute diarrhoea in several countries including Bangladesh (Chowdhury et al., 2011). The distribution of *V. fluvialis* is worldwide (Thekdi et al., 1982) and this organism is not only isolated from human diarrhoeal cases (Klontz et al., 1994; Hodge et al., 1995) but also from marine and estuarine environments (Morris and Black, 1985). There are reports of food poisoning caused by this organism (Thekdi et al., 1990), especially due to consumption of raw shellfish (Levine and Griffin, 1993).

The largest outbreak of *V. fluvialis* infection was reported in Bangladesh between October 1976 and November 1977, with more than 500 patients (Huq et al., 1980). In the United States, *V. fluvialis* has been associated with enterocolitis in infants (Bellet et al., 1989). In Indonesia, *V. fluvialis* has been recognized as one of the major enteric pathogens causing cholera-like diarrhoea (Lesmana et al., 2002). Recently, an examination of 400 non-agglutinating *Vibrio* species collected from patients with diarrhoea in the period 2002-2009 in Kolkata, India, identified 131 strains of *V. fluvialis*, of which 43 strains were suggested to be the sole pathogen and the remaining 88 strains were co-pathogens with other prominent enteric pathogens (Chowdhury et al., 2012). In 2009, an episode of massive diarrhoea broke out in coastal regions of India following the cyclone Aila. Further investigation confirmed *V. fluvialis* as the predominant pathogen responsible for this diarrhoeal outbreak. Furthermore, *V. fluvailis* behaved more aggressively than *V. cholerae* O1 in an epidemic

situation with a higher attack rate and a different clinical pictures (Bhattacharjee et al., 2010).

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 (Igbinosa and Okoh, 2010). In addition, *V. fluvialis* associated extraintestinal infections, such as haemorrhagic cellulites and cerebritis (Huang and Hsu, 2005), peritonitis (Ratnaraja et al., 2005), acute otitis (Cabrera et al., 2005), biliary tract infection (Liu et al., 2011), bacteraemia (Lai et al., 2006) and even ocular infections (Penland et al., 2000) were also reported.

Several toxins that may be important in pathogenesis have been reported in *V. fluvialis* including a Chinese hamster overy (CHO) cell elongation factor, CHO cell killing factor, enterotoxin-like substance, lipase, protease cytotoxin and haemolysin (Rahim and Azia, 1996; Kothary et al., 2003). None of these factors, however, has definitely been correlated with the diarrhoeal activity, and similarity and dissimilarity between the related substances and the factors are also unclear. The organism contains El Tor-like haemolysin (Kothary et al., 2003) and also exhibits cytotoxic and cell-vacuolating activity on HeLa cells (Chakraborty et al., 2005).

However, question regarding the microbiological characteristics, mechanism of pathogenicity, and ecology of the organism remain mostly unanswered. More studies are necessary to define risk factors and to determine the pathogenesis of the organism.

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1.2 Review of Literature

1.2.1 *V. fluvialis* Organisms

1.2.1.1 Historical Overview

V. fluvialis was broadly defined as a nonagglutinating *Vibrio* species (Chowdhury et al., 2012). It was first isolated in 1975 from patients with severe diarrhoea (Huq et al., 1980). These vibrios were designated 'group F' vibrios in England (Furniss et al., 1977) and 'CDC group EF6' in the USA (Huq et al., 1980). Taxonomic studies concluded that these organisms represented a new species, and they were designated as *V. fluvialis* (Lee et al., 1981). Both phenotypic tests and DNA relatedness indicated that these organisms were much closer to the genus *Vibrio* than to the genus *Aeromonas* (Seidler et al., 1980).

In some taxonomic studies, group F was separated into two subgroups based on gas production during glucose fermentation (Lee et al., 1978). The aerogenic group F strains were in a different DNA relatedness group from the anaerogenic strains, and the two biogroups were separated into two species within the genus *Vibrio* (Seidler et al., 1980). The name *V. fluvialis* was proposed for both aerogenic and anaerogenic strains of group F and the synonymous group EF-6. However, only anaerogenic strains have been isolated from human samples, even though both aerogenic and anaerogenic strains of *V. fluvialis* have been found in the environment (Lee et al., 1981).

Subsequently, the aerogenic strains of group F were confirmed to be a separate species from *V. fluvialis* and were named a new species, *V. furnissii* (Brenner et al., 1983). *V. furnissii* produces gas from D-glucose, while *V. fluvialis* does not.

1.2.1.2 Microbiology of the Organisms

Vibrio is a genus of Gram-negative bacteria possessing a curved rod shape (comma shape), several species of which can cause food borne infection, usually associated with eating undercooked seafood. Typically found in saltwater, *Vibrio* spp. is facultative anaerobes that test positive for oxidase and do not form spores. All members of the genus are motile and have polar flagella with sheaths.

Recently *V. fluvialis* strains PG41 and 121563 were sequenced on the Illumina HiSeq 1000 platform to obtain draft genomes of 5.3 Mbp and 4.4 Mbp respectively (Khatri et al., 2013).

1.2.2 Cultural Characteristics

1.2.2.1 Culture Medium

Several specialized selective bacteriological culture media have been developed for isolating *Vibrio*. These media include Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar, Taurocholate Tellurite Gelatine (TTGA - also known as Monsur's media) agar, Sucrose Tellurite Teepal (STP) medium etc. (Kobayashi *et al*., 1963; Monsur, 1961). Some of the non-selective plating media are also used for cultivation of *Vibrio* such as gelatine agar (GA), trypticase soy agar (TSA) etc. (Finkelstein and Gomes, 1963).

The medium CHROM agar is another selective media for *Vibrio,* helps to easily differentiate *V. parahaemolyticus, V. vulnificus* and *V. cholerae* from other *Vibrio* spp. directly at the isolation step by colony colour with sensitivity higher than conventional methods. *V. parahaemolyticus* colonies are purple; *V. vulnificus* and *V. choleare* appear as blue colonies while *V. alginolyticus* colonies are colourless. Colony colour for *V. fluvialis* is white. This medium is selective against most major enterobacteriaceae and Gram negative bacteria.

1.2.2.2 Salt Tolerance

As they are halophilic group of organism so they have a tendency to grow under a wide range of salt. But too much concentration of salt in water is inhibitory to their growth. They can not grow at 0%, 10% and 12% NaCl but can grow at 1%, 6% and 8% NaCl concentration in nutrient broth (Brenner et al., 1983)

1.2.3 Survival of Organisms

V. fluvialis has the capacity to survive in the seawater microcosm for more than 15 days at ambient temperature regardless of carbonated substrate uptake (Munro et al., 1994). In microcosms, *V. fluvialis* has been shown culturally viable for a year without losing its virulence and in sediments this organism was recovered from viable but non-culturable (VBNC) stage, even after 6 years (Amel et al., 2008). VBNC state was described for many *Vibrio* species such as *V. anguillarum, V. campbellii, V. cholerae, V. fischeri, V. harveyi, V. mimicus, V. natriegens, V. parahaemolyticus, V. proteolyticus, V. vulnificus* (McDougald et al., 1998). *V. fluvialis* exist naturally in warm, salty and brackish water and survive in

temperature between 9°C and 31°C, but thrives when water temperature rises above 18°C (Igbinosa and Okoh, 2010). The optimum growth temperature for *V. fluvialis* is 37°C. Under optimal condition growth can be very rapid.

1.2.4 Phenotypic and Genetic Characteristics

Several serotypes of *V. fluvialis* have been identified based on the somatic antigen variation. Though Shimada et al. (1999) identified more than 50 somatic antigens; the serological based typing of *V. fluvialis* remains non-customary. *V. fluvialis* strains belonging to serogroup O19 possessed the C (Inaba) antigen of *V. cholerae* O1, but not the B (Ogawa) or A (common) antigens (Kondo et al., 2000). In the crossed immuno-electrophoresis, antibodies against the oral cholera vaccines containing killed whole cells (WC) of *V. cholerae* O1 Inaba El Tor reacted with a few strains of *V. fluvialis* (Ciznar et al., 1989). Shinoda et al. (1984) demonstrated independently that anti-L-flagella antisera of *V. fluvialis* did not agglutinate other *Vibrio* species in the H-agglutination tests. Further studies placed *V. fluvialis* and *V. furnissii* in the same lateral flagellar serogroup-HL8 (Shinoda et *al.*, 1992). However, in practice, serotyping based on H-flagella is also not in use.

A chemotaxonomic study based on sugar composition of the polysaccharide portion of their lipopolysaccharide (LPS) has divided 35 O-antigen groups of *V. fluvialis* into 21 chemotypes (Iguchi et al., 1993). This seems to be a unique finding since the D-glycero-D-manno-heptose, and two kinds of uronic acids, i.e., galacturonic and glucuronic acids are rare in Gram-negative bacteria. In addition, 2-keto-3-deoxyoctonate, which is a typical sugar component of Gram-negative bacterial LPS was not detectable in any of the chemotypes.

Of all the molecular typing methods, the pulsed-field gel electrophoresis (PFGE) has proven to be highly useful in typing the bacterial isolates. ToxR is an ancestral gene of the family Vibrionaceae which encodes a transcriptional activation domain (TAD), a transmembrane domain (TMD) and a periplasmic domain (PD) (Osario and Klose, 2000). Among vibrios there is a high level of homology within the TAD of the ToxR protein and relatively conserved homology in the TMD and PD (Osario and Klose, 2000). Interestingly, there is a high level of homology with in the TAD and TMD. This region connects the TAD to the cytoplasmic membrane and it was therefore named the membrane tether region. *toxR*-based species-specific identification has been developed for *Vibrio parahaemolyticus* (Kim et al.,

1999) and *Vibrio hollisae* (Vuddhakul et al., 2000). PCR-based assay for the specific identification of *V. fluvialis* exploiting the sequence divergence within the membrane tether region of the *toxR* gene (Chakraborty et al., 2006).

Chakraborty and other co-workers (2006) reported that the VF-*toxR* PCR showed 100% sensitivity for all the *V. fluvialis* strains tested and more importantly, the VF-*toxR* primers did not give positive amplicon with *Aeromonas* strains or with other vibrios. Unlike *V. cholerae* O1 and pandemic *V. parahaemolyticu*s, the isolates of *V. fluvialis* from acute diarrhoeal patients exhibited large genetic diversity (Chowdhury et al., 2013).

1.2.5 Sporadic Cases and Outbreaks of Diarrhoea

V. fluvialis was first isolated in Bangladesh during 1976-77, when it caused an epidemic of diarrhoea (Furniss et al., 1977). Early reports from the US indicated involvement of *V. fluvialis* with gastroenteritis among infants (Kolb et al., 1997). Since 1979, *V. fluvialis* was isolated as one of the important pathogens in Tenri Hospital, Japan (Aihara et al., 1991). Recent studies found that the prevalence of *V. fluvialis* among diarrhoeal patients were progressively increased in India. (Chowdhury et al., 2012). During 1996–1998, prevalence of *V. fluvialis* was 9.4% among hospitalized diarrhoeal patients in North Jakarta (Lesmana et al., 2002). In Zhejiang Province, China, *V. fluvialis* was identified as the second most pathogen (12%) among acute diarrhoeal cases but next to *V. parahaemolyticus* (64%; Jiang, 1991). Investigations carried out after the 1998 floods in Bangladesh showed involvement of *V. fluvialis* in a diarrhoeal outbreak (Tanabe et *al.*, 1999). However, the number of cases was less compared to *V. cholerae* O1 and O139 infections.

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 diarrhoea (Allton et al., 2006). Between 1982 and 1988, 10 gastroenteritis cases of *V. fluvialis* have been reported in Florida due to consumption of contaminated seafood (Klontz and Desenclos, 1990). 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 (Levine and Griffin, 1993). Foodborne outbreaks were reported in several communities implicating *V. fluvialis* alone or with either *V. parahaemolyticus/Salmonella* spp. (Tokoro et *al.*, 1984). Foodborne diarrhoeal outbreaks caused by *V. fluvialis* have been reported during 1981 in Maharashtra (Thekdi et al., 1990)

and 2012 in Kolkata (Chowdhury et al., 2013). In Brazil and USSR, the first report on the association of *V. fluvialis* with diarrhoea was reported during 1990 and 1991, respectively (Libinzon et al., 1991). Though the incidence of cholera among high socioeconomic population in Brazil was very low (0.07%), but the other vibrios including *V. fluvialis* comparatively prevailed more (1.2%; Magalhaes et al., 1993). In Volga delta, Russia, acute enteric infections caused by *V. fluvialis* reaches about 30% during the summer months, mainly due to consumption of water than sea/fresh water fishes (Boiko, 2000). Among travelers with diarrhoeal symptoms, the incidence of *V. fluvialis* seems to be low compared to other enteric pathogens. Early studies conducted with US Peace Corps volunteers in Thailand identified *V. fluvialis* in about 3% of the cases (Taylor et al., 1985).

1.2.6 *V. flluvialis* Enterotoxin

Enterotoxicity of the *V. fluvailis* organism was first reported by Sanyal et al., 1980 and later on confirmed by other workers (Agarwal and Sanyal, 1981; Lockwood et al., 1982; Kobayashi et al., 1983; Nishibuchi and Seidler, 1983; Chakraborty et al., 2005). Sanyal et al., 1980 demonstrated that enterotoxic substances is released by *V. fluvialis* organisms into the culture medium during 18 h period of incubation and this substances caused fluid accumulation into the rabbit gut. Agarwal and Sanyal (1981) characterized the nature of these enterotoxic substances to be heat labile (LT). The enterotoxic substances caused lysis of Chinese Hamster Overy (CHO) cells in tissue culture system. They also suggested that the enterotoxin elaborated by *V. fluvialis* organisms may act through mediation of cAMP. Chikahira and Hamada (1988) showed the diarrhoeal activity of *V. fluvailis* was not neutralized by anti CT.

Mc Nicol et al., (1980) studied two *V. fluvialis* organisms and found that neither *V. fluvialis* isolates elaborated an enterotoxin active in the Y1 adrenal cell assay. On the contrary, Seidler et al., (1980) observed that cuture supernatant of *V. fluvialis* organisms produced detectable responses in the Y1 mouse adrenal cell assay.

Nishibuchi and Seidler (1983) demonstrated that enterotoxin production by *V. fluvialis* organisms was culture medium dependent and found that clinical isolates produced detectable levels of enterotoxins when they had been grown in Brain Heart Infusion (BHI) broth but not when they had been grown in other media.

1.2.7. Possible Toxic Genes Associated with Enterotoxicity

1.2.7.1 Cholera toxin (CT) Gene (*ctxA*)

Cholera toxin (also known as choleragen and sometimes abbreviated to CTX, Ctx or CT) is a protein complex secreted by the bacterium *Vibrio cholerae*. CTX is responsible for the massive, watery diarrhoea characteristic of cholera infection. The cholera toxin is an oligomeric complex made up of six protein subunits: a single copy of the A subunit (part A, enzymatic), and five copies of the B subunit (part B, receptor binding), denoted as AB5.

Twenty five years after Koch's discovery, De (1959) was the first to demonstrate the existence of a toxin in a *V. cholerae* culture filtrate, by using rabbit ileal loops. Subsequently, many workers attempted to isolate and purify the cholera toxin that causes diarrhoea. Finkelstein and Lo Spalluto (1969) were the first to succeed. Their findings led to a series of studies by many researchers in the characterization of cholera toxin and its role in the pathogenesis of cholera caused diarrhoea (Takeda. 1984).

In the same year, 1959, researchers in Bombay described the production of diarrhoea in infant rabbits by a crude protein isolated from *V. cholerae* culture filtrate. A protein secreted by the *V. cholerae* was isolated and purified. Two factors were identified; the active one, denoted choleragen, was actually the AB5 form of the toxin, while the inactive one was the B5 oligomer, known as choleragenoid (Spangler, 1992).

The organization of the *ctxAB* gene in the *V. cholerae* O1 genome has been delineated (Pearson et al., 1993) and can be used as one of the differentiating criteria between the O1 biotypes (Mekalanos, 1983). The gene encoding cholera toxin (*ctx*) was located on a 4-5 kb DNA segment, flanked by two or more copies of a direct repeat sequence (RS) varying in length from 2.4 to 2.7 kb (Pearson et al., 1993). The 4.5 kb core region contains genes encoding the zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), an unknown ORF (*orf*U) and core-encoded pilus (*cep*), as well as *ctxAB* (Baudry et al., 1992). In most *V. cholerae* O1 strains this CTX genetic element was present in more than one copy. In strains of El Tor biotype the CTX genetic elements were arranged in tandem and in classical strains they were widely separated on the chromosome (Mekalanos, 1983). The organization of the CTX genetic elements in non-O1 vibrios was not known. Regarding the organization of the CTX genetic element in O139 strains, it had recently been reported (Lebens and Holmgren,

1994) that the *ctxAB* gene, together with the genes associated with the virulence cassette, was located at two loci, a situation similar to that in classical vibrios. In contrast, studies by Waldor and Mekalanos (1994) have indicated that the organization of the CTX genetic element in the genome of O139 strains was similar to that in El Tor vibrios.

The causative organism, *V. cholerae*, adheres to the brush border of villous absorptive cells. Adhesion was mediated by bacterial filaments that recognize carbohydrate receptors on microvillous membranes. Variations in specific types of filaments and their receptors account for host, site and age (neonate or adult) specificity. *V. cholerae* caused hypersecretion of electrolytes and water with little or no morphologic damage to the epithelium. The intestinal abnormalities causing diarrhoea were mainly regulatory, not structural. The toxin secreted by *V. cholerae* acts to alter regulation of secretion and absorption, with the net result being water and electrolyte loss through the intestines (Moon, 1997).

The cholera toxin (CT) produced by *V. cholerae* binds to gangliosides GM1 receptors. This, through an unknown mechanism, causes the enzymatic subunit to be clipped from the rest of the protein, and transferred into the cell. Once inside the cell, the subunit causes an increase in cellular cAMP, leading to over activity of luminal sodium pumps. The electrolytes in the gut cause water to leave the cells, and produce the characteristic diarrhoea.

The CT secreted by *V. cholerae* is composed of five identical B subunits and A subunit. The five B subunits form a pentagonal structure with a central hole. A subuit is composed of two parts. Subunit A2 is a long alpha-helix, which is anchored within the central hole of the B5 unit. Subunit A1 is, in the final secreted form, attached to A2 by single disulphide bind. The B pentamer is the receptor-binding portion of the molecule, while A1, once cleaved, is the enzymatically active portion. A2 appears merely to mediate the connection between the two (Spangler, 1992).

There was a delay of approximately 15 minutes between the binding of the holotoxin to the GM1 receptors on the cell surface and the rise in cAMP. It had been postulated that during that delay, A subunit was translocated across the membrane and reduced to form A1 (Kassis

et al., 1982). The disulphide bond between A1 and A2 keeps the protein inactive; this bond must be cleaved for the A1 subunit to become enzymatically active.

Bacterial toxins induced characteristic changes in the function of G proteins by catalyzing covalent modification of the alpha chains (Chang and Bourne, 1989). The G proteins were a family of membrane-bound nucleotide-binding proteins, which played key roles in transducing hormonal and sensory signals (Sugden et al., 1987). These proteins were heterotrimers of alpha, beta and gamma chains and are distinguished principally by their structurally distinct alpha chains and were distinguished principally by their structurally distinct alpha chains, which bound and hydrolyzed GTP. The more highly conserved beta and gamma chains serve to attach the G proteins to the cytoplasmic face of the plasma membrane and to present the alpha chain to the receptor. The hormone activated receptor promotes binding of GTP by the G protein alpha a chain, which in turn stimulates the appropriate effector enzyme; stimulation of the effector is terminated when the alpha chain hydrolyses its bound HTP (Chang and Bourne, 1989).

The A1 subunit of cholera toxin was an ADP-ribosyltranferase that catalyses the transfer of an ADP-ribose from NAD+ to residue Arg 187 in the alpha chain of Gs (Kassis et al., 1982). ADP-ribosylation of alpha chain of Gs by cholera toxin stabilizes the GTP-bound conformation of Gs and decreases its intrinsic GTPase activity, thereby producing increased stimulation of adenyl cyclase and elevated intracellular c-AMP (Chang and Bourne 1989; Merritt et al., 1994). The ribosylated G protein activated luminal sodium pumps via a cAMP-dependent protein kinase (Zhang et al., 1995). There was also electrogenic Cl- secretion leading to decreased electrolytes, and more electrolytes and water moved into the cell from the bloodstream. Eventually this depletes the body's electrolytes and causes dehydration.

Virulence genes, including *ctxAB*, were found among environmental strains. When a limited number of isolates was used, the occurrence of *ctxA* was also found among 10% of non-O1/O139 environmental isolates from coastal Brazil (Rivera et al., 2001). How *ctxA* genes are spread in an aquatic environment in an area of nonepidemicity is unclear.

1.2.7.2 Thermostable Direct Haemolysin (TDH) Gene (*tdh*)

Pathogenic strains of *V. parahaemolyticus* may have one or more distinctive traits compared with nonpathogenic strains, including the ability to produce themostable direct haemolysin (TDH, encoded by *tdh* gene), an extracellular protein, which is responsible for the Kanagawa phenomenon (a β-type haemolysis observed on Wagatsuma agar). TDH has been long suspected to be an enterotoxin involved in most cases of *V. parahaemolyticus* diarrhoea. TDH has been shown to have various biological activities, such as enterotoxigenicity, cytotoxicity, cardiotoxicity, lethality to small experimental animals, and ability to stimulate vascular permeability (Takeda, 1983).

Zen-Yoji *et al.* (1974) showed haemolysin might be the most important enteropathogenic factor for the symptoms caused by infection with *V. parahaemolyticus* that cause turbid, bloody fluid accumulated in the loop. Obara *et al.* (1974) showed that the haemolysin might be an important agent in causing gastroenteritis in food poisoning due to *V. parahaemolyticus* in human patients, although the sudden death of animals after administration of the haemolysin was not explained by these results. Sakazaki *et al.* (1974) studied the reactivity of ligated ileal loops of rabbits after injection of culture filtrates of various strains of *V. parahaemolyticus.* He found that culture filtrates of both Kanagawa phenomenon-positive and negative strains caused fluid accumulation in rabbit ileal loops. That was consistent with the fact that living cells of both Kanagawa phenomenon-positive and negative strains caused fluid accumulation in the rabbit ileal loop test although the rate of positive reactions were greater with the former than with the latter. It was well established that only Kanagawa phenomenon-positive strains produce the thermostable direct haemolysin (Sakurai *et al.,* 1974), so these results suggested that the thermostable direct haemolysin was not the only factor with enteropathogenic activity.

1.2.7.2.1 *tdh* gene in Other Vibrios

Like *V. parahaemolyticus, V. hollisae, V. mimicus* and *V. cholerae* non-O1 are commonly isolated from the marine environment and occasionally from diarrhoeal stool specimens. While all strains of *V. hollisae* so far examined, including an isolate from a coastal fish, were positive for *tdh* gene (Nishibuchi *et al.,* 1988). Only some strains of *V. mimicus* and *V. cholerae* non-O1 isolated from patients with diarrhoea contained sequences homologous to the *tdh* probe (Honda *et al.,* 1986). The *tdh* genes of the three species, like *V.*

parahaemolyticus tdh genes, had open reading frames of 567 bp, and the coding sequences were highly homologous to *V. parahaemolyticus tdh* genes.

1.2.7.3 TDH Related Haemolysin (TRH) Gene (*trh*)

TRH is a 48 kDa holotoxin with similar biological properties of TDH (Honda et al., 1988). TRH is immunologically similar biological properties of TDH, but the two haemolysin have significantly different physicochemical characteristics and lytic activities for various erythrocytes (Honda et al., 1988). The hemolytic activity of Vp-TRH was labile on heat treatment at 60^{o}C for 10 min, unlike that of Vp-TDH. The immunological similarities, but not the identities of Vp-TRH and Vp-TDH, were demonstrated by Oucherlony, neutralization, and latex agglutination tests.

The similarity of the two haemolysins was confirmed at the molecular genetic level, *tdh* and *trh* genes, encoding TDH and TRH respectively, shares approximately 70% nucleotide sequence homology (Kishishita et al., 1992;). The *trh* gene sequence varied considerably from strain to strain, but the *trh* gene sequences in various strains could be clustered into two subgroups represented by *trh1* and *trh2* genes, which shared 84% sequence identity (Kishishiya et al., 1992). Molecular epidemiological studies employed that the DNA probes specific to the *tdh* and *trh* genes revealed that not only strains carrying the *tdh* gene but strains carrying a *trh* genes were strongly associated with gastroenteritis; therefore, TDH as well as TRH were considered important virulence factors of *V. parahaemolyticus* (Shirai et al., 1990)

1.2.7.4 Shigella Toxin (STX) Genes (*stx1* and *stx2)*

Enterotoxigenic *E. coli* (EHEC) referred to a subset of Shiga toxin-producing *Escherichia coli* (STEC) strains found to cause human and sometimes animal disease (Terrance et al., 2002). They were linked to development of haemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) (Hussein et al., 2003) and thrombotic thrombocytopenic purpura (TTP), which required hospitalization and intensive care (Riley et al., 1983). STEC O157:H7 strains were first isolated from cattle in Argentina in 1977, although the strains were identified as such 10 years later (Bonardi et al., 1999). Several virulence factors had gained importance for the pathogenesis of the STEC infections (Paton and Paton, 1998). However, the complete list of bacterial virulence determinants necessary for STEC to cause EHEC-related disease was not known. Shiga toxin was the key factor in STEC pathogenesis

(Terrance et al., 2002). Shiga toxin was toxic to human colonic, ileal epithelial (Schmidt et al., 1999) and endothelial cells (Obrig, 1998).

Two main groups of Shiga toxins were harbored in STEC (Schmidt et al., 2000). Shiga toxin 1 (Ludwig et al., 2001) were 98% homologous to the Stx produce by *Shigella dysenteriae* type 1, while Stx2 was about 60% homologous with Stx1 and was antigenically different (Nataro and Kaper, 1998). Some studies had revealed that strains possessing only are potentially more virulent than strains harboring s*tx1* or even strains carrying both *stx1* and *stx2* (Ludwig et al., 2002)

The basic A-B subunit structure was conserved across all members of the Stx family. For the prototype toxin of the Shiga toxin family, the single 32-kDa A subunit is proteolytically nick to yield and approximately 28-kDa peptide (A1) and a 4-kDa peptide (A2); these peptides remain linked by a disulfide bond. The A1 peptide contains the enzymatic activity and the A2 peptide serves to bind the A subunit to B subunits (pentamer of five identical 7.2-kDa subunits). The B pentamer binds the toxin to a specific glycolipid receptor, globotriasosylceramide or Gb3 is the main receptor, which is present on the surface of eukaryotic cells. While Gb3, which is present on the surface of eukaryotic cells. While Gb3 is the main receptor for Stx, the Stx2e variant uses Gb4 as its receptor.

The structural genes for Stx1 and Stx2 were found on lysogenic lambdoid bacteriophages. Production of Stx1 from *E.coli* and *S. dysenteriae* was thought to be repressed by iron and reduced temperature but expression of Stx2 was unaffected by these factors.

1.2.8 Possible Other Toxin Production

1.2.8.1 Neurotoxin

Neurotoxins are substances that are poisonous or destructive to nerve tissue. Nerve tissue are found throughout the brain and nervous system, and the function of these unique cells is critical for a variety of tasks, ranging from autonomic nervous system jobs like swallowing to higher-level brain function. Neurotoxins were an extensive class of exogenous chemical neurological insults which adversely affected function in both developing and mature nervous tissue (Olney, 2002). The term also used to classify endogenous compounds which when abnormally concentrated that proved neurologically toxic. Though neurotoxins were

often neurologically destructive, their ability to specifically target neural components was important in the study of nervous systems (Kiernan et al., 2005).

Common examples of neurotoxins include:

- Chemical: lead, ethanol (drinking alcohol), glutamate, nitric oxide (NO).
- Biological: botulinum toxin (e.g. Botox), tetanus toxin, and tetrodotoxin.

Some substances such as nitric oxide and glutamate are in fact essential for proper function of the body and only exert neurotoxic effects at excessive concentrations. These toxic substances can eventually disrupt or even kill neurons, key cells that transmit and process signals in the brain and other parts of the nervous system. Neurotoxins inhibited neuron control over ion concentrations across the cell membrane (Kiernan et al., 2005) or communication between neurons across a synapse (Arnon et al., 2001).

1.2.8.1.1 Mechanisms of Activity

As neurotoxins are compounds which adversely affect the nervous system, a number of mechanisms through which they function are through the inhibition of neuron cellular processes. These inhibited processes can range from membrane depolarization mechanisms to inter-neuron communication. By inhibiting the ability for neurons to perform their expected intracellular functions, or pass a signal to a neighbouring cell, neurotoxins can induce systemic nervous system arrest as in the case of botulinum toxin (Arnon et al., 2001) or even nervous tissue death (Brocardo et al., 2011). The time required for the onset of symptoms upon neurotoxin exposure can vary between different toxins, being on the order of hours for botulinum toxin (Thyagarajan et al., 2009) and years for lead (Lewendon et al., 2001).

1.2.8.1.2 Neurotoxicity Studies Employing Intra Peritoneum (i.p.)

This is the most commom route being technically simple and easy. It allows quite long periods of absorption from the repository site. The rate of absorption by this route was usually one-half to one-forth the intravenous one (Woodard, 1965). Intra peritoneum injection studies attempt to determine the biochemical changes associated with the effects of repeat methanol exposures on the brain, retina, optic nerve (Rajamani et al., 2006) and the hypothalamus-pituitary-adrenal (HPA) axis of the neuroendocrine system (Parthasarathy et al., 2006). Another study determine whether a sustained increase in formate levels, at

concentrations below those known to produce toxic effects from acute exposures, could induced biochemical changes in the retina, optical nerve, or certain regions of the brain (Gonzalez-Quevado et al., 2002). Mice were treated with Methamphetamine MA using an experimental paradigm that produces substantial neurotoxicity (5 mg/kg, i.p.; three injections at 2 hr intervals) (Battaglia et al., 2002). There was some experimental evidence that the presence of methanol can affect the activity of acetylcholinesterase (Tsakiris et al., 2007). Although these experiments were carried out on erythrocyte membranes *in vitro*, the apparent compound-related changes may have implications for possible impacts of methanol and/or its metabolites on acetylcholinesterase at other centers, such as the brain.

1.2.8.1.3 Symptoms

Symptoms may appear immediately after exposure or be delayed. They may include limb weakness or numbness, loss of memory, vision, and/or intellect, uncontrollable obsessive and/or compulsive behaviors, delusions, headache, cognitive and behavioral problems and sexual dysfunction. In extreme cases, the results of exposure may include coma and eventual death as the nervous system shuts down. Especially when a neurotoxin inhibits the function of the autonomic nervous system, the body quickly starts to break down, because a number of important tasks are not being performed.

In the case of acute exposure, someone is exposed suddenly to a dose of a neurotoxin. A snake bite is an example of acute exposure. Chronic exposure involves slow exposure over time; heavy metals poisoning often takes the form of chronic exposure, with the unwitting victim taking in a small amount each day.

Additionally, neurotoxicity had been found to be a major cause of neurodegenerative diseases such as Alzheimer's disease (AD). Local pathology of neurotoxin exposure often included neuron excitotoxicity or apoptosis (Dikranian, 2001) but can also included glial cell damage (Deng et al., 2003). Macroscopic manifestations of neurotoxin exposure included widespread central nervous system damage such as mental retardation (Olney, 2002) persistent memory impairments, epilepsy and dementia.

1.2.8.2 Haemolysin

Haemolysins are certain proteins and lipids that cause lysis (destruction) of red blood cells and the release of their haemoglobin by damaging their cell membrane. Although the lytic activity of some microbial haemolysins on red blood cells may be important for nutrient acquisition or for causing certain conditions such as anemia, many haemolysin producing pathogens do not cause significant lysis of red blood cells during infection.

Haemolysis by *V. cholera*e has been the subject of a number of investigations because of its possible significance in differentiating this species from the less pathogenic vibrios, such as the El Tor *Vibrio*, and nonpathogenic water Vibrios. *Escherichia coli* haemolysin is potentially cytotoxic to monocytes, lymphocytes and macrophages, leading them to autolysis and death. Visualization of haemolysis of red blood cells in agar plates facilitates the categorization of *Streptococcus.*

1.2.8.2.1 Mechanism

1.2.8.2 .1.1 Pore Formation

Many haemolysins are pore-forming toxins (PFT) which is capable to cause the lysis of erythrocytes, leukocytes and platelets by producing pores on the cytoplasmic membrane. Lysis of erythrocytes by forming pores in phospholipid bilayers was described by Chalmeau et al., (2011). Formation of the pores by the *Staphylococcus aureus* was studied more precisely which causes many infectious diseases such as pneumonia and sepsis. Haemolysin is normally secreted by the bacteria in a water-resoluble way. Haemolysin can be segregated by many different kinds of bacteria such as *Staphylococcus aureus*, *Escherichia coli* or *Vibrio parahemolyticus* among other pathogens. *V. cholerae* cytolysin/haemolysin (VCC) is an extracellular membrane-damaging toxin expressed by the majority of *V. cholerae* El Tor and non-O1 strains. In infant mouse and rabbit ileal loop models, VCC was found to be responsible for the residual toxicity and diarrhoea observed after the administration of vaccine strains into the gastrointestinal system (Alm et al., 1991). Additionally, VCC seemed to be the major contributor to the lethality of streptomycin-fed adult mice after gastrointestinal exposure to high doses of El Tor strains (Olivier et al., 2007). One report found no biological, immunological, or physico-chemical differences between the haemolysins of non-O1 *V. cholerae* and O1 *V. cholerae* biotype El Tor (Yamamoto et al., 1986).

1.2.8.2.1.2 Enzymatic

Some hemolysins damage the erythrocyte membrane by cleaving the phospholipids in the membrane.

1.2.8.2.2 Classification

Visualization of haemolysis of red blood cells in agar plates facilitates the categorization of haemolysin. Depending upon the production of haemolysin microbes can be classified into 3 catagories :

a) Alpha (α) haemolytic : partial haemolysis of RBC form a green zone
b) Beta (β) haemolytic : complete haemolysis of RBC form a clear zone
c) Gamma (γ) haemolytic : no haemolysis

1.2.8.2.3 Role During Infection

Haemolysins are thought to be responsible for many events in host cells. For example, iron might be a limiting factor in the growth of various pathogenic bacteria (Sritharan, 2006). Red blood cells are rich in iron-containing heme. Lysis of these cells releases heme into the surroundings, allowing the bacteria to take up the free iron. But not only is haemolysin related to bacteria in this way but in some others. The main consequence of haemolysis is haemolytic anemia, condition that involves the destruction of erythrocytes and their later removal from the bloodstream, earlier than expected in a normal situation. As the bone marrow cannot make erythrocytes fast enough to meet the body's needs, oxygen do not arrive to body tissues properly. Consequently, some symptoms may appear such as fatigue, pain, arrhythmias, an enlarged heart or even heart failure, among others.

El Tor-like haemolysin was an enterotoxic factor for non-O1 *V. cholerae* gastroenteritis because (i) most non-O1 *V. cholerae* strains produced the haemolysin (Sakazaki et al., 1967), (ii) the purified haemolysin induced fluid accumulation in experimental animals, and (iii) the pathological features caused by the haemolysin were in good agreement with those in the infection model (Madden et al., 1984) and in patients with gastroenteritis (Blake, 1980). Bloody and mucous stool had been reported in non-O1 *V. cholerae* gastroenteritis (Blake, 1980). The accumulated fluid in the De tests was more or less, but invariably, mucous and bloody; and haemorrhage was observed in the mucosa. That indicated that the

haemolysin was cytotoxic to mucosal cells. The histological changes in the intestinal mucosa that were found in this study agree with previous observations. Also, it is possible that the bloody fluid accumulation was caused by haemolysin from El Tor vibrios. Histological findings of intestinal mucosa of ordinary positive loops challenged with CT indicated that they were almost intact.

Depending on the type of haemolysin and the microorganism that produces it, manifestation of symptoms and diseases may differ from one case to the other:

- Alpha-haemolysin from uropathogenic *E. coli* produces extra intestinal infections and can cause cystitis, pyelonephritis and septicemia.
  Alpha-haemolysin from *Staphylococcus aureus* can cause severe diseases, such as pneumonia.
- Aerolysin from *Aeromonas sobria* infects the intestinal tract, but it might also cause septicemia and meningitis.

(Both haemolysins mentioned above are synthetized by extracellular bacteria, which infect specific tissue surfaces.)

- Listeriolysin from *Listeria* monocytogenes (a facultative intracellular bacterium which thrives within host cells, mainly macrophages and monocytes) causes the degradation of phagosomes membranes, but they are not a potential danger for the cell's plasmatic membrane.

The haemolysin TDH, or Thermoestable Direct Haemolysin, is now being studied in the field of oncology. It's now said that Thermostable Direct Haemolysin (TDH), produced by *Vibrio parahaemolyticus*, regulates cell proliferation in colon carcinoma cells. Like *V. parahaemolyticus, V. hollisae, V. mimicus* and *V. cholerae* non-O1 are commonly isolated from the marine environment and occasionally from diarrhoeal stool specimens. While all strains of *V. hollisae* so far examined, including an isolate from a coastal fish, were positive for *tdh* gene (Nishibuchi *et al.*, 1988) only some strains of *V. mimicus* and *V. cholerae* non-O1 isolated from patients with diarrhoea contained sequences homologous to the *tdh* probe (Honda *et al.*, 1986;). TDH-related haemolysin (TRH) produced by a Kanagawa phenomenon-negative strain was discovered in the investigation of an outbreak of gastroenteritis in the Maldives Islands in 1985.

Kudoh et al. (1983) demonstrated haemolysin production by *V. flluvailis* strains and also

suggested that haemolysin produced by *V. fluvailis* organisms was immunologically different from Kanagawa haemolysin of *V. parahaemolyticus*. Chikahira and Hamada (1988) reported most strains of *V. fluvialis* regardless of origin liberated both haemolytic and mouse lethal toxins into the medium and the haemolysin is not so closely correlated with the cell-killing and mouse lethal activities. Haemolytic activity of on sheep erythrocytes was reported by Chakraborty and other co-workers (2005). Kothary and other co-workers (2003) reported that *V. fluvailis* haemolysin lysed a wide variety of erythrocytes and was cytotoxic towards CHO cells and also isolated 63 kDa molecular weight protein responsible for haemolytic activity.

1.2.8.3 Cytotoxin

Cytotoxicity is the quality of being toxic to cells.

1.2.8.3.1 Cell Physiology

Treating cells with the cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis).

Cells undergoing necrosis typically exhibit rapid swelling, lose membrane integrity, shut down metabolism and release their contents into the environment. Cells that undergo rapid necrosis *in vitro* do not have sufficient time or energy to activate apoptotic machinary and will not express apoptotic markers.

Apoptosis is characterized by well defined cytological and molecular events including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation and cleavage of DNA into regularly sized fragments.

Cells in culture that are undergoing apoptosis eventually undergo secondary necrosis. They will shut down metabolism, lose membrane integrity and lyse.

1.2.8.3.2 Measuring Cytotoxicity

Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity. Vital dyes, such as trypan blue or propidium iodide are normally

excluded from the inside of healthy cells; however, if the cell membrane has been compromised, they freely cross the membrane and stain intracellular components (Riss *et al.*, 2004). Alternatively, membrane integrity can be assessed by monitoring the passage of substances that are normally sequestered inside cells to the outside.

In this study, Baby Hamstar Kidney 21 (BHK 21) and HeLa cell line was used to observe to cytolytic effect. The BHK-21 fibroblast cell line was established in 1961. The widely used line was a subclone (clone 13) of a parental line derived from the kidneys of five unsexed, 1-day-old hamsters. The cultural properties of the cell line are adherent type. HeLa or hela cell, is a cell type an immortal cell line used in scientific research. The line was derived from cervical cancer cells taken from Henrietta Lacks, on 1951. This is epitheloid cell morphology and adherent type cell line.

The main difficulty is media exchange and adaptation to persuation systems. Cell metabolism is also affected by cell morphology. Recombinant Chinese Hamstar Overy (CHO) cells have been reported to have a 10 times lower specific productivity. These differences make microcarrier culture an attractive alternative. CultiSpher- G eliminates the major difficulties associated with microcarrier culture; scale up difficulties and low harvest yields. The large growth span obtained through macroporosity makes it possible to use scale up steps of more than 50 times. As CultiSpher- G is based on highly purified gelatin, enzymes that are very specific can be used for harvesting. This results in high cell yield with almost 100% viability.

Seidler et al. (1980) observed cytotoxic responses in Y1 adrenal cells. The same was observed cytotoxic by other workers (Nishibuchi and Seidler (1983). Agarwal and Sanyal (1981) observed lysis of CHO cells which indicated elaboration of cytotoxic factor(s) in *V. fluvailis* culture filtrates. Later on Lockwood et al. (1982) observed that cell-free extracts and culture supernatant fluid of *V. fluvialis*, grown in the absence and presence of lincomycin, indicated that the bacterium could produce a factor which causes CHO cell elongation, cytolysin(s) active against rabbit erythrocytes, non haemolytic, CHO cell killing factor(s) and proteases(s) active against azocasein. Wall et al. (1984) characterized the *V. fluvialis* cytotoxin. They found that the CHO cell killing factor was a heat labile and death by disruption of essential internal function(s) rather than by cytolysis. They also found *V. fluvialis* culture supernatant fluids to posses haemolytic and phospholipase A activities.

Cytotoxic and cell vacuolating activity of *V. fluvialis* on HeLa cells was first reported by Chakraborty et al. (2005).

1.2.8.4 Other Factors

Payne et al., (1984) reported some preliminary results of studies of an induration or permeability factor(s) presents in *V. fluvialis* filtrates, but could not establish if induration factor(s) was identical to enterotoxin activity.

Chikahira and Hamada, 1988 reported culture filtrates of most of the *V. fluvialis* had lethal activity and the mice injected died within 20 min.

1.2.9 Virulence Factors

The clinical as well as environmental *V. fluvialis* strains express many putative virulence factors. Several virulence factors have been identified in *V. fluvialis*, but the majority of them are only partially characterized and their precise role in virulence remains to be known. The common virulence factor in *V. fluvialis* reported in several investigations is the expression of haemolysin that can be easily identified in sheep-blood agar plates. Eukaryotic cell lines are being used *in vitro,* for most of the toxin detection assays. In cell-free extracts, *V. fluvialis* had expressed Chinese hamster ovary (CHO) cell elongation factor, CHO cell-killing factors, cytolysins against erythrocytes and proteases active against azocasein (Lockwood et al., 1982). Cytotoxic and vacuolating activity on HeLa cells by *V. fluvialis* was also reported (Chakraborty *et al.* 2005). Various putative virulence factors of *V. fluvialis* are presented in Table 1.1. However, the ability to produce these factors was not uniform in all the isolates (Liang et al., 2013).

Table 1.1: Different putative virulence factors described in *V. fluvialis*

Factor	Reference
Cytolysin	Lockwood et al. (1982)
Heat-labile cytotoxin	Wall et al. (1984)
Cytotonic	Venkateswaran et al. (1989)
Haemolysin	Wong et al. (1992)
Mucinase	Janda (1986)
Mannose sensitive	Rahman et al. (1992)
Cell adherence	Scoglio et al. (2001)
Cell vaculation	Chakraborty et al. (2005)

Purification of cytotoxin produced by *V. fluvialis* showed that the protein was heat-labile, and deactivated by proteases. The culture supernatant retained haemolytic and phospholipase A2 activities and were coeluted in the gel filtration (Wall et al., 1984). The purified extracellular haemolysin produced by *V. fluvialis* showed virulence features including lyses of erythrocytes of different animal origin and activation of fluid accumulation in suckling mice (Han et al., 2002). The haemolysin of *Vibrio fluvialis* (VFH) formed pores of 2.8–3.7 nm diameter in erythrocyte membrane that was larger than those formed by other *Vibrio* haemolysins such as *V. cholerae* (Zitzer et al., 1995), *V. parahaemolyticus* (Honda et al., 1992), *V. metschnikovii* (Miyake et al., 1989), *V. mimicus* (Shinoda et al., 1993), and *V. vulnificus* (Yamanaka et al., 1987). They suggested that VFH, a major haemolysin of *V. fluvialis*, was a pore forming toxin and induces osmotic lysis in erythrocytes (Han et al., 2002).

The utilization of heme compounds by *V. fluvialis*, although an iron acquisition system mediated by the catecholate siderophore fluvibactin has been reported (Yamamaoto et al., 1993). The identification of the heme utilization protein HupO, which mediates the acquisition of iron from hemin in *V. fluvialis* and had amino acid sequence homology to bacterial outer membrane heme receptors (Ahn et al. 2005). Currently, the recent characterization of an enterotoxigenic El Tor-like haemolysin in *V. fluvialis*, which represented one of the virulence factors of *V. cholerae* (Kothary et al., 2003), so the clinical symptoms of gastroenteritis are very similar to that of *V. cholerae.*

The transmembrane regulatory protein (ToxR) is essential for the expression of virulence factors in pathogenic vibrios. Similar to *V. cholerae*, the ToxR played a major role in bile resistance of *V. fluvialis*, which was an initial phase in the progression of vibrios as potential intestinal pathogens (Provenzano et al., 2000). Adaptability of vibrios to the intestinal environment, especially the bile salts favored colonization and expression of virulence factors. After initial adaptation to the bile salts under *in vitro* conditions, the *V. fluvialis* exhibited swarming mobility, biofilm formation and adherence (Pietro et al., 2004). In the animal models, *V. fluvialis* and the cholera toxin (CT) produced by *V. cholerae* O1 strains confirmed skin permeability factor (SPF). However, the antibodies against CT did not neutralize the SPF of *V. fluvialis* (Rodrigues et al., 1993).

The exocellular metalloprotease produced by *V. fluvialis* (VFP) was found to be similar to the one produced by *V. vulnificus*, which had also been used for the haemagglutination activity (Miyoshi et al., 2002). In addition, the amino acid sequence of VFP was found to be a member of the thermolysin family. It is interesting to note that most of the *V. fluvialis* isolated from the diarrhoeal patients harbored genes encoding haemolysin and metalloprotease (Chowdhury et al., 2012).

1.2.10 Sources and Route of Transmission

Poor sanitation and hygiene conditions as well as lack of or little environmental awareness among people is considered the major cause of source water contamination. Typical examples include agricultural practices that involve usage of sewage water and/or cattle manure on farms. Another practice is uncontrolled wastewater effluents discharges into water bodies, which serve as raw water sources to municipal water treatment systems; unhygienic/unconventional sanitation practices in fields; and grazing of cattle next to catchment areas (WHO, 2006). *V. fluvialis* infections are common in areas that have high levels of fecal contaminated water, food supplies and consumption of raw seafood or contaminated seafood products (WHO, 2006) as well as by person-to-person contact (Igbinosa and Okoh, 2008).

1.2.11 Pathogenesis

The symptoms of enteric disease attributed to *V. fluvialis* are similar to those caused by *V. cholerae* (Huq et al., 1980). Patients typically have watery diarrhoea with vomiting, abdominal pain, moderate to severe dehydration and often fever. From the enzyme-linked immunosorbent assay, reported that several *V. fluvialis* strains isolated from environmental and human sources produced an enterotoxin which is immunologically indistinguishable from cholera toxin (CT) (Chikahira and Hamada 1988). *V. fluvialis* produces several toxins that might be important in pathogenesis including an enterotoxin-like substance, lipase, protease, cytotoxin, and haemolysin (Kothary et al., 2003). It was reported that *V. fluvialis* had weak adhesiveness and no bacterial cytotoxicity, (Baffone *et al.*, 2001) but another report found it had strong haemolytic and proteolytic activity (Wong et al, 1992).

Two cases of fatal infection due to *V. fluvialis* had been reported (Klontz et al., 1994). It accounted for 10% of *Vibrio* gastroenteritis cases in a US survey (Altekruse et al., 2000). Unlike other *Vibrio* spp., which had commonly been reported to cause extra-intestinal infections, *V. fluvialis* was uniquely associated with gastroenteritis, with only rare reports of extra-intestinal infections such as haemorrhagic cellulitis with cerebritis, bacteremia, and peritonitis (Huang and Hsu, 2005, Lai et al., 2006, Ratnaraja et al., 2005, Tacket et al., 1982 and Lee et al., 2008).

1.2.12 Clinical Manifestations

1.2.12.1 Diarrhoea

V. fluvialis related illness is characterized by gastroenteritis, nausea, loss of appetite, vomiting, watery bloody diarrhoea with abdominal cramps or significant fever. 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 (Huq et al., 1980). Moderate to severe dehydration, hypokalemia, metabolic acidosis, and occasionally, hypovolemic shock can occur in 4 to 12 hours if fluid losses are not replaced. Stools are colourless, with small flecks of mucus and contain high concentrations of sodium, potassium, chloride, and bicarbonate (Igbinosa and Okoh, 2010).

1.2.12.2 Other Infections

V. fluvialis caused a variety of infections in immune-competent/HIV patients, including bacteremia, biliary tract infec-tion and acute diarrhoea (Liu et al., 2011). The other rarely reported infections caused by this pathogen include suppurative cholangitis (Yoshii et al., 1987), peritonitis (Lee et al., 2008), acute otitis (Chen et al., 2012) and endophthalmitis (Penland et al., 2000). In the wound infection (cellulitis) that was caused by direct inoculation of bacteria into the skin or exposure of a wound to contaminate water (Huang and Hsu 2005). The primary septicaemia syndrome consists of high fever and chills, often with vomiting, diarrhoea, abdominal pain and extremities pain (Morris and Black 1985) with no apparent focus of infection. *V. fluvialis* had been reported as causing necrotising fasciitis and septicaemia in the Gulf of Mexico and Southeast Asia, associated with minor trauma and exposure to fish, raw oyster, shellfish, crabs or seawater, especially in the summer months (Morris, 2003). Large numbers of (29%) endophthalmitis patients were reported to have mixed infection with *V. fluvialis* (Hassan et al., 1992). A report from Cuba showed that *V. fluvialis* was one of the predominantly identified pathogens from different extraintestinal samples (Cabrera et al., 2007). Cases of bacteremia with diarrhoea (Lai et al., 2006) haemorrhagic cellulitis and cerebritis (Huang and Hsu, 2005), peritonitis (Ratnaraja et al., 2005) had also been reported (Morris and Black, 1985). Major diagnostic clues for *V. fluvialis* sepsis syndrome are heamorrhagic bullae which can be seen both in sepsis and cellulitis.

1.3 Aims and Objectives

V. fluvialis is considered as major food borne pathogens that cause sporadic outbreaks of diarrhoea in several countries including Bangladesh. They are distributed in many countries in the world, predominantly in Asian countries, and the prevalence of *V. fluvailis* is also increasing in diarrhoeal patients over the year. But, the virulence factors and mechanism of pathogenecity of *V. fluvialis* are not yet clear.

Precisely, the study will address the following objectives:

- To isolate and identify the *V. fluvialis* from environmental sources
- To detect the enterotoxin
- To detect the *ctxA* and other toxic genes
- To detect the production of other toxin(s)

2. Materials and Methods

2.1 Sampling Area

Buriganga and Turag rivers surrounding the Dhaka city, shrimp field near Nalta area of Satkhira and ponds near Tala area of Khulna were selected for sample collection during the study. The study was carried out in the Department of Microbiology, University of Dhaka between July 2013 to December 2014.

2.2 Sampling Procedure and Transportation

For sample collection, 50 ml sterile falcon tubes, glass marker, field note book, polythene bags etc. were taken to the sampling sites. Samples were collected from different locations following methods described in Environmental Protection Agency (EPA) manual (Bordner and Winter, 1978).

After collection, all samples were labeled appropriately, kept in an insulated cool foam box in the field, and transported to the laboratory. Samples were processed within 8-12 h from the time of collection.

2.3 Sample Processing and Culture

Samples were processed following standard method as described by Refai (1979) and Islam *et al.* (1992). Fifty gm of sample was enriched in 50 ml of alkaline peptone water (APW).

The processed samples were incubated at 37^{o}C for 18-24 h. After incubation, loopfull of enriched samples were streaked on Thiosulfate Citrate Bile salt (TCBS) agar (Kobayashi *et al.*, 1963) and incubated at 37^{o}C for 18-24 h. After incubation, characteristic yellow colonies were isolated from TCBS agar plate and were subcultured on *Vibrio* Chromogenic agar for discrete white colonies, which was indicative of desired organisms.

2.4. Biochemical Identification of *V. fluvialis* Organisms

2.4.1 Oxidase Test

The enzyme oxidase, present in certain bacteria catalyses the transport of electron from donor bacteria to the redox dye tetra-methyl-p-phenylenediamine dihydrochloride. The dye in the reduced state has a deep purple colour.

For oxidase test, inoculated nutrient agar plates were incubated at 37^{o}C for 24 h. A portion of the colony was taken with a sterile glass rod and rubbed on a strip of a filter impregnated with freshly prepared solution of 1% N,N,N,N-tetra-methyl-p-phenylenediamine dihydrochloride (Appendix II). The positive result was indicated by the production of dark blue colour within 7 sec and no change in colour was interpreted negative for the test.

2.4.2 Triple sugar Iron (TSI) Agar Test

The triple sugar iron (TSI) agar test is designated to differentiate among the different groups or genera of the Enterobacteriaceae and to distinguish the Enterobacteriaceae from other Gram-negative intestinal-bacilli. This difference is made on the basis of difference in carbohydrate fermentation patterns and hydrogen sulfide production by the various groups of intestinal organisms. TSI agar medium contains lactose and sucrose in 1% concentrations and glucose in a concentration of 0.1%. To facilitate the observation in carbohydrate utilization pattern, TSI agar medium is made with slant and butt. TSI agar medium also contains sodium thiosulfate and ferrous sulfate for detection of hydrogen sulfide production, which is indicated by blackening of medium.

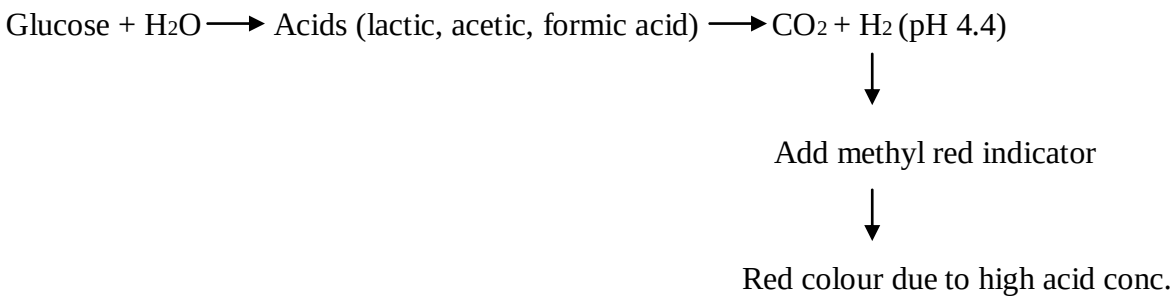
2.4.3 Citrate Utilization Test

The citrate agar test determines the ability of microorganisms to utilize citrate as the carbon source. Simmon's citrate agar contains sodium citrate as the sole source of carbon, ammonium dihydrogen phosphate as the sole source of nitrogen and the pH indicator bromothymol blue. When microorganisms utilize citrate, they remove the acid from the medium, which raises the pH and the pH indicator from green to blue. If there is no colour change, the organism is citrate negative.

2.4.4 Methyl Red (MR) Test

The methyl red test is used to determine the ability of microorganisms to oxidize glucose with the production and stabilization of high concentrations of acid as end products. Some bacteria ferment glucose and initially produce organic acid as end products that lower the pH of the medium below 5.0. During the later incubation period, bacteria convert these acids to non-acidic end products, resulting in an elevation of pH of approximately 6.0. The addition of pH indicator as methyl red is used to detect this acidity. The methyl red indicator in the pH range of 4.0 will turn red and pH 6.0 turns yellow.

For this test, MR-VP broth medium in each tube were inoculated and incubated at 37^{o}C for 24-48 h. After incubation few drops of methyl red solution (Appendix II) was added in each tube and shaken well. A distinct red colour throughout the broth indicated the positive result and yellow or any yellowish red colour indicated negative result.



2.4.5 Voges-Proskauer (VP) Test

The VP test is used to detect a specific organism that carry out 2, 3- butanediol fermentation. When bacteria ferment sugars, it produced 2, 3-butanediol that accumulate in the medium. The addition of 40% KOH and a 6% solution of alpha-naphthol in absolute ethanol will reveal the presence of acetoin (acetyl methyl carbinol). The acetoin in the presence of KOH, will develop a pink colour imparting a rose colour to the medium.

For this test, VP medium in each was inoculated with fresh culture and incubated at 37^{o}C for 24-48 h. After incubation 0.6 ml of 6% alcoholic α-naphthol solution was added to each tube followed by 1 ml of potassium hydroxide creatine solution. The tubes were then shaken vigorously for 1-2 min. Appearance of crimson ruby colour in the tube indicated positive result that indicated the production of acetyl methyl carbinol (acetoin).

2.4.6 Salt Tolerance Test

All isolates were tested for their salt tolerance in nutrient broth and agar containing 0%, 3%, 5%, 7% and 10% (w/v) sodium chloride. Fresh culture was inoculated into a nutrient broth and agar, and growth was observed visually after 24 h of incubation at 37^{o}C.

2.4.7 Fermentation of Carbohydrate

Fermentation test is of considerable significance in the identification and classification of bacteria and actinomycetes. The microorganisms differ in their ability to ferment different sugar.

1) Monosaccharide : Glucose
2) Disaccharide : Sucrose

To perform this test, methyl red was used as an indicator. One Durham's tube was used in each of the fermentation tubes. The tubes were then inoculated in duplicates with 24-48 h old cultures and incubated at 37^{o}C for 24 to 48 h. The change of the colour of indicator from red to colourless or yellow indicated the production of acid. Red colour indicated alkalinity. If carbon dioxide was produced it would be collected in the Durham's tube.

2.5 Anlytical Profile Index (API) 20E Identification of *V. fluvialis* Organisms

The API 20E system facilitates the 24 h identification of Enterocacteriaceae as well as 24 or 48 h identification of other Gram negative bacteria. The API 20E strip consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity and carbohydrate (CHO) fermentation. The biochemical tests investigated with API 20E system are: β-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H_2S production (H2S), urease (URE), tryptophane deaminase (TDA), indole production (IND), Voges-Proskauer (VP), gelatinase (GEL), glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), saccharose (SAC), melibiose (MEL), anygdalin (AMY), arabinose (ARA) and cytochrome oxidase (OX). The substrates are reconstituted by adding a bacterial suspension. After incubation, the metabolic end products are detected by indicator systems or the addition of reagents. CHO fermentation is detected by colour change in the pH indicator.

The procedure for the identification was described below:

2.5.1 Preparation of Inoculum

1. Five ml of 0.85% saline was added to a sterile test tube.
2. Then the center of a well isolated colony was carefully touched using a sterile inoculation loop.

2.5.2 Preparation of Strip

1. An incubation tray and lid were supplied for each strip.
2. Then 5 ml of water was dispensed in to the tray.

2.5.3 Inoculation of the Strip

1. Bacterial suspension was taken and inoculated into the strip with a 5 ml Pasteur pipette. Note: The ADH, LDC, ODC, H2S and URE reaction can be interpreted best if these microtubes are slightly underfiled.
2. Both the tube and cupule section of CIT, VP and GEL tubes were filled.
3. After inoculation, the cupule section of the ADH, LDC, ODC, H2S and URE tubes with mineral oil were filled.
4. An agar slant or plate was inoculated by using the excess bacterial suspension as a purity check and for oxidase testing, and additional biochemical reaction. Then incubated the slant or plate for 18-24 h at 37^{o}C.

2.5.4 Incubation of the Strip

1. After inoculation, the plastic lid was placed on the tray and incubated the strip for 18-24 h at 37^{o}C.
2. Weekend incubation: The biochemical reactions of the API 20E should be read after 18-24 h incubation. If the strips could not be read after 24 h incubation at 37^{o}C, the strips should be removed from the incubator and stored at 2-8^{o}C until the reactions could be read.

2.5.5 Reading the Strip

1. After 18 h of incubation and before 24 h incubation, all reactions not requiring the addition of reagents were recorded.

2. If the GLU tube was negative (blue or green), reagents was not added. It was re-incubated for further 18-24 h.
3. If the GLU is positive (yellow)
   i. Oxidase test was performed
   ii. The reagents to TDA and VP tubes were added. If positive, the TDA reactions will be immediate, whereas the VP reaction may be delayed up to 10 min.
   iii. The Kovac's reagent should then be added to the IND tube.
   iv. The nitrate reduction test should be performed on all oxidase positive organisms. The reagents should be added to the GLU tube after the Kovac's Reagents has been added to the IND tube.

2.6 Molecular Identification of *V. fluvialis* Organisms using Polymerase Chain Reaction (PCR)

PCR based method for species-specific identification was developed for the identification of organism that targeted to the *toxR* gene of *V. fluvialis*. The target sequence for *toxR* present in DNA was amplified by PCR with *in vitro* synthesis of DNA, complementary to 217 bp fragment of *toxR* gene (Chakraborty et al., 2006).

2.6.1 Preparation of Template DNA

One ml fresh culture grown at 37^{o}C for 18 h was transferred to a micro-centrifuge tube and centrifuged for 10 min at 13,000 rpm. Discarding the supernatant, the pellet was resuspended in 1 ml Phosphate buffer saline (PBS). Then the suspension was boiled in water bath at 100^{o}C for 10 min and immediately transferred into ice-tube for 30 minutes. When it was cooled down it was centrifuged at 10,000 rpm for 10 min and the supernatant was transferred into a sterile 1.5 ml micro-centrifuge tube. This DNA was used for PCR amplification and stored at -20^{o}C.

2.6.2 Preparation of Reaction Mixture

The primers used for PCR are given in Table 2.1. Oligonucleotide primers were obtained in a lyophilized form and resuspended in Tris-EDTA (TE) buffer or in water to get the working concentration. A master mix, including all the reagents for PCR reaction except the sample DNA, was prepared according to Table 2.2 and was aliquoted into PCR tubes before

adding different extracted DNA templates. After a brief spin, the PCR tubes were placed in a thermal cycler (Bio-Rad, USA).

Table 2.1: Primers used for PCR amplification of *toxR* gene sequences

Target gene	Nucleotide sequence (5´-3´)	Amplicon size (bp)
toxR $_{FWD}$	GACCAGGGCTTTGAGGTGGACGAC	217
toxR$_{REV}$	AGGATACGGCACTTGAGTAAGACTC	

Table 2.2: Components of PCR reaction mixture for amplification of *toxR* gene

Components	Volume (µl)
Sterile deionized water	14.6
Gene Taq Universal buffer (×10)	0.20
dNTP mixture (2.5 mM each)	2.5
Primer 1	2.5
Primer 2	2.5
Gene Taq DNA polymerase (1 units/µl)	0.2
Template DNA	2.50
Total	25.0

2.6.3 Thermocycling Parameters

The PCR reaction was performed according to the following program:

94°C…….5 min ……..initial denaturation

94°C…….... 40 sec

65°C…… 40 sec } 30 cycles

72°C..... 1 min

72°C…….... 3 min……final extension

After this, PCR tubes were stored at -20^{o}C until further analysis.

2.6.4 Post - PCR Detection of Amplified *toxR* Sequence by Electrophoretic Analysis

The PCR products were analyzed on a 2% agarose gel electrophoresis to detect specific band for *toxR* sequence.

2.6.4.1 Preparation of Agarose Gel

A 2% agarose gel was prepared by melting 2.0 gm agarose (Sigma, USA) in 100 ml 1× TBE buffer (Appendix-II). The melted agarose was allowed to cool to about 50°C and was poured into gel electrophoresis unit (Sigma, USA) with spacers and comb. After solidification of the gel, the comb was removed and wells were formed. Then the gel was submerged in 1× TBE buffer in a running gel tank.

2.6.4.2 Loading and Running the Sample

Five µl of PCR product was mixed with 1µl of 6× gel loading dye (Appendix-II). The mixture was slowly loaded into the well using disposable micro-pipette tips. Marker DNA of known size (Invitrogen, USA) was loaded in one well to determine the size of the PCR products. Electrophoresis was carried out at 90 volts.

2.6.4.3 Staining and Visualization of the Gel

After electrophoresis, the gel was submerged in staining solution containing ethidium bromide (Et-Br) (Appendix-II), for 15 minutes. Destaining was performed by submerging the gel in distilled water for about 15 minutes. The EtBr stained DNA bands were observed on a UV transilluminator (Vilber Lourmat, France). Photographs were taken using a gel documentation system (Vilber Lourmat, France) and bands were analyzed.

2.7. Preservation of *V. fluvialis* Isolates

2.7.1. Short-Term Preservation

Two ml of semi-solid preservation medium (pH 8.8) containing 0.5% APW, 1% NaCl and 0.45% agar was taken in one vial. Bacterial growth of each isolate was taken from nutrient agar plate with a sterile needle and the medium was inoculated by stabbing 2-3 times. Then the vial was incubated at 37°C for 6 h. After incubation, the surface of the medium was covered with sterile paraffin oil and the vial was stored at room temperature.

2.7.2. Long-Term Preservation

Five hundred µl of liquid preservation medium (pH 8.8) containing 0.5% APW and 1% NaCl was taken in acryovial and was inoculated with bacterial growth of each isolate. Then the cryovial was incubated at 37°C for 6 h. After incubation, 500 µl of sterile glycerol was added into the medium and the cryovial was stored at -20°C.

2.8. Preparation of Inocula

2.8.1 Preparation of Inocula of Live Cells

Inocula for live cells for all samples were prepared by incubating the cells in Brain Heart Infusion (BHI) Broth for 6 h at 37^{o}C with a rotation of 180 rpm (rotation per minute).

2.8.2 Preparation of Culture Filtrates

Culture filtrates were prepared by following the method of Sanyal *et al.* (1980).

Twenty five ml of BHI broth contained in a 100 ml Ehlenmeyer flask were inoculated with 5 ml of 6 h growth of the organisms in BHI broth with shaking at 37^{o}C and 180 rpm.

The cultures were centrifuged at 10,000 rpm for 10 minutes and the supernatant was filtered through membrane filter of 0.20 µm pore size and then preserved at -20^{o}C.

2.9 Detection of Enterotoxin

2.9.1 Adult Rabbit Ileal Loop (RIL) Assay

Methods described by De and Chatterje (1953) and Singh and Sanyal (1978) were used for the experiment. Albino rabbit (New Zealand strain) of 2-3 kg body weight was fasted overnight allowing water. After proper anesthesia with a lower dose of sodium pentobarbital (0.5 ml/kg weight, intravenous), the intestine were exposed and loops of 6-8 cm in lengths with 2 cm intervals (inter loops) between each were tied beginning near the ileocaecal junction. Usually 6-8 loops were made per rabbit. One ml of live cells grown in BHI broth and culture filtrates prepared in BHI broth were injected in each loop. After inoculation, the small intestine of the animal was carefully pushed back inside the abdomen and then the incision was sutured. The animal was kept in its cage and supplied with water. The animal was sacrificed after 18 h with excess (2× amount of dose used for anesthesia) of sodium

pentobarbital. Volume of the fluid accumulated and length of each loop was measured to determine the amount of fluid accumulated per unit length of gut. Inocula of toxigenic *V. cholerae* strain 569B and BHI broth used as positive and negative controls respectively. If there was any accumulation of fluid in any segment of intervals in between the loops or the negative control loop or negative reaction in positive control loop, the results were discarded. Each test was done in three rabbits and separate sets of rabbits were used for live cells and culture filtrates as inocula.

V.cholereae 569B was used as positive control and BHI broth was used as negative control.

2.10 Enhancement of Enterotoxicity by Passaging through Rabbit Gut

Two strains which gave poor reactions in ileal loops during the first series of experiments were serially passaged in rabbit gut loops following the methods of Singh and Sanyal (1978). The methodology for consecuive passaging in brief:

On sacrificed the of the animals after 18 h, the contents of the negative gut loops were streaked aseptically on a TCBS agar plate and incubated over night at 37^{o}C.

Five or six smooth colonies were inoculated in BHI broth, grown for 6 h at 37^{o}C and subjected to ileal loop tests as described earlier until positive responses were obtained.

2.11 Detection of Cholera Toxin Gene (*ctxA*) by DNA Amplification using Polymerase Chain Reaction (PCR)

The presence of *ctxA* was examined by DNA amplification described by (Keasler and Hall *et al.*, 1993). The target sequence for *ctxA* present in DNA was amplified by PCR with *in vitro* synthesis of DNA, complementary to 564 bp fragment of *ctxA* gene.

Template of DNA was prepared as mentioned before (Section 2.6.1).

2.11.1 Preparation of Reaction Mixture

The primers were used for PCR are given in the Table 2.3 Oligonucleotide primers were obtained in a lyophilized form and resuspended in Tris-EDTA (TE) buffer or in water to get the working concentration. A master mix, including all the reagents for PCR reaction except the sample DNA, was prepared according to Table 2.4 and was aliquoted into PCR tubes

before adding different extracted DNA templates. After a brief spin, the PCR tubes were placed in a thermal cycler (Bio-Rad, USA).

Table 2.3: Primers used for PCR amplification of *ctxA* gene sequences

Target gene	Nucleotide sequence (5´-3´)	Amplicon size (bp)
ctxA $_{FWD}$	CTCAGACGGGATTTGTTAGGCACG	564
ctxA$_{REV}$	TCTATCTCTGTAGCCCCTATTACG	

Table 2.4: Components of PCR reaction mixture for amplification of *ctxA* gene

Components	Volume (µl)
Sterile deionized water	16.875
Gene Taq Universal buffer (×10)	2.50
dNTP mixture (2.5 mM each)	2.00
Primer 1	0.5
Primer 2	0.5
Gene Taq DNA polymerase (5 units/µl)	0.125
Template DNA	2.50
Total	25.0

2.11.2 Thermocycling Parameters

The PCR reaction was performed according to the following program:

94°C…….3 min ……..initial denaturation

94°C……..... 1 min
55°C…… 1 min } 35 cycles
72°C..... 1 min

72°C……..... 3 min……final extension

After this, PCR tubes were stored at -20^{o}C until further analysis.

2.11.3 Post - PCR Detection of Amplified *ctxA* Sequence by Electrophoretic Analysis

The PCR products were analyzed by 1.5% agarose gel electrophoresis to detect specific band for *ctxA* gene sequence according to the protocol described in section 2.6.4.

2.12 Detection of Other Toxic Gene(s) Associated with Enterotoxin

After detection of *ctxA* gene, other common toxic genes associated with enterotoxicity were determined.

2.12.1 Detection of *tdh* and *trh* Virulence Gene using Polymerase Chain Reaction (PCR)

The presence of *tdh* and *trh* was examined by DNA amplification described by Tada and coworkers (1992). The target sequence for *tdh* and *trh* present in DNA was amplified by PCR with *in vitro* synthesis of DNA, complementary to 251 bp and 250 bp fragment of *tdh* and *trh* gene respectively.

Template of DNA was prepared as mentioned before (Section 2.6.1).

2.12.1.1 Preparation of Reaction Mixture

The primers were used in this experiment were given in Table 2.5 and Table 2.6. Master mix, including all the reagents was prepared as mentioned the Table 2.7. The master mix and template DNA containing PCR tubes were placed in thermal cycler (Bio-Rad, USA).

Table 2.5: Primers used for PCR amplification of *tdh* gene sequences

Target gene	Nucleotide sequence (5´-3´)	Amplicon size (bp)
tdh_{FWD}	GGTACTAAATGGCTGACATC	251
tdh_{REV}	CCACTACCACTCTCATATGC	

Table 2.6: Primers used for PCR amplification of *trh* gene sequences

Target gene	Nucleotide sequence (5´-3´)	Amplicon size (bp)
trh_{FWD}	GGCTCAAAATGGTTAAGCG	250
trh_{REV}	CATTTCCGCTCTCATATGC	

Table 2.7: Components of PCR reaction mixture for amplification of *tdh* and *trh* genes

Components	Volume (µl)
Sterile deionized water	17.375
Gene Taq Universal buffer (×10)	2.50
dNTP mixture (2.5 mM each)	2.00
Primer 1	0.25
Primer 2	0.25
Gene Taq DNA polymerase (5 units/µl)	0.125
Template DNA	2.50
Total	25.0

2.12.1.2 Thermocycling Parameters

The PCR reaction was performed according to the following program:

94°C…… 3 min ……..initial denaturation

94°C……..... 1 min
55°C…… 1 min } 35 cycles
72°C..... 1 min

72°C……..... 3 min……final extension

After this, PCR tubes were stored at -20°C until further analysis.

2.12.1.3 Post - PCR Detection of Amplified *tdh* and *trh* Sequences by Electrophoretic Analysis

The PCR products were analyzed by 1.5% agarose gel electrophoresis to detect specific band for *tdh* and *trh* genes sequence according to the protocol described in section 2.6.4.

2.12.2 Detection of *stx1* and *stx2* Virulence Gene using Polymerase Chain Reaction (PCR)

The presences of stx1 and stx2 genes were examined by DNA amplification described by (Vidal et. al. 2004). The target sequence for *stx1* and *stx2* present in DNA was amplified by PCR with *in vitro* synthesis of DNA, complementary to 348 bp and 584 bp fragment of *stx1* and *stx2* gene respectively.

Template of DNA was prepared as mentioned before (Section 2.6.1).

2.12.2.1 Preparation of Reaction Mixture

The primers used in this experiment were given in Table 2.8 and Table 2.9. Master mix, including all the reagents was prepared as mentioned the Table 2.10.

The master mix and template DNA containing PCR tubes were placed in thermal cycler (Bio-Rad, USA).

Table 2.8: Primers used for PCR amplification of *stx1* gene sequences

Target gene	Nucleotide sequence (5´-3´)	Amplicon size (bp)
stx1 $_{FWD}$	CAGTTAATGTGGTGGCGAAGG	348
stx1$_{REV}$	CACCAGACAAATGTAACCGCTC	

Table 2.9: Primers used for PCR amplification of *stx2* gene sequences

Target gene	Nucleotide sequence (5´-3´)	Amplicon size (bp)
stx2 $_{FWD}$	ATCCTATTCCCGGGAGTTTACG	584
stx2$_{REV}$	GCGTCATCGTATACACAGGAGC	

Table 2.10: Components of PCR reaction mixture for amplification of *stx1* and *stx2* genes

Components	Volume (µl)
Sterile deionized water	17.875
Gene Taq Universal buffer (×10)	2.50
dNTP mixture (2.5 mM each)	2.00
Primer 1	0.25
Primer 2	0.25
Gene Taq DNA polymerase (5 units/µl)	0.125
Template DNA	2.50
Total	25.0

2.12.2.2 Thermocycling Parameters

The PCR reaction was performed according to the following program:

94°C…… 3 min ……..initial denaturation

94°C……..... 1 min

55°C…… 1 min } 35 cycles

72 °C..... 1 min

72°C……..... 3 min……final extension

After this, PCR tubes were stored at -20^{o}C until further analysis.

2.12.2.3 Post - PCR Detection of Amplified *stx1* and *stx2* Sequences by Electrophoretic Analysis

The PCR products were analyzed by 1.5% agarose gel electrophoresis to detect specific band for *stx1* and *stx2* genes sequence according to the protocol described in section 2.6.4.

2.13 Detection of Other Toxin(s)

2.13.1 Detection of Neurotoxin

For the detection of neurotoxin, mouse lethality assay was done. This is called the indirect method for neurotoxin detection.

2.13.1.1 Mouse Lethality Assay

Swiss albino mice of 3-4 weeks were used for the assay. In this experiment, 0.1-0.5 ml of culture filtrate was injected intraperitonealy in each mouse and observed for 2-5 days for any physical change viz: paralysis of muscles. *E. coli* O157:H7 was used as positive control and BHI broth was used as negative control.

2.13.2 Detection of Haemolysin

Haemolysin detection was done by following two methods:

2.13.2.1 Tube Haemolysin Method

For the measurement of haemolytic 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^{o}C for 1 h followed by centrifugation at 2,000 rpm for 5 min. OD of the supernatant was measured for released hemoglobin with spectrophotometer at 450 nm.

2.13.2.2 Blood Agar Plate Method

Blood agar plate was used to demonstrate the alpha and beta haemolytic reaction of haemolytic organisms. It was performed by simply streaking the single isolated colony on the media followed by overnight incubation at 37^{o}C. Complete clear and partial clear zone around the colony was an indicative of beta and alpha haemolysin production respectively.

2.13.3 Detection of Cytotoxin

Culture filtrates of five *V. fluvialis* strains, as mentioned above, were tested for cytotoxin production in BHK-21 and HeLa cell monolayers. The assay in principle is that a monolayer of BHK-21 and HeLa cell was carried in a 25 cm^2 tissue culture flask under 4-5 ml of DMEM (Dulbecco Modified Egales) medium with 10% Fetal Bovine Serum (FBS) and antibiotics. When measured quantity of the prepared toxin is added to the cell suspension in a microtitre plate and incubated overnight, a toxin positive preparation will cause necrosis/elongation of the cells.

2.13.3.1 Propagation of BHK -21 and HeLa Cells

One ml of trypsinized cell suspension (from a stock BHK-21 cell and HeLa cell) was added to a 30 ml flat tissue culture flask containing 4.5 ml of DMEM medium (Appendix I) with 10% Fetal Bovine Serum and antibiotics (100 units/ml Penicillin, 100 µg/ml Gentamycin and 100 µg/ml Streptomycin) (Appendix II). On incubation at 37^{o}C with 5% CO_2, (Shel Lab CO_2 incubator) a monolayer of BHK-21 and HeLa cells spreaded all over the surface of the tissue culture flask within 3-4 days which could be seen under inverted microscope (KYOW Optilab TR-T).

2.13.3.2 Trypsinization of BHK-21 and HeLa Cells

After the cells grown for 3-4 days, the DMEM medium was decanted with Pasteur pipette. Then the cells were gently washed using 4-5 ml PBS (a balanced salt solution without calcium and magenesium). Washing solution was added gently to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth for several times. The wash step removes any traces of serum, calcium, and magnesium that would inhibit the action of the dissociation reagent. After that, wash solution was removed and discarded from the culture vessel and 800 µl of trypsin solution was added. The flask was then put in the incubator at 37^{o}C for 2-5 min with the monolayer side of the flask was in upper side for detachment of cell from the surface of the flask. If the cell not detached from the surface of the flask, it was tilted 10-12 times gently for 5-6 sec and observed under microscope.

2.13.3.3 Preparation of Inocula for Microtiter Plate Inoculation and for Assay

The flask was then taken out of the incubator and 3-4 ml of 10% DMEM media was added. Then the suspension was gently mixed and taken into 15 ml falcon tube. The suspension was centrifuged at 1000 rpm for 2-3 minutes. Then the supernatant was discarded and pellet was suspended with 10% DMEM. It was mixed properly with pipette to get uniform cell suspension. After proper mixing 10 µl of cell suspension was taken and poured on the counting chamber into the sample introduction point of Neubauer Hemocytometer by pipette. Cells were counted under an inverted microscope (KYOW Optilab TR-T) for the 4 cornered squares marked and diluted the cells to get 3.5×10^4 cells/450 µl.

Then 450 µl of cell suspension was inoculated into each well of 24 well cell culture plate, and gently shaken to cover the surface of well, observed under an inverted microscope and incubated at 37°C in a humid atmosphere with 4-5% CO_2.

2.13.3.4 Toxicity assay

After getting the confluent growth of cells, the culture filtrates of *V. fluvialis* were diluted to 10, 50, 100 folds in DMEM media. Then 50 µl of culture filtrate was delivered into each well of the cell culture plate in duplicate and mixed properly. The plate was then incubated for 18-20 h at 37°C in a humid atmosphere with 4-5% CO_2. The cells were then examined under an inverted microscope for necrosis, elongation, or rounding.

2.14 Concentration of Culture Filtrate

For the concentration of culture filtrate one representative strain (S-10) was taken which gave higher amount of toxic activities than others.

2.14.1 Concentration of *V. fluvialis* (S-10) Culture Filtrates by Ammonium Sulfate Precipitation Method

For the concentration of culture filtrate, we used synthetic medium in stead of BHI medium. One hundred ml of synthetic medium (Appendix I) was taken in a 500 ml of conical flask and was seeded with 5-6 colonies of the *V. fluvialis* (S-10), grown on TCBS agar plates. The flasks were incubated at 37°C for 42-44 h with constant shaking at 120 rpm and the cultures were then centrifuged at 8,000 rpm for 10 min. The supernatants were pooled and 80% saturated with ammonium sulphate, where the pH was maintained at 7.2 with ammonia solution. The suspension was held overnight at 4°C and centrifuged at 8,000 rpm for 10 min at 4°C. The precipitate was dissolved in 2-3 ml of 0.04 M phosphate buffer saline (PBS) at pH 7.2 and dialysed against the same buffer for 24-36 h at 4°C with 5-6 changes of buffer. The dialysate was passed through a membrane filter (Millipore) of 0.20 µm average pore diameter and stored at -20°C. The protein content was estimated by the method of Bio-Rad Protein Assay, based on Bradford method.

2.15 Protein Estimation of Concentrated Culture Filtrate

Bio-Rad Protein assay was used to estimate the protein concentration of concentrated culture filtrate.

2.15.1 Bio-Rad Protein Assay

The Bio-Rad Protein Assay, based on the method of Bradford, is a simple and accurate procedure for determining concentration of solublized protein. It involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a spectrophotometer. Comparison to a standard curve provides a relative measurement of protein concentration.

The procedure of the protein assay was described below:

1. At first, dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts distilled, deionized (DDI) water. Then it was filtered through Whatman #1 filter (or equivalent) to remove particulates. This diluted reagent may be used for approximately 2 weeks when kept at room temperature.
2. After that, five dilutions (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of a protein standard, BSA was prepared which was representative of the protein solution to be tested. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml.
3. Standard and sample solution of 100 µl of each was taken into a clean, dry test tube. Protein solutions were assayed in duplicate.
4. Then 5.0 ml of diluted dye reagent was added to each tube and vortex.
5. The mixtures were incubated at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
6. Abrobance was measured at 595 nm in spectrophotometer.

2.16 Determination of Toxic Activities of Concentrated Culture Filtrate

The toxic activities that we found for culture filtrates of *V. fluvialis* were also detected for concentrated culture filtrate.

2.16.1 Detection of Enterotoxin

Enterotoxic activity of concentrated culture filtrate was determined by rabbit ileal loop assay (RIL) as described in section 2.9.

2.16.2 Detection of Neurotoxin

Neurorotoxic activity of concentrated culture filtrate was determined by mouse lethality assay (indirect method) as described in section 2.13.1.

2.16.3 Determination of Haemolysin

Haemolytic activity of concentrated culture filtrate was determined by both tube method of hemolysin and in blood agar plate method as described in section 2.13.2.

2.16.4 Detection of Cytotoxin

Cytotoxic activity of concentrated culture filtrate was determined by cell culture assay as described in section 2.13.3.

2.17 Protein Profiling of Concentrated Culture Filtrate by SDS-PAGE Analysis

To determine the protein profile of the concentrated culture filtrate of *V. fluvilais*, they were subjected to SDS-PAGE analysis followed by Coomassie Blue Staining.

2.17.1 Preparation of Separating Gel

1. At first, clean and fresh glass plates were assembled in the casting chamber.
2. A 12.5% separating gel was constructed by primarily gently mixing the distilled water, lower gel buffer along with 10% SDS and 30% acrylamide-bisacrylamide solution according to table given bellow (Table 2.11):

Table 2.11: Composition of separating gel

Ingredients	Amount
Distilled water	2.4 ml
Lower gel buffer (pH 8.8)	1.875 ml
30% acrylamide bis-acrylamide solution	3.15 ml
10% SDS	0.15 ml
10% Ammonium per sulfate (APS)	45.0 µl
TEMED	16.0 µl

3. Then, the solution was mixed well and degassed. After making the solution gas-free, the beaker was not shaked.
4. Next step was followed by rapid addition of the 10% freshly prepared Ammonium per sulfate (APS) and N, N, N, N-Tetramethyl-ethyleneddiamine (TEMED).
5. As soon as APS and TEMED were added to the mixture the freshly mixed solution was carefully poured immediately into the glass plate chamber with a pipette. The gel mixture was then poured to a level of 3 cm below the top edge of the glass plates. Precaution was taken during pouring the gel mixture to avoid bubble production.
6. It was then carefully overlaid by saturated butanol to avoid gel drying which results in a roughness on the surface of the gel.
7. It was then left undisturbed for about 1-2 h to allow polymerization of the gel. A very sharp liquid-gel interface was visible with naked eye when polymerization was completed.
8. Then the butanol, excess water and any unpolymerized acrylamide were rinsed from the gel plates and extraneous moisture was removed with a piece of 1 mm Whatman filter paper was poured off or soaked.

2.17.2 Preparation of the Stacking Gel

After the polymerization of the separating gel has been completed, a stacking gel mixture was prepared.

1. Just like the separating gel, stacking gel mixture was also prepared by primarily mixing the distilled water, upper gel buffer along with 10% SDS and acrylamide bis-acrylamide solution according to the Table 2.12. Then it was mixed well and degassed as previously stated.
2. After that, 10% APS and TEMED was added promptly
3. The gel mixture was then rapidly poured above the previously constructed separating gel.
4. A 10 well comb was then placed between the two glass plates carefully so that no bubbles could take place inside the comb channels. The gel was then allowed to sit undisturbed for 1-1.5 h for polymerization.

Table 2.12: Composition of stacking gel

Ingredients	Amount
Distilled water	2.13 ml
Lower gel buffer (pH 8.8)	937.0 µl
30% Acrylamide bis-acrylamide solution	625.0 µl
10% SDS	37.0 µl
10% Ammonium per sulfate (APS)	22.0 µl
TEMED	8.5µl

2.17.3 Sample Preparation

1. The bacterial protein preparation was thawed just before loading.
2. The protein sample (10 µl) was mixed with 2×sample loading buffer at a ratio of 1:1 and was then boiled in water bath for 3 min for denaturation of the protein. During boiling, precaution was taken so that the sample does not bump up.
3. Five µl of tracking dye (0.1% Bromophenol blue) was then added to the boiled mixture.

2.17.4 Sample Loading

1. After the polymerization of the stacking gel has been completed, it is ready for sample loading.
2. Before loading the sample, the comb was removed from the glass chamber with soft hand, so that the well dividers do not crack.
3. The glass chamber was then fixed in the electrophoresis unit and gently placed in the buffer reservoir.
4. The wells were filled with the running buffer by pouring running buffer (Appendix II) inside two glass chamber of the electrophoresis unit (BioRad Mini-Protean II cell) up to the top edge of the glasses. Running buffer was also poured in to the main buffer reservoir up to 1/3rd of the height of the reservoir.
5. Twenty five µl of sample was then added to each wells with a micropipette.
6. The first column from left was loaded with 5 µl of Molecular weight standard (Thermo scientific, USA).

2.17.5 Running the Gel

1. After the loading process was done carefully, the electrophoresis unit was connected with the power pack adjusting the current at 15-20 mA keeping the voltage supply free.
2. The power supply was maintained for 1.5-2.0 h. As soon as the tracking dye reached the bottom level of the gel, the power supply was turned off. Time was depending upon the descent of the materials.

2.17.6 Staining and De-staining of the Gel

1. After the gel run had been completed, the gel was released from the glass plates and immersed in a fresh staining solution (0.1% Coomassie Brilliant blue). The gel was then shaken on a horizontal shaker for 1 h at room temperature.
2. Then the gel was taken out of the staining solution and flooded with de-staining solution (7% Acetic acid) and shaken overnight on a horizontal rotary shaker at room temperature. When the gel background became transparent, it was taken out of the de-staining solution and immersed in distilled water.

2.18 Determination of Molecular Weight

To determine the relative mobility (Rf) of the protein, distance of the protein migrated was divided by the distance of the tracking dye migrated.

$$\text{Relative mobility (Rf)} = \frac{\text{Distance of migrated protein}}{\text{Distance of the migrated tracking dye}}$$

The Rf were plotted (x-axis) against the known molecular weight (y-axis) in the graph paper. A calibration curve was prepared by using molecular weight standards. The molecular weight of the unknown protein was estimated from linear calibration curve.

3.0 Results

The present study has been delineated to isolate *V. fluvialis*, the causative agent of food borne gastroenteritis and diarrhoea, isolated from samples of the brackish environment as well as from the rivers, pond and shrimp field of Bangladesh and characterization of their toxin(s).

3.1 Primary Isolation of *V. fluvialis* from Environment

Over 100 samples were collected from Dhaka, Satkhira and Khulna area for the isolation of *V. fluvialis* from environmental samples. Details of sample collection are documented in Table 3.1.

After collection, samples were enriched in alkaline peptone water (APW) and from simple culturing on TCBS those appear as green colonies were presumptively *V. parahaemolyticus*, *V. mimicus* and were not of concern.

The main targets were the organisms giving initially yellow colonies on TCBS (Figure 3.1) and white on CHROM^{TM} agar for *Vibrio* (figure 3.2). The yellow colonies on TCBS agar are due to the organism's ability to ferment sucrose as a carbon source. However, *V. fluvialis, V. furnissii* and *A. hydrophila* give same colony making the identification difficult.

The colony properties of *V. fluvialis* on TCBS and CHROM^{TM} agar are given Table 3.2.

Table 3.1: Sampling date, location and types of samples

Sampling date	Location	Kinds	Salinity	Sample in saline water	Water Temperature (^{o}C)	Outside Temperature (^{o}C)
11.11.2013	Turag river	Root of water hyacinths	0.0%	100 ml	23.6	27.1
		Sediment		50 gm + 50 ml	20.5	25.7
15.11.2013	Satkhira (Near Nalta)	Sediment beside of shrimp field	0.4%	50 gm + 50 ml	24.7	29.1
15.11.2013	Satkhira (Near Nalta)	Shrimp of shrimp field	0.5%	One + 100 ml	24.7	29.1
		Sediment of shrimp field		50g + 50ml	24.6	
15.11.2013	Tala (Pond) 25 km far from Khulna	Root of water hyacinths	0.0%	100ml	22.5	31.1
		Sediment		50 gm + 50 ml		
21.11.2013	Buriganga river	Sediment	0.0%	50 gm + 50 ml	24.1	26.1

Table 3.2: Colony characteristics of *V. fluvialis* on TCBS and CHROM™ agar plate

Properties	Growth on TCBS agar	Growth on CHROM agar
Size	Moderate	Moderate
Elevation	Raised	Raised
Form	Circular	Circular
Margin	Entire	Entire
Optical characteristics	Opaque	Opaque
Color	Yellowish	White

3.2 Biochemical Identification of the *V. fluvialis*

After isolation of organisms, suspected colonies were subjected to biochemical identification including API 20E.

Yellow colonies from TCBS agar and white colonies from CHROM agar plates were subjected to extensive biochemical tests.

The main distinguishing feature between *V. fluvialis* and *V. furnissii* with *Aeromonas sp.* is that *Aeromonas sp.* is not capable of growing at 7% and 10% NaCl, while the *Vibrio*'s can. The growth of *V. fluvialis* on 7% NaCl is given in Figure 3.3. All the strains of *V. fluvialis* showed no growth on 0% NaCl containing media.

V. fluvialis and *V. furnissii* are simply differentiated from each other by the production of gas from simple sugar fermentation (Figure 3.4).

Identification based on conventional biochemical test perhaps not that trust worthy so the isolates that were confirmed as *Vibrio* were reconfirmed using the API 20E.

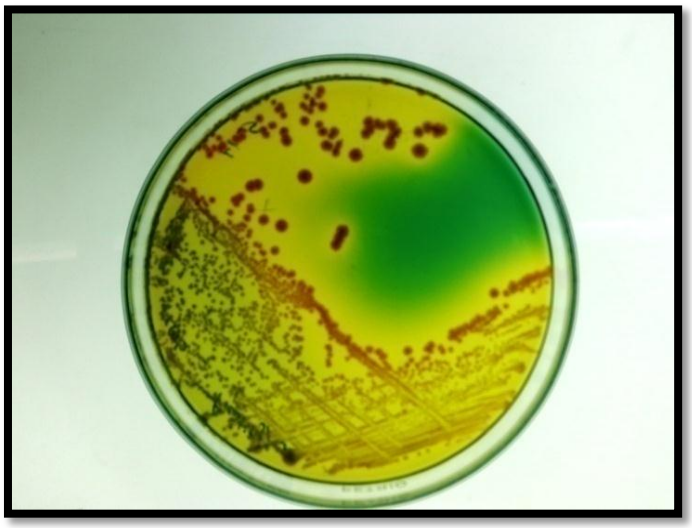


Figure 3.1: Characteristics growth of *V. fluvialis* on TCBS agar

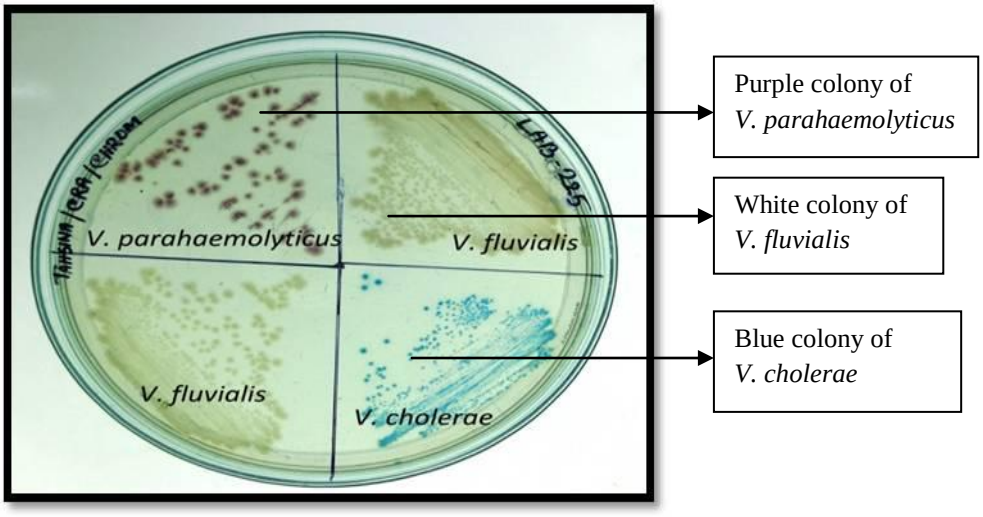


Figure 3.2: Characteristics growth of different *Vibrio sp.* on CHROM™ agar for Vibrios

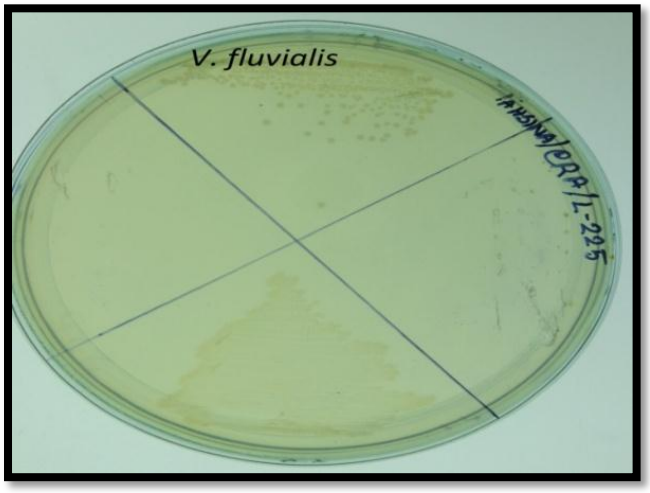


Figure 3.3: Growth of *V. fluvialis* on nutrient agar plate with 7% NaCl

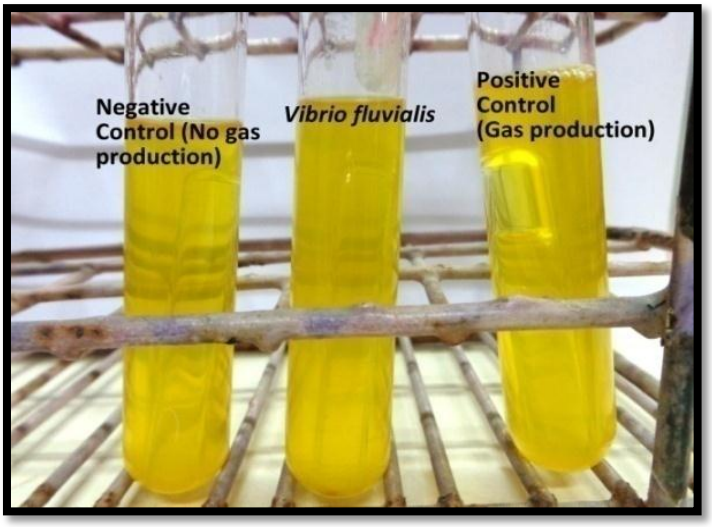


Figure 3.4: Glucose fermentation by *V. fluvialis* without gas production

3.3 Molecular Identification of *V. fluvialis*

Five isolates which showed positive results for *V. fluvialis* in the biochemical tests including API 20E, were tested for the presence of *toxR* gene, using its specific primers. All 5 isolates formed DNA bands (217 bp), specific for *toxR* after amplification and subsequent gel electrophoresis (Figure 3.5).

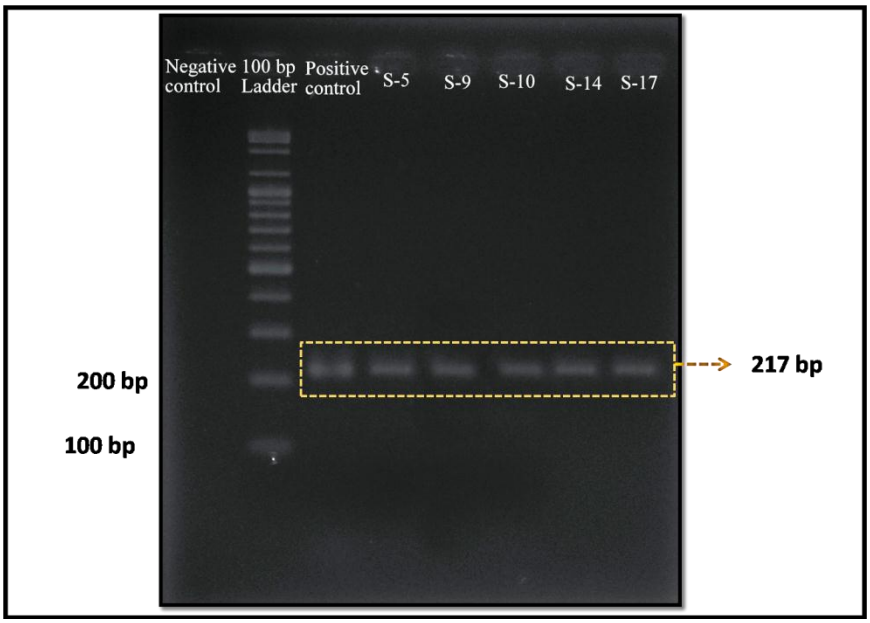


Figure 3.5: Agarose gel electrophoresis pattern of PCR amplicons of isolates with primers specific for *toxR* gene

Identification findings of isolates by biochemical characteristics including API 20E kit and molecular identification are given in Table 3.3.

From these five isolates were confirmed as *V. fluvialis* and taken for further study that indicated as S-5, S-9, S-10, S-14 and S-17. Among the *V. fluvialis*, S-5 was obtained from the downstream sediment of Turag river, S-9 and S-10 were from sediment of beside the shrimp field, S-14 was from sediment of pond in Khulna and S-17 was from the sediment of Buriganga river.

Table 3.3: Identification findings of *V. fluvialis, V. furnissii* and *Aeromonas hydrophila*

Isolate no.	TCBS	CV	TSI	Gas	Citrate	MR VP	Oxi dase	Growth in NaCl Conc.						VF-*toxR*	API 20E
								0%	3%	5%	7%	8%	10%		
S-3	Y	W	+/+	+	+	+	+	-	+	+	+	+	+		*V. furnissii*
S-5	**Y**	**W**	**+/+**	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	***V. fluvialis***
S-7	Y	WB	+/+	+	+	+	+	-	+	+	-	-	-		*A. hydrophila*
S-9	**Y**	**W**	**+/+**	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	***V. fluvialis***
S-10	**Y**	**W**	**+/+**	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	***V. fluvialis***
S-13	Y	WB	+/+	+	+	+	+	-	+	+	-	-	-		*A. hydrophila*
S-14	**Y**	**W**	**+/+**	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	***V. fluvialis***
S-16	Y	W	+/+	+	+	+	+	-	+	+	+	+	+		*V. furnissii*
S-17	**Y**	**W**	**+/+**	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	***V. fluvialis***
S-18	Y	WB	+/+	+	+	+	+	-	+	+	-	-	-		*A. hydrophila*
S-20	Y	WB	+/+	+	+	+	+	-	+	+	-	-	-		*A. hydrophila*
Ref. strain	**Y**	**W**	**+/+**	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	***V. fluvialis***

CV-Chrom *Vibrio*, Y-Yellow, W-White, WB-White Blue

3.4 Detection of Enterotoxin

3.4.1 Rabbit Ileal Loop (RIL) Assay with Live Cells

Live bacteria obtained from 4-6 h culture of *V. fluvialis* caused fluid accumulation in the loops (Figure 3.6). The range of mean ratio of fluid accumulation per cm of gut varied from strain to strain (Fluid accumulation ratio, FA 1.2-3.23) and rabbit loop to rabbit loop (Figure 3.7). All the strain of *V. fluvialis* caused fluid accumulation but the fluid accumulating activity by three isolates (S-5, S-9 and S-10) were comparable to the positive control *V. cholerae* 569B strain. The others (S-14 and S-17), however, caused relatively smaller amounts of fluid accumulations. Positive control was used as *V. cholerae* 569B in the first loop and BHI media which was used to prepare inoculums of live cells was the negative control. Purpose of using the medium as (-)ve control was to determine whether the medium itself has any such activity.

3.4.2 Passage in Ileal Loops

Two strains (S-14 and S-17) which gave little fluid accumulation in ileal loops during the first series of experiments became positive and accumulation of fluid was enhanced on three consecutive passsages in ileal loops (Figure 3.8), although the fluid accumulation was less dramatic.

3.4.3 Rabbit Ileal Loop (RIL) Assay with Culture Filtrates

Culture filtrates were prepared from all the 5 strains of *V. fluvialis* gave positive ileal loop reactions. The fluid accumulation varied from strain to strain (FA ratio 0.5-1.3) (Figure 3.9). But the culture filtrates gave less amount of fluid accumulation than the live cells.

A.

B.

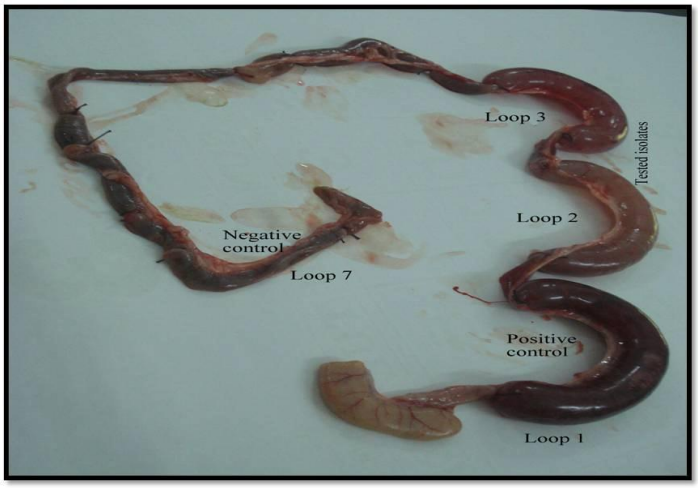


Figure 3.6: Rabbit Ileal Lop (RIL) assay

A) Inoculation in Rabbit ileal loop

B) Fluid accumulation in loops.

Loop 1 contains live cells of *V. cholerae* 569B

Loop 2, 3 contains live cells of *V. fluvialis* and

Loop 7 contains BHI medium

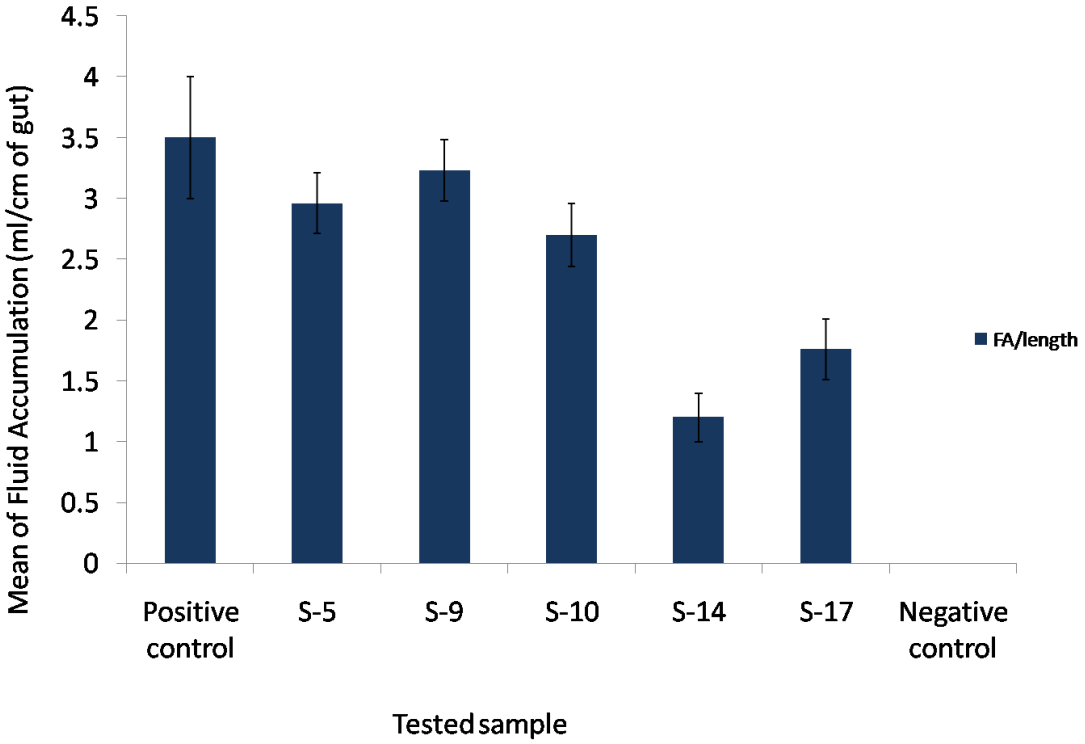


Figure 3.7: Graphical representation of mean ratio of fluid accumulation (FA) per length by live cells

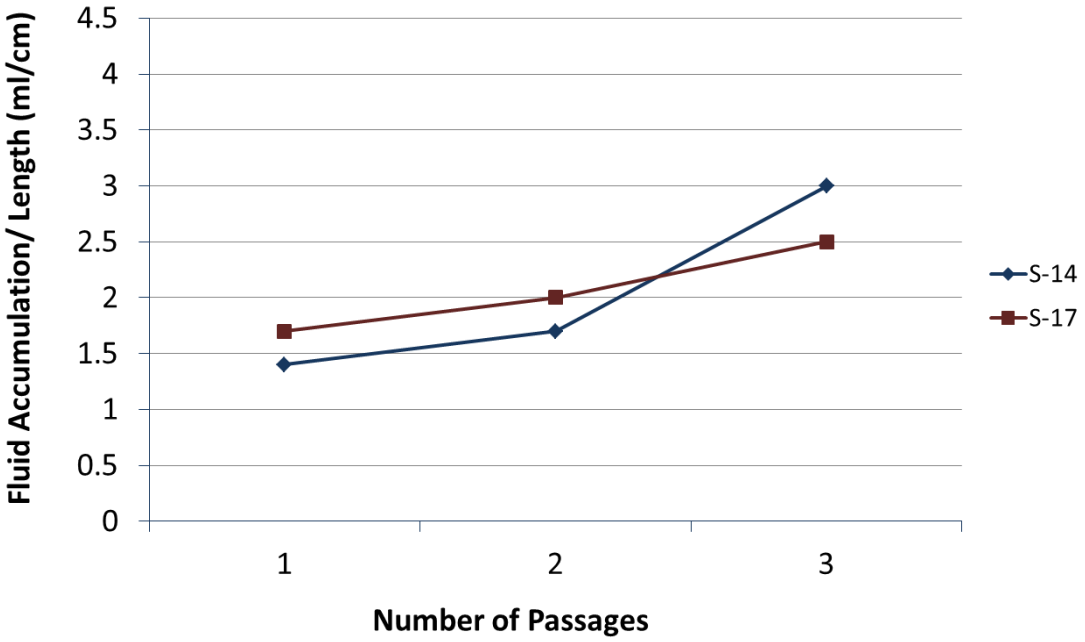


Figure 3.8: Graphical representation of the effects of consecutive passage on fluid accumulation through rabbit gut

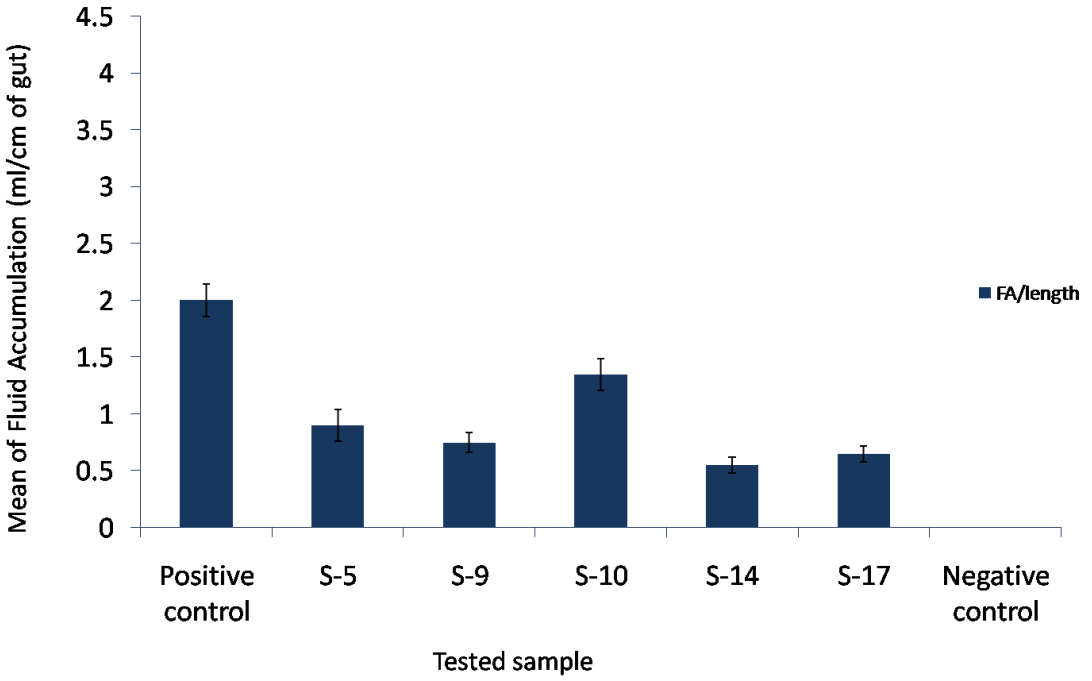


Figure 3.9: Graphical representation of mean ratio of fluid accumulation (FA) per length by culture filtrate

3.5 Detection of *ctxA* Gene

PCR analysis followed by agarose gel electrophoresis failed to provide the appearance of any desired band from the *V. fluvialis* organisms except in positive control, indicating lack of the *ctxA* gene in tested *V. fluvialis* organisms (Figure 3.10).

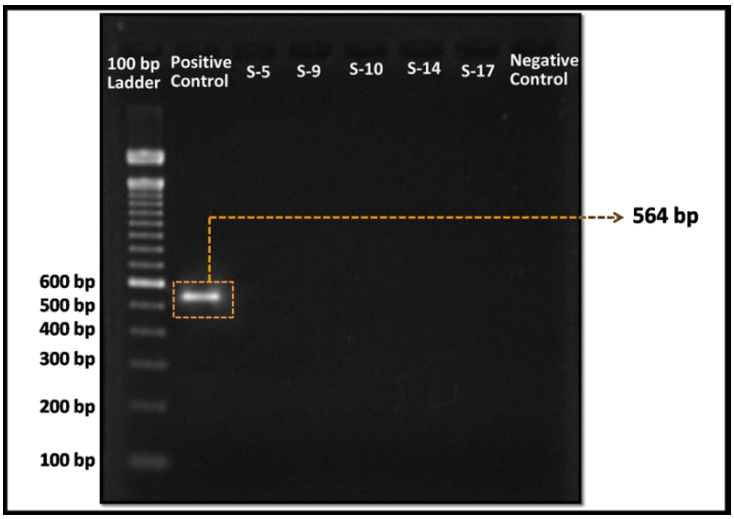


Figure 3.10: Agarose gel electrophoresis pattern of PCR amplicons with primers specific for *ctxA*

3.6 Detection of Other Toxic Gene(s)

Other toxic gene(s) that are commonly associated with enterotoxin were detected by PCR assay.

3.6.1 Detection of *tdh* and *trh* Genes

All five isolates showing negative results in *ctxA* gene were also observed for the presence or absence of *tdh* and *trh* virulence genes after 35 cycles of amplification using specific primer.

Amplified fragment of 251 bp was detected for *tdh* and 250 bp was detected for *trh* after agarose gel electrophoresis for positive controls only (figure 3.11).

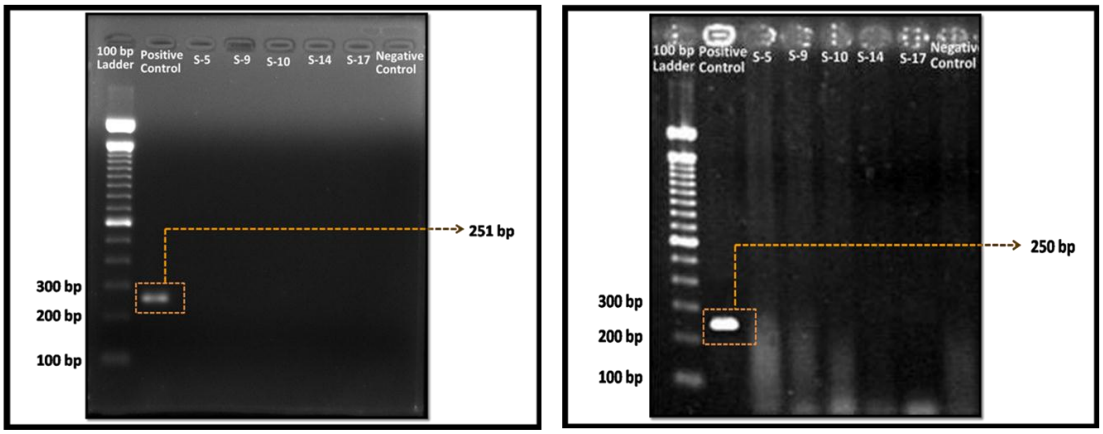


Figure 3.11: Agarose gel electrophoresis pattern of PCR amplicons with primers specific for (A) *tdh* (B) *trh*

3.6.2 Detection of *stx1* and *stx2* Genes

All five isolates showing negative results in *ctxA, tdh* and *trh* gene were also observed for the presence or absence of *stx1* and *stx2* virulence genes after 35 cycles of amplification using specific primer.

Amplified fragment of 348 bp was detected for *stx1* and 584 bp was detected for *stx2* after agarose gel electrophoresis only for positive controls (figure 3.12).

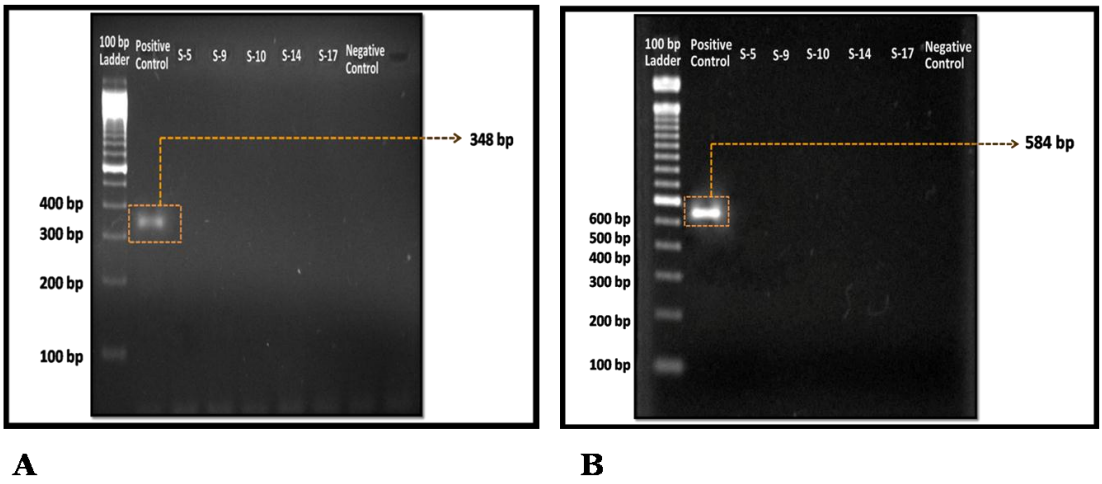


Figure 3.12: Agarose gel electrophoresis pattern of PCR amplicons with primers specific for (A) *stx1* (B) *stx2*

3.7 Detection of Other Toxin(s)

3.7.1 Detection of Neurotoxin

Neurotoxic activity was detected by mouse lethality assay that is the indirect method of neurotoxin detection.

3.7.1.1 Mouse Lethality Assay

Mice injected with culture filtrates of all isolates of *V. fluvialis* showed some sort of dizziness and paralysis of hind limb (Figure 3.13). Within 2-3 days, death of 14 mice out of 20 by *V. fluvialis* culture filtrates was also observed (Table 3.4). Here positive control was *E. coli* O157:H7.

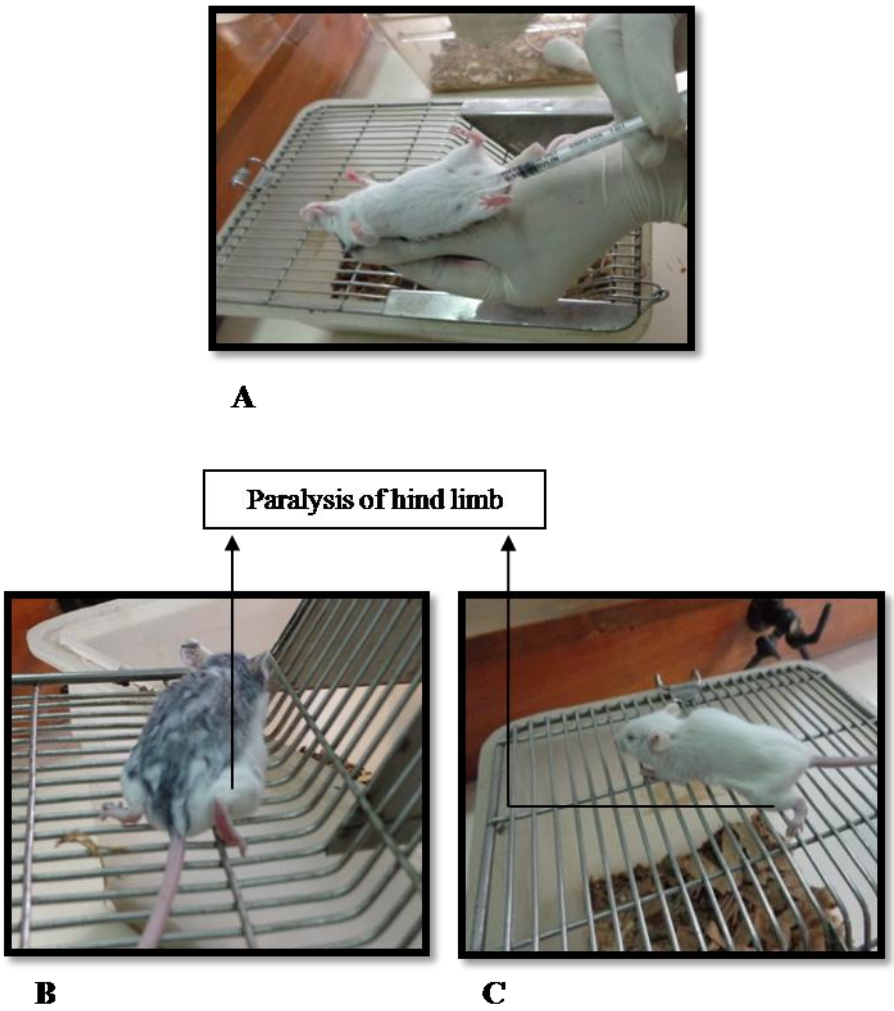


Figure 3.13: Mouse lethality assay

(A) Inoculation into mice (intraperitoneal)

(B) and (C) Paralysis of hind limb

Table 3.4: Results of mouse lethality assay

Isolates	Mice Lethality (Positive/ Tested)
Positive Control	4/4
S-5	2/4
S-9	2/4
S-10	4/4
S-14	2/4
S-17	4/4
Negative control	0/4

3.7.2 Detection of Haemolysin

3.7.2.1 Tube Haemolysin Method

Tube haemolysin method was used for culture filtrates and live cells. *V. fluvialis* showed positive results that is the lysis of RBC indicating production of haemolysin. Development of reddish colour through out the suspension indicated the production of haemolysin by the organism (Figure 3.14). Here, the release of haemolysin of sheep RBC was measured at 450 nm. The results of haemolysin assay by tube method were given in Table 3.5. Positive control was also *E. coli* O157:H7.

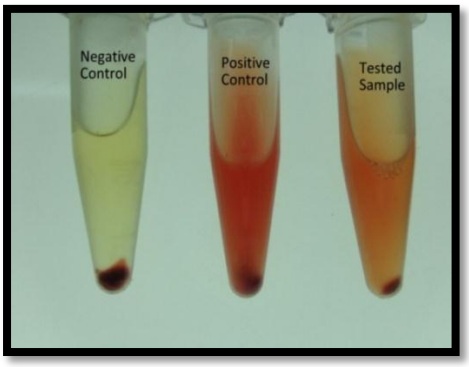


Figure 3.14: Haemolysin assay

Tube method of Haemolysin. Left tube with erythrocyte precipitated at the bottom (negative control), middle one with reddish colour through out the suspension due to haemolysin (positive control) and right one tested with *V. fluvialis*.

Table 3.5: Haemolysis of RBCs on Sheep Blood (OD at 450 nm)

Sample	O. D (450 nm)	
	LC	CF
Positive control	1.113	0.297
S-5	0.964	0.123
S-9	1.11	0.143
S-10	0.774	0.246
S-14	0.841	0.232
S-17	0.849	0.199
Negative control	0.00	0.00

LC = Live Cell, CF= Culture Filtrate

3.7.2.2 Blood Agar Plate Method

Blood agar plate was used for detection haemolysin. Inoculation of *V. fluvialis* culture on blood agar plate showed lysis of RBC around their growth. This is due to their ability to produce haemolysin that break down RBC. All the 5 strains of *V. fluvialis* showed zone of beta haemolysis around their growth. Zone of beta heaemolysis on blood agar plate was shown in Figure 3.15.

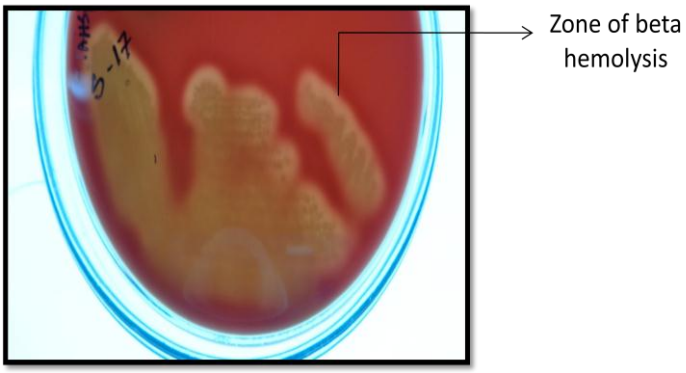


Figure 3.15: Haemolysin assay

Blood agar plate method: Zone of beta haemolysis on blood agar media.

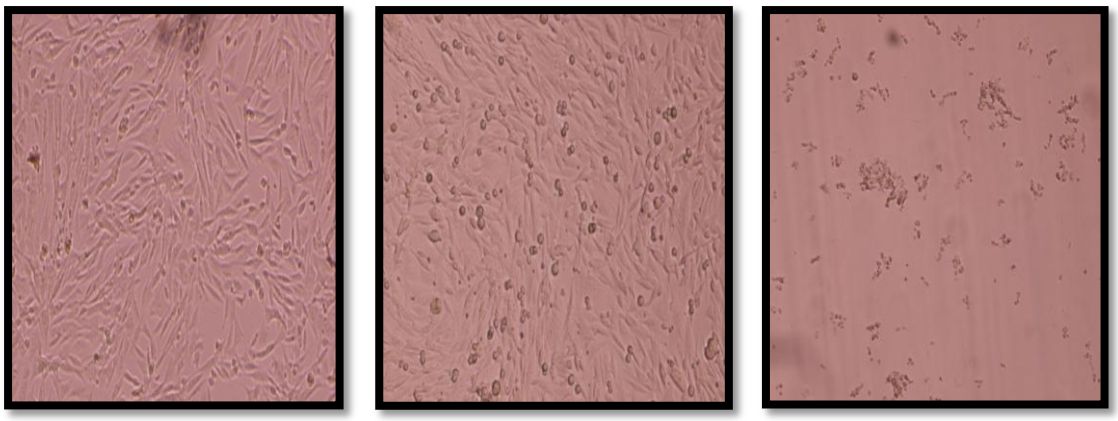
3.7.3 Detection of Cytotoxin

Cytotoxic activity of *V. fluvialis* culture filtrates were detected on both BHK-21 and HeLa cell line.

3.7.3.1 BHK-21 Cell Line

Culture filtrates of sample S-5, S-9, S-10, S-14 and S-17 were tested for cytotoxicity assay using BHK 21 cell line. Fifty µl of culture filtrate was added to 450 µl of cell. DMEM medium was used as negative control and culture filtrate of *E. coli* O157:H7 was used as positive control.

Culture filtrates of all *V. fluvialis* organisms showed 20-30% cell death on BHK-21 cell line (Figure 3.16) when compared with the controls.



A **B** **C**

Figure 3.16: Cytotoxicity assay on BHK-21 cell line

A. Negative control

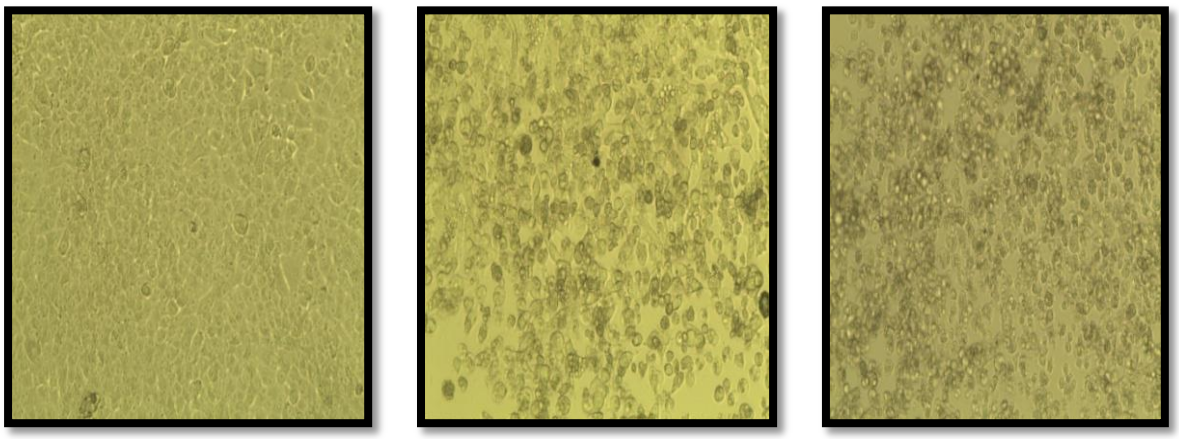
B. *V. fluvialis* culture filtrates

C. Positive control

3.7.3.2 HeLa Cell Line

Culture filtrates of sample S-5, S-9, S-10, S-14 and S-17 were tested for cytotoxicity assay using HeLa cell line. Fifty µl of filtrate was added with 450 µl of cell. DMEM medium was used as negative control and culture filtrates of *E. coli* O157:H7 was used as positive control.

However, the cell filtrate showed 20-40% cell death on HeLa cell line (figure 3.17) in comparison with the controls.



A B C

Figure 3.17: Cytotoxicity assay on HeLa cell line

A. Negative control B. *V. fluvialis* culture filtrates C. Positive control

3.8 Concentration of *V. fluvialis* Culture Filtrate by Ammonium Sulphate Precipitation Method

For the concentration of *V. fluvialis* toxin, one representative (S-10) strain was taken which showed higher amount of toxic activities (Table 3.6) among others.

Table 3.6: Cumulative results of toxic activities of all five *V. fluvialis* isolates

Experiment	*V. fluvialis* isolates				
	S-5	S-9	S-10	S-14	S-17
Entrotoxic activity	+++	+++	+++	+++	+++
Neurotoxic activity	+	+	+++	+	+++
Hemolytic activity	++	++	+++	+++	++
Cytotoxic activity	+	+	++	++	++

3.9 Protein Estimation of Concentrated Culture Filtrate

The concentration of protein was determined by Bio-Rad protein assay based on Bradford method. From this standard curve of BSA (Bovine Serum Albumin) (Figure 3.18), protein content of the concentrated culture filtrates were measured and found 2.6 mg/ml.

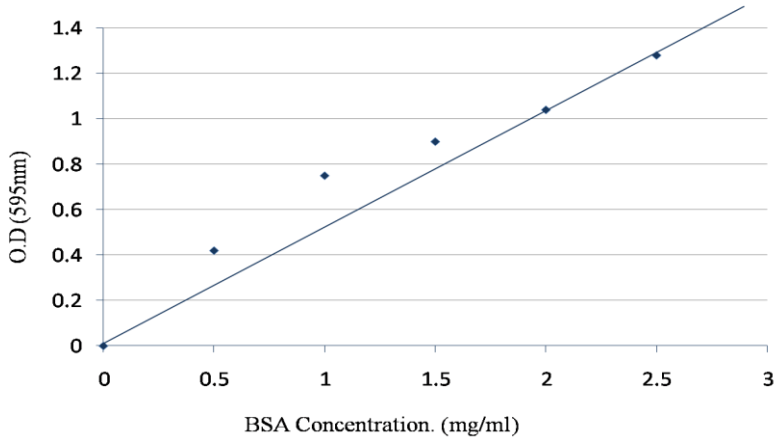


Figure 3.18: Preparation of standard curve for protein estimation

3.10 Detection of Toxic Activities of Concentrated Culture Filtrate

3.10.1 Detection of Enterotoxin

Concentrated culture filtrate that was prepared by ammonium sulphate precipitation method was subjected to detection of enterotoxin by rabbit ileal loop assay.

Concentrated toxins showed positive result in RIL assay with the mean of FA ratio 2.5 (Figure 3.19).

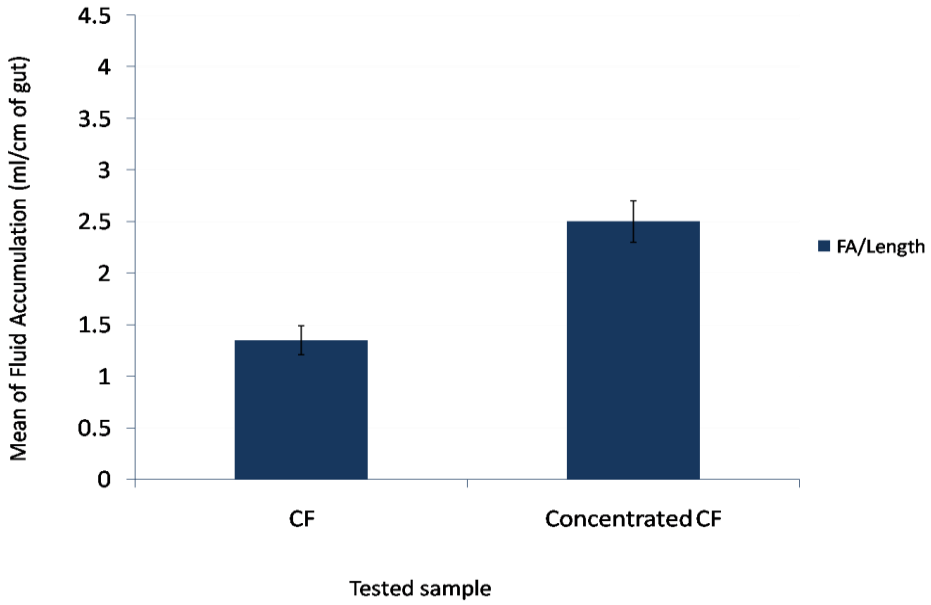


Figure 3.19: Graphical representation of mean ratio of fluid accumulation (FA) per length by culture filtrate (CF) and concentrated CF.

3.10.2 Detection of Neurotoxin

Upon intraperitoneal (IP) injection of 0.1 ml of concentrated culture filtrate, mice showed hind limb paralysis and death within 2-3 days.

3.8.3 Detection of Haemolysin

Tube method of haemolytic activity was performed for the detection of haemolytic activity of concentrated culture filtrate. Concentrated culture filtrate showed haemolytic activity on sheep RBC (O.D - 0.380 at 450 nm).

3.10.4 Detection of Cytotoxin

For the detection of cytotoxin production, HeLa cell line was used in cell culture assay. Concentrated culture filtrate showed 90-95% cell death on HeLa cell line when used 3.5×10^4 Cells/450 µL.

3.11 Protein Profiling of Concentrated Culture Filtrate by SDS-PAGE

After detection of toxic activities of concentrated culture filtrate, protein profiling was done by SDS-PAGE. In the SDS-PAGE analysis, a number of protein bands were obtained from the concentrated culture filtrate (Figure 3.20).

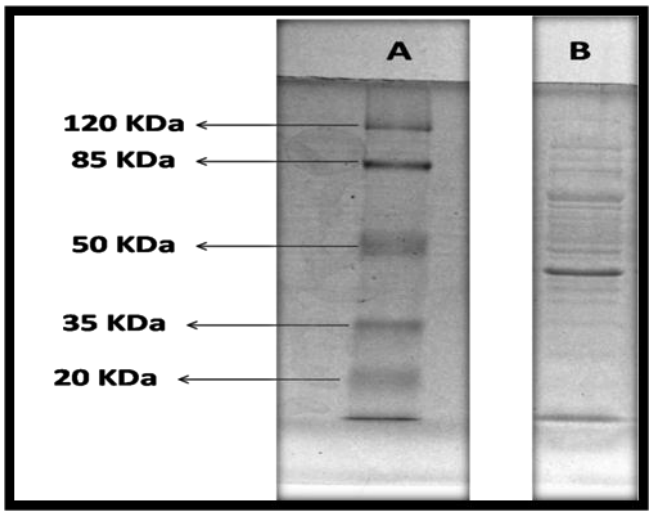


Figure 3. 20: SDS-PAGE analysis of concentrated culture filtrates

Lane A: Protein molecular weight marker

Lane B: Concentrated culture filtrate

3.12 Determination of Molecular Weight

For the detection of molecular weight, a calibration curve (Figure 3.21) was prepared by using molecular weight standards where Rf were plotted (x-axis) against the known molecular weight (y-axis).

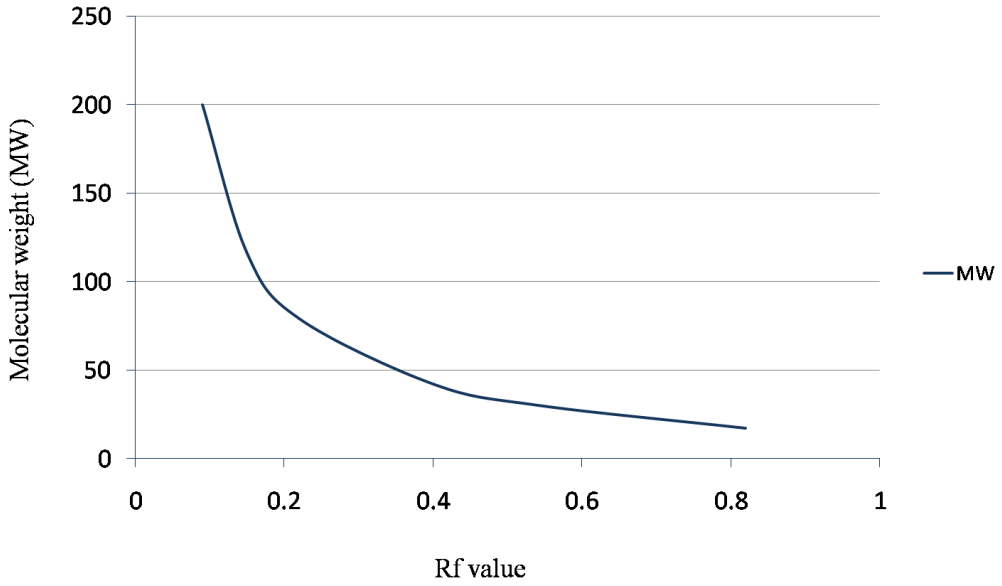


Figure 3.21: Preparation of standard curve for determination of molecular weight

We found 42 kDa, 43 kDa, 45 kDa, 50 kDa, 54 kDa, 60 kDa 64 kDa, 67 kDa, 78 kDa, 87 kDa and 102 kDa molecular weight of protein band for concentrated culture filtrates.

4. Discussion

The genus *Vibrio* is an ecologically and metabolically diverse group autochthonous to the marine, estuarine, and freshwater environment (Haley et al., 2010). This genus comprises of nearly 100 species of which, some members are capable of causing severe diarrhoeal diseases, thus posing a serious threat in the developing world (Morris, 2003, Thompson et al., 2004). Among these, *V. cholerae* O1/O139 and *V. parahaemolyticus* are considered major diarrhoeal pathogens and are responsible for several pandemics and epidemics (Kaper et al., 1995, Nair et al., 2007).

Previous research has focused mostly on *V. cholerae* in water because of the severity of the disease it causes (Mishra et al., 2004), but over the last decade, several studies have involved relatively minor *Vibrio* species of medical interest (Daniels et al., 2000), some of which are described as emerging pathogens, able to cause mild to severe human diseases (Tantillo et al., 2004). The members of the Vibrionaceae family namely *V. mimicus* and *V. fluvialis* are also frequently found to be associated in diarrhoeal outbreaks (Chitov et al., 2009, Chowdhury et al., 2012). *V. fluvialis* is directly associated with pollution or fecal waste and in most cases, causes disease in individuals, who eat contaminated seafood (usually raw or undercooked) or have an open wound that is exposed to seawater.

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 (Huq et al., 1980). In 2009, an episode of massive diarrhoea broke out in coastal regions of India following the cyclone Aila (Bhattacharjee et al., 2010). Prevalence of *V. fluvialis* among children with diarrhoea was very less during 1988 (0.6%) in Calcutta (now, Kolkata), India (Chatterjee et al., 1989). In the same region, progressive increased in the prevalence of *V. fluvialis* (>2%) among hospitalized acute diarrhoeal patients had been reported in the following years (Chowdhury et al., 2012). Even though the presence of Vibrios are mostly documented from coastal environs, the domination of a particular species depends on many physico-chemical and biological factors. In many investigations, the detection frequency of *V. fluvialis* is increased both in aquatic environment and in diarrhoeal cases (Ramamurthy et al., 2014). There are very few reports on characterization of *V. fluvialis* from environmental samples in Bangladesh. To our knowledge, this study was the first of its kind aimed at

isolation of *V. fluvialis* from rivers around Dhaka city and southern parts of Bangladesh and characterization of their toxins.

More specifically, we isolated *V. fluvialis* from the sediment and water hyacinth root samples, which included strains from the Turag and Buriganga rivers in Dhaka, two coastal areas include ponds in Khulna and shrimp field in Satkhira in November 2013 when water temperature lies between 20-25^{o}C.

V. fluvialis is a halophilic organism, which can not 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.

In a study carried out by Nishibuchi and Seidler (1983), *V. fluvalis* was reported to produce detectable level of toxins in Brain Heart Infusion (BHI) broth media but not in other media including Casamino Acid plus yeast extract, syncase and TSB. Hence, we prepared both our live cells and culture filtrates in BHI media for the toxin detection assay.

The isolates caused different ranges (Fluid accumulation ratio, FA 1.4-3.23) of fluid accumulation in Rabbit Ileal Loop assay; out of which three of them were comparable to the positive control, *V. cholerae* 569B. Fluid accumulation in the loop indicated that liberation of an enterotoxic-like substance(s) by the organisms during multiplication in the intestine. The differences in fluid accumulation between strains may have been due to variation in the release of toxin.

Two strains (S-14, S-17) which gave poor reaction initially (FA ratio 1.4 and 1.7) showed enhancement of enterotoxicity as judged by increased fluid outpouring (FA ratio 3.0 and 2.5) after serial passages in rabbit gut. Similar observations regarding the effect of passage on enterotoxicity were made earlier with *V. cholerae* (Blachman et al., 1970; Dutta et al., 1963; Singh and Sanyal, 1978) and *A. hydrophila* (Annapurna and Sanyal, 1977). Singh and Sanyal (1978) postulated that a mechanism of repression of toxin gene which becomes expressed on passage may account for this phenomenon, or the initial cultures were probably mixed population, containing a small proportion of toxigenic members and these probably increased in proportion during passage in ileal loops (Annapurna and Sanyal, 1977). Therefore, the effect of the passage or the enteric environment might also have

influence on the regulation of toxin production either in physiologic or genetic level as postulated by Singh and Sanyal (1978).

These observations may suggest that in a community where toxigenic organisms are in continuous circulation, the ratio of toxigenic to nontoxigenic organisms may be reduced under unfavorable condition, but enhanced by passage through susceptible hosts.

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* (Annapurna and Sanyal, 1977), *V. cholerae* serotypes other than O1 (Singh and Sanyal, 1978), etc. Many of these strains caused fluid accumulation comparable to that of *V. cholerae* culture filtrate. 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 (Shankar et al., 1982) and to biological variations in rabbits (Mekalanos et al., 1982). In our study, the fluid accumulating activity by culture filtrates were found to be less than the live cells. It may occur due to enterotoxin production which is medium dependent and also depends on other factors.

Many workers have shown that in *V. cholerae* O1, the toxin production *in vitro* depends on many variable factors (Karuna-sagar et al., 1979). Nishibuchi and Seidler (1983) observed 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. Other workers also used BHI broth as the medium for *V. fluvialis* organisms (Sanyal et al., 1980; Agarwal and Sanyal, 1981). The yield of the toxin has also been shown to be dependent on the critical balance between toxin production and concomitant production of toxin destroying substances (Kasuma and Craig, 1970) or BHI media may contain any protein that is toxin destroying or may inhibit the toxin secretion.

The isolates were next subjected to the molecular based assay – Polymerase chain reaction (PCR) to detect the presence of toxic genes. To determine the possible reason for fluid accumulation in rabbit ileal loop, PCR assay was performed to detect the cholera enterotoxin A gene (*ctxA)*. Though, the putative toxin is an A-B toxin but here primer used was to amplify the gene responsible for the A subunit (Active subunit) of the toxin. This is because it is known from the mode of action of the cholera toxin (CT) that the B subunit is only responsible for binding with the intestinal epithelia and forms a channel through the

membrane. Once binding occur the enzymatic cleavage of A subunit into two subunit A1 and A2 results in the inhibition of GTPase activity and left adenylate cyclase in the 'on' mode. As a result abnormal high rate of cAMP production causes loss of water and electrolyte that result in diarrhoea.

Hence, the main target to determine the presence of CT is gene for A subunit designated as *ctxA* though the target for vaccination is *ctxB* to prevent binding. PCR analysis shows that the isolates harbor no *ctxA* gene. So the fluid accumulation factors may be distinct from CT-like enterotoxin.

Since the isolates were negative for *ctxA* gene, we moved further to determine whether other known toxic genes accountable for enterotoxicity were responsible for fluid accumulation. Thus, detection of other toxic genes were also performed that were closely related with enterotoxicity.

Thermostable direct haemolysin (TDH) has been long suspected to be a toxin involved in most cases of *V. parahaemolyticus* diarrhoea. On injection of the purified haemolysin of *V. parahaemolyticus* into ligated rabbit ileal loops causes turbid, bloody fluid accumulation in the loop suggested that haemolysin might be the most important enteropathogenic factor (Zen-Yoji *et al.,* 1974). On the other hand, TDH-related haemolysin (TRH) produced by *V. parahaemolyticus* are also strongly associated with gastroenteritis. TDH-related haemolysin (TRH) produced by a Kanagawa phenomenon-negative strain was discovered in the investigation of an outbreak of gastroenteritis in the Maldives Islands in 1985 (Honda and Miwatani, 1988). In this study, all *V. fluvialis* showed negative result for both gene responsible for TDH and TRH.

Again, *stx1* and *stx2* are responsible for production of Shiga-like toxin of *E. coli* O157:H7. In this study, all *V. fluvialis* isolates also found to contain no genes responsible for shiga-like toxin (STX).

As far as the enterotoxicity of *V. fluvialis* isolates in our experiment is concerned, we established that all of them lacked the genes responsible for CT, TDH, TRH, STX. However, fluid outpouring in the ileal loop indicates the presence of other related gene encoding toxin that causes diarrhoea. This could possibly be due to the presence of a new

uncharacterized toxin. More investigation is required to find out the causative agent behind fluid accumulation.

Our next attempt was to see whether the *V. fluvialis* isolates produced any other related toxins. Neurotoxicity is not an inherited property of *Vibrios.* However, the culture filtrate prepared from all the *V. fluvialis* organisms showed hind limb paralysis in Swiss Albino mice within 2-3 days while some died within 2-4 days. Therefore, based on the experimental observation we believe that *V. fluvialis* produce neurotoxin like substances. Since, neurotoxicity assay was conducted by an indirect method (mouse lethality assay), further investigation has to be carried out to confirm these findings.

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 stool. Lysis of RBC is an indicative of haemolysin production. Since *V. fluvialis* is enterotoxigenic there is a possibility that this haemolysin may correlate with bloody diarrhoea. To determine the possible reason, haemolysin production assay was done by both blood agar plate and tube haemolysin method. Both live cells and culture filtrates of all isolates are capable of causing lysis of sheep red blood cells. These results are coherent with study which reported that *V. fluvialis* haemolysin lyses a wide variety of erythrocytes and is immunologically related to the El Tor haemolysin and is enterotoxigenic in suckling mice (Kothary et al., 2003). Among the *Vibrio* spp., the role of haemolysins in the pathogenesis of gastrointestinal diseases is not very clear, but evidence from their enterotoxigenic activity in various animal models suggested that in the absence of other known virulence factors, haemolysins may play a significant role in the disease process (Kothary et al., 2003).

Huq et al., (1980) and Seidler et al., (1980) attempted to detect enterotoxin of *V. flluvialis* by CHO (Chinese Hamster Overy) cell assay; they discussed the morphological changes of CHO cells evoked by enterotoxin without distinction between cell elongation factor (CEF) and cell killing factor (CKF). Lockwood et al., (1982) characterized partially purified CEF which was distinct from CKF against CHO cells, but they did not ascertain the direct enterotoxicity of the substances. To determine the toxin that has the cell killing ability, Baby Hamster Kidney (BHK-21) and HeLa cell line were used in the study. *V. fluvialis* showed about 20-30% and 20-40% cell killing ability on BHK-21 and HeLa cell lines, respectively, as compared to the positive control *E.coli* O157:H7, however, none of the

isolates showed any cell elongation factor (CEF). That is why we think that cell killing factor (CKF) probably masks cell elongation factor in BHK-21 and HeLa cells, because these cells would be broken down by cell killing factor before the cells elongated. This view was supported by Lockwood et al. (1982) on Chinese Hamster Overy (CHO) cells.

After the toxin assays, we concentrated the culture filtrates by ammonium sulphate precipitation method to find whether the toxic activities of *V. fluvialis* are increased or not. Synthetic media was used in this experiment to determine whether the toxic activities were of bacterial origin or proteins from the culture medium. We found that our concentrated culture filtrate was protein in nature and the protein content was 2.6 mg/ml.

The toxic activities that we found in culture filtrates prepared in BHI media, were also detected by the concentrated culture filtrate. In enterotoxicity assay, FA ratio of concentrated culture filtrate was 2.5, in contrast to 1.35 for culture filtrates. Similarly, in mouse lethality assay, neurotoxic activity was found with 0.1 ml of concentrated culture, compared to 0.5 ml of culture filtrate. Haemolytic activity was also increased with concentrated culture filtrate. In the cytotoxicity assay, 90-95% cell death found on HeLa cell line for concentrated culture filtrates, while for BHI culture filtrate found only 20-40% cell death.

Finally, in the analysis of protein profiling by SDS-PAGE, a number of protein bands were found in the concentrated culture filtrate namely the 67-68 kDa, 63-65 kDa and the 60 kDa protein bands. Kothary et al. (2003) purified and characterized the enterotoxigenic El Tor-like haemolysin by *V. fluvialis* and determined the molecular weight to be 63 kDa, that is same as the report for the *V. mimicus* (Miyoshi et al., 1997) but is slightly higher than the 60 kDa of the El Tor and El Tor-like haemolysin of *V. cholerae* (McCardell et al., 1999, Yamamoto et al., 1986).

In this study, we were able to isolate *V. fluvialis* from the environment having 0% salinity salinity from rivers around Dhaka and Khulna area and higher salinity in Satkhira area. These environmental *V. fluvialis* lacked *ctxA* gene, responsible for enterotoxicity. They also lacked other toxic genes *tdh, trh, stx1* and *stx2*. Although *ctxA* gene negative, these organisms showed rabbit ileal loop positive reaction which indicated that they might produce a toxin which is different from the known cholera toxin. Apart from the enterotoxic activity, these organisms also produced neurotoxin (mouse lethal activity), cytotoxin (BHK-21 and Hela cell line) and haemolysin (Blood agar plate and tube method of haemolysin).

All these results suggested that these environmental *V. fluvialis* are highly pathogenic which lacked *ctxA* gene but causes enterotoxicity and also produce other toxins which may be considered as potential threats for the society. To our knowledge this is the first report on fluid accumulation by *V. fluvialis* in absence of the gene responsible for CT.

V. fluvalis is considered as an emerging pathogen. However, not so much work has been done with its virulence factors. Hence our study focused on these bacteria, particularly on characterizing the enterotoxic and other toxic activities. In future, more studies should be needed about the purification of toxins to confirm the findings that can evaluate the role of the toxin(s) in the pathogenesis of the enteric disease and to determine its mechanism of action on target cells.

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Appendix-I

Media Composition

Unless otherwise mentioned, all media were sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this thesis have been given below:

1. Alkaline peptone water (APW) "*Nissui*"

Ingredients	Amount (gm/L)
APW dehydrated media	20.0 gm
Distilled water	1.0 L
P^H	8.8

2. Blood agar media

Anhydrous blood agar base (OXOID) (40.0 gm/L) supplemented with 5-7 ml defibrinated Sheep erythrocytes. Available in anhydrous from Diffco.

3. Brain heart infusion (BHI) broth

Ingredients	Amount (gm/L)
BHI dehydrated media	37.0 gm
Distilled Water	1.0 L
P^H	7.2

Available in anhydrous from Diffco.

4. Carbohydrate fermentation (Phenol Red Broth)

Ingredients	Amount (gm/L)
Tryptone/Tripticase	10.0 gm
NaCl	5.0 gm
Sugar	5.0 gm
Phenol red	0.2 gm
Distilled water	1 L
P^H	7.3

Phenol red should be prepared in a stock solution and added by drop.

5. CHROMagar™ Vibrio (74.7 gm/L)

Ingredients	Amount (gm/L)
Agar	15 gm
Peptone and Yeast extract	8.0 gm
Salts	51.4 gm
Chromogenic mix	0.3 gm
Distilled water	1.0 L
P^H	9.0 ± 0.2

* Media was not autoclaved. It was boiled for few minutes

6. DMEM (Dulbecco Modified Egales) Media

Ingredients	Amount (gm/L)
DMEM	6.73 gm
$NaHCO_3$	1.85 gm
HEPES	2.38 gm
Glutamine	0.58 gm
Deionized water (18Ω)	Up to 500 ml
P^H	7.2-7.4

* Media was not autoclaved. It was filtered through Millipore membrane of 0.20 µm average pore diameter.

7. Luria-Bertani (LB) Broth

Ingredients	Amount (gm/L)
Bacto-tryptone	10.0 gm
Bacto-yeast extract	5.0 gm
NaCl	10.0 gm
Distilled water	1.0 L
P^H	7.0

8. McConkey Agar media (OXOID)

Ingredients	Amount (g/L)
Dehydrated media	51.5 gm
Distilled Water	1.0 L
P^H	7.1±0.2

9. MR-VP Broth

Ingredients	Amount (gm/L)
Peptone	7.0 gm
Dextrose	5.0 gm
K_2HPO_4	5.0 gm
Distilled water	1.0 L
P^H	6.9

10. Nutrient agar

Ingredients	Amount (gm/L)
Peptone	5.0 gm
NaCl	5.0 gm
Beef extract	3.0 gm
Agar	15 gm
Distilled Water	1.0 L
P^H	7.0

11. Simmons' Citrate Agar, (Simmons', 1926, Modified)

Ingredients	Amounts (gm/L)
NaCl	5.0 gm
$MgSO_4$	0.2 gm
NH_4PO_4	1.0 gm
K_2HPO_4	1.0 gm
Sodium Citrate	2.0 gm
Agar	15.0 gm
Water	1 L
P^H	6.8

12. Synthetic media

Ingredients	Amount (g/L)
K_2HPO_4	10 gm
$MgSO_4$	0.2 gm
NH_4Cl	2.0 gm
Na_2HPO_4	5.0 gm
Glucose	5.0 gm
$FeCl_3$	0.005 gm
dl-Aspertic acid	2.0 gm
dl-Serine	1.5 gm
Glutamic acid	2.5 gm
Arginine	1.0 gm
dH_2O	1L
P^H	7.56

* Media was not autoclaved. It was filtered through Millipore membrane of 0.20 µm average pore diameter.

13. Thiosulphate Citrate Bile salt Sucrose (TCBS) Agar "*Nissui*"

Ingredients	Amount (gm/L)
TCBS dehydrated media	86.0 gm
Distilled water	1.0 L
P^H	8.6

* Media was not autoclaved. It was boiled for few minutes

14. Triple Sugar Iron (TSI) agar

Ingredients	Amount (gm/L)
Casein digest	10.0 gm
Animal tissue	10.0 gm
Lactose	10.0 gm
sucrose	1.0 gm
Dextrose	1.0 gm
$Fe(NH_{4)2}(SO_4)_{2.}.6H_2O$	0.2 gm
Sodium Chloride	5.0 gm
Sodium Thiosulfate	0.9 gm
Phenol Red	0.025 gm
Agar	15 gm
Distilled water	1 L
P^H	6.9

Appendix-II

Solution and Reagent

A. 10x-TBE (Tris-borate –EDTA pH 8.0)

108 gm of Tris-base, 55 gm of boric acid and 40 m1 of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 1000 ml. The buffer was stored at room temperature.

B. 0.5 M EDTA

18.61 gm of $Na_2EDTA.2H_2O$ (disodium ethylene diamine tetra-acetic acid) was dissolved in 80 ml of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. The final volume was made up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

C. 1x-TE buffer (Tris-EDTA)

TE buffer (10 mM Tris-Cl/1mM EDTA, pH 8.0) was prepared was prepared by diluting concentrated stocks of 1 M Tris-Cl and 0.5 M EDTA in distilled water. For making 1000ml or 1 L of TE buffer, 10 ml of 1 M Tris-HCl (pH 8.0) and 2 ml EDTA (0.5 M) and distilled water added up to 1000 ml. The buffer was autoclaved and stored at room temperature.

D. EtBr (0.5 µg/ml)

10 µl of 10 mg/ml Ethidium bromide solution was added to 200 ml distilled water. This solution was stored at room temperature and covered with aluminum foil.

E. 6×-Gel loading buffer (Glycerol & bromophenol blue)

Glycerol (30%)	3 ml
Bromophenol blue (0.25%)	25 mg
Distilled H_2O	Up to 10ml

F. Normal saline (0.85% NaCl)

0.85 gm of NaCl, upto 100 ml by distilled water.

G. Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na_2HPO_4 and 2.0 gm of KH_2PO_4 in 800 ml distilled water. pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 L by distilled water.

The solution was sterilized by autoclaving and was stored at room temperature.

H. Composition of Taq 2X Master Mix (New England Biolabs)

Reagents	Concentration
Tris-HCl	10 mM
KCl	50 mM
$MgCl_2$	1.5 mM
dNTPs	0.2 mM
Glycerol	5%
IGEPAL® CA-630	0.08%
Tween® 20	0.05%
Taq DNA Polymerase	25 units/ml

I. Alsever's Solution

NaCl	4.2gm
Citric Acid.3Na.$2H_2O$	8.0 gm
Citric Acid.H_2O	0.55 gm
D-Glucose	20.5 gm
Distilled water	1000 ml

The pH was adjusted to 6.1 with 10% Citric acid and autoclaved at 121^{o}C for 15 min. The solution was placed in a refrigerator at 2-8°C for 2 weeks.

J. Kovac's reagent

1.25 gm of para-dimethylaminobenzaldehyde was dissolved in 18.75 ml of amylalcohol. Then concentrated HCI was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4°C.

K. Methyl red reagent

0.01 gm of methyl red was dissolved in 30 ml of 95% ethanol. Then distilled water was added to make the final volume 50 ml. This reagent was covered with aluminum foil and stored at 4°C.

L. Oxidase reagent

100 mg of N,N,N^1,N^1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4°C.

M. Antibiotic stock

Penicillin	50 mg
Streptomycin	50 mg
Gentamycin	50 mg
dH_2O	10 ml

The solution was filtered through Millipore membrane of 0.20 µm average pore diameter and aliquote into 1 ml in each ependrof. Then it was stored at -20^oC

N. SDS-PAGE Analysis stock solution

i. 30% acrylamide-bis acrylamide solution

Acrylamide	14.5 gm
Bis-acrylamide	0.5 gm
Distilled water	Up to 50 ml

The solution was stored at 4^oC. The powder is neurotoxic. Mask was used during handling.

ii. Upper gel buffer (0.5 M Tris HCl, pH 6.8)

Tris-base	6.57 gm [0.5×MW (121.14)/10]
Distilled water	up to 100 ml
pH	adjusted to 6.8 with concentrated HCl

The solution was stored at 4^oC.

iii. Lower gel buffer (1.5 M Tris HCl, pH 8.8)

Tris-base	36.34 gm [1.5×MW (121.14)/20]

Distilled water	up to 200 ml
pH	adjusted to 8.8 with concentrated HCl

The solution was stored at 4^{o}C.

iv. 10% SDS

SDS	5.0 gm
Distilled water	Up to 50 ml

The solution was stored at room temperature.

v. 10% ammonium per sulfate (APS)

Ammonium per sulfate (APS)	5.0 gm
Distilled water	Up to 5 ml

The solution was aliquot into 0.5 ml in each eppendrof tube and stored at -20^{o}C.

vi. TEMED

Readymade and was stored at room temperature.

vii. Saturated butanol

Fiflty ml of distilled water was taken in a beaker with a magnetic stirrer. Butanol was adding to water provided the machine was turned on. Then adding butanol was stopped when the solution got saturated. The solution was stored at 4^{o}C.

viii. 0.1% BPB (Bromophenol blue solution) or Tracking dye

At first, 2 ml of 50% glycerol solution was to be made by mixing 1 ml glycerol with 1 ml dH_2O. Then 2 mg of Bromophemol blue was weighted and added 50% glycerol up to 2 ml mark. It was stored at 4^{o}C. Five µl of 0.1% BPB was to be added per sample.

ix. 2× Sample loading buffer

0.5 M Tris- Cl (pH 6.8)	0.4 ml
10% SDS	0.4 ml
2-mercaptoethanol	0.04 ml
Glycerol	0.4 m

Distilled water	0.76 ml

The solution was stored at 4^{o}C.

x. Running/Electrophoresis buffer (pH 8.3)

Tris base	3.0 gm
Glycine	14.4 gm
10% SDS	10 ml
Distilled water	1000 ml

The pH of the solution should be 8.3, which may range from 8.1 to 8.5. If unsuccessful, the solution was made again. The solution was to be stored at 4 ^{o}C. The bottle was to be marked after every usage. It should not be used more than 8-10 times.

xi. Staining solution (0.1% Comassie blue solution)

Coommassie brilliant blue G-250	100 mg
7 % acetic acid	Up to 100 ml

The solution was stored at room temperature.

xii. Destaining solution (7 % acetic acid)

Glacial acetic acid	7 ml
Distilled water	Up to 100 ml

The solution was stored at room temperature.

Appendix-III

Apparatus used

Autoclave	HL-42AE, Hirayama corp, Japan
Balance	Adventurer AR1140, Mettler Toledo EL202
Centrifuge	Eppendorf Centrifuge 5804, Germany Hettich Universal 320R, Germany
Class II Biosafety Cabinet	Lab Caire, USA
CO_2 Incubator	Shel Lab
Electrophoresis Unit	Sigma E0638, Consort N.V.
Freezer (-30^{o}C)	Liebherr comfort, Germany, Siemens
Gel Documentation System	Vilber Lourmat Doc Print VX05
Heater/Magnetic Stirrer	Spinot
Heat Sealer	Hulme Martin
Incubator	Japan
Inverted Microscope	KYOW Optilab TR-T
Laminar Air Flow	HF – 48 Flow laboratories, Japan
Microcentrifuge	Hettich, Tarsons Spinwin MC-02
Microwave Oven	Butterfly, China
pH Meter	Hanna HI2211
Power Pack	Biometra Standard Power Pack
Refractometer	2442-W05 Atago, Japan
Refrigerator	Royal Frestech, Vestfrost,
SDS-PAGE Unit	BioRad Mini-Protean II cell
Shaker	Electro Plus, Schuttelmaschine LS10
Spectrophotometer	Genesys 5
Sterilizer	NDS-600D, Japan
Thermomixer	Eppendorf
Thermocycler	Biorad C1000, Biorad MJ Mini, USA
Vortex	IKA MS3 Basic
Vacuum Pump	Sartorius GMBH
Waterbath	Grant SUB6, England