Understanding the effect of exposure of human pathogens to polluted surface water: *Vibrio cholerae* as a model organism

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Declaration

I hereby declare that the thesis project titled **"Understanding the effect of exposure of human pathogens to polluted surface water: *Vibrio cholerae* as a model organism"** has been written and submitted by me, Syeda Sumaiya Islam and has been carried out under the supervision of Mahbubul Hasan Siddiqee, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

It is further declared that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. All explanations that have been adopted literally or analogously are marked as such.

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Abstract

Urbanization and industrialization results in the fecal organisms contaminating the water-bodies which are important sources of recreational activities. This eventually increases the risk of humans getting infected during accidental ingestion or contact. The purpose of the study was to detect the presence of *Vibrio cholerae* in water bodies contaminated with industrial pollutants. For this purpose Hatirjheel Lake and Buriganga River, the two prominent water bodies of our country were chosen for sampling. A total of 40 samples were collected aseptically from Hatirjheel Lake and 20 samples from Buriganga River within duration of three months. Then, they were processed for isolation of culturable *Vibrio cholerae*. After that several biochemical tests were done for presumptive identification of isolates. For further confirmation, RT-PCR was done targeting CqsS gene. While many isolates were found to be positive with biochemical tests, after RT-PCR only 5% of samples from both water bodies gave positive result. Further, three strains from both water bodies were tested for survival potential under starvation stress. The survival patterns between the lake and river showed slight difference but both showed a gradual decrease in culturable count over the time. Next, the strains from those water bodies were checked for their susceptibility to an array of eight antibiotics. The antibiogram revealed that isolates from Hatirjheel showed complete resistance to tetracycline, ampicillin, ceftriaxone while the Buriganga isolates showed complete resistance to only ampicillin, tetracycline, nalidixic acid, co-trimethoprim and ceftriaxone. While no conclusion could be drawn on the actual presence of *Vibrio cholerae* in Hatirjheel or Buriganga, isolation of this organism from heavily polluted water bodies could be difficult.

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Chapter 1

Introduction

1.0 INTRODUCTION

Water is essential to life, but many people do not have access to clean and safe drinking water and many dies of waterborne bacterial infections (Cabral, 2010). The most important bacterial diseases transmitted through water are Cholera, Typhoid fever, bacillary dysentery e.t.c. Two and a half billion people have no access to improved sanitation and more than 1.5 million children die each year from diarrheal diseases (Fenwick, 2006). According to the WHO, the mortality of water associated diseases exceeds 5 million people per year. From these, more than 50% are microbial intestinal infections, with cholera standing out in the first place (Cabral, 2010).

Diarrheal diseases remain a leading cause of preventable death, especially among children under five in developing countries (Peabody, Taguiwalo, Robalino, 2006). *Vibrio* species is one of the main causative agents of Diarrheal diseases. Cholera is endemic in countries of the world where socioeconomic conditions are poor, sanitary systems and public hygiene are rudimentary, and safe drinking water is not available, especially during floods (A. Huq et al., 1996). *Vibrio cholerae* is autochthonous to natural waters and can pose a health risk when it is consumed via untreated water or contaminated shellfish (Louis et al., 2003). *Vibrio cholerae* serogroups O1 and O139 are associated with epidemic and pandemic cholera (M. Alam et al., 2007). *Vibrio cholerae* non-01 is a causative agent of moderate-to severe, cholera-like diarrhea in Bangladesh and other parts of the world (Islam, Alam, & Neogi, 1992). Epidemics due to *V. cholerae* non-01 have also been reported during cholera outbreaks(Islam et al., 1992). The enterotoxin produced by *V. cholerae* non-01 is closely related to cholera toxin produced by classical *V. cholerae* 01 (Islam et al., 1992).

The main regions of cholera endemicity include the coasts surrounding the Bay of Bengal, both Bangladesh and the Indian subcontinent, and coastal Latin America. In each of these three geographical regions, patterns of disease frequency follow similar trends and are most likely explained by the same physical or environmental drivers. (Lipp, Huq, & Colwell, 2002)

Cholera incidences in the Bengal Delta region, the native homeland of cholera, show bi-annual peaks (Akanda, Jutla, & Islam, 2009). Cholera is endemic in the Ganges delta, occurring twice yearly in epidemic form (Munirul Alam et al., 2006). First peak time is just after the monsoon during September to December and smaller peak of cholera cases also is observed during the spring, between March and May(Faruque et al., 2005). Extensive studies have shown that *V. cholerae* O1 becomes coccoid and enters into a non-culturable state in the environment when conditions are not conducive to active growth (Xu et al., 1982). However, in most cases *V. cholerae* has been found to persist in its natural environment mainly in two forms: viable but not culturable (VBNC) and conditionally viable environmental cells (CVEC). VBNC is a dormant state that *V.cholerae* enters in response to nutrient deprivation and other environmental conditions. These forms cannot be recovered by culture techniques but are still able to cause infection and under certain conditions can revert to the culturable form. It was recently shown that *V.cholerae* can also enter a CVEC state in which it can be recovered after the appropriate enrichment culture techniques are applied(Almagro-Moreno & Taylor, 2013).When *V.cholerae* enters the viable-but-non-culturable state, it loses its flagellum and changes to a smaller, spherical form, in a spore-like stage. This dormant state serves as a survival strategy as cells survive changes in temperature, salinity, or availability of organic matter and remain infectious(Trinidad & Sedas, 2000).The VBNC cells have the capacity to revert to the culturable state and colonize the intestine the mechanism of which is largely unknown. These organisms may go undetected if conventional culture based methods are used(Begum, 2017).

Surface water refers to river, lakes, ponds, streams and other reservoirs. Due to rainfall a diverse amount of pollutants flowed into water bodies. In developing countries, because of industrialization many waste products are being dumped into water bodies. The unprecedented urbanization and industrialization is associated with exerting pressure on the quality of water resources placing human health at risk (Rabbani, Chowdhury, & Khan, 2017). Local communities are suffering from a variety of health problems that could be a direct or indirect result of the activities of local factories. These problems include skin diseases, diarrhea, dysentery, respiratory illnesses, anemia and complications in childbirth (Rabbani et al., 2017). The rapid construction of commercial buildings, housing, roads and other infrastructure are reducing the permeability of the surrounding land which results in alteration of hydrological

characteristics of water flowing to those lakes and rivers. As a result, runoff volumes increase carrying more discharges into the water bodies (Unless, Act, Rose, If, & Rose, 2013).

Bangladesh is the world's largest delta having a high density of river system criss-crossing the whole country. Its capital city Dhaka lies beside one of the major rivers named Buriganga. Due to unplanned urbanization, deforestation the environment of Dhaka city is degrading day by day and this climate change is one of the main causes of Buriganga river pollution. Dhaka city is the capital of Bangladesh, which largely depends on the Buriganga River's water for drinking, fishing, carrying merchandise and transportation (Uddin, Afrin, & Alam, 2018). The surface water area of Dhaka city is about 10-15% of the total land area. Hatirjheel, Gulshan Lake, Banani Lake, Ramna Lake and Dhanmondi Lake are the popular relaxation spots in the city, is being polluted by slums and sewages, the business firms and industries operating in the area, as the locals and environmentalists alleged. Hatirjheel lake plays a vital role in maintaining the only drainage system of those areas (Miah, Majumder, & Latifa, 2016). Because of consumption and using this polluted water by the marginal people who are leaving on the bank of the Buriganga River especially children facing different types of water borne diseases, skin sore, irritation in respiratory tract, typhoid, dysentery, cholera, viral hepatitis etc. and loss their life (Uddin et al., 2018).

Several physiochemical parameters such as: pH, DO, BOD, COD, TSS, SS, total coliforms, heavy metals, turbidity temperature etc. determines water quality. The water quality of Hatirjheel Lake and Buriganga River are different because of the difference in their location and the sources of pollution present in them. Severe pollution has reduced the river Buriganga into a 'dumping drain' of toxic refuse, threatening millions of people living on its banks with serious health hazards and a loss of their livelihoods (M. Huq, Ahmed, Tabassum, & Miah, 2013).Significant sources of pollution for Buriganga are from the tanneries and as a result, it has been found that the water of Buriganga contains 2.6 to 28.0 mg/L of hexavalent chromium where 0.1 mg/L is the permissible limit. There is a mixture of other heavy metals such as arsenic, lead, mercury, cadmium, chromium, nitrates, nitrites etc. in the polluted waters some of which are believed to be cancer causing agents (M. Huq et al., 2013).

Parameters	DOE standards to maintain the aquatic ecosystem	Compliance with standards (Yes/No)	
		Dry season	Wet season
Temperature	20 to 30°C	Y	Y
pH	6.5 to 8.5	Y	Y
EC	350 µs/cm	N	Y
DO	5 mg/L	N	N
BOD_5	2 mg/L	N	N
COD	4 mg/L	N	N
PO_4-P	6 mg/L	Y	Y
NH_3-N	0.5 mg/L	N	N
Pb	0.05 mg/L	Y	Y
Cr	0.05 mg/L	N	N

Figure 1: Compliance of Buriganga River water quality parameters with DOE guidelines (Rahman & Bakri, 2010)

High BOD level clearly indicates that the River Buriganga is polluted with the organic chemical as well as bacterial pollutants and is unsuitable for fisheries (Saifullah, Kabir, Khatun, Roy, & Sheikh, 2012). Low DO is probably due to the easily oxidized industrial and municipal organic wastes which are directly linked to the high turbidity of the water. This results in low photosynthesis and a negligible replacement of oxygen (Rahman & Bakri, 2010). High COD and ammonia values are a result of severe pollution from the chemical and sewage discharges in the area.

Due to the lack of proper design and improvement the water quality of Hatirjheel Lake is getting more polluted and results in high BOD and COD values of 90 mg/l and 175 mg/l, respectively which improve a bit in the dry seasons compared to wet season. Unlike BOD and COD, ammonia concentration continues to increase. Anytime free ammonia (NH3) is higher than 0.05 mg/l, the fish are being damaged and the free ammonia concentration throughout Hatirjheel was more than the standard value with 2.5 mg/l being the highest recorded. Nitrate concentration increases after wet season when the organic compounds are degraded into ammonia which oxidizes to nitrate. On the other hand sulphide concentration remains relatively high throughout the seasons increasing more during the wet seasons. The average concentration of DO vary from 1.6 to 3.6ppm when the standard is 5ppm. This parameter is required for the survival and decomposition of compounds by microorganisms (Islam, Rehnuma, Tithi, Kabir, & Sarkar, 2015).

According to El Niño-Southern Oscillation (ENSO), it has been shown that Climate variability has an influence over the year-to-year variation of seasonal outbreaks of cholera in Bangladesh and the inter-annual variation of the disease have been shown to be positively associated with sea surface temperatures (Martinez et al., 2017). An estimated 28,000 to 1,42,000 people die of cholera every year (icddr,b report). This is why it is important to know if these two prominent water bodies contaminated with *Vibrio* or not.

Several physicochemical conditions affect *V. cholerae* populations in the natural environment, such as water temperature, salinity, oxygen tension, sunlight, rainfall, pH, and the availability of trace elements and chemical nutrients. Although there are strong correlations between the changes in the physicochemical conditions in the environment of *V. cholerae*, the mechanisms by which some of them affect the population dynamics of *V. cholerae* remain unknown(Almagro-Moreno & Taylor, 2013).

Our aim of the study is to detect the level of pollution in the Hatirjheel Lake as well as Buriganga River and whether their occurrence varies or not. There is difference in the presence and amount of pollutant present in Hatirjheel Lake and Buriganga River, which can have an effect on the characteristics of isolates. According to our hypothesis, the waters will be contaminated with *Vibrio cholerae*and the contaminants will have different characteristics between the Lake and River.

Chapter 2

Materials and Methods

2.0 Methods and materials

2.1 Sampling sites:

Hatirjhil is considered as one of the recreational sites of Dhaka city. The Lake is about 302 acres of Tejgaon, Moghmazar, Rampura region and has been playing a vital role in maintaining the only drainage system of those areas (Miah et al., 2016). It contains few bridges and has a open space which attracts a large of visitors every day. On the other hand, Buringanga is a tide-influenced river which passes through Dhaka city but at present the level of pollution in Buriganga is at its highest. These two surface water bodies were chosen as the sampling site of this study. These points were chosen as people often visits there as well as to know about the safety measurements.

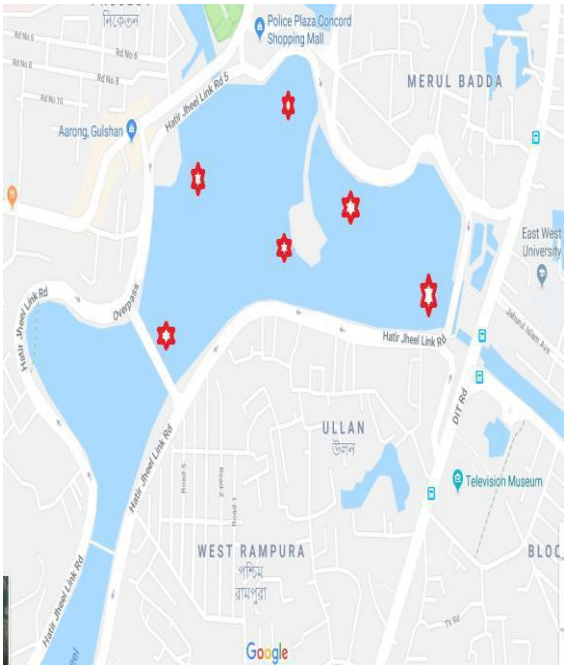

Figure 2: Hatirjheel Lake sampling site

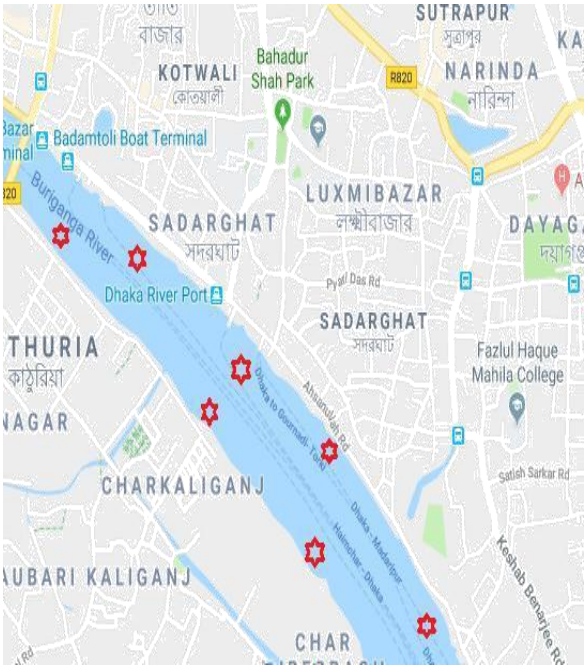

Figure 3: Buriganga River Sampling Site

2.2 Water sampling:

In total 60 samples were collected from Hatirjheellake and Buriganga river. 40 samples were from Hatirjhil and 20 samples were from Buriganga. Sample collection procedure started from October 2017 to January 2018. For water sample collection, autoclaved plastic bottles were used. During sample collection gloves were used to collect water into bottles. Small boats and water taxis were used to reach the sample site. Within 2 hours of sample collection, samples were transported to laboratory for further processing.

2.3 Isolation:

For environmental samples, it is highly recommended to use pre-enrichment step to improve detection or isolation (Anwar Huq et al., 2012). For enrichment purpose, alkaline peptone water (APW) was used. Fifty milliliter of APW was taken to flask and then 50 ml of water sample was poured into flask. For measurement autoclaved measuring cylinder was used. If the water sample had any sediment then it was removed using cheese cloth. Then, the flasks were kept in incubator at 37°c for overnight.

After enrichment in APW, collected surface growth (may be present as a whitish film) from the enrichment flask with an inoculating loop and streaked onto TCBS (Thiosulfate citrate bile salts

sucrose) agar(Choopun, Louis, Huq, & Colwell, 2002; Anwar Huq et al., 2012). The plates were then kept in incubator at 37°c overnight.

After incubation, smooth, flat, sucrose-fermenting, yellow colonies from TCBS were sub cultured to modified nutrient agar. If plates are heavily overgrown with bacteria, try to touch the top-center of a presumptive *V. cholerae* colony and re-streak onto selective media again to isolate a single colony(Anwar Huq et al., 2012). For stock, MNB (modified nutrient broth) was used. One milliliter of MNB was transferred to autoclaved eppendorf tubes and then one loopful of colonies was inoculated from MNA. After inoculation, eppendorf tubes were kept in incubator at 37°c. The next day, 300µl of autoclaved glycerol was added to it and kept at -20°c.

Physiochemical parameters such as pH and temperature of the samples were measured on the day of sampling.

2.4 Identification:

The suspected colonies were then tested for the following biochemical tests to presumably conclude whether they are *Vibrio cholera*

Gram staining

Gram staining was done to confirm whether the organism is gram positive or gram negative. *Vibrio cholerae* is a gram negative, comma shaped, motile bacteria which can be observed under microscope through gram staining.

Biochemical identification of *Vibrio cholerae*

Several biochemical tests were performed for presumptive identification of *Vibrio cholerae.* These tests include- oxidase test, Triple sugar iron agar test (TSI), IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, and Citrate utilization test), MIU test (Motility test, Indole test and Urease test. The protocols were according to the microbiology laboratory manual (Cappuccino & Sherman, 2005).

Oxidase test

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the **cytochrome c oxidase** oxidizes the reagent **(tetramethyl-p-phenylenediamine)** to **(indophenols)** purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless. Bacterial colonies were picked from freshly culture plate using toothpick and touched onto filter paper and then oxidase

reagent (0.5% tetramethyl-*p*-phenylenediamine hydrochloride) was added using dropper. Formation of purple color is considered as positive result. (Cappuccino & Sherman, 2005).

Triple Sugar Iron Agar test

Triple sugar iron test was done to differentiate among the different groups or genera of the *Enterobacteriaceae*based on the ability to reduce sulfur and ferment carbohydrates. Triple sugar iron slants were prepared in the test tubes by autoclaving at 15 psi 121°C. Using sterile technique; small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab and streak inoculation method with an inoculating needle. The screw caps were not fully tightened and the tubes were incubated for 24 hours at 37°C (Cappuccino & Sherman, 2005).

MIU (Motility, Indole and Urease) test

MIU test was done to simultaneously determine the ability of the bacteria to produce indole, check motility and degrade urea by means of the enzyme urease. MIU media was prepared by autoclaving at 15 psi 121◦cand the media was cooled to about 50-55◦C and 100 ml of urea glucose solution was added aseptically to 900 ml base medium. After that, 6 ml solution was transferred to each sterile test tube and allowed to form a semi solid medium. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37°C (Acharya, 2015).

Indole Production test

Indole production test was done to determine the ability of the bacteria to degrade the amino acid tryptophan by the enzyme tryptophanase. Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 48 hours at 37◦C. In order to test for indole production, 5 drops of Kovac's reagent was added directly into the tubes (MacWilliams, 2009).

Citrate utilization test

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrate permease. Simmons citrate agar slants of 2 ml in each vials were prepared by autoclaving at 15 psi 121^{0}C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37^{0}C (Cappuccino & Sherman, 2005).

MR-VP (Methyl Red- VogesProskauer) test

The Voges-Proskauer test determines the capability of some organisms to produce non acidic or neutral end products, such as acetyl methyl carbinol, from the organic acids that result from glucose metabolism. The reagent used in this test is Barritt's reagent, consists of a mixture of alcoholic a-naphthol and 40% potassium hydroxide solution. Detection of acetyl methyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of the a-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium. As a result, a pink complex is formed, imparting a rose color to the medium. Development of a deep rose color in the culture 15 minutes following the addition of Barritt's reagent is indicative of the presence of acetyl methyl carbinol and represents a positive result. The absence of rose color is a negative result.

Methyl Red test determines whether the microbe performs mixed acids fermentation when supplied glucose. Types and proportion of fermentation products produced by anaerobic fermentation of glucose is one of the key taxonomic characteristics which help to differentiate various genera of enteric bacteria. If the culture medium turns red after addition of methyl red indicates positive result and yellow color indicates negative result.

A loopful of bacterial culture was inoculated test tubes containing MR-VP Broth and incubated at 37°c for overnight. After incubation, 5ml of broth was transferred to another test tube for MR test. Next, 5 drops of the methyl red indicator solution was added to the first tube (for Voges-Proskauer test, Barrit's reagent is added to another tube). A positive reaction is indicated, if the colour of the medium changes to red within a few minutes (Cappuccino & Sherman, 2005).

2.5 Gene Specific PCR

2.5.1 DNA extraction by Boiling method:

Preparation of DNA by boiling lysis of bacteria isolated from the sample. DNA from broth was prepared by boiling. The samples were centrifuged at 15,000*g* for 15 min. The supernatant was eliminated, and the pellet was resuspended in molecular biology-grade water and centrifuged at 15,000 *g* for 10 min. The supernatant was eliminated, and the pellet was resuspended in 40 µl of autoclaved distilled water, subjected to boiling at 100°C in a water bath for 10 min, cooled on ice foe further 10 minutes and centrifuged at 15,000*g* for 10 s before it was stored at -20°C. Aliquots of 2 µl of template DNA were used for PCR(Queipo-Ortuño, De Dios Colmenero, Macias, Bravo, & Morata, 2008).

Polymerase Chain Reaction (PCR) is an in vitro technique based on the principle of DNA polymerization reaction by which a particular DNA sequence can be amplified and made into multiple copies. It relies on thermal cycling consisting of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using thermostable DNA polymerase, primer sequence (complementary to target region) and dNTPs.

2.5.2 RT-PCR:

Compared to conventional PCR, real-time PCR is less labor intensive, more safe, and rapid due to the elimination of gel electrophoresis. It has greater sensitivity and can detect minute amounts of target amplicons that might be missed by the conventional PCR. Real-time PCR can directly target genomic DNA and thus eliminate extensive incubation periods. Furthermore, VBNC cells can be detected which might be missed by culture-based methods(Begum, 2017).Real time PCR was done with SYBR green probe according to the manufacturer protocol

Reactionmixture:

5ul SYBR green mix

$MgCl_2$ (1.2 ul)

0.5 ul forward primer (10 pmol)

0.5 ul reverse primer (10 pmol)

1 ul water

2ul DNA template

Table 1: Primer used for gene specific PCR

Target Gene	Primers	Sequence (5'-3')	Amplicon Size(bp)	Annealing Temp(°C)	References
CqsS	Primer F	5'-TGGCTTTCCTGCCTACTACTTCATCT-3'	670bp	58°C	icddr,b
	Primer R	3-GAGTCGCGAAACTTTTGCAGCTAAC-5'			

2.6 Determination of the Antibiotic Resistance Pattern of the Target

After presumptive identification of suspected colonies the strains were used to compare the antibiotic susceptibilities as well as resistance among the strains available in lakes and in rivers via Kirby-Bauer disc diffusion method.

2.6.1 Kirby-Bauer Disc diffusion:

The isolates were subjected to antimicrobial susceptibility testing by disk diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI, 2017) using commercial antimicrobial disks. The antibiotic disks used in this study were: Ampicillin (25µg), Chloramphenicol (30µg), Gentamycin (10µg), Ciprofloxacin (5µg), Ceftriaxone (30 µg), Ceftazidime (30µg), Chloramphenicol (30µg), Cotrimoxazole (sulfamethoxazole 23.75 µg + trimethoprim 1.75 µg), Nalidixicacid(30µg), Tetracycline (30µg)

The method described by Bauer and Kirby (1969) was followed. An inoculating needle was touched to a freshly grown, well isolated colony on plate and then inoculated into 1 ml of Muller-Hinton Broth (MHB). The culture were then incubated in a shaker at 37°C for 4 hours to obtain the actively growing culture, equivalent to 0.5 McFarland standard ($1.5x\ 10^8$ CFU/mL). A sterile cotton swab was dipped into the standard suspension, excess broth was purged by pressing and rotating the swab firmly against the inside wall of the tube above the fluid. The swab was then streaked evenly in three directions over the entire surface of the agar plate to obtain a

uniform inoculum. A final sweep was made of the agar rim with the cotton swab. This plate was then allowed to dry for three to five minutes before the disks were applied. Antibiotic impregnated disks were then applied to the surface of the inoculated plates with sterile syringe needle. All disks were gently pressed down onto the agar with sterile forceps to ensure complete contact with the agar surface. Within 15 minutes after the disks were applied, the plates were inverted and placed in an incubator at 37°C. After overnight incubation, the plates were examined for zone of inhibition and the diameter of the zone of inhibition was measured to the nearest whole millimeter by a ruler. The zone diameters for individual antimicrobial agents were then translated into susceptible, intermediate, or resistant categories according to the CLSI guidelines (2017).

2.6.2 Plasmid Profiling

After the antibiogram, plasmid profiling was further performed with the isolates used there in order to determine whether the antibiotic resistance gene is present in the plasmid or not. Alkaline lysis method was used to extract the bacterial plasmid (Plasmid DNA Extraction From)

Procedure:

1. The bacterial cells were grown overnight in NB broth overnight.

2. 1.5ml of the broth was transferred to an Eppendorf in order to harvest by centrifugation at 10000 rpm for 2 min (repeated this step 3 times).

3. The pellet was resuspended in Solution 1 (200 µl) and then freshly prepared solution 2 (400 µl) was added; mixed by gentle inversion.

4. The cells were incubated at room temperature for 5 mins for cell lysis.

5. To this, ice cold solution 3 (300 µl) was added and mixed by inversion and incubated on ice for 10 mins.

6. The mixture was then centrifuged at 12000 rpm for 15 mins.

7. The supernatant was transferred in a fresh tube.

8. To this, equal volume of phenol: chloroform: IAA (25:24:1) was added, mixed by vortexing and centrifuged at 12000 rpm for 2 min.

9. The supernatant was carefully collected an equal volume of choloroform : IAA (24:1) was added to it, followed by mixed by vortexing and centrifuged at 12000 rpm for 5 mins.

10. The supernatant was collected and then 0.6 volume of isopropanol was added to this, then mixed by inversion and centrifuged at 12000 rpm for 15 minutes.

11. The DNA pellet was washed in 70% ethanol, air-dried and resuspend in 20 µl of TE buffer.

Separation of plasmid DNA by Gel Electrophoresis

Plasmid DNA was separated by horizontal electrophoresis in 1% agarose slab gels in TBE buffer at room temperature at 70 volts for 60 minutes. 8µl of plasmid DNA solution was mixed with 2µl of tracking dye and was loaded into individual well of the gel. The DNA bands were seen under UV light and a positive control was used as known size marker.

2.7 Survival Assay

In order to carry out survival assay, three isolates were randomly selected from each river and lake in order to determine their die-off rate in autoclaved tap water. The purpose of the assay was to compare how these isolates can survive under starvation stress and to see if their characteristics are different or not due to belonging in different water bodies. As a positive control, clinical strain of *Vibrio cholerae* (Ogawa) was used while as a negative control blank water was used.

2.7.1 *Vibrio cholera* culture and preparation of inoculum

Vibrio cholerae were revived from the stock culture in NA (Nutrient Agar) and incubated at 37^{0}C for 24 hours. Then two colonies were taken from each sample and inoculated in 10ml NB (Nutrient Broth) and incubated at 37^{0}C for approximately 1.5 to 2 hours in a shaking incubator. The optical density of the broths was measured till it reached 0.1 OD at 600nm wavelength using a spectrophotometer. The cultures were then centrifuged at 14,500 rpm for 5 minutes to harvest the cells. The cells were then washed with sterile saline twice and resuspended in it to get a final

concentration of approximately 10^8 cfu/ml which was then inoculated in the microcosms. (Fernández-delgado, García-amado, & Contreras, 2015)

2.7.2 Preparation of microcosm

Tap water was taken in plastic bottles and autoclaved at 121°C along with the glass beakers which served as the microcosm for the entire experiment. 100ml of the autoclaved tap water was poured into the 250ml glass beakers using sterile measuring cylinder. After inoculation into the microcosm, they were kept covered at room temperature.

Figure 4: Microcosm prepared for isolates

2.7.3 Enumeration of survivors

Plate count in order to determine the colony forming units was taken starting from day 0 the day of inoculation followed by day 1, day 3 and then day 7 on selective media TCBS agar. On day 0, the samples were diluted up to 6 times and then 100µl of it was spread plated. On the next count, the samples were diluted up to 4 times while on the 3rd day samples were diluted upto 2 times and lastly, on the 7th day one fold dilution of the sample was made and then spread plated on TCBS agar.

Chapter 3

Results

3.0 Results

3.1 Qualitative analysis

After collection of the samples it was immediately brought to the laboratory where 50ml of the sample was taken and inoculated in 50ml of alkaline peptone water (APW) and incubated at 37°C for 24 hours in incubator. After incubation, whitish layer was collected and streaked onto TCBS agar and then kept in incubator at 37°c.

The highest temperature recorded was from Hatirjheel Lake at 29.4°C while the lowest was 20.8°C. On the other hand, the temperature in Buriganga River remained almost constant at 25°C. The pH in both water bodies remained with the range of 6 to 7.

3.2 Identification:

After presumptive isolation of *Vibrio*, the samples were subjected to 8 standard biochemical tests for the identification along with gram staining. According to standard chart *Vibrio* spp is a gram negative comma shaped organism which is supposed to test positive for motility, methyl red from MR-VP, indole and oxidase while negative for urease, citrate and vogesproskauer from MR-VP. In TSI it is supposed to give yellow slant, yellow and black butt due to the production of hydrogen sulphide. The following table describes the results of the samples tested for the aforementioned biochemical tests.

Table 2: Biochemical test chart

Name	Oxidase	Motility	Indole	Urease	MR	VP	Citrate	Slant+Butt	Gas	H_2S
***V.cholerae* (Ogawa)**	+	+	+	-	R	-	-	B+S=Y	-	-
HJ-2 (Y)	+	+	+	-	R	-	-	B+S=Y	+	-
HJ-5 (Y)	-	+	-	+	Y	-	-	B+S=Y	-	-
HJ-16 (Y)	-	+	-	-	R	-	-	B+S=Y	-	-

HJ-18 (Y)	-	-	-	+	Y	-	-	B+S=Y	-	-
HJ-19 (Y)	+	-	-	-	Y	-	-	B+S=Y	+	-
HJ-21 (Y)	-	+	+	-	R	-	-	B+S=Y	-	+
HJ-22 (Y)	-	+	+	-	R	-	-	B+S=Y	+	+
HJ-23 (Y)	+	-	-	-	R	-	-	B+S=Y	+	-
HJ-25 (Y)	+	+	+	-	R	-	-	B+S=Y	+	+
HJ-27 (Y)	+	+	+	-	R	-	-	B+S=Y	-	+
HJ-28 (Y)	+	+	+	-	R	-	-	B+S=Y	-	+
HJ- 30 (Y)	+	+	+	-	R	-	-	B+S=Y	-	-
BR-13(Y)	+	+	+	+	R	-	+	B+S=Y	-	+
BR-1 (Y)	+	+	+	+	+	-	-	B+S=Y	+	+
BR-2 (Y)	+	+	+	-	+	-	-	B+S=Y	+	+
BR-3 (Y)	+	+	+	+	+	-	-	B+S=Y	+	+
BR-4 (Y)	+	+	+	-	+	-	-	B+S=Y	-	+
BR-5 (Y)	+	+	+	+	+	-	-	B+S=Y	-	+
HJ-10 (Y)	+	+	-	-	+	-	-	B+S=Y	-	+
HJ-15 (Y)	+	+	+	+	+	-	-	FULL BLACK	-	+
BR-6 (Y)	+	+	-	-	+	-	-	S=R FULL BLACK	-	+

3.3 Gene Specific PCR

After the presumptive identification of the samples, genetic amplification of CqsS gene was performed using RT-PCR in order to determine whether the isolates present in the environment are carrying this gene or not as well as to confirm if these are *Vibrio cholera*e or not. The primer used in the PCR mixture was specific for CqsS gene and the bands are usually approximately 670 bp long DNA. Very few of the isolates gave positive bands for the specific gene which means that the isolates were *Vibrio cholera*e that were present in the surface of the water bodies.

CqsSPCR result of BRAC University samples

SL	ID		CqsS
1. positive control	N16961		positive
2. Sample	HJ2Y		Negative
3. Sample	HJ3Y		Negative
4. Sample	HJ5Y		Negative
5. Sample	HJ10Y		Negative
6. Sample	HJ15Y		Negative
7. Sample	HJ18Y		Negative
8. Sample	HJ19Y		positive
9. Sample	HJ21Y		Negative
10. Sample	HJ22Y		Negative
11. Sample	HJ23Y		Negative
12. Sample	HJ25Y		Negative
13. Sample	HJ27Y		positive
14. Sample	HJ28Y		Negative
15. Sample	HJ30Y		Negative
16. Sample	BR1Y		Negative
17. Sample	BR2Y		Negative
18. Sample	BR4Y		positive
19. Sample	BR5B		Negative
20. Sample	BR6Y		Negative
21. Sample	BR13Y		Negative
22. negative control	HB101		Negative

Figure 5: RT-PCR Result using CqsS gene

Table 3: Sampling month, number of samples and percentage of positive sample

Month of collection	Sampling site	Number of samples	Number of Presumptive samples	Number of confirmed samples	Positive sample (%)
October 2017	Hatirjheel Lake	5	2	0	0%
November 2017	Hatirjheel Lake	17	7	1	5.88%
December 2017	Hatirjheel Lake	6	4	1	16.66%
	Buriganga River	10	6	1	10%
January 2018	Hatirjheel Lake	12	1	0	0%
	Buriganga River	10	1	0	0%

A total of 40 samples were collected fromHatirjheel Lake out of which 14 isolates were presumptively positive for *Vibrio*. After PCR 2 isolates were positive which means that 18% of the samples were positive. On the other hand, 20 samples were collected from Buriganga River from which 7 isolates were presumptively positive. After PCR only one isolate was positive which accounts for 16% of positive sample.

3.4 Antibiogram

Randomly selected 10 isolates, 5 from each lake and river were tested for antibiotic susceptibility against 8 antibiotics. The zones of inhibition were measured and compared with the standard CLSI chart to determine if they are resistant, intermediate or sensitive. Patterns of antibiogram showed slight difference between Hatirjheel Lake and Buriganga River.

Sample no.	Tetracycline	Ciprofloxacin	Nalidixic acid	Ampicillin	Gentamicin	Chloramphenicol	Co-trimethoprim	Ceftazidime
HJ-19Y	R	S	I	R	R	S	S	R
HJ-27Y	R	R	R	R	S	S	R	R
BR-4Y	R	S	R	R	S	I	R	R

Table 4: Antibiotic Resistance pattern in Hatirjheel Lake and Buriganga River

The isolates from Buriganga showed complete resistance against the antibiotics: ampicillin, tetracycline, Nalidixic acid and ceftriaxone and Co-trimethoprim. On the other hand, Ciprofloxacin and Gentamycin showed 100% sensitivity while Chloramphenicol showed 100% intermediate zone.

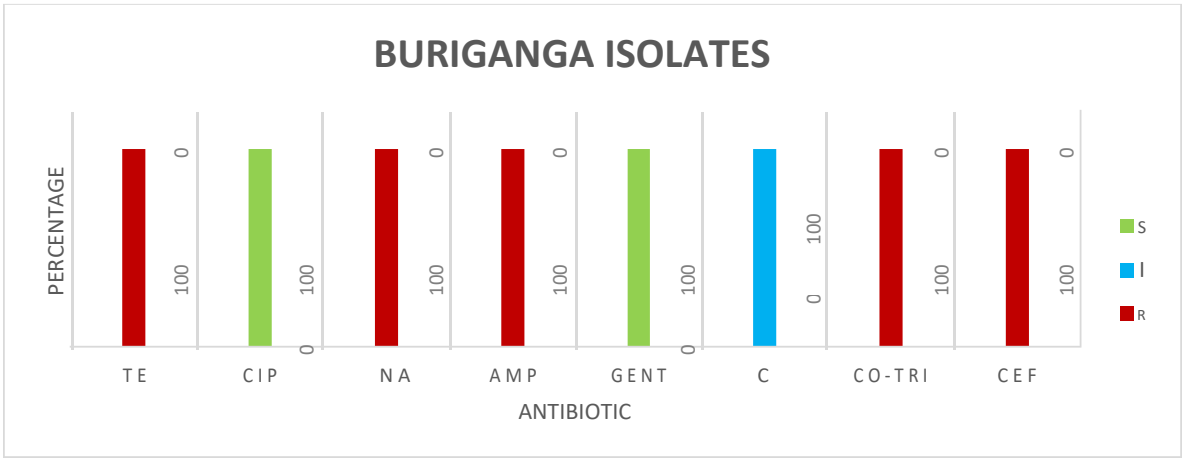

Figure 6: Graphical translation of the susceptibility and resistance pattern of the Buriganga isolates to different antibiotics.

The isolates from Hatirjheel showed complete resistance against Ampicillin, Tetracycline and Ceftriaxone. InCiprofloxacin (50%), nalidixic acid (50%), gentamycin (50%) and Co-trimethoprim (50%) resistance was seen. Amongst all the antibiotics chloramphenicol showed 100% sensitivity followed by Co-trimethoprim, Gentamycin, and Ciprofloxacinat 50%. Intermediate zone was seen only against nalidixic acid at 50% of the isolates.

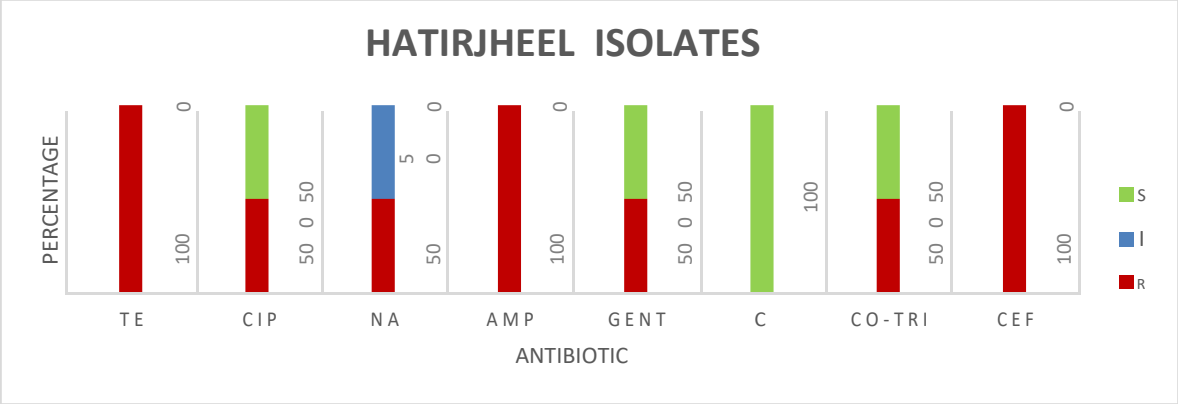

Figure 7:Graphical translation of the susceptibility and resistance pattern of the Hatirjheel Lake isolates to different antibiotics.

Overall, there was not much difference between the antibiograms however, isolates from Buriganga showed 100% resistance towards co-trimoxazole which was 50% sensitive for the Hatirjheel isolates. On the other hand, isolates from Hatirjheel showed 100% sensitivity towards chloramphenicol but Buriganga isolates showed an intermediate zone of 100%.Isolates of Buriganga showed 100% sensitivity towards Gentamycin which was 50% in Hatirjheel isolates.

3.5 Plasmid Profiling

Both the isolates of Hatirjheel and Buriganga showed bands for plasmid along with positive control. From the results it can be assumed that the antibiotic resistance of the isolates came from plasmids.

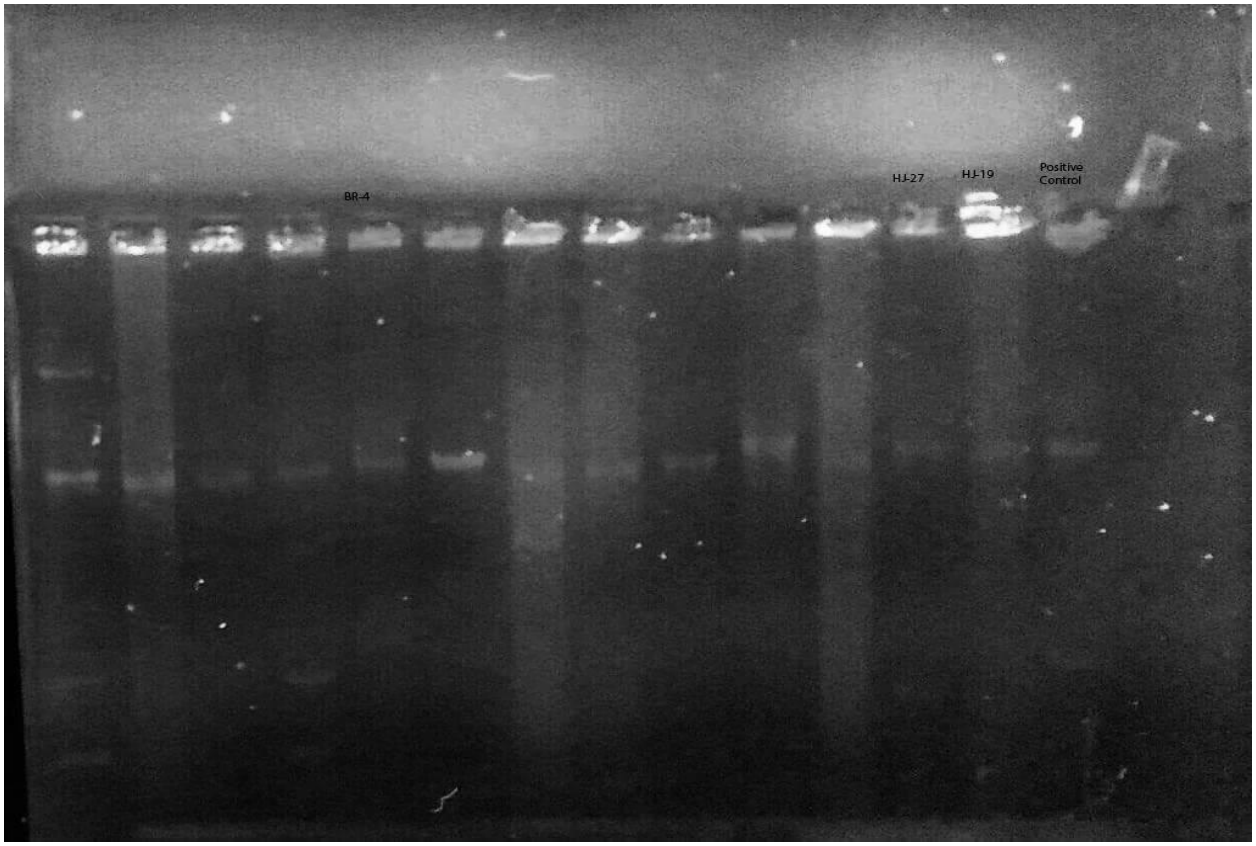

Figure 8:Agarose gel electrophoresis band for plasmid extracted from both Buriganga River and Hatirjheel Lake.

3.6 Survival Assay

The survival pattern of *Vibrio* between Hatirjheel Lake and Buriganga River showed difference in their die-off rate. The isolate of Buriganga died faster than the isolates of Hatirjheel which indicates that there was an effect of pollutants on those isolates. Both of the isolates decreased in cell number rapidly within the days observed. However, the rate of death for the Buriganga isolates were little bit faster than the Hatirjheel isolates as shown in the graphs below. As a positive control, clinical *V.cholerae* (Ogawa) strain was used and its die-off rate was slower

compared to the environmental isolates. Cell count was taken by direct plating on selective media TCBS. Initially 10^8 cfu/ml isolates were inoculated in the autoclaved tap water which decreased to approximately 10^1 cfu/ml in both the isolates on day 7.

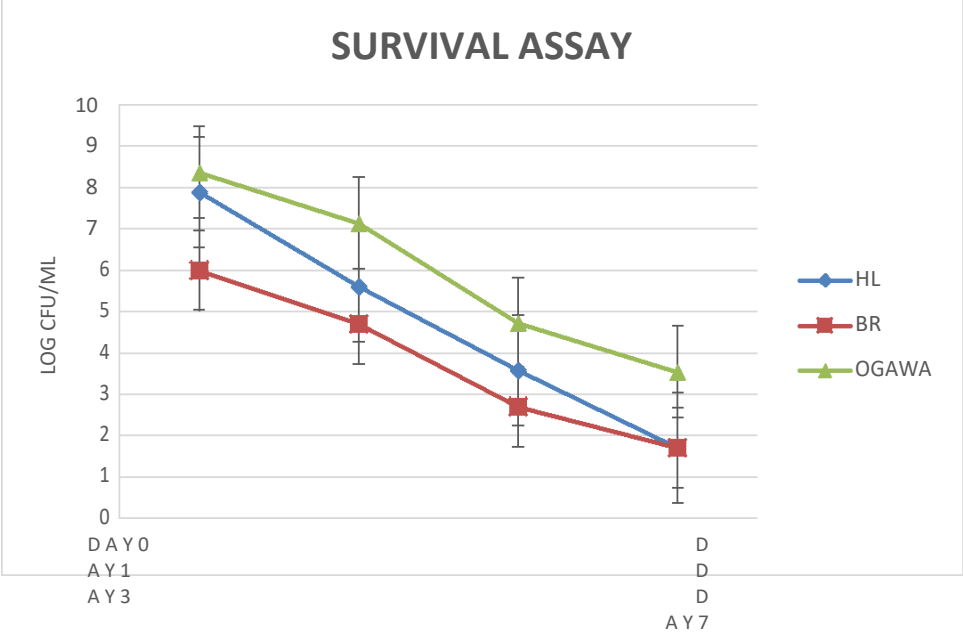

Figure 9:Die-off rate comparison between the isolates of Hatirjheel as well as Buriganga and positive control which shows a gradual decrease in growth

Chapter 4

Discussion

4.0 Discussion

The purpose of this study was to isolate and identify the presence of *Vibrio cholerae*from two of the most prominent water bodies in the Dhaka city; Hatirjheel Lake and Buriganga River. The organism *Vibrio cholerae* is responsible for cholera. The growth in the urban population caused by internal migration can cause increased contamination of water and Global warming due to climate change might influence the transmission dynamic of cholera. There are estimated to be between 1.4 and 4.3 million cases of cholera every year. An estimated 28,000 to 1,42,000 people die of cholera every year (icddr,b report). This is why it is important to know if these two prominent water bodies contaminated with *Vibrio* or not.

At first, the water samples were collected from two water bodies and presumptive isolation and identification of *Vibrio cholerae* was done. After that, gene specific RT-PCR was done. CqsS gene was targeted as the gene is considered to be present in non01 *Vibrio cholerae* as it's assumed that most of the *Vibrio* from environmental samples is non01 *Vibrio.* After confirmation of pathogenic *Vibrio cholerae* isolates from PCR it can be said that these isolates derived from water bodies are *Vibrio Cholerae*and they can cause cholera among people.

The number of positive isolates after RT-PCR was very less. Only 18% of isolates from Hatirjheel and 16% of isolates from Buriganga River was identified. There can be few reasons behind such result. First of all, there might be some problem in media composition for which non*Vibrio* isolates gave colonies that resembles*Vibrio*.*V. cholerae* has been shown to possess similar biochemical properties with other species in the Genus *Vibrio* and *Aeromonas*, hence complicating an accurate identification. (Begum, 2017). Secondly, while picking colonies from selective media there is a possibility that mistakenly that colony was not chosen which actual *Vibrio* was. Biochemical result suggests, 35% of the isolates were presumptive Vibrio but after PCR the percentage decreased. This is because might be the isolates are showing variations or there was a mutation in that specific gene which was targeted in PCR. There is significant genetic diversity among environmental and non-O1, non-O139 strains of *V. cholera*. Most *V. cholerae* strains, especially those from the environment, lack the genes required to produce CT, but the possibility of genetic exchange in the environment allows the potential emergence of new toxigenic clones. While horizontal gene transfer is common among *V. cholerae,* as well as

between and among other bacteria, In particular, horizontal transfer and genetic reassortment have been a key mechanism for the emergence of new toxigenic strains of *V. cholera* as well as clonal diversity(Lipp et al., 2002).

Physiochemical parameters of the water such as pH and temperature were measured for both water bodies. The average temperature for Hatirjheel Lake was 25.8°C while for Buriganga River it was 25.0°C. The average pH for lake water was 7.0 while the pH for river water was 6.74. According to the standard chart (Islam et al., 2015) both values fall within the range.

Cholera has reemerged as a global killer with the world witnessing an unprecedented rise in cholera infection and transmission since the 1990s (Akanda et al., 2009). After the completion of identification of the organisms, antibiogram was performed in order to determine their resistance pattern.

From antibiogram result it is visible that isolates from Buriganga showed 100% resistance towards co-trimoxazole which was 50% sensitive for the Hatirjheel isolates. On the other hand, isolates from Hatirjheel showed 100% sensitivity towards chloramphenicol but Buriganga isolates showed an intermediate zone of 100%. Isolates of Buriganga showed 100% sensitivity towards Gentamycin which was 50% in Hatirjheel isolates.So, it can be said that isolates of Burigangaare less affected by Co-trimethoprim and Chloramphenicol compared to Hatirjheel isolates. The difference can be a result of the increased exposure to various stresses in river water from the industries. The effluents from industrial wastes contain metals such as mercury and chromium which can trigger the development of metal resistance along with various antibiotic resistances (Baker-austin et al.).

For further analysis of the antibiotic resistance, plasmid extraction of the isolates was performed to see if the resistance gene was plasmid mediated or not. However, after performing gel electrophoresis it was seen that the isolates contain plasmid. From this result we can assume that the antibiotic resistance came from plasmids. *V. cholerae* becomes drug resistant by exporting drugs through efflux pumps, chromosomal mutations or developing genetic resistance via the exchange of conjugative plasmids, conjugative transposons, integrons or self transmissible chromosomally integrating SXT elements (Kitaoka, Miyata, Unterweger, &Pukatzki, 2011).

Generally the wild strains of *Vibrio cholerae* are a poor host for plasmids carrying genes that encode antimicrobial resistance. The frequency of plasmid carriage in clinical and environmental strains of *Vibrio cholerae* 01 is less (2%) compared with non-01 strains (25%). In some studies it is shown that MDR resistant environmental non-01 *V.cholerae* strains harboured a large plasmid carrying plasmid resistance (Ramamurthy, 2008). The plasmid, with a molecular size of 72 megadaltons, belonged to incompatibility group 6-C and conferred resistance to ampicillin, chloramphenicol, sulfonamide, and trimethoprim(Dupont, Jouvenot, Couetdic, & Michel-briand, 1985). From these aspects we cannot surely say that antibiotic resistance came from the plasmids but there is a possibility that resistance came from plasmids.

Due to the difference in the rate of pollution and contaminants between the two water bodies it was hypothesized that the characteristics of the organisms will be different. Organisms are constantly under pressure from the pollutants present in the water and these pollutants vary between the lake and river which can affect the adaptability of the organisms in different way.. To observe the die-off rate of the selected isolates, they were tested by placing in autoclaved tap water and taking their viable count by spread plate method. The autoclaved tap water provides a nutrient free media that acts as a source of starvation stress for the organisms.

There was difference in the die off rate of isolates from both water bodies. The isolate of Buriganga died faster than the isolates of Hatirjheel which indicates that there was an effect of pollutants on those isolates. The causative agent of cholera, *Vibrio cholerae*, can enter into a viable but non-culturable (VBNC) state in response to unfavorable conditions (Fernández-delgado et al., 2015). Microcosm studies demonstrated that these cells could remain viable in the environment for years and continue to be capable of causing disease (Lipp et al., 2002). So, there is high possibility that a portion of the bacterial population entered the VBNC state as they were in a nutrient limited environment for few days. Due to this VBNC state the isolates from two water bodies died gradually and couldn’t survive in that nutrient limited condition.

Conclusion and Future Direction:

The purpose of the study was to identify the presence of *Vibrio cholerae* in two main water bodies of Dhaka city (Hatirjheel Lake and Buriganga River) as well as determine their survival and the pattern of antibiotic resistance. There was difference in antibiotic resistance pattern of isolates and in future further research can be done to determine from where exactly this resistance came from. On the other hand, in survival there was slight difference between and isolates from both water bodies. In future, survival can be conducted using different concentration of isolates for better comparison. Finally, more work should be done on the molecular level to confirm isolates as they are not being isolated using cultural method.

Chapter 5

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5. 0 References

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Appendix

Media compositions

The composition of all media used in the study is given below:

All the media used were from Himedia

TCBS Agar:

Composition	**Amount (g/L)**
Yeast extract	5.000
Peptic digest of animal tissue	10.000
Sodium citrate	10.000
Sodium thiosulphate	10.000
Sodium cholate	3.000
Oxgall	5.000
Sucrose	20.000
Sodium chloride	10.000
Ferric citrate	1.000
Bromothymol blue	0.040
Thymol blue	0.040
Agar	15.000

Alkaline Peptone Water:

Composition	**Amount (g/L)**
Peptone	20
NaCl	20
Distilled Water	20

Modified Nutrient Agar:

Composition	**Amount (g/L)**
Beef Extract3.0 g	3.0
Peptone	5.0

Agar	15.0
NaCl	1.0
Distilled Water	1000

Nutrient Broth:

Component	Amount (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH at 25^0C	7.4±0.2

Mueller- Hinton Agar:

Component	Amount (g/L)
Beef, dehydrated infusion form	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH at 25^0C	7.3± 0.1

Saline:

Component	Amount (g/L)
Sodium chloride	9.0

Motility, Indole, Urease Agar:

Component	Amount (g/L)
Peptone	3%
Sodium chloride	0.5%
Urea	2%
Mono Potassium Phosphate	0.2%
Phenol Red	0.0005%
Agar	0.4%
pH	7

Simmons Citrate Agar:

Component	Amount (g/L)
Magnesium Sulfate	0.02%
Sodium chloride	0.5%
Sodium Citrate	0.2%
Di potassium Phosphate	0.1%
Mono potassium phosphate	0.1%
Bromothymol Blue	0.008%
Agar	2%
pH	7

Triple Sugar Iron (TSI):

Component	Amount (g/L)
Beef extract	3.0
Peptone	20.0
Yeast extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose monohydrate	1.0
Ferrous sulfate	0.2
Sodium chloride	5.0
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	12

Indole Broth:

Component	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

Methyl Red and VogesProskauer Media (MR-VP):

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Reagents and Buffer

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of pdimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

1M TrisHCl:

In a McCartney bottle, 1.576g TrisHCl was added. Then 10 ml distilled water was added to prepare 10 ml 1M TrisHCl. After that pH was adjusted to 8. Then it was stored at 4ºC.

0.5M EDTA:

In a McCartney bottle, 1.861 g EDTA was added. Then 10 ml distilled water was added to prepare 10 ml 0.5M EDTA. After that pH was adjusted to 8. Then it was stored at room temperature.

1X TBE Buffer:

In a Durham bottle, 5.4 g of Tris base, 2.75 g of Boric Acid, 2ml of 0.5M EDTA were added. Then 500 ml distilled water was added to prepare 500 ml 1X TBE Buffer. After that pH of the buffer was adjusted to 8. Then it was autoclaved at 15psi 121ºC. After autoclave, it was stored at room temperature.

Supplementary

PCR mixture preparation

Reaction mixture:

5ul SYBR green mix

$MgCl_2$ (1.2 ul)

0.5 ul forward primer (10 pmol)

0.5 ul reverse primer (10 pmol)

1 ul water

2ul DNA template

Recipe of solutions used for Plasmid Extraction

Plasmid DNA extraction of the isolates were performed using the alkaline lysis method. For this technique, essentially 3 solutions were used which were added to the pellet after centrifugation.

1. Solution I:
    - Tris
    - EDTA
    - Glucose
2. Solution II:
    - NaOH
    - SDS
3. Solution III: (ICE COLD)
    - Sodium Acetate
    - Acetic acid