

**Study of the persistency of an environmental isolate of
Vibrio cholerae under different biotic and abiotic
conditions**



**A thesis
Submitted to**

**Biotechnology and Genetic Engineering Discipline, Life Science School,
Khulna University, Khulna**

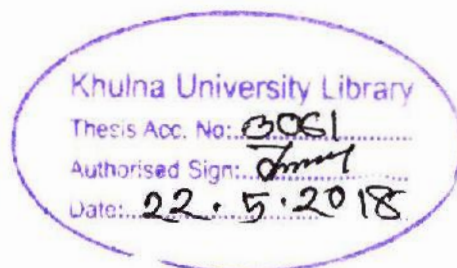
**For the partial fulfillment of requirement's for the degree of one and half years
Master of Science (M.Sc.) in Biotechnology and Genetic Engineering**

Submitted by

Sabiha Rahman

Examination Roll No: MS-110716

Session # 2011-2012



**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY
KHULNA-9208, BANGLADESH**

September, 2014

Study of the persistency of an environmental isolate of *Vibrio cholerae* under different biotic and abiotic conditions

**A Thesis
Submitted By
Sabiha Rahman
Examination Roll No: MS-110716
Session # 2011-2012**

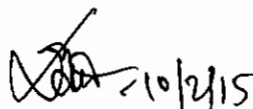
Approved As To The Style And Content By:



.....

**Supervisor
Dr. Dilara Islam Sharif
Professor
Biotechnology & Genetic Engineering
Discipline
Life Science School
Khulna University**

Chairman of the Examination Committee



10/2/15

**Dr. Sheikh Md. Enayetul Babar
Professor
Biotechnology and Genetic Engineering Discipline, Life Science School
Khulna University**

**BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
LIFE SCIENCE SCHOOL, KHULNA UNIVERSITY
KHULNA-9208, BANGLADESH**

September, 2014

Study of the persistency of an environmental isolate of *Vibrio cholerae* under different biotic and abiotic conditions

DECLARATION

This thesis report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/will not be published anywhere without the written concurrence of the concerned supervisor. Legal action will be taken in any case of infringement regarding the declaration.

Sabiha Rahman
.....

Sabiha Rahman
Student ID: MS-110716

10.02.2015
.....

Date:

**DEDICATED
TO
MY BELOVED PARENTS**

ACKNOWLEDGEMENT

First of all, the author would like to praise to the supreme of the Universe, the almighty Allah, for enabling the author to complete this task smoothly.

The author would like to express her deep sense of gratitude and sincere thanks to her honorable supervisor Dr. Dilara Islam Sharif, Professor of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh for her ingenious supervision, sharing knowledge, valuable suggestions, constructive comments and kind inspiration throughout this research work. The author really owe to them for giving him the opportunity to work in their close association. Without their kind co-operation, it would not be possible for her to complete this research work successfully.

The author would like to express the deepest respect and appreciation to her parents for their unlimited inspiration, affection and co-operation without which everything would have been impossible.

The author immensely proud to express her thanks to friends Meshu, Keya, sadia, juniors and seniors for their inspiration, constructive suggestions and countless help during her research work.

The author

ABSTRACT

Vibrio cholerae was identified in water, sediment and water hyacinth samples from different locations of Moyuri River. Among them *V. cholerae* from water hyacinth was detected throughout various seasons. A *V. cholerae* isolate from surface water was used for further studies to determine its persistency under different biotic and abiotic conditions. The persistence of *V. cholerae* at different detergent, pesticides concentrations and in association (direct and indirect) with *Anabaena spp.* was investigated. The growth of *V. cholerae* was not found to be affected in the presence of pesticides both in LB and MS media during the first 4 hours of incubation. However, 24 hour of treatment showed slight reduction in growth. The growth of *V. cholerae* was found to be reduced at a higher detergent concentration (40%) compared to lower concentrations (10%, 20%, and 30%). The growth in the presence of detergent was further reduced over time with initial 6×10^4 c. f. u on Day 1 to 2×10^4 c. f. u on Day 4 at 40% detergent concentration. The growth of isolated *V. cholerae* was greatly enhanced in direct association with two and three week's old culture of *Anabaena sp. PCC 7120*. It can be anticipated that biotic and abiotic conditions may have some effect on growth of *V. cholerae*.

3.4.1	Growth of <i>V. cholerae</i> in association with two weeks culture of <i>Anabaena sp. strain PCC 7120</i>	43-44
3.4.2	Growth of <i>V. cholerae</i> in association with three weeks culture of <i>Anabaena sp. strain PCC 7120</i>	45-46
	CHAPTER 4: DISCUSSION	47-50
	CHAPTER 5: REFERENCES	51-65
	APPENDICS	a-1

LIST OF FIGURES

	Page
Fig. 1: Classification of <i>Vibrio cholera</i>	4
Fig. 2.1: Enrichment of <i>V. cholerae</i> in APW	19
Fig 2.2: Anabaena sp. strain PCC 7120 (three weeks culture) with <i>V. cholera</i>	29
Fig 3.1: Colony characteristics on TCBS-agar plate for water sample	31
Fig 3.2: Colony characteristics on TCBS-agar plate for sediment sample	32
Fig 3.3: Colony characteristics on TCBS-agar plate for water hyacinth sample	32
Fig 3.4: Agarose gel electrophoresis for PCR amplicons of <i>virA</i> gene from the vibrio isolates	33
Fig 3.5: Two different colony morphology observed on LB agar plate during March	34
Fig 3.6: Smooth and rugose. colonies under stereo binocular microscope	35
Fig 3.7: Growth of isolated <i>V. cholerae</i> in the presence of pesticides	36
Fig 3.8: Growth of <i>V. cholerae</i> with and without pesticides in LB media	37
Fig 3.9: Growth of <i>V. cholerae</i> with and without pesticides in MS media	38
Fig 3.10: CfU/ml of isolated <i>V. cholerae</i> in MS media	39
Fig 3.11: Slightly rugose and smooth colony appeared onto LB agar plate	40
Fig 3.12: CfU/ml of <i>V. cholerae</i> measured at different detergent concentration with control (no detergent)	41
Fig 3.13: CfU/ml of <i>V. cholerae</i> measured at different detergent concentration	42
Fig 3.14: Persistence of <i>V. cholerae</i> following exposure to 10% and 20% detergent solution	42
Fig 3.15: Persistence of <i>V. cholerae</i> following exposure to 30% and 40% detergent solution	43

Fig 3.16: Cfu/ml with period of time for direct and indirect association

in two weeks culture 44

Fig 3.17: cfu/ml with period of time for direct and indirect association

in three weeks culture 46

LIST OF TABLES

	Page
Table 2.1: Types of samples were collected	18
Table 2.2: Specific genes for pathogenic bacteria detection	22
Table 2.3: Primers and expected size of PCR amplified gene targets of pathogenic bacteria	23
Table 2.4: The component of reaction mixture and their compositions in 25 µl reaction mixture together with final concentration for the PCR	24
Table 2.5: Percentage of detergent solution and amount of detergent used	28
Table 3.1: Colony characteristics of vibrio isolates from different samples	29
Table 3.2: The summery of the PCR test results	32
Table 3.3: Overview of colony morphology analysis with seasonal variability	35
Table 3.4: Persistence of <i>V. cholerae</i> in different concentrations of detergent solutions during 4 day period	40
Table 3.5: Enumeration (Cfu/ml) of <i>V. cholerae</i> in association (direct and indirect) with two weeks culture of <i>Anabaena sp. PCC 7120</i>	44
Table 3.6: Enumeration (Cfu/ml) of <i>V. cholerae</i> in association (direct and indirect) with three weeks culture of <i>Anabaena sp. PCC 7120</i>	45

ABBREVIATIONS

APW	Alkaline peptone water
BG11 media	Blue-Green media
cm	Centimeter(s)
Conc.	Concentration(s)
CT	Cholera toxin
DNA	Deoxyribo Nucleic Acid
e.g.	exempli gratia=for example
<i>et al.</i>	<i>et alii/alia</i> =and other people
etc	etcetera=the others
gm	Gram(s)
i.e.	<i>id est</i> =that is
l	Liter
ppm	parts per million
LB	Luria Bertani
mg	Milligram(s)
ml	Mililiter
°C	Degree Celsius
OD	Optical Density
OmpU	Outer membrane porin
OmpT	Outer mambrane porin
PCC 7120	Pasteur Culture Collection 7120
PCR	Polymerase Chain Reaction
PEG	Poly ethylene glycol

pH	Acid Base Measurement
WHO	World Health Organization
SLS	Sodium Lauryl Sulphate
SDS	Sodium Dodecyl sulphate
TCBS	Thio-sulphate Citrate Bile Salts Sucrose
TcpA	Toxin coregulated pilus
UV	Ultra violet
VBNC	Viable But Non Culturable



CHAPTER 1: INTRODUCTION

1. INTRODUCTION:

1.1 Overview:

Vibrio cholerae is a devastating bacterium causing rigorous diarrheal disease known as cholera. Cholera is a waterborne disease and its occurrence associates with prevalence of causative *V. cholerae* strain in aquatic environment (Khan, M.U., et al. 1984). It causes severe threat in country where sanitation and hygiene is very poor (Faruque, S. M., et al. 1998). Cholera is a very common disease in Asia, Africa, and America. It is transmitted through food and drinking water contaminated with this bacterium. Contamination with *V. cholerae* most often occurs due to the lack of basic sanitation, contaminated drinking water supplies, crop treatment with infected human feces or by some sea foods and fishes if consumed raw or in an undercooked form. In Europe, the risk of *Vibrio*- associated infectious diseases is limited to a few cases each year but it is estimated to increase in the future due to raw shellfish consumption, the increase in immune-compromised people and also the impact of anthropogenic activities and global warming on the marine environment (Baker-Austin, C., et al. 2010). Every year worldwide about 120,000 people die because of epidemic cholera (WHO, 1995). It has been estimated that every year 4, 50,000 – 1,000,000 cases of cholera occurs in Bangladesh (WHO, 2009; Rahman, M., 2011). Bangladesh is considered as a meadow of *V. cholerae* due its geographical position. Generally *V. cholerae* survive and thrive well on coastal and estuarine water. As Bangladesh is a coastal country its aquatic environment is abundant of *V. cholerae*. The occurrence of cholera is closely associated with biotic and abiotic parameters such as precipitation, floods, seasonality, river level, sea surface temperature, coastal salinity, dissolved organic material and fecal contamination. In Bangladesh cholera out breaks occurs mainly two seasons during pre and post monsoon seasons in the country that is influenced by the interaction of *V. cholerae* with environment and climate. Apart from this, Water salinity in the coastal area are increasing gradually with the increase of sea level rise (Faisal and Parveen, 2004; IPCC, 2001; World Bank, 2000) that makes favorable environment for *V. cholerae* and its spread in coastal area. Previously, different rural areas of Bangladesh have been studied for outbreak of cholera during different seasons (Sack, R. B., et al. 2003). But the south part of Bangladesh including western coastal zone for abundance of *V. cholerae* and their surveillance is not extensively studied before. The huge amount of river discharge releases on the coastal zone of southern part of

Bangladesh that makes it one of the most dynamic ecosystems of the world. This zone covers the Sundarbans mangrove forest, covering greater Khulna. Mangrove swamps, tidal flats, natural levees and tidal creeks are distinctiveness of the zone. It is also considered as a hotspot of flora and fauna. Phytoplankton concentrations are found to be considerably higher near the coastal zone of the Bay of Bengal (BoB). So it can be say that the aquatic environment of this zone is highly enriched in microbial community. The rivers of Khulna are interconnected with the coastal area. In Khulna District annual average highest temperature is 35.5°C and lowest is 12.5°C and annual rainfall is 1710 mm (Banglapedia 2005). It is observed that, since last three decades monsoon was the highest rainfall occurring season in four seasons at Khulna. In Khulna, Myuri River nearby Gollamari is outskirts of the town. Every day vast amount of daily waste of whole city is dumped in this river. Beside these, a big bazar and locality is grown up adjacent to the river. That makes the river water and sediments affluent in nutrients for microorganisms. Additionally, sewage line of most of the house is directly connected with this river that increases the availability of *V. cholerae* in this environment. Moreover, peoples wash their cloths, dishes and other daily works in the river. On the other hand, in rainy season, the pesticides run off from nearby agricultural field also mixed with the river water. Due to these anthropogenic activity and natural environment of this zone, it is important to study the abundance of *V. cholerae*, their persistence in diverse conditions like detergents, pesticides or other chemicals and its association with reservoir such as blue green algae (*Anabaena spp.*) in non epidemic season in the aquatic environment of Khulna.

1.2 Classification:

The genus of *Vibrio* contains several species, of which *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are the most important pathogens of humans. *V. mimicus* has also been involved in diarrheal disease. *V. cholerae* belongs to *Vibrionaceae* family. *V. cholerae* was first classified by Gardner & Venkatraman (1935) based upon its serogroups. Serogroups can be referred as distinct variation within a species of bacteria of different individuals with common antigens. Serogroups differ significantly in its sugar lipopolysaccharide (LPS) composition of the heat-stable surface somatic “O” antigen which shows no evident cross-reactivity between them. LPS consists of three domains: (a) oligosaccharide part (O-SP) composed of glycosidically-linked sugars (15–18 substituted perosamine residues); (b) the core sugars (branched

heteropolysaccharides) connecting O-SP to lipid A; (c) lipid A (phosphoglycolipid) part attaching to bacterial membrane (Chatterjee & Chaudhuri, 2003). To date, 206 serogroups have been documented, and out of these only two serogroups, known as O1 and O139, are responsible for epidemic cholera (Tamang, M. D., et al. 2005). *V. cholerae* strains on biochemical tests which do not agglutinate with “O” antiserum are collectively known as non-O1 *V. cholerae*. The O1 serogroup further subdivided into two biotypes on the basis of phenotypic differences including classical and El Tor. The term biotype refers to a group of organisms having the same genetic makeup that can be distinguished from the physical appearance of a group of organisms. The O-SP and core region differentiate O1 and O139 serogroups, whereas the terminal sugar of the O-SP differentiates the two O1 serotypes, Ogawa and Inaba (Cox, A. D., et al. 1996; Kondo, s., et al. 1996). Strains of the Ogawa serotype expresses the A and B antigens and a small amount of C antigen, whereas Inaba strains express only the A and C antigens. Separately from these serotypes, Hikojima a third serotype expresses all three antigens that are rare and unstable. It has been reported that, El Tor biotype of the O1 serogroup first emerged in Indonesia at the beginning of the seventh pandemic in 1961 (Glass, R. I., and R. E. Black., 1992). It has then been ubiquitous in the Ganges delta of the Indian subcontinent, and subsequently spread into other countries (Faruque, S. M., et al. 1998; Siddique, A. K., et al. 1992). In 1992, a new serogroup, O139 also called “Bengal” that cause epidemic cholera came insight (ICDDR, 1993; Nair GB, et al. 1994; Ramamurthy T, et al. 1993), posses major outbreaks in India and Bangladesh, spread to neighboring countries, and continues to cause epidemic cholera in adjacent areas. *V. cholerae* O139 contamination had been reported from India, Bangladesh, Nepal, Burma, Thailand, Malaysia, Saudi Arabia, China, and Pakistan (Albert, M. J., 1993; Chongsa-nguan, et al. 1993; Fisher-Hoch, et al. 1993). *Vibrio cholera* O139 has characteristics in common with the El Tor biotype but differs from O1 in its polysaccharide surface antigen. *V. cholera* other than O1 and O139 strains called non-O1, non- O139 strains do not have potential for epidemic cholera. The non-O1 serogroups can cause small sporadic cases of diarrhea and milder extraintestinal infections (Janda, J. M., et al. 1988). The majority of *V. cholerae* strains isolated from the environment are non-O1 serovars or serogroups, although O1 serovars have also been observed in areas where limited outbreaks of cholera has occurred (Singleton, F. L., et al. 1982c). Despite their nonpathogenecity, a large outbreak of cholera in India and Bangladesh

which subsequently spread into several parts of the subcontinent was originated by non-O1 strain, O139 Bengal (Ramamurthy, T., et al. 1993).

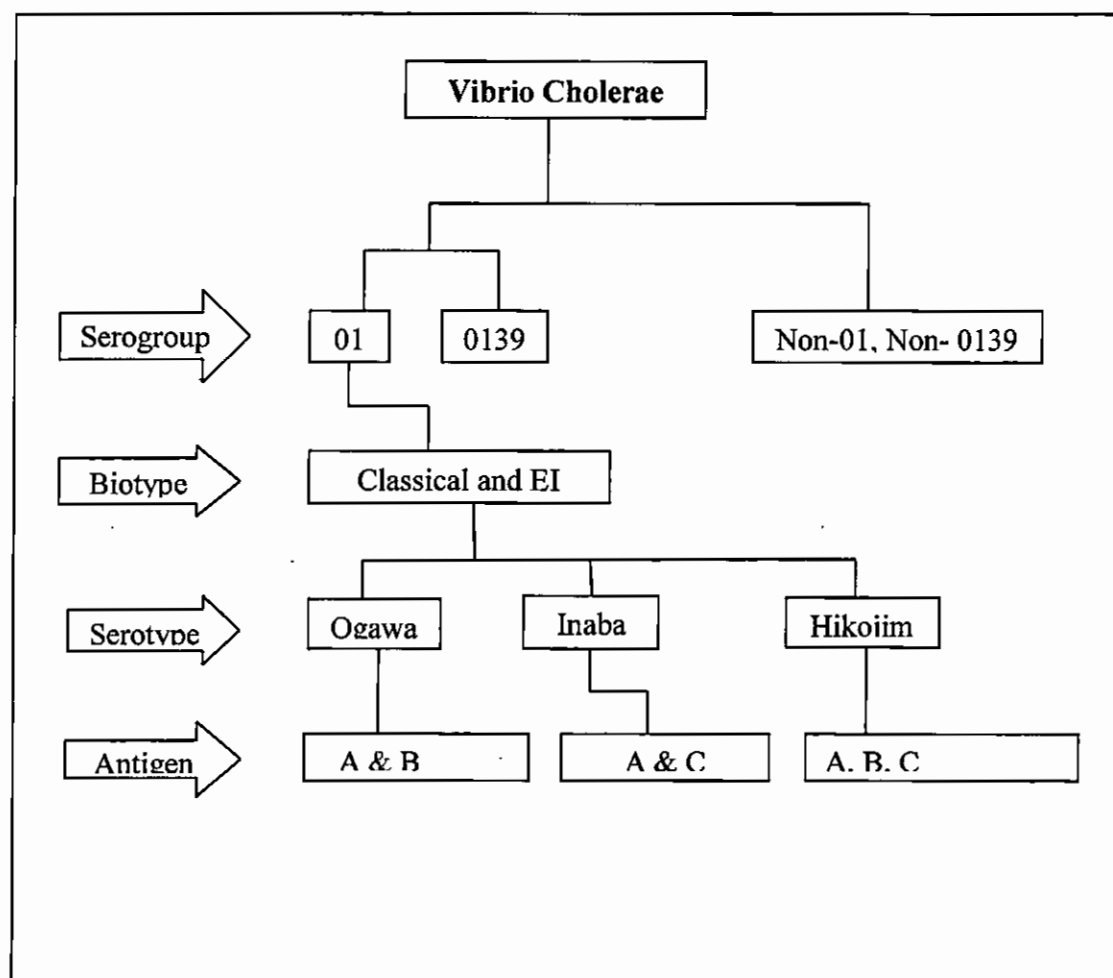


Fig. 1: Classification of *Vibrio cholerae*

1.3 Characteristics:

Vibrio cholerae is a gram negative, non-spore forming, and facultative, anaerobic bacterium. This bacterium is motile which depends on its single polar flagellum (Albert, M. J., 1994). It is small, straight, comma shaped, slightly curved rod measuring 2- 3 μm by 0.5 μm . It belongs to *Vibrionaceae* family but it also shows some characteristics of *Enterobacteriaceae* family (Adagbada, et al. 2012). Fimbriae are produced by *V. cholerae* 01 and non-01. These fimbriae are 5-6 nm wide and form bundles of parallel undulating filaments up to 15 μm long. *V. cholerae*

serogroup O1 does not have a polysaccharide capsule, but in serogroup O139, polysaccharide capsule have been detected. *V. cholerae* have adherence factors to adhere to microvilli where several hemagglutinins and the toxin-coregulated pili said to be involved in adherence but actual mechanism is not known. The motility of the vibrios may influence virulence by penetrating the mucus layer. *V. cholerae* produces enterotoxin called cholera toxin, which act on the mucosal epithelium is responsible for the characteristic diarrhea of the disease cholera (Kenneth Todar, 2008-2012). During the logarithmic phase of growth, they show little or no change to the cellular envelope. Conversely, towards the end of logarithmic phase and into the beginning of stationary phase cholera toxin is produced, which is accompanied by internal swelling of the cell and permeability changes to the cellular envelope. The toxin coregulated protein (TcpA) from pili is very important because its formation is coregulated with cholera toxin expression and is a key determinant for colonization. The gene encoding TcpA reside on a pathogenicity island. Pathogenic serogroups produce cholera toxin (CT) while non pathogenic strains may or may not produce this toxin (Ryan, K. J., and Ray, C. G., 2004).

1.3.1 Cultural characteristics and biochemical characteristics:

V. cholerae are well defined on the basis of biochemical tests (Baumann, et al., 1984). *V. cholerae* produces convex, smooth, round colonies that are opaque and granular in transmitted light. They have relatively simple growth factor requirements, grow in synthetic media containing glucose as a carbon and energy source, inorganic nitrogen source and require salt or sea-water based medium for optimal growth. In sufficient media, they grow rapidly with a generation time of less than 30 minutes. Their population densities increases when grown with vigorous aeration, but they can also grow anaerobically (Finkelstein, R. A., 1988). *V. cholerae* and most other *Vibrios* grow well at 37 °C but growth temperature varies from 30-40°C. No growth occurs at 4°C. Characteristically, *Vibrios* grow at a very high pH (8.5–9.5), tolerate alkaline media and are rapidly killed by acid. Most frequently used selective media is thiosulphate-citrate-bile salts- sucrose (TCBS) agar for isolation of *V. cholerae* after enrichment in alkaline peptone water (APW). Strains of *V. cholerae* produce yellow colonies on TCBS agar because of fermentation of sucrose. For the metabolism of *Vibrios*, sucrose is added as a fermentable carbohydrate and *Vibrio* able to utilize sucrose will form yellow colonies. Bromothymol blue and thymol blue are also added in TCBS agar that is pH indicators. The

alkaline pH of the medium improves the revival of *V. cholerae*. A few strains of *V. cholerae* may appear green or colourless on TCBS agar due to late sucrose fermentation (MacFaddin J. F., 1985). Generally, *V. cholerae* regularly ferments sucrose and mannose but not arabinose. *Vibrios* are oxidase-positive, which differentiates them from enteric gram-negative bacteria. A positive oxidase test is a key step in the identification of *V. cholerae* and other vibrios. Most *Vibrio* species are halotolerant, and NaCl often stimulates their growth. Some vibrios are halophilic, requiring the presence of NaCl to grow. *Vibrio cholerae* is also capable of changing its colony morphology. In adverse condition, they go in viable but non culturable state. Viable but non culturable form indicates that, bacteria cannot be recovered by standard culture techniques. In this state they can cause infection and can revert to the culturable form (Colwell, R. R., and Huq, A., 1994).

1.4. Identification:

V. cholerae can be isolated and detected from environment in many ways. The process involves either direct plating from the study sample (water, plankton, sediment, shellfish) onto thiosulphate citrate bile-salts sucrose (TCBS) agar and taurocholate tellurite gelatin agar (TTGA) or pre-enrichment of the sample in alkaline peptone water followed by streaking for isolation onto agars. In case of environmental samples, a pre-enrichment step is highly suggested to improve detection or isolation. Following an incubation period, presumptive *V. cholerae* can be isolated from these media which can be further confirmed either by biochemical analyses (Huq, A., et al. 2006) or directly by PCR. Isolates can then be serogrouped as O1, O139, or non-O1/non-O139 by a slide agglutination assay using antisera for the O1 and O139 antigens or by PCR using primers developed to target O1 and O139 coding regions of the genomic DNA. These *V. cholerae* isolates can then be stored for prolonged periods in nutrient agar containing 0.5% NaCl overlaid with sterile glycerol stock at -70°C (Huq, A., et al. 2012). Another technique developed for distinguish colonies of *Vibrio cholerae* species from others obtained on thiosulphate citrate bile salts sucrose agar after isolation from different environmental species. It was demonstrated here that the yellow colonies (sucrose-fermenting), which are able to grow on nutrient agar without added NaCl and which present a positive oxidase reaction, can be confidently considered as presumptive *V. cholerae*. Confirmation of the identification was carried out using the API 20E microtest and by species-specific *ompW*-based polymerase chain

reaction. This procedure used for reliable identification of *V. cholerae* species and is adapted to the capabilities of routine clinical, food-testing and environmental microbiology laboratories (S. Baron, s., et al. 2007). Further, the increase in genome sequences now available has allowed development of PCR-based typing schemes for analysis of variants of strains, genes, and mobile genetic elements, including pathogenicity islands (Huq, A., et al. 2012). From environmental water samples, *V. cholerae* can be detected by using fluorescent labeling technique. In Bangladesh, Brayton, P. R., et al. (1987) established a fluorescent-antibody (FA) direct viable count (FA-DVC) assay to detect culturable and nonculturable *V. cholerae* O1. The direct fluorescent antibody (DFA) assay is used to detect viable but nonculturable *V. cholerae* O1 in environmental water (Binsztein, N., et al. 2007) and in studies on the role of *V. cholerae* biofilm on the transmission of cholera (Alam, M., et al. 2007).

1.5 Environmental Survival and Persistence under biotic condition

V. cholerae is a natural inhabitant of aquatic environment. It can survive in a free living state in both fresh water and salt water. Although human is a key reservoir for disease cholera but this bacterium has also been found to survive in association with various algae, copepods (zooplankton), crustaceans and insects (Krauss, H., et al. 2003; Bronze, M.S., and Greenfield, R.A, 2005). Senderovich, Y., et al. (2010) showed that fish digestive tract can be a reservoir and vector of *V. cholerae*. The deep ground water, riverine, estuarine, and coastal water is an important reservoir of *V. cholerae* in endemic areas where a significant member of zooplankton community inhabit these areas as well (Magny, C. G. D., et al. 2011). *V. cholerae* has a biofilm- or aggregate-associated lifestyle and can also persist intracellularly in amoebae. Although the major habitat of *V. cholerae* is the aquatic environment, little is known about concentrations of the pathogen in different water types. Although disease is clearly known to be caused in most cases by consumption of contaminated water, apparent concentrations in surface water are well below the infectious dose (Faruque, S. M., et al., 2006). During epidemic periods, toxigenic *V. cholerae* have been isolated by cultivation only infrequently (Islam, M. S., et al. 1994a) and in interepidemic periods isolation is extremely rare (Huq, A., et al. 1990).

1.5.1. Aquatic microphytes:

Toxigenic *V. cholerae* O1 biotype El Tor has been isolated from macrophytes in both seawater and fresh water. In a study carried out in Bangladesh by Spratt et al., toxigenic *V. cholerae* O1 biotype El Tor was isolated from the roots of *Eichhornia crassipes*, a fresh water macrophyte also known as water hyacinth. The authors observed that adhesion of the microorganism to the plant roots favors its survival. *V. cholerae* O1 biotype El Tor survived for longer periods when they were attached to the freshwater macrophyte *Lemna minor* than when they were suspended in the water (Islam, M. S., et al., 1990).

1.5.2 Phytoplankton and zooplankton:

A laboratory study by Tamplin, M., et al. (1990) in Bangladesh indicated that five strains of *V. cholerae* O1 of clinical origin (three of the classic biotype and two of the El Tor biotype) adhered primarily to the exoskeleton of copepods (*Acartia* sp., *Acartia chilkaensis*, *Acartia sewelli*, and *Cyclops* sp.), cladocerans (*Bosmina* sp., *Ceriodaphnia* sp., *Diaphanosoma* sp., and *Bosminopsis* sp.), and rotifers (*Brachionus* sp.). They were also able to attach to two species of green algae (*Volvox* sp. and *Pediastrum simplex*) and to two species of blue-green algae (*Spirulina* sp. and unicellular cyanobacteria). *V. cholerae* O1 secrete an enzyme called chitinase, which is associated with chitinous fauna, mainly zooplankton. Mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to Zooplankton (Chiavelli, D. A., et al. 2003). Zooplankton may provide attachment sites for *V. cholerae* to multiply and serve as a vector to transmit infection to humans. It has been proved the presence of *V. cholerae* O1 and O139 in the cyanobacteria of the aquatic environment in Bangladesh (Islam, M. S., et al., 1992). *V. cholerae* have been detected in phytoplankton specimens of the species *Anabaena variabilis*, a cyanobacterium collected from a pond in Dhaka, Bangladesh in the viable but nonculturable state. Naturally in non-epidemic seasons these pathogenic bacteria remain in dormant state. It's called viable but non culturable state (Roszak, D. B., and Colwell, 1987) or inter-epidemic period. In the inter-epidemic period, cyanobacteria can provide micro environment for the persistence of *V. cholerae* and can act as a reservoir (Islam, M. S., et al. 1994b). Islam, M. S., et al. (1994b) hypothesized that *V. cholerae* O1 uses extracellular products released from *A. variabilis* for nutrition and that the availability of salts on the mucilaginous surface of this blue-green alga enables *V. cholerae* survive for a long periods in fresh water environments. In winter season, organic nutrients in pond, lakes, and

canals become increased and temperature, daylight becomes optimal for algal bloom formation. In the aquatic environment of Bangladesh, cyanobacteria are available and they maintain a symbiotic relationship with *V. cholerae* that causes epidemic cholera (Ahmed, M. S., et al., 2007). No association has been observed with species of genera such as *Euglena* and *Phacus*.

1.5.3 Fish, crustaceans and mollusks:

Fish act as an important reservoirs and vectors of *V. cholerae*. Fish of various species and habitats contain *V. cholerae* in their digestive tract. In Israel, it has found that, *V. cholerae* isolates have the ability to degrade chitin, indicating a commensal relationship between *V. cholerae* and fish. Nontoxigenic strains of serogroup O1 biotype El Tor have been isolated from oysters (*Crassostrea virginica*) found in estuarine waters in the state of Florida. In 1991, *V. cholerae* O1 (of unresearched toxigenicity) was detected in fish and mollusks in the coastal waters of Lima, Peru (Boroto, J. R., 1997). Senderovich, Y., et al. (2010) suggested that *V. cholerae* proliferates by attaching or associated with eukaryotic organisms, likely copepods (Crustacea). Chironomids (non-biting midges, Diptera) also serve as an intermediate host reservoir for *V. cholerae*.

1.5.4 Biofilm community:

Biofilm community is a supplementary reservoir of *V. cholerae* within aquatic environment during interepidemic period in Bangladesh (Alam, M., et al. 2006). Several studies have revealed that environmental non-O1 and non-O139 *V. cholerae* strains and *V. cholerae* O1 El Tor and O139 produce a three-dimensional biofilm on surfaces which creates a microenvironment (Broza, M., and Halpern, M., 2001; Watnick, P. I., et al. 2001). Biofilm production is a survival strategy and persistence of *V. cholerae* in marine environment (He, H., et al. 2012). Biofilms are microbial environment, attached to living and nonliving surfaces and produce the extrapolymeric substances. Johnson LR (2008) found that, the exopolymeric wall act as a shield for *V. cholerae* by protecting from toxic compounds, such as antibiotics, thermal stress, and predation. Biofilm formation is dependent on the expression of an exopolysaccharide (EPS) for both O1 El Tor and O139 strains of *V. cholerae* (Watnick, P. I., et al. 2001; Wai, S. N., et al. 1998). EPS matrix production depend on expression of the *Vibrio* polysaccharide synthesis genes (*vps*), encoded in two gene clusters on the larger chromosome (*vpsA-K* and *vpsL-Q*). To form mature

stable biofilm EPS matrix production is prerequisite. If EPS production is in excess, it causes resistance to osmotic and oxidative stress and bacteriocidal agents (Yildiz, F. H, et al. 1999). In dynamic environment, biofilm may add the survival of cells by colony formation that contains thousands of cells (Mizunoe, Y., et al. 1999).

1.5.5 Morphological alteration:

In nutrient stress conditions, non-spore-forming, heterotrophic bacteria adapt some morphological, physiological, and chemical changes to survive in those conditions (Dawson, M. P., et al.1981; Marden, P., et al. 1985). Several studies have proposed that the persistence of *V. cholerae* in the environment is due to a viable but non culturable state (VBNC) in response to starvation and cold (Colwell, R. R., et al. 1985; Roszak, D. B., et al. 1984; Shiba, T., et al. 1995; Xu, H.-S., et al. 1982). It has been depicted that *V. cholerae* can switch between the well defined off white smooth and rough shrink white known as rugose colony morphotypes to survive in the environment. Bruce White (late 1930s) first coined the term ‘rugose’ colony phenotype and is characterized by wrinkled colonies with the expression of EPS. Under nutrient deprived conditions smooth *V. cholerae* cells can spontaneously shift at a low frequency to express “rugose” exopolysaccharide (rEPS) and exhibit wrinkled (rugose) colonies (Mizunoe, Y., et al. 1999; Morris, J. G., et al. 1996). Rugose colony can be characterized by EPS layer that consist of heavy, fibrous, electron-dense, ferretin-stained surrounding the cells, but smooth colony *V. cholerae* do not have this EPS layer (Wrangstadh, M., et al. 1986). Bruce White (1938) described that, *V. cholerae* can form “wrinkled” rather than typical smooth colonies on nonselective media such as LB agar (White, P. B. 1938). Rugose variants produce plentiful amounts of exopolysaccharide, which gives resistance to chlorine, acid pH, serum killing, and osmotic and oxidative stresses (Morris, J. G., et al. 1996; Wai, S. N., et al. 1998; Yildiz, F. H., et al. 1999). So it can anticipate that rugose phenotype's characteristics, plays a vital role in the survival and persistence of *V. cholerae* in the environment predominantly under stressful growth conditions (Ali, A., et al. 2002; Rice, E. W., et al. 1993)

1.6 Effect of abiotic parameters on growth of *V. cholerae*:

V. cholerae is widely distributed in temperate and tropical aquatic environments. The West Bengal of India and Bangladesh has been known as “Homeland of cholera” (Barua, D., 1992).

Geographically Bangladesh is a riverine country and located in delta region. Flood, natural disaster occurs rotationally in this region that causes abundance of *V. cholerae* in the environment. Biotic and abiotic factors significantly enhance the growth of *V. cholerae*. Abiotic factors include: Water temperature, salinity, and nutrient concentration known to have impact on distribution of *V. cholerae* (Juras, H., et al., 1979; Lee, J. V., et al., 1982). The natural inhabitant of microorganisms such as; the surface water and ground water are polluted by daily discharge of industrial effluents, detergents, pesticides run off, alkanoids, and hydrocarbons. These extreme conditions interrupt the natural growth of microorganisms.

1.6.1 Temperature:

Temperature fluctuation has direct correlation with seasonal variability and climate change. The effect of temperature on growth of *V. cholerae* is immense. Generally, high temperatures during summer months are suitable for survival of *V. cholerae* in the environment (Kaper, J., et al., 1979; Lee, J. V., et al., 1984; Roberts, N. C., et al., 1984). Lobitz et al. (2000) conducted an experiment and revealed a correlation between sea surface temperature, sea surface height, and occurrence of cholera in the Bay of Bengal in Dhaka, Bangladesh from 1992 to 1995. The increasing of water temperature in ponds and rivers enhance the cholera cases through the faster growth rate of the pathogen in aquatic environments. The increase of sea surface temperature enhances the growth of algae and zooplankton that results the abundance of *V. cholerae* (Burke, D., et al. 2001). Cholera outbreaks mainly two season of the year in Bangladesh (Khan, M.U., et al. 1984). In Bangladesh, most of the cholera epidemic occurred during either the pre- or the post-monsoon season (The monsoon season is approximately June–September, throughout the country) (Sack, R. B., et al. 2003). Huq, A., et al. (1984) reported that, November month after monsoon is susceptible for cholera epidemic in Bangladesh. Besides this, climatic factors change such as; floods and droughts affect the abundance of the bacterium in the environment as well as its survival by affecting other environmental factors (Colwell, R. R., 1996).

1.6.2 P^H & Salinity:

V. cholerae thrives under high-pH conditions. Huq, A., et al. (1984) found that, alkali condition (pH 8.5) is favorable for rapid multiplication of *V. cholerae* with copepods and their association with planktonic crustaceans but in acidic condition (pH 6.5); the same rate of multiplication is not

possible. Salinities stimulate the growth of *V. cholerae* in inland coastal areas and estuaries. Optimal salinity for *V. cholerae* growth ranges from 5 and 25 psu, or 2.0‰ and the optimal pH for survival is from 7.0 to 9.0, depending on salinity. *V. cholerae* can tolerate salinities near 45 psu (Singleton, F. L., et al., 1982a).

Although only salinity cannot influence the growth of *V. cholerae* at low temperature but with the increase of temperature its impact is enormous (Singleton, F. L. et al., 1982a). It has been demonstrated that, at an estuarine salinity (25‰) and a temperature of 10° C, *V. cholerae* survived (i.e., was culturable) for less than 4 days (Singleton, F. L., et al., 1982b).

1.6.3 Detergents:

Detergents are organic compounds, which have both polar and non-polar characteristics. It is also a surfactant or a mixture of surfactants which has cleaning properties. Detergents can be classified into three classes depending on their electric charges: anionic, cationic, and non-ionic. Anionic and cationic have permanent negative or positive charges, attached to non-polar (hydrophobic) C-C chains. If the surfactant carries positive charge then it's called cationic. Typical anionic detergents are alkylbenzene sulfonates. Cationic surfactant carries negative charge and most commonly used surfactant includes alkyl ammonium chlorides. A non-ionic surfactant has no charge groups in its head (common types include polyethylene ethoxylates).

- Sodium lauryl sulphate (SLS):

Sodium lauryl sulfate (SLS) is an anionic surfactant, detergent, and emulsifier used in thousands of cosmetic products, as well as in industrial cleaners. In solution, the sodium cation (Na⁺) separates from the anionic part of the compound (lauryl or dodecyl sulfate), and this anionic compound is the active chemical. It is used in many cleaning and hygiene products. One end of the molecule is charged and therefore has an affinity for water, and the other end is nonpolar and soluble in fats/oils. The salt is of an organosulfate consisting of a saturated 12-carbon tail for its "lipophilic" end attached to a sulfate group for its "lipophilic" end which is negatively charged, giving the material the amphiphilic properties required of a detergent. SLS has a faint odor of fatty substances and at room temperature, occurs as white or cream-colored crystals, flakes, or powder (Chemfinder, 2006), or a clear to yellowish thick fluid. SLS is stable under normal



It present in higher concentrations with industrial products including engine degreasers, floor cleaners, and car wash soaps, hair color and bleaching agents, toothpastes, shampoos, shaving creams, laundry detergents, and bath oils/bath salt.

- 3



secreted and encountered by enteric *V. cholerae* bacteria in human. Bile is anionic surfactants and affects the growth of this pathogen. It also facilitates the expression of virulence factors, induction of efflux pumps, biofilm formation, motility, production of the outer membrane proteins OmpU and OmpT in *V. cholerae*. If *V. cholerae* grows in the presence of bile, expression of the major virulence factors CT and TCP repressed, motility enhanced, biofilm formation is facilitated, and intracellular production of OmpU is stimulated with associated repression of OmpT synthesis. Beside the intracellular environment in many natural environment like ponds, rivers huge amount of detergents are dumped by dying industries, household works. *V. cholerae* are found to survive by themselves in extreme detergent concentrations by changing their cell surface characteristics. The outer membranes of environmental *V. cholerae* is resistant to hydrophobic compounds, detergents etc (Chaudhuri, K., et al. 1992). Amund et al. (1997) found that SDS with an alkyl sulfate moiety as surfactant supported the highest growth of the detergent-utilizing organisms and the *Vibrio* is the most frequently encountered organism of utilization while *Proteus* was the rarest. The transmembrane regulatory protein ToxR of *V. cholerae* mediates enhanced bile resistance. Classical and El Tor *toxR* mutant strains of *V. cholerae* exhibits reduced growth rates at subinhibitory concentrations of SDS (Provenzano, D., et al. 2000). *Enterobacter cloacae* can grow in 25% sodium dodecyl sulphate and can metabolize it (Kramer, C. V., et al. 1980). It was found that a *Klebsiella* isolate (G1) capable of survival and growth in an environment containing high levels of anionic (15.6%) detergent (Beadle, I. R., and Verran, J., 1999). Other isolates belonged to *Pseudomonas aeruginosa*, *Pseudomonas mendocina*, *Pseudomonas stutzeri*, *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas putida* and *Pseudomonas otitidis* have been shown to degrade and metabolize SDS (Venkatesh, C., et al. 2011). Cationic detergent alkaline PEG reagent may inactivate common pathogens. *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus cereus* could not survive in alkaline 60% PEG reagent at room temperature. The alkaline PEG interacts with water at alkaline pH, binds H⁺ and dissociates water resulting increase in the concentration of free OH⁻ (Chomczynski, P., and Rymaszewski, M., 2006).

1.6.4 Pesticides:

Pesticide is a chemical or biological agent (such as a virus, bacterium, antimicrobial, or disinfectant) that deters, incapacitates, and kills pests. Target pests can include insects, plant pathogens, weeds, mollusks, birds, mammals, fish, nematodes (roundworms), and microbes that

destroy property, cause nuisance, or spread disease, or are disease vectors. Chemical pesticides can be divided into three groups include

Organochlorines, Organophosphates and Carbamates.

- **Organochlorines:**

Organochlorine hydrocarbons (e.g., DDT) disrupt the sodium/potassium balance of the nerve fiber, forcing the nerve to transmit continuously. They were commonly used in the past, but many have been removed from the market due to their health and environmental effects and their persistence (e.g., DDT and chlordane).

- **Organophosphates:**

Organophosphates are quite toxic to vertebrates, mammals, birds and fish (generally 10-100 times more poisonous than most chlorinated hydrocarbons) (Cunningham et al., 2003). They usually are not persistent in the environment. They degrade easily, so their bioaccumulation is rare. Some examples are parathion, malathion, dichlorvos, dimethyldichlorovinylphosphate (DDVP) and tetraethylpyrophosphate (TEPP).

- **Carbamates:**

There are several subgroups within the carbamates. Thiocarbamate and dithiocarbamates are subclasses of carbamates. They are toxic to bees. They include carbaryl, aldicarb, aminocarb and carbosulfun.

1.6.4.1 Effect of pesticides on growth of bacteria:

In agricultural field, chemical pesticides are daily used in greater amount throughout the world. In 1992 the total consumed pesticide was 7400 metric tons in Bangladesh (BRRI). But in 2010, the consumed amount was 53460 metric ton which has increased more than 6 times in 2 decades (BBS). To meet up the food demand, pest control is obligatory. For this reason, farmers of the developing country apply more toxic strong pesticides without following the instructions of proper amount and use. So in monsoon season, this chemical pesticides run off comes in contact of Pond, River, lakes, sea water and then become threats for our aquatic environment. Some microorganisms of soil such as, *Pseudomonas* are able to metabolize pesticides in the

environment. Murugesan, A. G., et al. (2010) identified five bacterial isolates (*Pseudomonas aeruginosa*, *Klebsiella* sp., *Escherichia coli*, *Bacillus* sp. and *corynebacterium*. sp.) from Brinjal cultivated field that are capable of degrading cypermethrin. Chanika, E., et al. (2011) identified bacterial isolates *Pseudomonas putida* that are able to degrade both organophosphates and carbamates pesticides. Organophosphate esters that interfere with nerve signal transmission in insects do not affect microbial processes. Microorganisms able to hydrolyze phosphotriesters by the help of enzyme and utilizes them as source of carbon and phosphorus. Bacteria are more likely to contain such enzymes because of their tendency to develop new enzymes and metabolic pathways to supply nutrients (Copley, S. D., 2009). They use these pesticides for their energy. The degradation rates depend on many microbiological, physical and chemical properties of the soil, as well as the properties of the pesticide. It has been found that, *Pseudomonas aeruginosa* is able to degrade some pesticides like malathion, cypermethrin and carbofuran in different concentrations (Wathik, A. H., et al. 2013). Not much has been studied on the effect of pesticide on *V. cholerae* bacteria. It has reported that, exposure of strains of El Tor *Vibrio cholerae* to different concentrations of pesticides (fazolone, treflane, prometrine, magnesium chlorate, omait and gardon) and mineral fertilizers lowers the antibiotic sensitivity (Ziiaev, ShI., et al. 1991).

Aims and objectives of this study were:

Aims-

- To study the association of an environmental isolate of *V. cholerae* with *Anabaena* sp. strain PCC 7120.
- To study the effect of pesticides and detergent solution in persistence of the environmental isolate of *V. cholerae*.

Objectives-

- Detection and isolation of *V. cholerae* from environmental sample.
- Determine the persistence of isolated *V. cholerae* in the direct and indirect association with *Anabaena* sp. strain PCC 7120.
- Determine the persistence of isolated *V. cholerae* in different concentrations of pesticides and detergent solution.

CHAPTER 2: MATERIALS AND METHODS

2. Materials and Methods

2.1 Preparation of Culture Media:

- **Alkaline Peptone Water (APW):**

APW (3g) (Oxoid LTB, England) was dissolved in 150 ml of distilled water and dissolved completely by autoclaving for 15 psi (121°C). Cooled at room temperature prior to use.

- **Thio-sulphate Citrate Bile Salts Sucrose Agar (TCBS):**

TCBS agar (10.8 g) (Biolife, Italy) was boiled with 120 ml of distilled water for 5 minutes. Then poured into Petri dishes and allowed to solidify.

- **Luria-Bertani (LB) broth preparation:**

LB broth was prepared by dissolving 1 g of trypton, 1 g of sodium chloride crystal and 0.5 g of yeast extract on 100 ml distilled water and dissolved completely and sterilized by autoclaving for 15 psi (121°C). Cooled at room temperature prior to use.

- **Preparation of Blue-Green media:**

For 1 L media preparation, 9 components of BG-11 media (appendix) were added in 900 ml of distilled H₂O and autoclaved. Cooled at room temperature prior to use.

- **Nutrient broth preparation:**

Nutrient broth (2.5 g) was dissolved in 100 ml of distilled water and sterilized by autoclaving for 15 psi (121°C). Then cooled at room temperature prior to use.

- **MS (mineral salt) media preparation:**

MS media was prepared by taking 2 ml from stock solution I (appendix) and 1 ml from stock solution II-VI (appendix) each in conical flask. Then autoclaved distilled water was added to the flask to give 100 ml solution. Then autoclaved for 15 psi (121°C) and cooled prior to use.

- **Microscopy:**

Two colonies were picked up from each streaked plates of three different types of samples and observed under microscope to determine morphological characteristics. Gram staining was also done in order to determine whether the bacteria were gram positive or gram negative.

Gram staining was done as below:

First 1/2 drop of sterilize distilled water was transferred on a clean glass slide with an inoculation loop and added a drop of the suspended culture of the test organism to be examined and spread over a small area. Then the smear was allowed to air dry. After drying the smear on the slide fixed the dried film by passing it briefly through the Bunsen flame two or three times without exposing the dried film directly to the flame. The following steps are done then respectively:

- Flooded the fixed smear with crystal violet solution and allowed to remain for 1 minute.
- Rinsed off the crystal violet with distilled or tap water.
- Flooded the slide with iodine solution. Allowed to remain for one minute.
- Rinsed off the iodine solution with distilled or tap water.
- Flooded the slide with decolorizer for one to five seconds.
- Rinsed off the decolorizer with distilled or tap water.
- Flooded the slide with safranin. Allow to remain for 30 seconds.
- Rinsed off the safranin with distilled or tap water.
- Dried the slide on absorbent paper and place in an upright position.

Microscopically the slide was examined for bacterial organisms under a 100X objective. Several fields were observed on the slide for bacterial organisms.

In the gram staining test the Gram-positive bacteria stain deep violet to blue and gram-negative bacteria stain pink to red.

2.2.4.2 Stock culture preparation:

For storage, pure culture of each selected colony was done by streaking in LB agar media. Colonies were picked and stock cultures were made for further studies.

For long term storage colony from TCBS agar plate was picked up and inoculated in LB broth by sterile loop. Then incubate for overnight at 37°C. The overnight culture was centrifuged at 10000 rpm at 37°C. The pellet was then washed with autoclaved distilled water. After that, 30%

autoclaved glycerol was added and vortex vigorously to mix properly. Then samples were stored in refrigerator at -20°C.

For short term storage, slants of nutrient agar were prepared. Then colonies from TCBS agar plate were picked up and streaked on slant by sterile loop and capped carefully on sterile condition. Then incubate overnight at 37°C. Finally, they were stored at -4°C.

2.2.4.3 Confirmative PCR test

2.2.4.3.1 Culture of organism

Five ml autoclaved LB broth was taken into test tube where 10 µl samples (water and sediment) were added and incubated in a shaking incubator at 37°C overnight. Following overnight culture, the sample was used for DNA extraction

2.2.4.3.2 DNA Extraction

DNA was isolated by using DNAzoL Reagent. Bacterial DNA was extracted from the cultured bacteria as described in Invitrogen manual for DNAzoL Reagent. DNAzoL Reagent is a complete and ready-to-use reagent for the genomic DNA isolation from small and large volume of solid and liquid samples that normally combines with both reliability and efficiency with simplicity of isolation protocol. The DNAzoL Reagent procedure is based on the use of a novel guanidine-detergent lysing solution which permits selective precipitation of DNA from a cell lysate. During the isolation, sample was lysed (or homogenized) in DNAzoL Reagent and the genomic DNA was precipitated from the lysate with ethanol. Following an ethanol wash, DNA was solubilized in 8 mM NaOH. The whole procedure was done in about 30 minutes with DNA recovery of 70-100%.

2.2.4.3.3 Qualitative and quantitative determination of extracted DNA

According to Sambrook *et al.* (1989), agarose gel electrophoresis helps to study the integrity of extracted DNA sample. Briefly, electrophoresis was performed using a horizontal electrophoresis apparatus (Bioneer, Korea) at a constant volt of 100 with a 1.5 % agarose gel containing 2 µl ethidium bromide in 150 ml 1x TAE buffer. DNA migrated near about the two-third of the gel towards the

positive electrode and power supply for electrophoresis was maintained at this time. DNA size was determined by comparison with a DNA size marker (100 bp DNA ladder, Bioneer, Korea). DNA bands were visualized by UV illumination and photographed using the High Performance UV Transilluminators, (Ultra-Violet Products Ltd., UK).

2.2.4.3.4 Components for DNA amplification in PCR

2.2.4.3.4.1 Description of Target genes and primers

- **Target genes:**

The target genes were selected for this study included: *virA* gene for *Vibrio*. One pair (forward and reverse) of specific primer was designed from above mentioned gene sequence.

Table 2.2: Specific genes for pathogenic bacteria detection.

Species	Specific Gene	Accession No.	References
<i>Vibrio</i>	<i>rpoB</i> , <i>toxR</i> gene, <i>vvhA</i> gene, <i>virA</i> gene	D26468	Tarr <i>et al.</i> ,2007

- **Oligonucleotide primers:**

Primer selection is the preliminary step for the DNA fragment amplification. Primers are short, artificial DNA strands that are complementary to the beginning and the end of the DNA fragment to be amplified. They anneal to the DNA template at these starting and ending points, where the DNA-polymerase binds and begins the synthesis of the new DNA strand. In this study, one pairs of primers were used. Sequences of the one PCR primer pairs and size of expected amplification products are shown in Table 2.3.

Table 2.3: Primers and expected size of PCR amplified gene targets of pathogenic bacteria.

specie	Primers	Sequence (5' →3')	Product size (bp)	Temp
<i>Vibrio</i>	Vibr-F	CGGTGAAATGCGTAGAGAT	663	49
	Vibr-R	TTACTAGCGATTCCGAGTTC		

2.2.4.3.5 Taq DNA polymerase enzyme

The enzyme was isolated from recombinant *E. coli*. Strain containing the DNA polymerase gene from *Thermus thermophilus*. It exhibits highest activity at pH 9.0 and 72°C (Bioneer, Korea). To prepare 1 unit Taq DNA Polymerase from purchased 5 units, it was diluted with enzyme dilution buffer (50 mM Tris-HCL, 0.1 mM EDTA, 1mM DTT, Stabilizers, 50% Glycerol, pH 8.2) at 4: 1 ratio (Buffer : Enzyme).

2.2.4.3.6 Deoxynucleotide Triphosphates (dNTPs) mixture

In the present experiment, 10 mM dNTP mixture (each dNTP 2.5 mM) was used (Bioneer, Korea).

2.2.4.3.7 Buffer used for PCR

10x Reaction buffer (Tris; pH 9.0, 15 mM MgCl₂) was used for PCR test.

2.2.4.3.8 Target gene amplification by Polymerase Chain Reaction (PCR):

At first concentrations of the entire extracted DNA samples were adjusted. Total 25µl reaction mixture was prepared in a 0.2 ml PCR tube (Eppendrof, Humburg, Germany) which contain 2 µl DNA sample, 2 µl oligonucleotide primers, 5 µl 5X green reaction buffer, 2 µl 10 mM dNTPs mixture, 1 µl Taq DNA polymerase and 13 µl de-ionized distilled water. The 0.2 ml PCR tube containing reaction mixtures were then placed in a DNA thermal cycler (C1000™, BIO-RAD, USA) for polymerase chain reaction.

Table 2.4: The component of reaction mixture and their compositions in 25 µl reaction mixture together with final concentration for the PCR

Component	Final volume	Final concentration
Di-ionized water	13 µl	
5X Green Reaction Buffer (Tris; P ^H 9.0, 15 mM MgCl ₂)	5 µl	1×
10 mM dNTP mixture (each dNTP 2.5 mM)	2 µl	1.0 mM
Primers	2 µl (Forward 1 µl + Reverse 1 µl)	1.0 µM
Template DNA	2 µl	0.25 µg/ µl
Taq DNA polymerase* (5 units/ µl)	1 µl	1.25 unit

*To prepare 1 unit *Taq* DNA polymerase, it was 3 times diluted with di-ionized water.

PCR amplification was performed in a Gene Cyclor under the following conditions: In brief, heat denaturation at 94°C for 2 min followed by 35 cycles of heat denaturation at 94°C for 1 min. primer annealing at 58°C for 1 min and DNA extension at 72°C for 1 min. This is followed by incubation at 72°C for 10 min followed by cooling at 4°C. While running PCR reaction a positive control was kept.

2.2.4.3.9 Evaluation of PCR products:

After completion of the thermal cycling, a sample of 10 µl from each PCR products was analyzed electrophoretically by running on a 1.5 % agarose gel and the amplified product size was determined by comparison with a 100 bp DNA size marker.

2.3 Analysis of colony morphology of *V. cholerae* with seasonal variation:

2.3.1 TCBS agar plating:

To analysis the colony morphology of *V. cholerae* with seasonal variation water hyacinth samples were used. Four roots of water hyacinth plants were prepared separately as described in 2.2.3. Then, drops of 5 µl enrichment (APW) culture were given onto TCBS plate by micropipette and incubated at 37°C for 24hour.

2.3.1 Colony transfer onto LB agar plate:

Colonies were transferred in March, April, November and January. During March, colonies were picked up from TCBS agar plate with a sterile loop and inoculated into LB broth and incubating for overnight at 37°C. After incubation, 5µl of culture was inoculated onto LB agar plate and incubated overnight. Subculture of two different colonies was done from LB agar plate in one plate by streaking with sterile loop. In April and January each colony of every TCBS agar plate was transferred in test tube of 5 ml LB broth with sterile inoculating loop and incubated for 4 hour at 37°C. After incubation 5 µl drops of fresh culture were given in LB agar plate and duplicate plates were also prepared and incubated at 37°C for 10 days. During November colonies were transferred by velveteen cloths. For colony transfer replica discs were prepared by covering the discs with velveteen cloth and sealed by gum. Then the disc was slightly touched on the surface of master TCBS agar plate for colony transfer to the discs. The replica disk was then slightly pressed against LB agar plates and incubated at 37°C for 10 days.

2.4 Growth of *V. cholerae* in different pesticides solutions:

2.4.1 Preparation of Seed culture of *V. cholerae*:

Seed culture of isolated *V. cholerae* and referred *V. cholerae* collected from Biochemistry Lab was prepared by centrifuging overnight culture at 1500 rpm for 15 minutes. Then supernatant was discarded and the pellet was washed twice with sterile distilled water. Then resuspended in distilled water and the optical density of both cultures were measured to give an optical density of 1.0 at 600 nm.

2.4.2 Preparation of Stock pesticides solution:

For preparation of stock pesticides solution 5ml of pesticides (Cypermethrin, Milathion, Carbosulfun) solution was prepared according to Appendix 4A. For studies 100 µl from stock pesticides solution was used.

2.4.3 Analysis of persistence of *V. cholerae*:

For analysis of persistence of *V. cholerae* in pesticide solution two different media were used, such as LB and MS media. 100 µl of mixed pesticides solution prepared (described in section 2.4.2) was added to 50 ml LB of MS and LB media. 1 ml of seed culture (OD = 1.0) of isolated environmental *V. cholerae* (described in section 2.4.1) was also added to each of LB and MS media containing pesticides. A similar set was also prepared for reference *V. cholerae*. For control experiments, flasks were prepared similarly without adding the pesticides solution. After adding bacterial culture the flasks were incubated in rotary incubator at 120 rpm at 28°C. For measurement of growth OD was taken at 0, 4 and 24 hour respectively. Viable cell counts were carried out only in case of MS media after 48 hour by serial dilution and spreading a 100 µl of aliquot on LB-agar plate. Then incubation was done for overnight and colonies were counted and expressed as c. f. u/ml.

2.4.4 Colony morphology analysis in presence of pesticides:

The above mentioned culture of 48 hour in MS media was used for analysis of colony morphology and was compared with fresh culture of enriched environmental isolate of *V. cholerae* in APW media. In order to study the colony morphology of environmental (isolated) *V. cholerae*, MS media was prepared with mixed pesticides solutions containing bacterial culture. A culture grown overnight was inoculated in APW media for enrichment and incubated for 24 hour in rotary incubator. Then drops of 5 µl overnight APW culture and culture with mixed pesticides on MS media were given on LB agar plate and incubate for 8 days. In this case as control APW culture without pesticides and only culture on MS media was kept for comparison the effect.

2.5 Persistence of isolated *V. cholerae* in detergent solution:

2.5.1 Detergent solution preparation:

Detergent solution of different percentages (10%, 20%, 30%, 40%)(w/v) was prepared by dissolving an anionic detergent, Sodium lauryl sulphate (SLS) and a non ionic detergent, poly ethylene glycol (PEG) in 100 ml distilled water (Table 2.5) and autoclaved at 121°C for 15 minutes. For each experiment duplicate plates were prepared.

2.5.2 Inoculum preparation:

An overnight grown *V. cholerae* stock culture inoculated in 100 ml LB broth and incubate on orbital shaker at 28°C for 24 hours. The culture was then centrifuged at 1500 rpm for 15 minutes and media was discarded. The pellet was washed twice with sterile distilled water and resuspended in sterile distilled water to give an optical density of 1.0 at 600 nm. This culture was used as inoculum for studying of persistence.

2.5.3 Studies of persistence:

In ten glass vials 1ml (OD = 1.0) of bacterial inoculums was transferred. The effect of different percentages of detergent solution on *V. cholerae* was determined by directly adding different amount of detergent to the vials according to the table (Table: 2.5) and a control without detergent was kept. Then vial were capped loosely with enough air and incubate at 37°C for 96 hours. Viable cell counts were carried out by serial dilution and spreading a 100 µl of aliquot on LB-agar plate after 24 hours and 96 hours incubation. Plates were incubated at 37°C for 24 hours.

Table 2.5: Percentage of detergent solution and amount of detergent used:

Percentage of detergent	Amount of detergent used in each vial (W/V)
Control	No detergent
10%	0.05 g of SLS and PEG each
20%	0.1 g of SLS and PEG each
30%	0.15 g of SLS and PEG each
40%	0.2 g of SLS and PEG each

2.6 Association of isolated *V. cholerae* with *Anabaena* sp. PCC 7120:

2.6.1 Preparation of *Anabaena* sp. PCC 7120:

Eight flasks of 100 ml BG-11 media were prepared by inoculating 1ml of cyanobacterial culture. For experimental studies four flasks of culture were grown for two weeks and other four flasks of culture were grown for three weeks at 25°C.

2.6.2 Preparation of seed culture of isolated *V. cholerae*:

For experimental studied stock cultures were received by overnight incubation. Then 50 µl of overnight culture was inoculated in 30 ml autoclaved LB broth and culture was shaken in room temperature at 120 rpm for 4 hours to make seed culture of *V. cholerae*.

2.6.3 Determination of association of *V. cholerae* with *Anabaena* sp. PCC 7120:

For determination of association similar experiments were performed for two and three week's cyanobacterial old culture.

- For direct association, 2 ml prepared bacterial seed culture was inoculated in 2 flasks of 2 weeks culture of *Anabaena* sp. PCC 7120.
- For indirect association, 2 flasks of 2 weeks cyanobacterial culture were centrifuged at 4000 rpm for 30 minutes. Then 80 ml supernatant was taken in a sterile flask by

micropipette from centrifuge tube to prepare cyanospent culture and 1.6 ml bacterial seed culture was inoculated into the flasks.

- Control cultures were prepared by replacing the cyanospent culture with BG-11 media and 2 ml of bacterial seed culture was also added in 2 flasks of 100 ml BG-11 media and kept as control. Then all flasks were grown for 12 days in a shaker incubator at 100 rpm and plating was done every 2 days later by spreading 200 μ l aliquot on TCBS-agar plate.



Fig 2.2: *Anabaena* sp. strain PCC 7120 (three weeks culture) with *V. cholerae*.

CHAPTER 3: RESULTS

3. RESULTS:

3.1 Detection of *V. cholerae* in environmental sample:

3.1.1 Isolation of *V. cholerae* from environmental samples:

- **Thio-sulphate Citrate Bile Salts Sucrose Agar (TCBS) plate:**

To isolate *V. cholerae* from environment samples were streaked on TCBS agar plate and the colonies were observed for typical *V. cholerae*. In case of water samples only yellow colored colonies observed on TCBS agar plate (Fig: 3.1). For sediment and water hyacinth samples two different (yellow & green) colonies appeared on TCBS agar plate (Fig: 3.2 & 3.3). Among two different colonies that appeared on TCBS agar plate, only yellow colored colonies were selected from TCBS plate for further identification. All colonies appeared smooth, round with opaque center and translucent peripheries. Yellow colored colonies were as large as 2-3mm, but green colored colonies were larger than yellow (Fig: 3.2).

Table 3.1: Colony characteristics of vibrio isolates from different samples:

Sample	Color on TCBS	Transparency	Colony morphology (from TCBS agar plates)			Assumed bacteria
			Shape	Elevation	Surface	
Water	Yellow colonies	Opaque center and translucent peripheries	Small and round (2-3mm)	Raised	Smooth	<i>V. cholerae</i>
Sediment	Yellow colonies	Opaque center and translucent peripheries	Small and round (2-3 mm)	Raised	Smooth	<i>V. cholerae</i>
	Green colonies	Deep green centered	Small and slightly flattened (3-4mm)	Raised	Smooth	<i>V. mimicus</i> <i>V. damsela</i> <i>V. vulnificus</i> <i>V. hollisae</i>
Water hyacinth	Yellow colonies	Opaque center and translucent peripheries	Small and round (2-3 mm)	Slightly elevated	Smooth and rough	<i>V. cholerae</i>

- Growth on TCBS plate:



Fig 3.1: Colony characteristics on TCBS-agar plate for water sample.

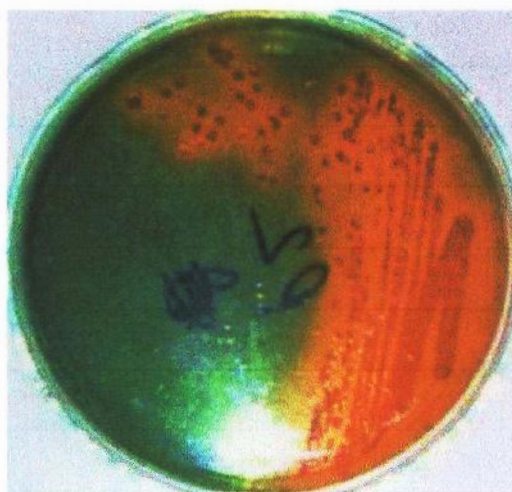
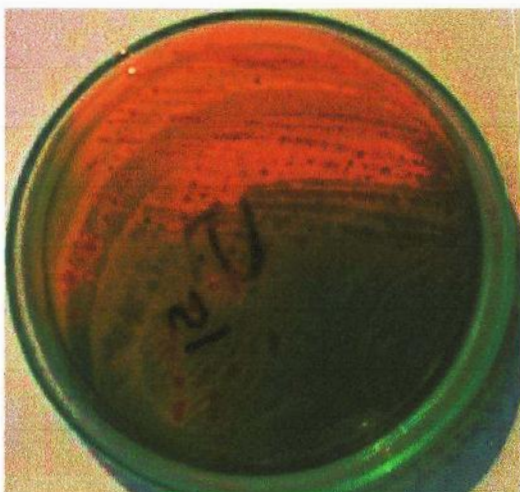


Fig 3.2: Colony characteristics on TCBS-agar plate for sediment sample.

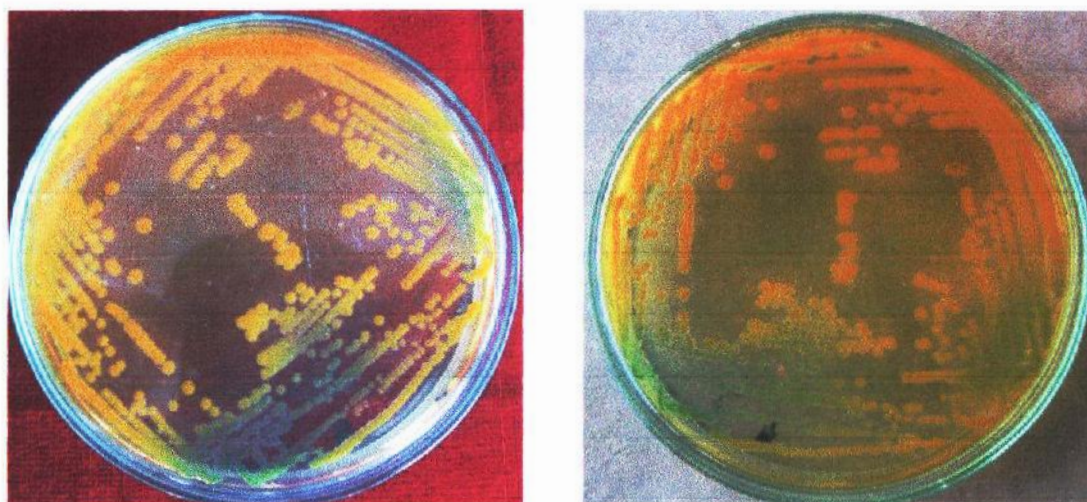


Fig 3.3: Colony characteristics on TCBS-agar plate for water hyacinth sample.

- **Microscopic observation:**

Under the microscope the bacteria were seen as slightly curved rod or “comma” shaped. After gram staining the bacteria appeared as red that representing the characteristics of gram negative bacteria.

3.1.2 Confirmation of isolated samples by PCR test:

To confirm the presence of *V. cholerae* in collected sample, PCR test was carried out. In the PCR test, the PCR product was amplified with designed primers directed towards virulence gene. The summary of the results were shown in Table 3.2.

Table 3.2: The summary of the PCR test results:

Sample	Result of PCR
Water-1	Positive (+)
Water- 2	Positive (+)
Sediment- 1	Positive (+)
Sediment- 2	Negative (-)
Water -3	Negative (-)
sediment-3	Negative (-)

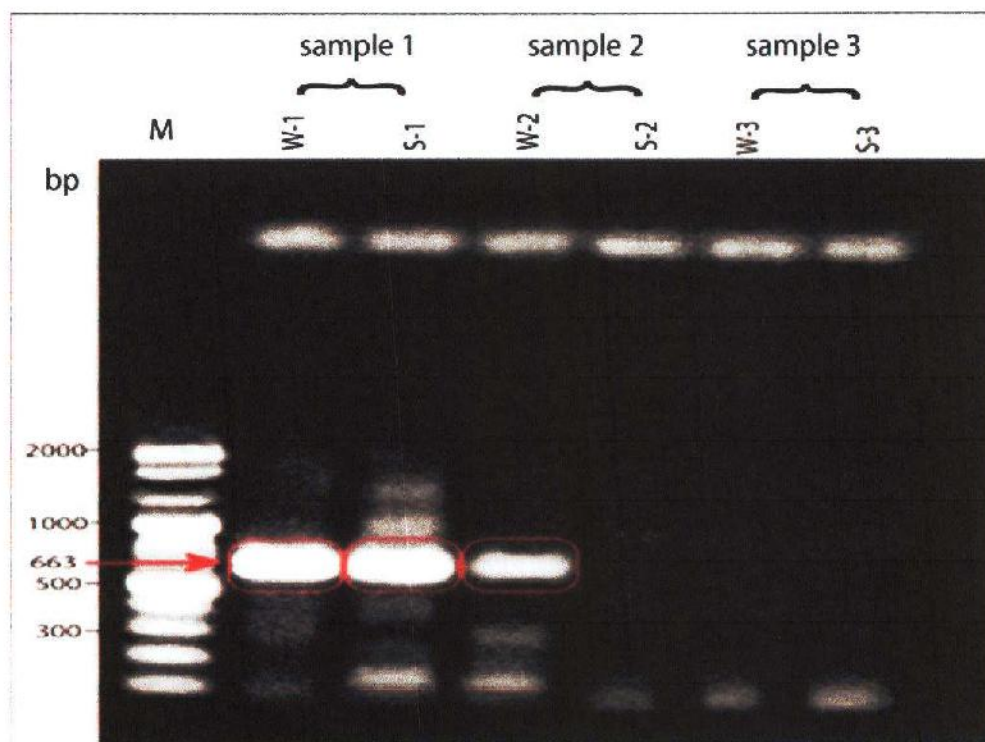


Fig 3.4: Agarose gel electrophoresis for PCR amplicons of *virA* gene from the vibrio isolates. Lane W-1 and W-2 represent *V. cholerae* isolates from water samples of two different location show 663 bp amplicon of *virA* gene. Lane W-3 represents water isolate from third location shows no amplicon. Lane S-1 shows PCR amplicon of *virA* gene from sediment isolate of *V. cholerae*. Lane S-2 and S-3 represent sediment isolate of *V. cholerae* from location-2 and -3 shows no amplicon.

The water samples showed two bands on electrophoresis gel at 663 bp positions indicating characteristic two bands that indicated the presence of *V. cholerae* in water of sample 1 and 2. For sediment samples, one band was observed in the sediment sample that indicated the presence of *V. cholerae* in sediment of sample 1. In case of water and sediment of sample 3, both in water and sediment no band was observed (Fig: 3.4) that indicated the absence of *V. cholerae* in these samples.

3.2 Analysis of colony morphology of *V. cholerae* with seasonal variability:

The colony characteristics from water hyacinth on TCBS agar plate were analysed. The colonies appeared yellow, round and smooth (Fig: 3.5) which showed the characteristics of *V. cholerae* colonies (Appendix 3). In order to analyse seasonal variation, colonies from TCBS were transferred on LB agar plate.

3.2.1 Colony morphology on transferred LB plates at different month:

Two different types of colony smooth and rugose appeared on LB media after colonies were transferred from TCBS agar plate. During March, both smooth and rugose colonies were observed onto LB agar plate (Fig: 3.5). The colonies were subcultured further analysed under electron binocular microscope that showed the characteristics smooth and rugose colony morphology of *V. cholerae* (Fig 3.6).

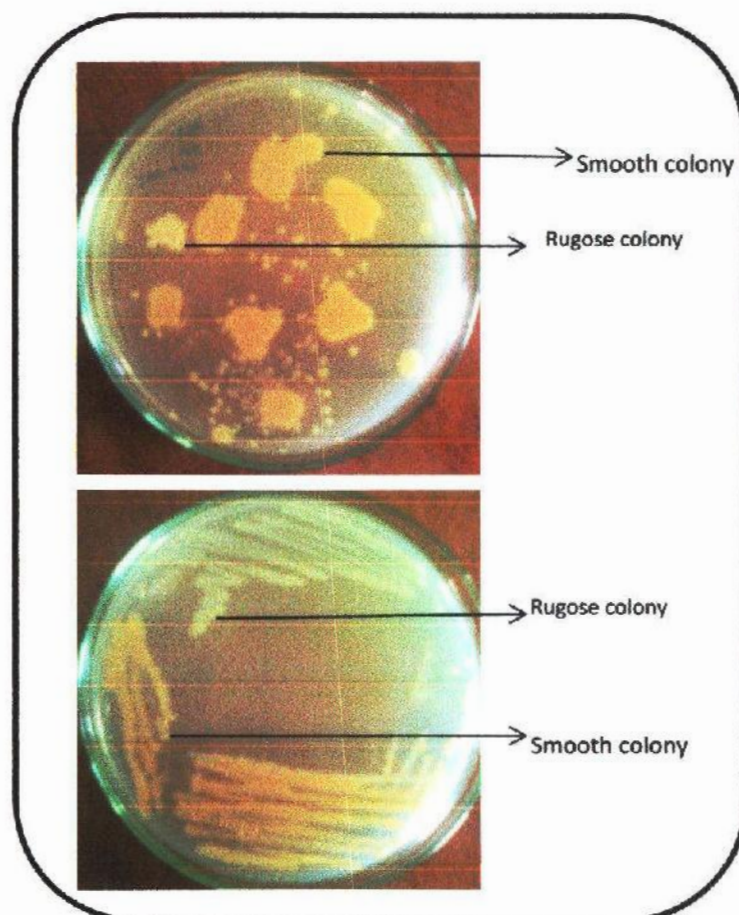


Fig 3.5: Two different colony morphology observed on LB agar plate during March.

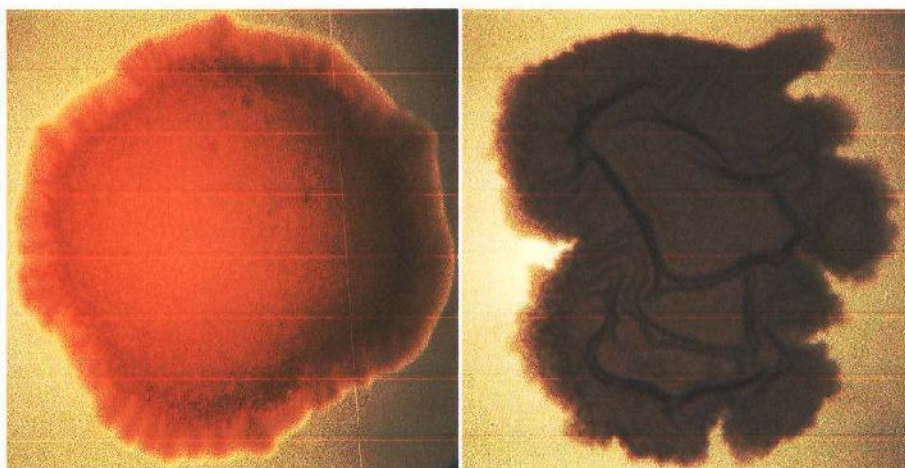


Fig 3.6: Smooth and rugose colonies under stereo binocular microscope.

In month of April and November smooth, yellowish coloured colonies appeared on LB agar plate. In January smooth and slightly rugose colonies were observed. The overall results of different months are summerized in Table 3.3.

Table 3.3: Overview of colony morphology analysis with seasonal variability.

Month	Part of water hyacinth plant	Colony morphology
March	root	Smooth and ruguse
April	root	smooth only
November using velveteen cloths	root	Smooth but flattened
January	root	Smooth and slightly rugose

3.3 Study of persistence of isolated *V. cholerae* in different pesticides solutions:

The growth of isolated *V. cholerae* from water of sample 1 (described in section 3.1.2) in the presence of pesticides was analyzed by measuring the optical density (OD₆₀₀) using different media (LB broth and MS) over 24 hour time interval. Control culture were grown in absence of pesticides using LB and MS media on different period of time and compared to that of the pesticides (Appendix 4)

3.3.1 Growth of *V. cholerae* on LB and MS media

Growth of both isolated and reference *V. cholerae* was increased with time in LB media in compared with that of MS media in presence of pesticides.

Comparison between LB and MS media in presence of pesticides

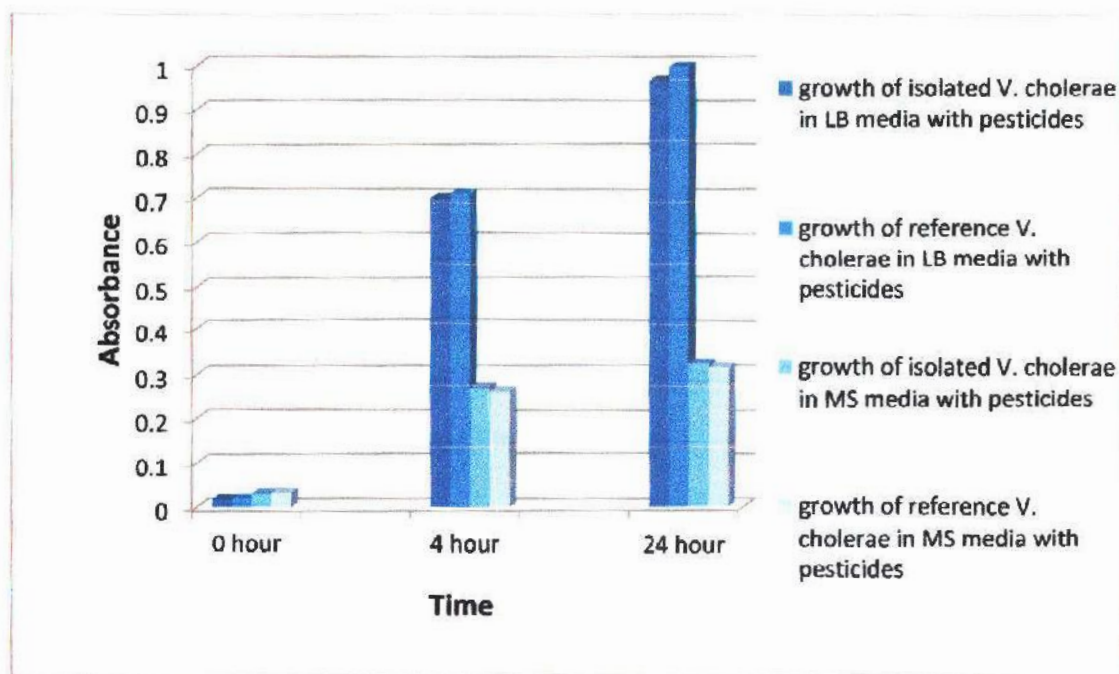


Fig 3.7: Growth of isolated *V. cholerae* in the presence of pesticides. The graph shows growth of isolated and reference *V. cholerae* (OD_{600}) measuring absorbance in 600nm in presence of pesticides at time interval of 0, 4, 24 hour in LB media and MS media.

A higher growth was observed in LB media compared to that of MS media both in presence of pesticides. No difference in growth was observed between the isolated strain and reference strain in both media and in the presence of pesticides.

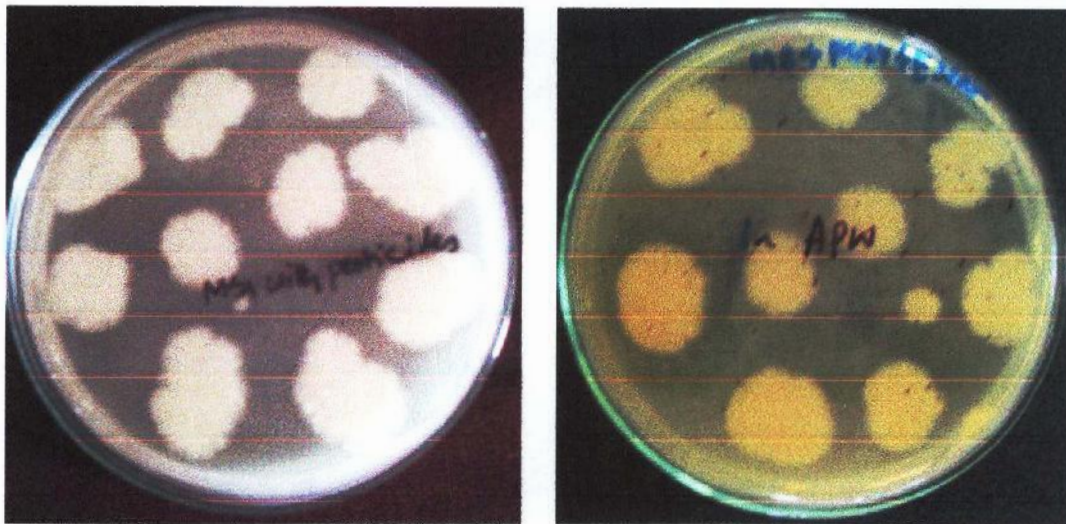


Fig 3.11: Slightly rugose and smooth colony onto LB agar plate.

3.4 Study of persistence of isolated *V. cholerae* in detergent solution:

Persistence of isolated *V. cholerae* was tested using different concentrations of detergent solution. Persistence was determined by counting number of cfu/ml and comparing of *V. cholerae* with that of the control (no detergent) (Table: 3.4).

Table 3.4: Persistence of *V. cholerae* in different concentrations of detergent solutions during 4 day period.

Days	Cfu/ml				
	Control (No detergent)	Detergent solution (10%)	Detergent solution (20%)	Detergent solution (30%)	Detergent solution (40%)
Day 1	3×10^8	1.7×10^7	4.8×10^6	2.6×10^5	6×10^4
Day 4	3.6×10^7	6.1×10^6	4.6×10^5	2.2×10^5	2×10^4

Persistence calculated as cfu/ml at 10%, 20%, 30% and 40% detergent solution. Bacterial cfu/ml at different detergent concentration decreased compared to the respective control cultures (Table 3.4). Colony count was found to decrease with increasing detergent concentration from 10-40% on both Day 1 and Day 4 (fig: 3.12).

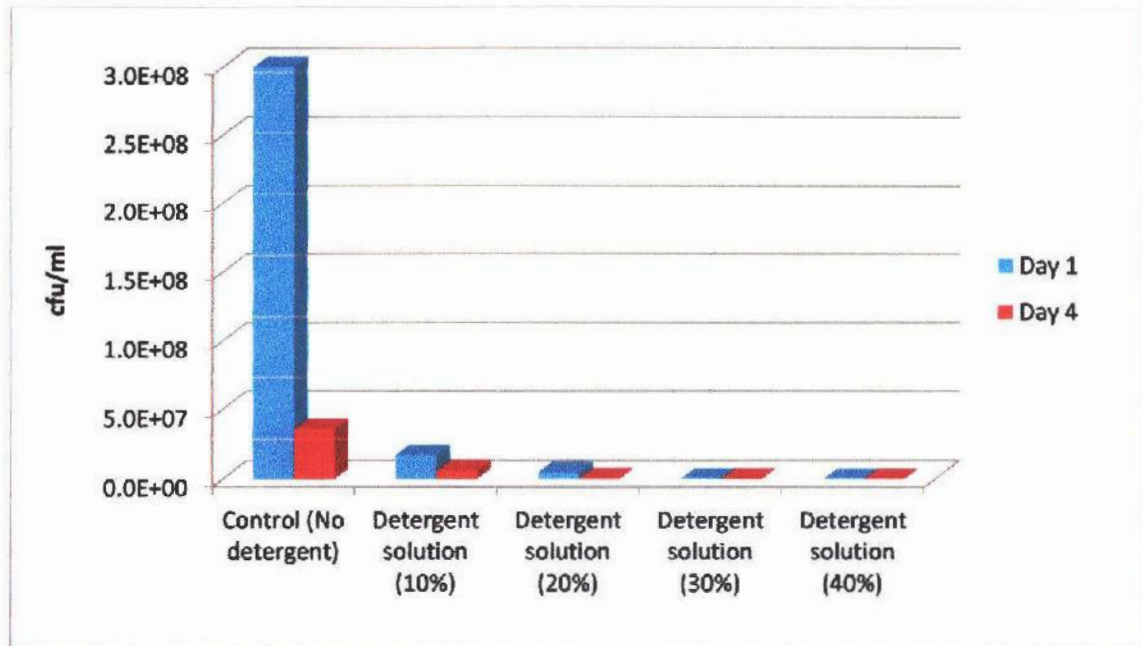


Fig 3.12: Cfu/ml of *V. cholerae* measured at different detergent concentration with control (no detergent).

In control cultures (no detergent) the growth of *V. cholerae* showed higher growth in Day 1 compared to Day 4. Cfu/ml of *V. cholerae* was found to decrease with increasing detergent concentration both in Day 1 and Day 4 in comparison to the control (Fig. 3.12).

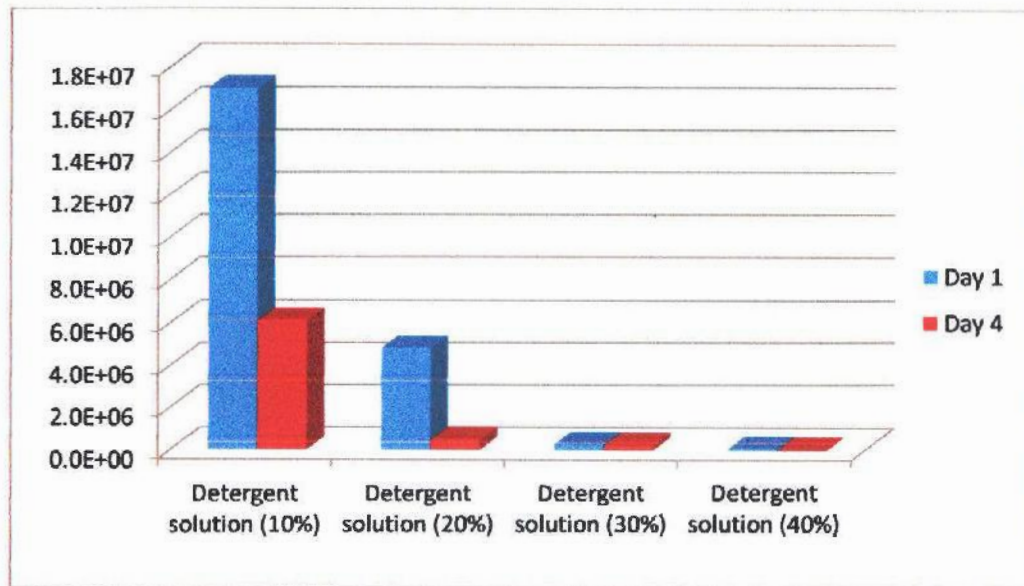


Fig 3.13: Cfu/ml of *V. cholerae* measured at different detergent concentration. Detergent concentration 10%, 20%, 30% and 40% were used and cfu calculated on Day 1 and Day 4.

The number of c.f.u/ml decreased on Day 4 compared to Day 1 with increasing detergent solution.



A



B

Fig 3.14: Persistence of *V. cholerae* following exposure to 10% and 20% detergent solution. **A** and **B** represent counts of colonies from 100 μ l aliquets following 4 days exposure to 10% and 20% detergent respectively.

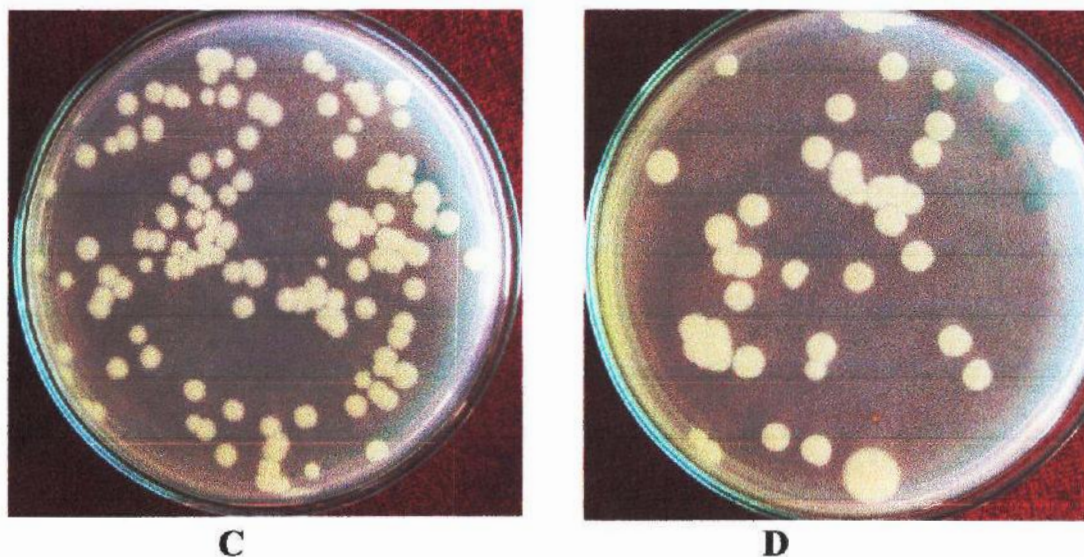


Fig 3.15: Persistence of *V. cholerae* following exposure to 30% and 40% detergent solution. C and D represent counts of colonies from 100 μ l aliquets following 4 days exposure to 30% and 40% detergent respectively.

3.5 Study of association of isolated *V. cholerae* with *Anabaena* sp. PCC 7120:

3.5.1 Growth of *V. cholerae* in association with two weeks culture of *Anabaena* sp. PCC 7120:

In the study of two weeks culture of *V. cholerae* in direct association with *Anabaena* sp. strain PCC 7120, the cfu/ml of *V. cholerae* increased gradually compared to that of the indirect association and control. The highest of cfu/ml was observed in direct association with *Anabaena* sp. PCC 7120 on day 12 (Table: 3.5). In case of indirect association, different trend was observed compared to direct association. The cfu/ml was found to gradually decrease reaching lowest on day 12. In control cultures the cfu/ml was found to remain almost constant throughout the 12 days period (Fig: 3.16).

Table 3.5: Enumeration (Cfu/ml) of *V. cholerae* in association (direct and indirect) with two weeks culture of *Anabaena sp. PCC 7120*:

Two weeks	Cfu/ml		
	Control	Direct association	Indirect association
	BG-11 media with <i>Vibrio cholerae</i>	<i>Anabaena sp. PCC 7120</i> with <i>Vibrio cholerae</i>	Cyanospent culture with <i>Vibrio cholerae</i>
Day3	12 cfu/ml	9 cfu/ml	15 cfu/ml
Day6	8 cfu/ml	15 cfu/ml	13 cfu/ml
Day9	10 cfu/ml	55 cfu/ml	10 cfu/ml
Day12	10 cfu/ml	85 cfu/ml	8 cfu/ml

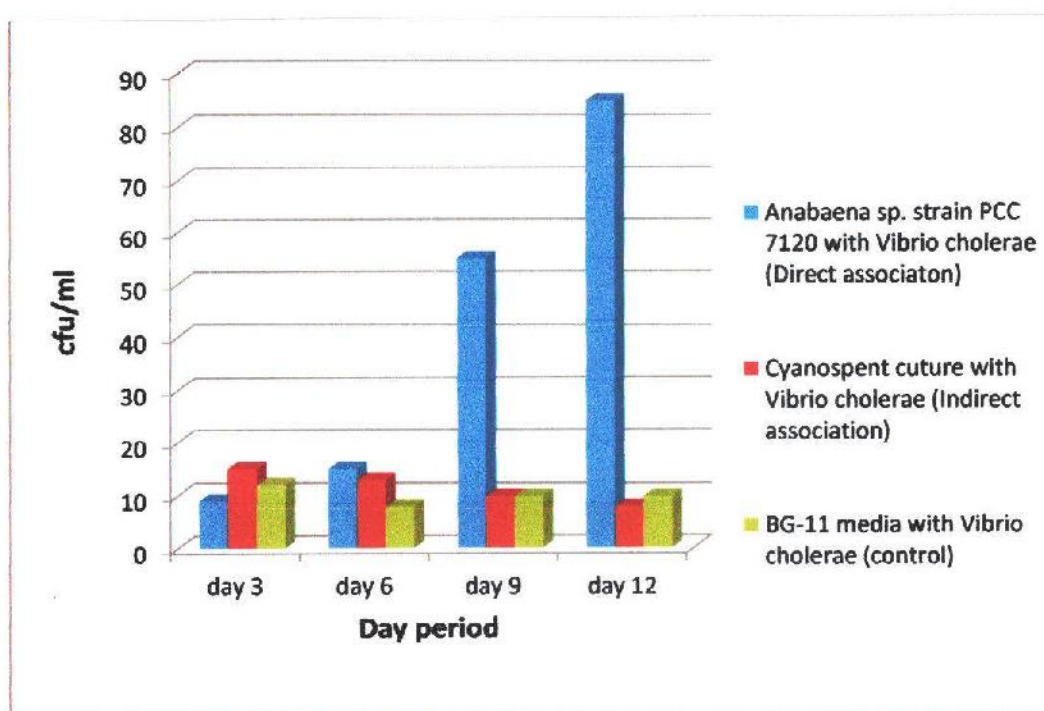


Fig 3.16: Cfu/ml with period of time for direct and indirect association in two weeks culture.

3.5.2 Growth of *V. cholerae* in association with three weeks culture of *Anabaena* sp. PCC 7120:

Anabaena is a slow growing bacteria so they were grown for three weeks. In the direct association of *V. cholerae* with *Anabaena* sp. PCC 7120 it was found that, cfu/ml increased gradually through 12 days compared to that of the indirect association and control. The highest of cfu/ml was observed in direct association with *Anabaena* sp. PCC 7120 on day 12 (Table: 3.6). In case of indirect association, the cfu/ml slightly decreased but almost remained same during the 12 days period. In control cultures the cfu/ml was found to increase steadily throughout the period. (Fig: 3.17).

Table 3.6: Enumeration (cfu/ml) of *V. cholerae* in association (direct and indirect) with three weeks culture of *Anabaena* sp. PCC 7120:

3 weeks	Cfu/ml		
	Control	Direct association	Indirect association
	BG-11 media with <i>Vibrio cholerae</i>	<i>Anabaena</i> sp. PCC 7120 with <i>Vibrio cholerae</i>	Cyanospent culture with <i>Vibrio cholerae</i>
Day 3	4 cfu/ml	10 cfu/ml	15 cfu/ml
Day 6	9 cfu/ml	25 cfu/ml	12 cfu/ml
Day 9	10 cfu/ml	35 cfu/ml	10 cfu/ml
Day 12	13 cfu/ml	50 cfu/ml	15 cfu/ml

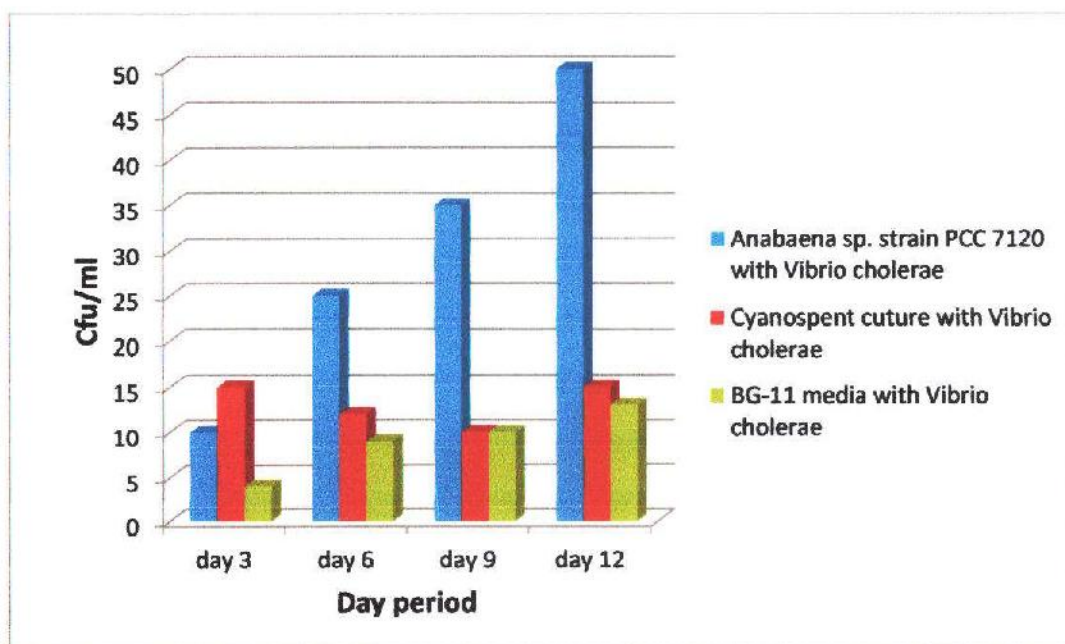


Fig 3.17: cfu/ml with period of time for direct and indirect association in three weeks culture. The graph shows that, cfu/ml was comprehensively high in presence of *Anabaena sp. PCC 7120* *PCC 7120* (direct association) in comparison with cyanospent culture with *V. cholerae* (indirect association) during 12 days time period for two and three weeks culture.

CHAPTER 4: DISCUSSION

4. Discussion

Microbial species can grow in a large amount in waste enriched aquatic environment. It is evident that, *Vibrio cholerae* have been identified from aquatic environment containing high quantity of waste materials and detergents from Mozambique and Nigeria. But detection and isolation of *V. cholerae* from polluted aquatic environment in southern part of Bangladesh is not well known. The river Moyuri of Khulna, from where the samples were collected is highly contaminated. So the organic content of the river is very high. Here, Algae, cyanobacteria, phytoplankton, zooplankton and water hyacinth are detected in a large amount. Three locations of Moyuri river which is more or less polluted were selected for sample collection to represent the whole river. *V. cholerae* was isolated from samples of water, sediment and water hyacinth. *V. cholerae* was isolated throughout the year from water hyacinth of polluted area.

In Bangladesh cholera occurs twice a year and in epidemic seasons they isolated from surface water of pond, river, lake etc. apparently in interepidemic period they disappears from environments (khan, M. U., et al. 1984). In interepidemic period they may hide in microenvironment for survival and multiplication. It has been suggested that blue green algae may act as a reservoirs (Islam, M. S., et al. 1994). *V. cholerae* has been detected in mucilaginous sheath of blue green algae *Anabaena variabilis* (Islam, M. S., et al. 1990). In aquatic environment of Bangladesh about 307 different species of cyanobacteria has been reported from all kinds of water sources (river, canal, ponds, ditches, lakes etc.).

Direct attachment or formation of biofilm is well known as prerequisite in the survival at unfavorable environmental condition. It has been found from the experiment that cfu/ml of isolated *V. cholerae* from Moyuri river increased in direct association with *Anabaena sp. strain PCC 7120* compared to the indirect association. It may be that in direct association, the bacteria attaches to the filament of algae and utilizes metabolites as nutrients (Islam, M. S., et al. 1994). In such association bacteria can supply carbon dioxide and produce an environment of reduced oxygen that facilitates photosynthesis and nitrogen fixation by *Anabaena*. It is also possible that *Anabaena* produce a balance P^H environment by secreting nutrient in surrounding aquatic environment forming a symbiotic relationship. *Anabaena* can secrete nutrients and other substances in surrounding environment in indirect association. *V. cholerae* with cyanobacteria

was tested to see whether any cyanobacterial extracellular substances promote growth of *V. cholerae*. It was observed that in indirect association c. f. u of *V. cholerae* increased compared to the control indicating that extracellular substances secreted by *Anabaena* can also enhance growth. However, since c. f. u of *V. cholerae* was higher in direct association compared to indirect it can be possible that *V. cholerae* relatively can persist in association with *Anabaena*.

In Bangladesh cholera out breaks mainly in pre and post monsoon season. Seasonal outbreaks closely related with survival strategies of *V. cholerae* in aquatic environment. Alteration of smooth to rugose colony morphotypes of *V. cholerae* plays a critical role to survive in the environment. Smooth and rugose variants of *V. cholerae* are able to form biofilm. Biofilm of rugose variant composed of microorganisms and extrapolymeric substances that are not present in smooth variant (Wrangstadh, M., et al. 1986). The extrapolymeric substances of rugose variant shows resistance to cold, osmotic, acid, nutrient deprived and oxidative stresses in nonepidemic seasons (Wai et al. 1998). *V. cholerae* attaches water hyacinth plants by forming biofilm. To see whether *V. cholerae* were smooth or rugose variant when attached with water hyacinth, colony morphology was analyzed.

To analyze colony morphology experiments were carried out in different months. During March and January both smooth and rugose colonies of *V. cholerae* were found from water hyacinth of Moyuri river. Interestingly in April and November we observed only smooth colonies. It can be say that in April prior to monsoon and November after monsoon the aquatic environment remains nutrient rich conditions for *V. cholerae* whereas in March and January the non epidemic season nutrients becomes lower in aquatic environment. To survive this condition they may switch to form rugose colony.

Bangladesh is an agricultural country and every year huge amount of pesticides are used in this field. Pesticides are mixed with pond, lake or river water by runoff resulting from rainfall or irrigation. The presence of milathion, cypermethrin and carbamate pesticides residue was found in some pond water of Bangladesh (Uddin, M. A., et al. 2012). Bacteria can metabolize pesticides for integration of essential nutrients and energy.

In the study it was observed that environmental isolates of *V. cholerae* can grow in presence of pesticides both in LB and MS media. In our experiment pesticides mixture of 50 ppm was used.

In other study pesticides of 50 ppm was also used to assess the growth of pesticides degrading bacteria (S. KaviKarunya and D. Reetha 2012). In presence of pesticides the growth of *V. cholerae* was reduced slightly but not markedly, which may be due to resistance of *V. cholerae* to pesticides. It has been found that different species of *Enterobacteriaceae* are involved in the degradation of organophosphorous insecticides like chlorpyrifos (Singh, K., et al. 2004), phosphonate (Lee, K. S., et al. 1992) and glyphosate (Dick, R. E, and Quinn J. P. 1995) but whether *V. cholerae* degrade the pesticides is not known.

In Moyuri River *V. cholerae* comes in contact with detergents in aquatic environment. Since, everyday people on the river side use river water for various washing purposes. It has been found that environmental *V. cholerae* are much more resistant to hydrophobic compounds, detergents etc than *V. cholerae* isolated from clinical sample (Chaudhuri, K et al., 1992). Detergents are lipophilic compound that have various effects on cell membranes. Detergents can disrupt the organization of the cell membranes. The detergent concentration is very high of Moyuri river and bacterial population is found to survive in presence of this constrains. For this reason persistence in presence of detergent solution of isolated *V. cholerae* from this river was studied. Detergent solution containing same amount of Sodium lauryl sulphate (SLS) and polyethylene glycol (PEG) was considered as the test detergent solution because SLS is one of the major surfactant used in our country in washing up liquids, soaps, shampoos etc. PEG is a mild surfactant used in toothpaste.

Vibrio is the most frequently encountered organism for detergent degradation and utilization (Amunt et al., 1990). In the study of the persistency of *V. cholerae* to different concentrations of detergent solution it was found that, the colony count was decreased at detergent concentration up to 40% both on Day 1 and Day 4. Colonies were found to be round and smooth with opaque center and translucent periphery. At higher concentrations it may provide adverse environment for the growth and survival of *V. cholerae* resulting decreased number of colonies. In extended period of incubation it may undergoes stress conditions that resulted in lower growth on Day 4 compared to Day 1. Report shows that inhibition of growth occurs when cells of *V. cholerae* were grown in the presence of a detergent (0.01% SDS) (Hung, D. T., and Mekalanos, J. J. 2005).

Conclusion

In this study it can be concluded by that, environmental isolate of *V. cholerae*, from Moyuri river can survive immediate disposal of pesticides, detergent in the river. It can also grow favorably in association with *Anabaena sp. strain PCC 7120*. Everyday huge amount of industrial waste, agricultural waste, human waste are disposed and available water hyacinth are detected in Moyuri river. In our experiment *V. cholerae* showed considerable growth in different pesticides mixture and higher concentrations of detergent. These biotic and abiotic conditions may affect the growth of *V. cholerae*. Study of persistency of *V. cholerae* in biotic and abiotic conditions have greater importance to reveal the actual mechanism of their adaptation in these conditions. Further studies are needed regarding these that may help us to understand the mechanisms of survival of *V. cholerae* in biotic and abiotic stress.

CHAPTER 5: REFERENCES

6. REFERENCES

- Adagbada, A. O., Adesida, S. A., Nwaokorie, F. O., Niemogha, M. T., Coker, A. O. (2012) "Cholera epidemiology in Nigeria: an overview" *The Pan African Medical Journal* - ISSN 1937-8688.
- Ahmed, S. M., Raknuzzaman M., Akther, H., and Ahmed S. (2007) "The Role of Cyanobacteria Blooms in Cholera Epidemic in Bangladesh" *Journal of Applied Sciences* 7 (13):1785-1789.
- Alam, M., M. Sultana, G. B. Nair, A. K. Siddique, N. A. Hasan, R. B. Sack, D. A. Sack, K. U. Ahmed, A. Sadique, H. Watanabe, C. J. Grim, A. Huq, and R. R. Colwell. 2007. Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proc. Natl. Acad. Sci. U. S. A.* 104:17801-17806.
- Alam, M., Sultana M, Nair, G. B., Sack, R. B., Sack, D. A., Siddique, A. K., Ali, A., Huq, A., and Colwell R. R. (2006) "Toxigenic *Vibrio cholerae* in the Aquatic Environment of Mathbaria, Bangladesh" *Appl. Environ. Microbiol.* 72(4):2849-2855.
- Alam, M., Sultana, M., Nair, G. B., Siddique, A. K., Hasan, N. A., Sack, R. B., Sack, D. A., Ahmed, K. U., Sadique, A., Watanabe, H., Grim, C. J., Huq, A., and Colwell, R. R. (2007) "Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission" *Proc. Natl. Acad. Sci. U. S. A.* 104:17801-17806.
- Albert, M. J. (1993) "Personal reflections on the discovery of *Vibrio cholerae* 0139 synonym Bengal: a tribute to team work and international collaboration" *J. Diarrhoeal Dis. Res.* 11:207-210.
- Albert, M. J. (1994) "*Vibrio cholerae* O139 Bengal" *J. Clin. Microbiol.* 32(10): 2345.
- Albert, M. J., A. K. Siddique, M. S. Islam, A. S. G. Faruque, M. Ansaruzzaman, S. M. Faruque, and R. B. Sack. 1993. A large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. *Lancet* 341:704.

Ali, A., Rashid, M. H., and Karaolis, D. K. R. (2002) "High-frequency rugose exopolysaccharide production by *Vibrio cholerae*" *Appl. Environ. Microbiol.* 68:5773-5778.

Amund, O. O., Ilori, M. O., Odetundun, F. R. (1997) "Degradation of commercial detergent products by microbial populations of the Lagos Lagoon" *Folia Microbiologica* 42(4):353-356.

Baker-Austin, C., L. Stockley, R. Rangdale, and J. Martine Urtaza (2010) "Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective" *Environ. Microbiol. Reports* 2:7-18.

Banglapedia, (2005)

Baron, S., Chevalier, S., and Lesne, J. (2007) *J Health Popul Nutr.* 25(3):312-318

Barua, D. (1992) "History of cholera" In: Barua D, Greenough WB, eds. *Cholera*. New York: Plenum Medical Book, 1-5.

Baumann, P., Furniss A. L., Lee, J. V (1984) Genus 1, *Vibrio*. In: Krieg PNR, Halt JG, eds. *Bergey's manual of systematic bacteriology*. Baltimore, Williams & Wilkins 1: 518-538.

Beadle, I. R., and Verran, J. (1999) "The survival and growth of an environmental *Klebsiella* isolate in detergent solutions" *J. App. Microbiol.* 87:764-769

Binsztein, N., M. C. Costagliola, M. Pichel, V. Jurquiza, F. C. Ramirez, R. Akselman, M. Vacchino, A. Huq, and R. Colwell. 2004. Viable but nonculturable *Vibrio cholerae* O1 in the aquatic environment of Argentina. *Appl. Environ. Microbiol.* 70:7481-7486.

Blackstone, G. M., J. L. Nordstrom, M. D. Bowen, R. F. Meyer, P. Imbro, and A. DePaola. 2007. Use of a real time PCR assay for detection of the *ctxA* gene of *Vibrio cholerae* in an environmental survey of Mobile Bay. *J. Microbiol. Methods* 68:254-259.

Borrito, J. R. (1997) "Ecology of *Vibrio cholerae* serogroup O1 in aquatic environments" *Rev Panam Salud Publica* 2 (5):105-108.

Brayton, P. R., Tamplin, M. L., Huq, A., and R. R. Colwell (1987) "Enumeration of *Vibrio cholerae* O1 in Bangladesh waters by fluorescent-antibody direct viable count" *Appl. Environ. Microbiol.* 53:2862-2865.

Bronze, M.S., and Greenfield, R.A (Ed.). (2005). "Biodefence Principles and Pathogens horizon bioscience"

Broza, M., and Halpern, M. (2001) "Pathogen reservoirs: Chironomid egg masses and *Vibrio cholerae*" *Nature* 412:40.

Burke, D., Carmichael, A., Focks, D., Gimes, D., Harte, J., Lele, S., Martens, P., Mayer, J., Mearns, L., Pulwarty, R., Leal, L., Ropelewski, Ch., Rose, J., Shope, R., Simpson, J., Wilson, M. (2001) *Under the Weather: Climate, Ecosystems, and Infectious Disease*. Committee on Climate, Ecosystems, Infectious Diseases, and Human Health, Board on Atmospheric Sciences and Climate, National Research Council, Division on Earth and Life Studies, National Research Council. National Academy of Sciences. Washington DC, U.S.A.: The National Academy Press.

Chakraborty, S., J. Khanam, Y. Takeda, and G. B. Nair. 1999. Application of PCR for detection of toxigenic *Vibrio cholerae* O1 in water samples during an outbreak of cholera. *Trans. R. Soc. Trop. Med. Hyg.* 93:527-528.

Chanika, E., Georgiadou, D., Soueref, E., Karas, P., Karanasios, E., Tsiropoulos, N.G., Tzortzakakis, E. A., Karpouzias, D.G. (2011) "Isolation of soil bacteria able to hydrolyze both organophosphate and carbamate pesticides" *Bioresour Technol.* 102(3):3184-92.

Chatterjee & Chaudhuri, (2003) "Lipopolysaccharides of *Vibrio cholerae*. I. Physical and chemical characterization" *Biochim Biophys Acta.* 1639(2):65-79.

Chaturvedi, V., and Kumar, A. (2011) "Diversity of culturable sodium dodecyl sulfate (SDS) degrading bacteria isolated from detergent contaminated ponds situated in Varanasi city, India" *International Biodeterioration & Biodegradation* 65:961-971.

Chaudhuri, K., Bhadra, R. K., and Das, J. (1992) "Cell surface characteristics of Environmental and clinical isolates of *V. cholerae* Non-01" *Appl. Environ. Microbiol.* 58(11):3567-3573.

Chaudhuri, S., Masterson, C., Boehmer, P. E., McDonald, F., Hickson, I. D., Emmerson, P. T. (1992) "Reconstitution of the activities of the RecBCD holoenzyme of *Escherichia coli* from the purified subunits" *J Biol Chem.* 267(19):13564-72.

Chemfinder. 2006. http://chemfinder.cambridgesoft.com/result.asp?mol_rel_id=151-21-3

Chiavelli, D. A., Marsh, J. W., and Taylor, R. K. (2001) "The Mannose-Sensitive Hemagglutinin of *Vibrio cholerae* Promotes Adherence to Zooplankton" *Appl. Environ. Microbiol.* 67(7):3220-3225.

Cholera Working Group, International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR) (1993) 'Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal" *Lancet* 342:387-90

Chomczynski, P., and Rymaszewski, M. (2006) "Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood" *BioTechniques* 40:454-458

Chongsa-nguan, M., Chaicumpa, W., Moolasart, P., Kandhasingha, P., Shimada, T., Kurazono, H., and Takeda, Y. (1993) "*Vibrio cholerae* 0139 Bengal in Bangkok" *Lancet* 342:430-431.

Chun, J., A. Huq, and R. R. Colwell. 1999. Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus*. *Appl. Environ. Microbiol.* 65:2202-2208.

Colwell, R. R. (1996) "Global climate and infectious disease: the cholera paradigm" *Science* 274:2025-2031.

Colwell, R. R., and Huq, A. (1994) "Vibrios in the environment: viable but non-culturable *Vibrio cholerae*. In: *Vibrio cholerae and cholera: molecular to global perspectives*" Wachsmuth I K, Blake P A, Olsvik O, editors. Washington, D.C: American Society for Microbiology:117-133.

Colwell, R. R., P. R. Brayton, D. J. Grimes, D. R. Roszak, S. A. Huq, and L. M. Palmer (1985) "Viable, but non-culturable *Vibrio cholerae* *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms" *Bio/Technology*. 3:817-820.

Copley, S. D. (2009) "Evolution of efficient pathways for degradation of anthropogenic chemicals". *Nature Chemical Biology* 5(8): 559-66.

Cox, A. D, Brisson, J. R, Varma V, Perry, M. B (1996) "Structural analysis of the lipopolysaccharide from *Vibrio cholerae* O139" *Carbohydr Res*. 290(1):43-58

Cunningham, C. C, and Van Horn, C. G. (2003) "Energy availability and alcohol-related liver pathology" 27(4):291-9.

Dawson, M. P., Humphrey, B., Marshall, K. C. (1981) "Adhesion:a tactic in the survival strategy of a marine *Vibrio* during starvation" *Curr. Microbiol*. 6:195-201

Dick, R. E, and Quinn J. P. (1995) "Glyphosate-degrading isolates from environmental samples: Occurrence and pathways of degradation" *Appl. Microbiol. Biotechnol*. 43(8): 545-550

Faisal, I.M., and Parveen, S. (2004) "Food Security in the Face of Climate Change, Population Growth and Resource Constraints: Implications for Bangladesh" *Environmental Management* 34(4):487-498.

Farmer J J, Hickman-Brenner F W, Kelly M T 1985 *Vibrio*. In: Lennette E H et al. (eds) *Manual of clinical microbiology* 4th edn. American Society for Microbiology, Washington DC, pp 282-301.

Faruque, S. M., Islam J., Ahmad, S. Q., Biswas, K., Faruque, A. S. G., Nair, G. B., Sack, R. B., Sack, D. A., and Mekalanos, A. j. (2006) "An improved technique for isolation of environmental *Vibrio cholerae* with epidemic potential: monitoring the emergence of a multiple- antibiotic-resistant epidemic strain in Bangla" *J. Infect. Dis.* 193:1029-36.

Faruque, S. M., Albert, M. J., and Mekalanos, J. J. (1998) "Epidemiology, Genetics, and Ecology of Toxigenic *Vibrio cholerae*" *Microbiol Mol Biol Rev.* 62(4):1301-1314.

Faruque, S. M., Asadulghani, Alim, A. R., Albert, M. J., Islam, K. M., and Mekalanos, J. J. (1998) "Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic *Vibrio cholerae* O1 and O139" *Infect. Immun.* 66:3752-3757.

Finkelstein, R. A. (1988) Chapter 24 Cholera, *Vibrio cholerae* O1 and O139, and Other Pathogenic Vibrios *Medical Microbiology*. 4th edition.

Fisher-Hoch, S. P., Khan, A., Haq, I. U., Khan, M. A. and Mintz, E. D. (1993) "*Vibrio cholerae* O139 in Karachi, Pakistan" *Lancet* 342:1422-1423.

Fraga, S. G., Pichel, M., Costagliola, M., Cecilia, M., Jurquiza, V., Peressutti, S., Caffer, M. I., Aulet, O., Hozbor, C., Tracanna, B. C., de Gamundi, A.V., Hernández, D., Ramírez, F. C., Akselman, R., Binsztein, N. (2007) "Environment and virulence factors of *Vibrio cholerae* strains isolated in Argentina." *J Appl Microbiol.* 103(6):2448-56.

Glass, R. I., and R. E. Black (1992) "The epidemiology of cholera" 129-154. In D. Barua and W. B. Greenough III (ed.), *Cholera*. Plenum, New York, NY.

He, H., Cooper, J. N., Mishra, A., Raskin, D. M. (2012) "Stringent response regulation of biofilm formation in *Vibrio cholerae*" *J Bacteriol.* 194(11):2962-72.

HSDB (Hazardous Substances Data Bank). 2002 Sodium Lauryl Sulfate. <http://toxnet.nlm.nih.gov/>.

Hung, D. T., and Mekalanos, J. J. (2005) "Bile acids induce cholera toxin expression in *Vibrio Cholerae* in a ToxT-independent manner" *PNAS* 102(8):3028–3033.

Huq, A., Colwell, R. R., Rahman, R., Alt, A., Chowdhury, M. A. R., Parveen, S., Sack, D. A., and Cohen, E. R. (1990) "Detection of *Vibrio cholerae* 01 in the aquatic environment by fluorescent-monoclonal antibody and culture methods" *App. Environ. Microbiol.* 56:2370-2373.

Huq, A., Haley, B. J., Taviani, E., Chen, A., Hasan, N. A., and Colwell, R. R. (2012) *Current Protocols in Microbiology* 6A.5.1-6A.5.51

Huq, A., West, P. A., Small, E. B., Huq, M. I., and Colwell, R. R. (1984) "Influence of Water Temperature, Salinity, and pH on Survival and Growth of Toxigenic *Vibrio cholerae* Serovar 01 Associated with Live Copepods in Laboratory Microcosms" *Appl. Environ. Microbiol.* 48(2):420-424.

Huq, A., Grim C., Colwell, R. R., Nair, G. B. (2006) "Detection, isolation, and identification of *Vibrio cholerae* from the environment" *Curr Protoc Microbiol.* Chapter 6: Unit 6, A.5. In R. R. Colwell (ed.), *Vibrios in the environment*. John Wiley & Sons, inc., New York.

IPCC (2001) *Climate Change 2001: Mitigation, Contribution of Working Group III to the Third Assessment Report of the Intergovernmental Panel on Climate Change (IPCC)* Cambridge University Press, UK.

Islam, M. S. (1990) "Effect of various biophysicochemical conditions on toxigenicity of *Vibrio cholerae* O1 during survival with a green alga, *Rhizoclonium fontanum*, in an artificial aquatic environment" *Canadian Journal of Microbiology* 36:464–468.

Islam, M. S., Alam M. J., and Neogi, P.K.B. (1992) "Seasonality and toxigenicity of *V. cholera* non 01 isolated from different components of pond ecosystems of Dhaka city, Bangladesh" *World J. Microbiol. Biotechnol.* 8:160-163.

Islam, M. S., Drasar, B. S., and Sack, R. B. (1994a) "The aquatic flora and fauna as reservoirs of *V. cholera* A review." *J. Diarrhoea Dis. Res.* 12:87-96.

Islam, M. S., Drasar, B. S., Sack, R. B. (1994b) "Probable role of blue green algae in maintaining endemicity and seasonality of cholera in Bangladesh: A hypothesis." *Journal of Diarrhoeal Disease Research* 12:245-256.

J. Glenn Morris, Jr., Marcelo B. Sztein, Eugene W. Rice, James P. Nataro, Genevieve A. Losonsky, Pinaki Panigrahi, Carol O. Tacket, and Judith A. Johnson *Vibrio cholerae* 01 Can Assume a Chlorine-Resistant Rugose Survival Form that Is Virulent for Humans *The Journal of Infectious Diseases* 1996; 174:1364-8

Janda, J. M., Powers, C., Bryant, R. G., and Abbott, S. L. (1988) "Current perspective on the epidemiology and pathogenesis of clinically significant *Vibrio* spp." *Clin. Microbiol. Rev.* 1:245-267.

Juras, H., Futh, U., Winkler, D., Friedmann, J., and Hillig, J. (1979) "Vorkommen von NAG-vibrio en in Berliner gewässern" *Z. Allg. Mikrobiol.* 19:403-409.

Kaper, J. B., Morris, J. G., and Levine, M. M. (1995) "Cholera" *Clin. Microbiol. Rev.* 8:48-86.

Kaper, J., Lockman, H., Colwell, R. R., and Joseph, S. W. (1979) "Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay" *Appl. Environ. Microbiol.* 37:91-103.

KaviKarunya, S., and Reetha, D. (2012) "Biological Degradation of Chlorpyrifos and Monocrotophos by Bacterial Isolates" International Journal of Pharmaceutical & Biological Archives 3(3):685-691.

Kenneth Todar (2008-2012) "Textbook on bacteriology: *Vibrio cholerae* and Asiatic Cholera"

Khan, M. U., Shahidullah, M., Haque, M. S., Ahmed, W. U. (1984) "Presence of vibrios in surface water and their relation with cholera in a community" Trop. Geogr. Med. 36:335-40.

Kondo, S., Sano, Y., Isshiki, Y., Hisatsune, K., (1996) "The O polysaccharide chain of the lipopolysaccharide from *Vibrio cholerae* O76 is a homopolymer of N-[(S)-(+)-2-hydroxypropionyl]-alpha-L-perosamine" Microbiology. 142 (Pt 10):2879-85

Kramer, V. C., Calabrese, D. M., and Nickerson, K. W. (1980) "Growth of *Enterobacter cloacae* in the presence of 25% sodium dodecyl sulfates" Environ. Microbiol. 40:973-976.

Krauss, H., Weber, A., Appel, M., Enders, B., Isenberg, H. D., Schiefer, H. G., Slenczka, W., von Graevenitz, A., & Zahner, H. (Eds.). (2003) "Zoonoses Infectious Diseases Transmissible from Animals to Humans" (3rd ed.). Washington: ASM press

Lee, J. V., Bashford, D. J., Donovan, T. J., Furniss, A. L., and West, P. A. (1982) "The incidence of *Vibrio cholerae* in waters, animals and birds in Kent, England" J. Appl. Bacteriol. 52:281-291.

Lee, J. V., Bashford, D. J., Donovan, T. J., Furniss, A. L., and West, P. A. (1984) "An incidence and distribution of *Vibrio cholerae* in England" p. 427-450. In R. R. Colwell (ed.), *Vibrios in the environment*. John Wiley & Sons, Inc., New York.

Lee, K. S., Metcalf, W. W. and Wanner, B. L. (1992) "Evidence for two phosphonate degradative pathways in *Enterobacter aerogenes*" J. Bacteriol. 174(19):2501-2510.

Lobitz, B., Beck, L., Huq, A., Wood, B., Fuchs, G., Faruque, A. S., Colwell, R.R. (2000) "Climate and infectious disease: use of remote sensing for detection of *Vibrio cholerae* by indirect measurement" *Proc Natl Acad Sci U S A.* 97(4):1438-43

MacFaddin J. F., (1985) "Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria" Williams & Wilkins, Baltimore, Md. Vol. 1,

Magny, C. G. D., Mozumder, P. K., Grim, C. J., Hasan, N. A., Naser, M. N., Alam, M., Sack, R. B., Huq, A., and Colwell, R. R. (2011) "Role of zooplankton diversity in *Vibrio cholerae* population dynamics and in the incidence of cholera in the Bangladesh Sundarbans" *Appl. Environ. Microbiol.* 77:6125–6132.

Marden, P., Tunlid, A., Malmcrone-Friberg, K., Odham, G., Kjelleberg, S. (1985) "Physiological and morphological changes during short term starvation of marine bacteria isolate" *Arch. Microbiol.* 149:326–332.

Matin A., Auger E. A., Blum P. H., Schultz J. E. (1989) Genetic basis of starvation survival in nondifferentiating bacteria. *Annu. Rev. Microbiol.* 42:293–316.

Mizunoe, Y., Wai, S. N., Takade, A. and Yoshida, S-I (1999) "Isolation and Characterization of Rugose Form of *Vibrio cholerae* O139 Strain MO10" *Infect. Immun.* 67(2): 958-963

Morris, G. K., Merson M H, Huq I, Kibrya A K M G, Black R 1979 Comparison of four plating media for isolating *Vibrio cholerae*. *Journal of Clinical Microbiology* 9: 79-83.

Morris, J. G., Sztein, M. B., Rice, E. W., Nataro, J. P., Losonsky, G. A., Panigrahi, P., Tacket, C. O., and Johnson, J. A. (1996) "*Vibrio cholerae* O1 can assume a chlorine-resistant rugose survival form that is virulent for humans" *J. Infect. Dis.* 174:1364-1368.

Murugesan, A. G., Jeyasanthi, T., and Maheswari, S., (2010) "Isolation and characterization of cypermethrin utilizing bacteria from Brinjal cultivated soil African" *J. Microbiol. Res.* 4 (1): 010-013.

Nair, G. B., Ramamurthy, T., Bhattacharya, S. K., et al. (1994) "Spread of *Vibrio cholerae* O139 Bengal in India" *J. Infect. Dis.* 169:1029–34.

Nelson, E. J., Harris, J. B., Morris, J. G., Jr, Calderwood, S. B., & Camilli, A. (2009). Cholera transmission: the host, pathogen and bacteriophage dynamic. *Nature Reviews Microbiology*, 7(10), 693–702.

Provenzano, D., Schuhmacher, D. A., Barker, J. L., and Klose, K. E. (2000) "The Virulence Regulatory Protein ToxR Mediates Enhanced Bile Resistance in *Vibrio cholerae* and Other Pathogenic *Vibrio* Species" *Infect. Immun.* Mar. 68(3): 1491–1497.

PTCL (Physical and Theoretical Chemistry Laboratory Oxford University). 2005. Chemical and Other 456 Safety Information: Safety (MSDS) Data for Sodium Dodecyl Sulfate. http://ptcl.chem.ox.ac.uk/MSDS/SO/sodium_dodecyl_sulfate.html

Rahman, M (2011) Cholera burden in Bangladesh, in *Introduction to cholera*

Ramamurthy, T., Garg, S., Sharma, R., et al. (1993) "Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India" *Lancet* 341: 703–4.

Rice, E. W., Johnson, C. H., Clark, R. M., Fox, K. R., Reasoner, D. J., Dunnigan, M. E., Panigrahy, P., Johnson, J. A., and Morris, J. G. (1993) "Chlorine and survival of "rugose" *Vibrio cholerae*" *Int. J. Environ. Health Res.* 3:89-98.

Roberts, N. C., Bradford, H. B., and Barbay, J. A. (1984) "Ecology of *Vibrio cholerae* in Louisiana Coastal waters" 389-398.

Roszak, D. B., and Colwell (1987) "Survival strategies of bacteria in the natural environment" *Microbiol. Rev.* 51:365-379.

Roszak, D. B., D. J. Grimes, and R. R. Colwell. (1984) "Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems" *Can. J. Microbiol.* 30:334-338.

Ryan, K. J., and Ray, C. G. (Eds.) (2004) "Sherris Medical Microbiology: An Introduction to Infectious Disease" (Fourth Edition. ed.). New York.: McGraw-Hill.

Sack, R. B., Siddique, A. K., Longini, I. M., Nizam, A., Yunus, Md., Islam M. S., Morris, J. G., Ali, A., Huq, A., Nair, G. B., Qadri, F., Faruque, S. M., Sack, D. A., and Colwell, R. R. (2003) "A 4-Year Study of the Epidemiology of *Vibrio cholerae* in Four Rural Areas of Bangladesh" . *Infect. Dis.*187:96-101

Senderovich, Y., Izhaki, I., Halpern, M., (2010) "Fish as reservoirs and vectors of *Vibrio cholerae*" *PLoS* 5(1):8607.

Shiba, T., R. T. Hill, W. L. Straube, and R. R. Colwell. (1995) "Decrease in culturability of *Vibrio cholerae* caused by glucose" *Appl. Environ. Microbiol.* 61:2583-2588.

Siddique, A. K., Zaman, K., Baqui, A. H., Akram, K., Mutsuddy, P., Eusof, A., Haider, K., Islam, S., and Sack, R. B. (1992) "Cholera epidemics in Bangladesh: 1985-1991" *J. Diarrhoeal Dis. Res.* 10:79-86.

Singh, K., Brajesh, K., Walker, A., Alum, J., Morgan, W., Wright, D. J. (2004) "Biodegradation of chlorpyrifos by *Enterobacter* strain B-14 and its use in biodegradation of contaminated soils" *Appl. Environ. Microbiol.* 70:4855-4863.

Singleton, F. L., Attwell, R., Jangi, S., Colwell, R. R (1982a) "Influence of salinity and organic nutrient concentration on survival and growth of *Vibrio cholerae* in aquatic microcosms" *Appl. Environ. Microbiol.* 43:1080-1085.

Singleton, F. L., Attwell, R., Jangi, S., Colwell, R. R (1982b) "Effects of temperature and salinity on *Vibrio cholerae* growth" *Appl. Environ. Microbiol.* 44(5): 1047-1058.

Singleton, F. L., Huq, A., W. R., Jangi, M. S., and Colwell, R. R. (1982c) "Studies on the ecology of *Vibrio cholerae*" Proceedings of the Seventh Annual Tropical And Subtropical Fisheries Technological Conference of the Americas. TAMU-SG-82-110. Marine Information Service. Sea Grant College Program. Texas A&M University, College Station, Tex. p. 259-282

Tamang, M. D., Sharma N, Makaju, R. K, Sarma, A. N, Koju, R., Nepali, N., Mishra, S. K. (2005) "An outbreak of El Tor cholera in Kavre district, Nepal." KUMJ 3(2):138-142.

Tamplin, M., Gauzens, A., Huq, A., Sack, R., Colwell, R. R. (1990) "Attachment of *Vibrio cholerae* serogroup 01 to zooplankton and phytoplankton of Bangladesh waters" Appl. Environ. Microbiol. 56:1977–1980

Uddin, M. A., Auwal, M .A., Chowdhury, M.A. Z., Rahman, M. A., and Alam, M. K. (2012) "pesticide residue in some selected pond water samples of Bangladesh Bangladesh" J. Sci. Res. 25(1): 93-98.

Vaccine, Bangladesh. Sasakawa Auditorium, Dhaka.

Wai, S. N., Mizunoe, Y., Takade, A., Kawabata, S-I, and Yoshida, S-I (1998) "*Vibrio cholerae* O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation" Appl. Environ. Microbiol. 64: 3648-3655.

Wathik A. H., Safaa A. A., Kais, K. G., Maher, M. M. (2013) "Study the Growth Kinetics of *Pseudomonas aeruginosa* Degrading Some Pesticides which Isolated from Cultivated Soll" Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ University of Bagdad

Watnick, P. I., Lauriano, C. M., Klose, K. E., Croal, L. and Kolter, R. (2001) "The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* 0139" Molec Microbiol 39(2):223-235.

West, P. A., and J. V. Lee. 1982. Ecology of *Vibrio* species, including *Vibrio cholerae*. in natural waters of Kent, England. *J. Appl. Bacteriol.* 52:435-448.

White, P. B. (1938) "The rugose variant of vibrios" *J. Pathol. Bacteriol.* 46:1-6.

WHO (2009) Diarrhoeal diseases.

World Bank (2000) Bangladesh: Climate Change & Sustainable Development. Report No. 21104 BD, Dhaka.

World Health Organization. (1995) Meeting on The Potential Role of New Cholera Vaccines in the Prevention and Control of Cholera Outbreaks during Acute Emergencies. Document CDR/GPV/95.1. World Health Organization, Geneva, Switzerland.

Wrangstadh, M., Conway, P. L., Kjelleberg, S. (1986) "The production and release of an extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. and the effect thereof on adhesion" *Arch. Microbiol.* 145(3):220-227.

Xu, S-H., Roberts, N., Singleton, F. L., Attwell, R. W., Grimes, D. J., and Colwell, R. R. (1982) "Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment" *Microb. Ecol.* 8:313-323.

Yildiz FH and Schoolnik GK (1999) *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the

Yildiz, F. H., and G. K. Schoolnik. 1999. *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc. Natl. Acad. Sci. USA* 96:4028-4033. [

Yildiz, F. H., and Schoolnik, G. K. (1999) "Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation" *Proc. Natl. Acad. Sci. USA* 96:4028-4033.

Ziiaev, ShI., Vallamatov, ST., Zaĭtseva, TS., Ismailov, II. (1991) "Antibiotic sensitivity of El Tor *Vibrio cholerae* after exposure to various pesticides and mineral fertilizers" *Antibiot Khimioter* 36(8):37-9.

Appendices

Appendix 1

Composition of Agar powder:

Component	Quantity
Foreign insoluble matter	1%
Loss on drying	18%
Sulphated ash	5%
Acid insoluble ash	1%
Arsenic (As)	0.0003
Heavy metals (as Pb)	0.004%
Foreign starch passes	1%
Foreign organic matter	1%

Typical Composition of Nutrient agar (g/liter)

Ingredients	g/liter
Beef Extract	3g
Peptone	5g
Agar	15g

Typical Composition of Luria Broth (LB) (g/liter)

Ingredients	g/liter
Tryptone	10g
Yeast	5g
NaCl	10g

Typical Composition of Minimal minerals salt nutrient culture medium (Bacteria) (mg/liter)

Stock 1	
chemicals	mg/l
NH ₄ NO ₃	1650
KNO ₃	1900
Stock 2	
MgSO ₄ .7H ₂ O	370
MnSO ₄ .4H ₂ O	22.3
ZnSO ₄ .7H ₂ O	8.6
CuSO ₄ .5H ₂ O	0.025
Stock 3	
CaCl ₂ .2H ₂ O	440
CoCl ₂ .6H ₂ O	0.025
KI	0.83
Stock 4	
KH ₂ PO ₄	170
H ₃ BO ₃	6.2
Na ₂ MoO ₄ .2H ₂ O	0.25
Stock 5	
Na ₂ EDTA	37.3
FeSO ₄ .7H ₂ O	27.8
Stock 6	
Inocitol	100
Glycin	2
Nicotinic acid	0.5
Pyridoxin HCl	0.5
Thiamin	0.1

- d. 1.5 µl Ethidium bromide was added to the solution and mixed well;
 - e. The solution was poured into the gel casting tray containing sample comb and were allowed to solidified at room temperature; (Note: with decreasing temperature gel will be solidify rapidly thus step 1.4 to 1.6 was done quickly without consuming high time.
 - f. After gel has solidified, the comb was removed, using care not to rip the bottom of the wells.
 - g. The gel could be stored at -4°C for further use.
3. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and was covered by adding approximately 450 ml TAE buffer;
 4. 4 µl of the extracted DNA sample was mixed with 2 µl of loading buffer on a parafilm and were poured into a sample wells. By maintaining sample's serial number, this was done for every sample separately. To avoid cross-contamination, separate micro-tips were used for every sample.
 5. Approximately 2 µl of colored DNA size marker was poured into first sample wells on gel ;
 6. The electrophoresis chamber was covered with the lid;
 7. The power supply was switched on, only volt was fixed at a constant value of 50, the machine was run until DNA migrated to the two-third of the gel towards the positive electrode (DNA was usually blue colored based on loading buffer);
 8. After 25 to 30 minutes The power supply was switched off and the lid of the electrophoresis chamber was removed;
 9. The gel, still in its plastic tray, was removed from the electrophoresis chamber and placed on an ultraviolet (UV) transilluminator.
 10. The UV transilluminator was switched on, DNA banding pattern was visible;
 11. The resulting banding pattern was captured by using the digital camera and software.

100µl of stock solution was added in 51 ml media containing culture.

So, in 51 ml or 0.051L contains 0.0003 gm cypermethrin

Then 1L contains $0.0003/0.051$ gm cypermethrin

$$= 5.88 \times 10^{-3} \text{ gm cypermethrin}$$

We know, $100\text{gm/L} = 100114.2303 \text{ ppm}$

Then, $5.88 \times 10^{-3} \text{ gm/L} = 100114.2303 \times 5.88 \times 10^{-3} / 100 \text{ ppm}$

$$= 5.89 \text{ ppm of cypermethrin.}$$

- **Carbosulfun 20 E.C. or emulsifying concentrates (Sandat, Singapur):**

In 1L or 1000000 µl solution carbosulfun present 200gm

So, in 150 µl solution carbosulfun present $= 200 \times 150 / 1000000$

$$= 0.03\text{gm}$$

150µl of carbosulfun solution was added in 4850µl distilled water to make 5000µl or 5ml stock pesticide solution.

So, in 5000µl stock pesticide solution carbosulfun present 0.03gm

100 µl stock pesticide solution carbosulfun present $0.03 \times 100 / 5000$

$$= 6 \times 10^{-4} \text{ gm}$$

100µl of stock solution was added in 51 ml media containing culture.

So, in 51 ml or 0.051L contains 6×10^{-4} gm carbosulfun

Then 1L contains $6 \times 10^{-4} / 0.051$ gm carbosulfun

$$= 0.012 \text{ gm carbosulfun}$$

We know, $200\text{gm/L} = 200228.4606 \text{ ppm}$

Then, $0.012 \text{ gm/L} = 200228.4606 \times 0.012/200 \text{ ppm}$

$= 12.01 \text{ ppm of carbosulfun}$

- **Milathion 57 E.C. or emulsifying concentrates (Cheminova, Denmark):**

In 1L or 1000000 μl solution milathion present 570gm

So, in 150 μl solution milathion present $= 570 \times 150 / 1000000$

$= 0.0855 \text{ gm}$

150 μl of milathion solution was added in 4850 μl distilled water to make 5000 μl or 5ml stock pesticide solution.

So, in 5000 μl stock pesticide solution milathion present 0.0855gm

100 μl stock pesticide solution milathion present $0.0855 \times 100 / 5000$

$= 1.71 \times 10^{-3} \text{ gm}$

100 μl of stock solution was added in 51 ml media containing culture.

So, in 51 ml or 0.051L contains $1.71 \times 10^{-3} \text{ gm}$ milathion

Then 1L contains $1.71 \times 10^{-3} / 0.051 \text{ gm}$ milathion

$= 0.033 \text{ gm milathion}$

We know, $570 \text{ gm/L} = 570651.11275 \text{ ppm}$

Then, $0.033 \text{ gm/L} = 570651.11275 \times 0.033 / 570 \text{ ppm}$

$= 33.03 \text{ ppm of milathion}$

Total ppm of three pesticides $= 5.89 + 12.01 + 33.03$

$= 50 \text{ ppm}$

Appendix 4B:

Growth of *V. cholerae* without pesticides in LB and MS media.

Time	Growth of environmental <i>V. cholerae</i> in LB media	Growth of referred <i>V. cholerae</i> in LB media	Growth of environmental <i>V. cholerae</i> in MS media	Growth of referred <i>V. cholerae</i> in MS media
0 hour	0.012	0.018	0.025	0.034
4 hour	0.740	0.745	0.285	0.270
24 hour	1.140	1.377	0.391	0.374

Appendix 4C:

Growth of *V. cholerae* in presence of pesticides in LB and MS media

Time	Growth of environmental <i>V. cholerae</i> in LB media	Growth of environmental <i>V. cholerae</i> in MS media	Growth of referred <i>V. cholerae</i> in LB media	Growth of referred <i>V. cholerae</i> in MS media
0 hour	0.017	0.030	0.019	0.032
4 hour	0.70	0.268	0.710	0.260
24 hour	0.964	0.324	0.997	0.316

Appendix 4C:

Growth of *V. cholerae* with pesticides vs without pesticides in LB media

Time	Growth of environmental <i>V. cholerae</i> with pesticides	Growth of environmental <i>V. cholerae</i> without pesticides	Growth of referred <i>V. cholerae</i> with pesticides	Growth of referred <i>V. cholerae</i> without pesticides
0 hour	0.017	0.012	0.019	0.018
4 hour	0.70	0.740	0.710	0.745
24 hour	0.964	1.140	0.997	1.377