Studying *Vibrio* spp. in industrially polluted surface waters : Difficulties and uncertainities during characterization

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

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Declaration

I hereby declare that the thesis project titled **"Studying *Vibrio* spp. in industrially polluted surface waters : Difficulties and uncertainities during characterization"** has been written and submitted by me, Sadia Alam 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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Acknowledgement

First and foremost, I would like to express my thanks to Almighty Allah because He has given me the opportunity and strength to finish this research. I am also thankful for His blessings to my daily life, good health and healthy mind. I acknowledge my esteem to Professor **A F M Yusuf Haider** and Chairperson of MNS Department and Professor **Mahboob Hossain** for allowing me and encouraging me to complete my undergraduate thesis.

My regards, gratitude, indebtedness and appreciation go to my respected supervisor **Mahbubul Hasan Siddiqee**, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for his constant supervision, constructive criticism, expert guidance, enthusiastic encouragement to pursue new ideas and never-ending inspiration throughout the entire period of my research work. I would like to thank and express my deepest gratitude for guiding me in my report writing and providing time to time suggestions regarding setting of experimental designs, interpretation of results and subsequent directions for the whole work without being a bit of impatient. It would have been impossible to submit my report without his cordial help.

I would like to extend my appreciation to the respective Lab officers **Asma Binte Afzal** and Shamim Akhter Chowdhury, Teaching assistants **Maisha Mosharrat Chowdhury,** Nahreen Mirza, Salman Khan and Akash Ahmed for their suggestions and moral support during my work. I am specially thankful to the respective Lab officer **Asma Binte Afzal** for guiding and supporting me devoutly in every single steps throughout my whole work.

I also appreciate my thesis partners **Raghib** and **Mahmudul** for their kind cooperation and active support throughout my work.

Sadia Alam
November, 2018

Abstract

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Being the capital of Bangladesh Dhaka city faces multiple pollution problems due to rapid urbanization leading to unplanned industrialization. This often results in the contamination of water bodies with bacteria pathogenic to humans. 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* spp. in water bodies contaminated with industrial pollutants. For this purpose Gulshan Lake and Buriganga River, the two prominent water bodies of our country were chosen for sampling. A total of 80 samples were collected aseptically. In total, 70 samples were from Gulshan Lake and 20 samples were from Buriganga River within duration of three months. Then, they were processed for isolation of culturable *Vibrio* spp. using culture-based method. Of these water samples, 67% were found positive for *Vibrio* spp. and the rest were found to be non-*Vibrio.*Biochemical tests were then performed to confirm DNA extraction in boiling method was performed before the Genus specific PCR targeting the *rpoA* gene for further confirmation. Then helomysis test was performed where 86% isolates gave beta hemolysis and 14% isolates gave gamma hemolysis. Anbibiotic susceptibility and resistant test was done against ten antibiotics where Nalidixic acid (30µg) showed highest(86%) resistance and Tetracycline (30µg) showed lowest (14%) resistance. This study reveals that both Gulshan Lake and Buriganga river are contaminated with *Vibrio* spp. and can be highly virulent that may lead to serious health hazard in near future.

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CHAPTER 1

INTRODUCTION

1.0 Introduction

Water is a necessity for life and an adequate, safe and accessible supply is of paramount importance. The impact of water on health is associated with unwholesome sources contaminated with feaces (from humans or animals) containing pathogenic microorganisms. The quality of drinking water is closely associated with human health and providing safe drinking water is a major public health priority .Traditionally, water has been considered to be the most important vehicle for cholera transmission (Bulus, H., Ado, A., Yakubu, E. and Ella, E., 2015). Pathogens such as bacteria, viruses, and parasites can spread by water and cause water-borne diseases and they are also known as communicable diseases because they can spread from one person to another. So water is the vehicle for spread of the pathogens and other health hazards (Gasana, 2014).

The World Health Organization says that every year 3.4 million people die as a result of water related diseases. According to an assessment commissioned by the United Nations, 4,000 children die each day as a result of diseases caused by ingestion of filthy water. The report says four out of every 10 people in the world, particularly those in Africa and Asia, do not have clean water to drink (Berman, 2009). The most important bacterial diseases transmitted by water are Cholera, bacillary dysentery. diarrhea, typhoid fever etc. *Vibrio cholerae* causes cholera in human. It spreads indirectly through faecal contaminated water and foods which are undercooked or consumed raw (Jayasinghe, C., Ahmed, S. and Kariyawasam, M.,2007). Approximately, 1.8 percent people die annually from diarrheal diseases including cholera. Of which, almost 90 percent are children under five years old. Up to 88 percent of water-borne diseases arise from unsafe water supplies, inadequate sanitation and hygiene (Ameer, 2017).

History of Cholera: During the 19th century, cholera spread across the world from its original reservoir in the Ganges delta in India. Six subsequent pandemics killed millions of people across all continents. The current (seventh) pandemic started in South Asia in 1961, and reached Africa in 1971 and the Americas in 1991. Cholera is now endemic in many countries (World Health Organization, 2018).

The genus *Vibrio* includes gram-negative, oxidase-positive, rod or curved rod-shaped, facultative anaerobes (FDA, 1992) (Jayasinghe, Ahmed, Kariyawasam, 2007). The family Vibrionaceae and is known worldwide as the etiological agent of cholera. Over 200 serogroups of *V. cholerae* are known of which only O1 and O139 serogroups have been associated with the diarrhoeal disease generally known as cholera. *V. cholerae 01* and *V. cholerae 1039* colonizes the small intestine and produces an enterotoxin responsiblefor a watery diarrhea. Without prompt treatment, a person with cholera may die of dehydration in a matter of hours after infection. Cholera outbreaks are generally associated to contaminated food and water supplies. Appropriate sanitation and safe water are the main weapons against the disease (Codeco, 2001).

Symptoms of Cholera: It takes between 12 hours and 5 days for a person to show symptoms after ingesting contaminated food or water (2). Cholera affects both children and adults and can kill within hours if untreated.Most people infected with V. cholerae do not develop any symptoms, although the bacteria are present in their faeces for 1-10 days after infection and are shed back into the environment, potentially infecting other people (World Health Organization, 2018).

Once there, the bacteria begin to produce cholera toxin (CT), which is responsible for the copious secretory diarrhoea that is the hallmark of cholera (Kaper *et al*., 1994). CT is encoded by the *ctxAB* genes present within a filamentous bacteriophage that is integrated in the *V. cholerae* genome (Waldor and Mekalanos, 1996). CT acts as a classical A–B type toxin, leading to ADP-ribosylation of a small G protein, GSa, and constitutive activation of adenylatecyclase, thus giving rise to increased levels of cyclic AMP within the host cell (reviewed in Kaper *et al*., 1994). This results in the rapid efflux of chloride ions and water from host intestinal cells. The ensuing diarrhoeal disease can lead to death by dehydration within hours if not properly treated (Merrell, Hava & Camilli, 2002).

When the low pH of the stomach is buffered with sodium bicarbonate prior to the oral administration, the required dose to elicit the diarrhea decreases several logs. These results indicate that it is unlikely for *V. cholerae* in the free-living state to be the major source of epidemic cholera, as the stomach barrier appears to be a major hindrance to its survival.

Thus,the association of the bacterium with other organisms and/or abiotic surfaces facilitates the ability of *V. cholerae* to cause the disease (Almagro-Moreno & Taylor, 2013).

All other serogroups termed non-O1/non O139 are reported as causative agents of sporadic and localized outbreaks of a cholera-like disease (Bulus, G.H, Ado, S.A, Yakubu, S.E, Ella, 2015). Two species, *V.cholerae* and *V.parahaemolyticus* are well documented human pathogens (Jayasinghe, Ahmed, Kariyawasam, 2007).

V. cholerae regulates its virulence genes with two inner membrane-localized regulators, ToxR and TcpP, are required to transcribe the *toxT* gene, which encodes the master regulator of virulence in choleragenic *V. cholerae* (Almagro-Moreno & Taylor, 2013). ToxR has since been well characterized as a transmembrane DNA binding protein that regulates expression of many of the known virulence factors of *V. cholera* (Merrell, Hava & Camilli, 2002).

Further, *V.cholerae* cells encoding genes and mobile elements known to cause disease in humans and confer resistance to antibiotics are found in the absence of cholera. In developing countries, exposure to *V.cholerae* typically occurs via consumption of natural surface water containing *V.cholerae* in sufficient numbers to cause disease, whereas in developed countries exposure to *V.cholerae* often occurs via consumption of raw or undercooked shellfish or travel to regions where cholera outbreaks occur frequently (Huq, Haley, Taviani, Chen, Hasan, Colwell, 2012).

There are 12 *Vibrio* species which cause human disease; the most important of them are *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. The clinical signs may range from gastroenteritis to wound infection, otitis and septicaemia depending on the bacterial species which cause disease (Ulusarac and Carter, 2004).

Consumption of foods contaminated with high concentration of total *V. parahaemolyticus* and/or Pathogenic *V. parahaemolyticus* can cause gastrointestinal infections. An open wound in skin comes in contact with *V. parahaemolyticus* is suggested as an infection pathway as well. Major syndromes caused by *V. parahaemolyticus* include gastroenteritis, wound infection, and septicaemia. Typical symptoms of the gastroenteritis may include abdominal pain, diarrhea, nausea, and fever (Tan et al., 2017).

Among *Vibrio* and related genera, *V.anguillarum, V.parahaemolyticus* and*V.vulnificus* are the main pathogenic species involved in salt water, and *V.mimicus* and *V.cholerae* in fresh water culture. The *Vibrio* species pathogenic formarine fish are *V.alginolyticus, V.anguillarum, V.carchariae V.cholerae, V.ordalii, V.vulnificus* and *V.parahaemolyticus* (Jayasinghe, Ahmed, Kariyawasam, 2007).

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).

Dhaka is the capital of Bangladesh and one of the major cities of South Asia, located at the banks of the Buriganga river. According to the National Water Management Plan (NWMP), the population of Dhaka city might cross 26 million by 2025. It is increasing and putting pressure on drinking water requirement of the city. According to Dhaka Water Supply and Sewerage Authority (DWASA), it can currently supply 75% of water demand,out of which 85% is from groundwater sources (Deep Tubewells) (Rahman, Rummana, Aziz & Nishat, 2008).

Surface water is water on the surface of the planet such as in a river, lake, wetland, or ocean. It can be contrasted with groundwater and atmospheric water. In Bangladesh, rainfalls and trans-boundary rivers flows are the main sources of surface water. The most important recreational water sites in Dhaka include Dhanmondi lake, Gulshan lake, Ramna lake and Hatirjheel lake. Gulshan Lake is a lake in Dhaka, Bangladesh, that borders Gulshan Thana, Shahjadpur, and Baridhara Diplomatic Zone.

Gulshan Lake is the northernmost lake in a chain of water bodies (Gulshan Lake, Hatirjheel, Begunbari Khal, BaluRiver and Shitalakhya River) in Dhaka, suffering from highly significant pollution thus increasing pollution from north to south (Ahmed, 2013). Gulshan-Baridhara Lake

was declared an Ecologically Critical Area (ECA) in 2001. Continuation of all sorts of banned activities in the ECA has turned it into an ecologically dead lake. This has been manifested recurrently through the death of fishes, receiving media coverage vigorously each year (Ali, 2007). The poor water quality of Gulshan Lake is also contributing to the pollution of Hatirjheel, where a restoration project is already implemented and the downstream water bodies. It is very important to take immediate steps to restore the water quality of Gulshan Lake (Ahmed, 2013)

Importance of the lake: In the context of very scarce recreational opportunity in Dhaka city, this lake could be an important recreational point for city dwellers. Despite being a source of ground water recharge, the lake has a very important cooling effect on the city environment. Also this lake is a source of drinking water for wild fowl and other animals in the area (Ali, 2007).

The lake is fragmented into four parts by culvert type structures. Openings of such structures appear to be very insufficient for free flow of water among different parts of the lake. This badly affects the dilution of pollution loads, dissolution of oxygen into lake water and as a result, the localised effect of pollution sometimes becomes so severe that it causes death of aquatic organisms, such as fish (Ali, 2007).

It is observed that pH has slight seasonal variation with the values of 5.23 to 7.42. In dry season average pH value is 7.36 while in wet season value is 6.80.The pH of Gulshan lake water is high in dry season. The spatial variation shows the pH increase slightly from upstream (Near Madani-avenue.) to downstream (Gulshan-Badda link road) (Ahmed, 2013).

The lake water has been characterized by very low DO (mostly below 5ml/l) and the high BOD (up to 101.0 mg/l) indicates significant organic pollution. It was observed that the concentration of color and Total Dissolved Solids TDS increased in dry season and concentration of Total Suspended Solids TSS and turbidity increased during the wet season. During dry season, the maximum value of TDS is 334mg/l and in wet season the minimum TDS value is near 212mg/l (Ahmed,2013).

On the other hand,the Buriganga is economically very important to Dhaka. The bank of Buriganga was a major trade center when the Mughals made Dhaka their capital in 1610. During the British regime, the bank of the Buriganga River was considered to be the most scenic place in Dhaka for its natural beauty. However, many people still use the river for recreational purposes, such as walking along the river side, swimming and boating. The river was also the city's main source of drinking water. Today, the Buriganga River is afflicted by the serious problem of pollution. The chemical wastes of mills and factories, household wastes, medical wastes, sewage, dead animals, plastics, and oil are some of the Buriganga's pollutants. Dhaka city discharges thousands of tons of solid wastes every day and most of it is released into the Buriganga. According to the Department of the Environment (DOE), 22,000 cubic liters of toxic wastes are released into the river by the tanneries everyday. The Buriganga River was once used as the main source of water supply for Dhaka's residents. It provides an extensive network of inland waterways all over Bangladesh, particularly within the southern districts. Hundreds of launches and thousands of country and engine boats ply on this river daily to transport goods and passengers. Most of the people who live within the catchment area of the river and directly use the water for washing, bathing and other domestic purposes on a daily basis. and socially and environmentally degraded industries poses a new challenge to Bangladesh. The River management is now very much necessary to protect the river. The rehabilitation option is the best solution of the river management (Kibria, Kadir, Alam, 2015).

The two main water bodies of Dhaka city – Gulshan lake and Buriganga river are selected for this experiment. The aim of the study is to determine the presence of *Vibrio spp.* and to observe the antibiotic resistant pattern in Gulshan lake and Buriganga river. According to the hypothesis, the water will be contaminated with *Vibrio* genus and the contaminants will have different characteristics in Gulshan lake and Buriganga river.

CHAPTER 2

MATERIALS and METHOD

2.0 Materials and Method

2.1 Sampling sites

Gulshan Lake is the northernmost lake in a chain of water bodies (Gulshan Lake, Hatirjheel, Begunbari Khal, Balu River and Shitalakhya River) in Dhaka, suffering from highly significant pollution. Gulshan lake is located 23º48' N and 90º25' E of Dhaka city. The length of the lake is 3.8 km which covers an area of 0.0160 km2. It has an average depth 2.5 m and volume 12 × 105m3 (Nishat A, 2000). The lake is a channel-like elongated water body. The peripheral sides are, northern at Baridhara, southern at Tejgaon- Hatirjheel, western at Gulsan-Banani and eastern at Badda area (Razzak, Siddik, Ahmeduzzaman, 2013).

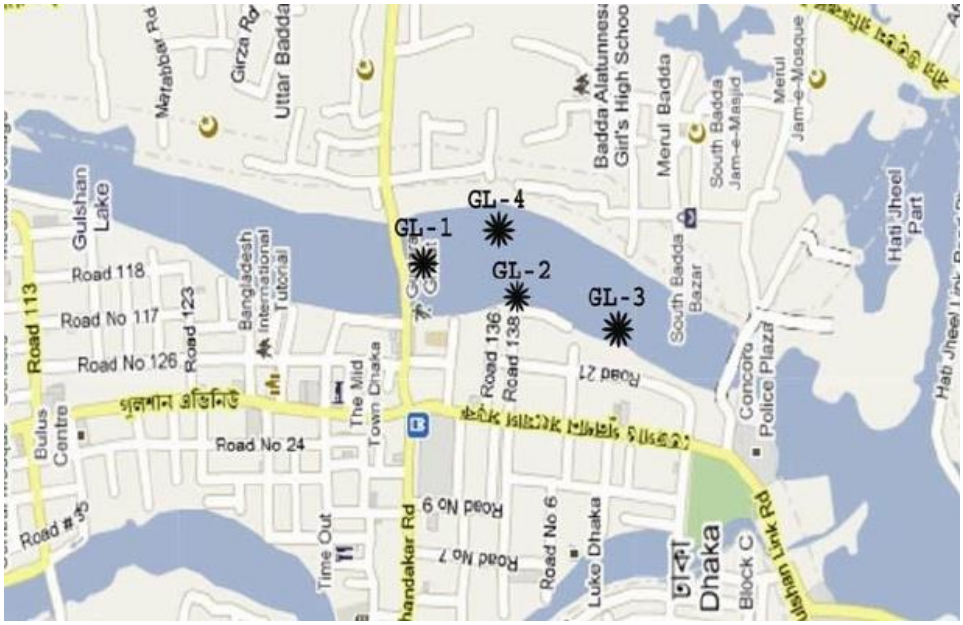

Figure 2.1: Sampling sites of Gulshan lake **(Source: Google image)**

The Buriganga river is located in the southern part of the north central region of Bangladesh and close to the confluence of the Padma (Ganges) and upper Meghna river (Kibria, Kadir, Alam, 2015). This river is only 27 km long. at present, it lies about 3.22 km southwest of Fatullah (Chowdhury, Banglapedia, 2015)

Figure 2.2: Sampling sites of Buriganga River **(Source: Google image)**

2.2 Water Sampling

In total, 80 samples are collected from Gulshan lake and Buriganga river. Among them, 70 samples were from Gulshan lake and 10 samples were from Buriganga river. Sample collection procedure started from November 2017 to January 2018. Autoclaved plastic bottles are used to collect the samples aseptically from different parts of Gulshan lake and Buriganga river. Small boats are used to reach the sample site. After the sample collection, the samples were transported to the laboratory for further processing.

2.3 Isolation

Pre-enrichment of the environmental water samples is required to improve isolation. For enrichment purpose, Alkaline Peptone Water (APW) was used. The pH was measured with a pH meter (pH 8.4). 50ml of autoclaved APW was taken to a conical flask and then 50ml of sample water was poured into it making it total 100ml. Autoclaved measuring cylinder was used for measurement. Then the flasks were kept in an incubator at 37°C for overnight.

After enrichment in APW, next day the surface growth was collected that present as a whitish film from the enrichment flask with an inoculating loop and streaked onto TCBS (Thiosulfate citrate bile salt sucrose) agar plates. The plates were kept in an incubator at 37°C for overnight.

After incubation, two colors (yellow, green) of colonies were obtained. Smooth and flat colonies were taken by a sterile inoculating loop from TCBS and subcultured to modified nutrient agar (MNA) that contains 3% salt in it and again kept for incubation same as the above.

For stock, modified nutrient broth (MNB with 3% salt) was used. 1ml of MNB was transferred to each autoclaved microcentrifuge tubes. Then, one loopful of colonies from MNA was inoculated into MNB. After inoculation, microcentrifuge tubes were kept in an incubator at 37°C. The next day, 300µl of autoclaved glycerol was added to each tube and kept at -20°C for storage.

2.4 Identification

The suspected colonies were then tested for the following biochemical tests to presumably conclude whether they are *Vibrio* spp. or not.

Gram staining

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

Biochemical tests

Several biochemical tests were performed to accurately identify the presumptive bacteria chosen previously. These tests include- oxidase test, Triple sugar iron agar test (TSI), Methyl red test (MR), Voges- Proskauer test (VP), Citrate utilization test and MIU test (Motility 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. The ability of the bacteria to produce cytochrome oxidase is determined by the addition the test reagent (p-aminodimethylaniline oxalate) to colonies grown on a plate medium. Following the addition of the test reagent, development of purple color end product on the surface of the colonies is indicative of cytochrome oxidase production and is a positive result. No color change or presence of light pink color of the colonies is indicative of the absence of oxidase activity and is a negative result. Bacterial colonies were picked from freshly culture plate using toothpick and touched onto filter paper and then oxidase reagent was added using dropper. Formation of purple color is considered as positive result. (Cappuccino & Sherman, 2005).

Triple sugar iron agar test (TSI)

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^{o}C. Using sterile technique; a 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).

Methyl red test (MR)

In this test, the pH indicator methyl red detects the presence of a large concentration of acid end products in presence of glucose. A loopful of bacterial culture was inoculated in test tubes containing 10 ml of MR-VP broth and incubated at 37°c for overnight. After incubation, 5ml of broth was transferred to another test tube for MR test (other 5ml for Voges- Proskauer test) and then 5 drops of the methyl red indicator solution were added in the test tubes. A positive reaction is indicated if the color of the medium changes to red within a few minutes (Cappuccino & Sherman,2005) and yellow color indicates a negative result.

Voges- Proskauer test (VP)

The Voges-Proskauer test determines the capability of some organisms to produce non acidic or neutral end products, such as acetylmethyl 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 α-naphthol and 40% potassium hydroxide solution. Detection of acetylmethyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of the α-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 (Cappuccino & Sherman,2005).

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. This ability depends on the presence of citrate permease enzyme that facilitates the transport of citrate in the cell. Simmons citrate agar slants of 2ml in each vial were prepared by autoclaving at 15 psi 121°C. Using sterile technique, a 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 24-48 hours at 37°C (Cappuccino & Sherman, 2005).

MIU test (Motility test 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◦C and 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).

Motility test

- Non-motile bacteria generally give growths that are confined to the stab-line, have sharply defined margins and leave the surrounding medium clearly transparent.
- Motile Bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque (Acharya, 2015)

Urease test

- If a microorganism produces urease enzyme, the color of the slant changes from light orange to magenta.
- If microorganism does not produce urease the agar slant and butt remain light orange (medium retains original color) (Acharya, 2015)

2.5 DNA Extraction (Boiling method)

Preparation of DNA is done by boiling lysis of bacteria isolated from the sample. DNA from APW broth was prepared by boiling method. Samples were inoculated in 50ml APW broth and kept at shaker for 48 hours. For 50ml, 15ml falcon tubes were used.

Procedure :

1. Centrifuge 48 hours of culture of *V. cholerae* in APW 13,000 rpm for 10 minutes
2. Removed the supernatant
3. Add 1.5ml of autoclaved distilled water stored at room temperature (gentle inversion for washing)
4. Centrifuge and remove the supernatant
5. Add 1.5ml of autoclaved distilled water and re-suspend the pellet
6. Lyse cells at 98°C for 7 minutes
7. Immediate cold shock with ice for 10 minutes
8. Centrifuge at 13,000 rpm for 5 minutes
9. Take the supernatant in a new micro-centrifuge tube
10. Store at -20°C

2.6 Genus specific PCR

A polymerase chain reaction (PCR) method based on the RNA polymerase alpha subunit (rpoA) gene was developed for the detection of the *Vibrio* genus. The specific primers were designed aligning the rpoA gene sequences of all *Vibrio* species. The rpoA primers correctly amplified the *Vibrio* species confirmed by ABIS online software. rpoA is a housekeeping gene of *Vibrio* spp. (Dalmasso et al., 2009)

Table 2.1: Reaction mixture for a 25µl reaction volume

Component	Volume
Master Mix, 2X	12.5µl
Upstream primer,10µM	2.5 µl
Downstream primer,10 µM	2.5µl
DNA template	5µl
Nuclease-Free Water	25µl

Primers were designed as follows: 5′-AAATCAGGCTCGGGCCCT-3′ (sense) and 5′GCAATTTT(A/G)TC(A/G/T)AC(C/T)GG-3′(antisense) corresponding, respectively,

to positions 294 to 311 and 519 to 535 of *V. parahaemolyticus* (GenBank accession number no. AJ842676) (Dalmasso et al. 2009)

Thermocycling conditions were the following: initial denaturation at 94°C for 3 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. The final extension was carried out at 72°C for 5 min (Dalmasso et al. 2009).

Table 2.2: Primer sequence of rpoA gene used in this study

Organism	**Primer sequence**	**Denaturation temparature**	**Annealing temparature**	**Extension**	**Amplicon size**
Vibrio genus	5'- AAATCAGGCTCGGGCCCT-3' 5'- GCAATTTTGTCGACCGG-3' 242bp band indicates *Vibrio*	94℃	55℃	72℃	456 bp & 242bp

Then, Gel electrophoresis was done on 1.5% agarose gel.

2.7 Kirby-Bauer Disc Diffusion

The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds in order to assist a physician in selecting treatment options for his or her patients (Hudzicki,2009). It was used to test whether the experimental bacteria were susceptible to specific antibiotics as recommended by the Clinical Laboratory Standard Institute (CLSI) using commercial antimicrobial discs. The antibiotic discs used in this study are: Ampicillin (25µg), Co-Trimoxazole(25µg), Nalidixic acid (30 µg), Doxycycline (30µg), Erythromycin (15µg), Chloramphenicol (30µg), Tetracycline (30µg), Gentamycin (10µg), Streptomycin (10µg), Norfloxacin (10µg).

MH agar plates were used to make lawn culture of *Vibrio*. With a sterile inoculating loop 3-4 isolated colonies of the microorganism were taken to be tested and suspended in 5ml of sterile saline. The saline tubes were vortexed to create a smooth suspension. The turbidity of the

suspension was adjusted to a 1.0 McFarland standard by adding more organism if the suspension was too light or, diluting with sterile saline if the suspension was too heavy. The suspension was used within 5 minutes of preparation. Then, autoclaved cotton swab was dipped into the saline suspension and the excess liquid from swab was removed by pressing it against the side of the test tubes. The dried surface of MH agar plates was inoculated by streaking with that cotton swab three times over the entire agar surface. The plate was rotated each time to ensure even distribution the inoculums and allowed to dry for 3-5 minutes before the antibiotic discs were applied. Each antibiotic-impregnated disc must be pressed down with sterile forcep to ensure complete contact with the MH 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).

2.8 Hemolysis Test

The Hemolytic test is also known as blood agar culturing method and is used for the identification of forms of hemolysis from pathogenic microorganisms. The blood agar plate (BAP) contains mammal blood (5% sheep blood) and is used to identify bacteria that produce the hemolytic enzyme 'hemolysin', an exotoxin by nature and disrupt membrane of the host likely erythrocytes. The mechanism of action of hemolysin is that it disrupts RBCs and increases the content of free iron and is also involved in dermonecrosis and vasoconstriction. Sodium Citrate anti-coagulant was added to fresh autoclaved Duran bottle before blood is placed in the bottle.

Blood agar base was prepared and autoclaved. After cooling down, 5ml of blood was added to 100ml of blood agar base. Vigorous shaking was done to prepare blood agar and the agar was poured into fresh petri dish. A small amount of bacteria from 24 hours of old pure culture was streaked onto the blood agar plate with a sterile loop. Then the plates were kept for incubation for 24 hours at 37°C.

If the bacterial colonies create a clear and transperant area, it is beta hemolysis that indicates that the bacteria growing on blood agar completely broke down RBCs. Brown colored area appeared without transparacy when the bacteria partially broke the blood cells, it is alpha hemolysis..And

if the bacteria is not able to break down blood cells then no color change in the medium will occur and this is gamma hemolysis (Cappuccino & Sherman, 2005).

CHAPTER 3

RESULTS

3.0 Results

3.1 Identification

After presumptive isolation of *Vibrio*, the samples were subjected to 6 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 and oxidase while negative for urease, citrate and Voges-Proskauer from MR-VP. In TSI it is supposed to give yellow slant, yellow and black butt due to the production of hydrogen sulfide. The following table describes the results of the samples tested for the mentioned biochemical tests.

Table 3.1: Biochemical Test Result

Sample	Methyl Red	Voges-Proskauer	Oxidase	Citrate utilization	Motility	Urea hydrolysis	H_2S	Gas	D-glucose	Lactose	Sucrose	ABIS Result
2	+	-	-	-	+	-	+	-	+	-	-	*Photobacterium leiognathi* 81.9% *Vibrio parahaemolyticus* 81.2%
3	+	-	-	-	+	-	-	-	+	-	-	*Photobacterium leiognathi* 90.1% *Vibrio parahaemolyticus* 89.3%
8	+	-	-	-	+	-	+	-	+	+	+	*Vibrio metschnikovii* 88.5% *Vibrio cincinnatiensis* 82.5%
10	+	-	-	-	+	-	+	-	+	+	+	*Vibrio metschnikovii* 80.3% *Vibrio cincinnatiensis* 74.3%
11	+	-	-	-	-	-	-	-	+	-	-	*Vibrio gallicus* 82.7% *Photobacterium leiognathi* 81.9%
12	+	+	-	-	+	-	-	-	+	-	-	*Photobacterium angustum* 90.1% *Photobacterium leiognathi* 90.1%
13	+	-	-	-	+	-	+	-	+	-	-	*Photobacterium leiognathi* 81.9% *Vibrio parahaemolyticus* 81.2%
15	+	+	-	-	+	-	-	-	+	-	-	*Photobacterium angustum* 90.1% *Photobacterium leiognathi* 90.1%
16	+	-	-	-	+	+	-	-	+	-	-	*Vibrio parahaemolyticus* 82.7% *Photobacterium leiognathi* 81.9%
17	+	-	-	-	+	+	-	-	+	+	+	*Vibrio metschnikovii* 80.3% *Vibrio cincinnatiensis* 74.3%
20	-	-	-	-	+	-	+	-	+	-	-	*Photobacterium leiognathi* 81.9% *Vibrio parahaemolyticus* 81.2%
21	-	+	-	-	+	-	+	-	+	-	-	*Salinivibrio proteolyticus* 82.7%

												Enterovibrio norvegicus	*74.5%*
23	-	-	-	-	+	+	+	-	+	-	-	*Aliivibrio fischeri* *Enterovibrio norvegicus*	*80.3%* *74.3%*
24	-	-	-	-	+	-	+	-	+	-	-	*Enterovibrio norvegicus* *Grimontia hollisae*	*82.7%* *82.7%*
25	+	-	-	-	+	-	+	-	+	-	-	*Photobacterium leiognathi* *Vibrio parahaemolyticus*	*81.9%* *81.2%*
27	+	+	-	-	+	-	+	-	+	-	-	*Photobacterium angustum* *Photobacterium leiognathi*	*81.9%* *81.9%*
30	-	-	-	-	+	-	+	-	+	-	-	*Enterovibrio norvegicus* *Grimontia hollisae*	*82.7%* *82.7%*
31	+	-	-	-	+	-	-	-	-	-	-	*Photobacterium leiognathi* *Vibrio parahaemolyticus*	*81.9%* *81.2%*
36	-	-	-	-	-	-	-	-	+	+	+	*Aeromonas salmonicida subsp. Achromogenes* *90%* *Salinivibrio siamensis*	*72.3%*
41	+	-	-	-	+	+	-	-	+	+	+	*Vibrio metschnikovii* *Vibrio cincinnatiensis*	*80.3%* *74.3%*
44	+	-	-	+	+	-	+	+	+	+	+	*Vibrio gazogenes* *Vibrio furnissii*	*86.7%* *74.5%*
48	+	-	-	-	+	-	+	-	+	+	+	*Vibrio metschnikovii* *Vibrio cincinnatiensis*	*80.3%* *74.3%*
61	+	-	-	-	+	-	-	-	+	-	-	*Photobacterium leiognathi* *Vibrio parahaemolyticus*	*90.1%* *89.3%*
63	+	-	-	-	+	-	-	-	+	+	+	*Vibrio metschnikovii* *Vibrio cincinnatiensis*	*88.5%* *82.5%*
78	+	-	-	-	+	-	-	-	+	-	-	*Photobacterium leiognathi* *Vibrio parahaemolyticus*	*90.1%* *89.3%*
83	+	+	-	-	+	-	-	-	+	+	+	*Vibrio metschnikovii* *Photobacterium angustum*	*96.8%* *89.3%*
R-2	-	-	-	-	+	-	+	+	+	-	-	*Enterovibrio norvegicus* *Grimontia hollisae*	*82.7%* *82.7%*

In total, 80 samples were collected. 70 from Gulshan lake and 10 from Buriganga river. 56 isolates were survived and biochemical tests were performed for identification. Among them, 27 isolates were confirmed by ABIS online software (Shown in the table above) after the biochemical test and 21 of them are *Vibrio* genus.

Table 3.2: Isolated different specieses of *Vibrio* genus

Organism Name	**Percentage**
Vibrio gazogenes	4%
Vibrio parahaemolyticus	33%
Vibrio metschnikovii	26%
Vibrio galicus	4%
Non-vibrio	33%

Total 67% of *Vibrio* spp. and 33% of non-vibrio are present in Gulshan lake. Among the *Vibrio* spp. *Vibrio parahaemolyticus* was obtained in highest percentage (33%).

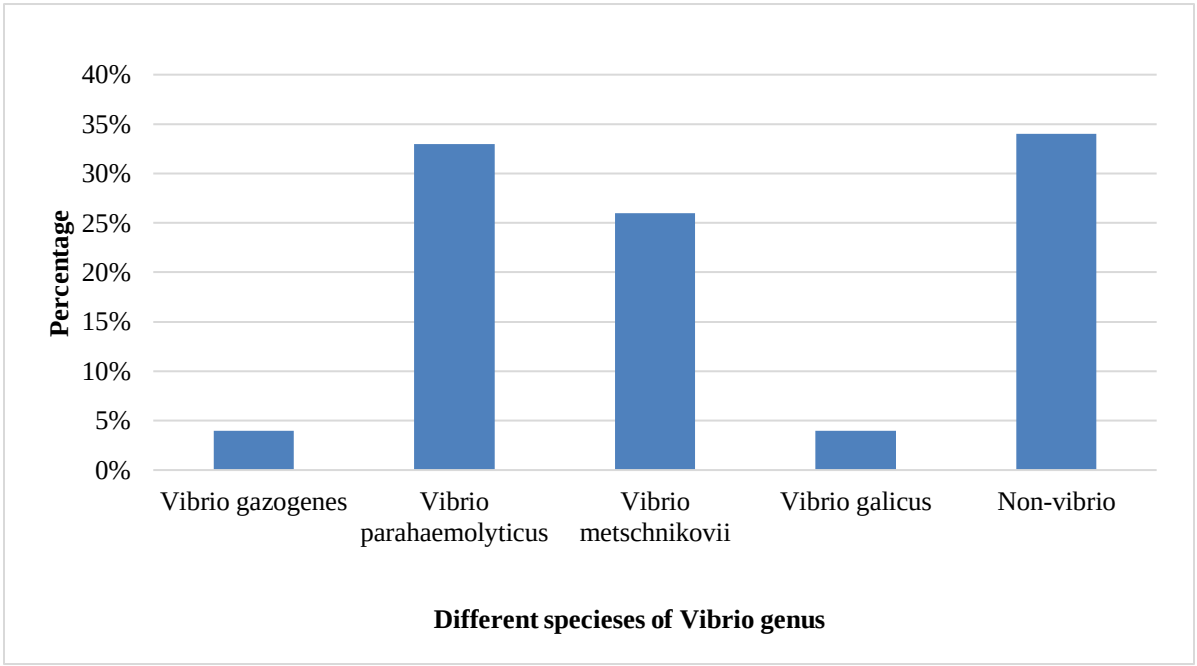

Figure 3.1: Graphical representation of *Vibrio* spp. and Non-*Vibrio* spp.

3.2 Genus specific PCR

The specificity of the primers was tested against 21 *Vibrio* spp. confirmed by ABIS online software. These 21 isolates are all from Gulshan lake. Buriganga river isolates did not survived till the last. The Genus specific PCR result in shown below:

Table 3.3 : Genus specific PCR result

Sample Number	Genus specific PCR result
Ogawa control	**Positive**
2	Negative
3	Negative
8	Negative
10	Negative
11	Negative
13	**Positive**
16	Negative
17	Negative
20	Negative
21	Negative
23	Negative
25	Negative
31	Negative
36	Negative
41	**Positive**
44	**Positive**
48	**Positive**
61	**Positive**
63	Negative
78	Negative
83	**Positive**

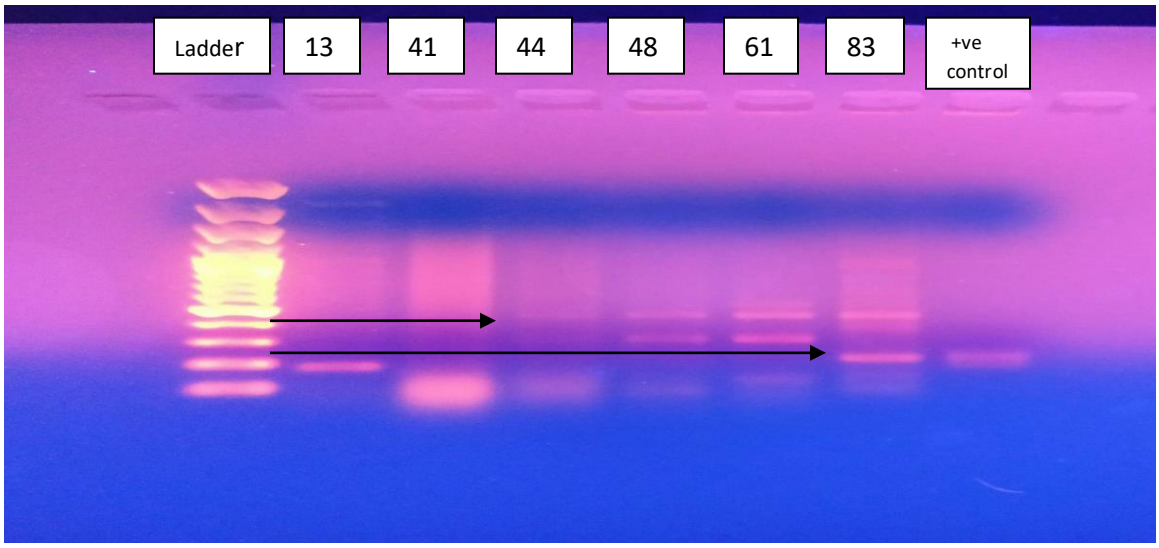

Figure 3.2: Agarose gel electrophoresis of isolates from Gulshan lake showing positive results for *rpoA* gene.

All bacteria isolates give a 456bp band while *Vibrio* spp. give an additional 242bp band along with that.

Table 3.4: Sampling date of the isolates of Genus specific PCR positive results

Sample no.	Date of sampling
13	12.11 2017
41	26.11.2017
44	26.11.2017
48	26.11.2017
61	26.11.2017
83	22.01.2018

3.3 Antibiogram

The 6 isolates (Gulshan lake) which gave PCR positive result were taken for antibiobic susceptibility test against 10 antibiotics : Ampicillin (25µg), Co-Trimoxazole(25µg), Nalidixic acid (30 µg), Doxycycline (30µg), Erythromycin (15µg), Chloramphenicol (30µg), Tetracycline (30µg), Gentamycin (10µg), Streptomycin (10µg), Norfloxacin (10µg).

Table 3.5 : Antibiogram result of 7 Genus specific PCR positive isolates

Sample no.	AMP 25	COT 25	NA 30	DO 30	E 15	C 30	TE 30	GEN 10	NX 10	S 10
Ogawa control	S	S	R	S	S	S	S	S	I	S
13	S	S	S	S	S	S	S	S	S	S
41	R	I	R	S	I	S	S	S	S	I
44	S	S	R	I	R	I	R	I	R	R
48	R	R	R	I	R	S	I	S	I	R
61	R	I	R	I	R	S	I	I	I	R
83	R	R	R	S	R	S	S	I	R	R

Table 3.6: Antibiotic resistant and susceptibility percentage in Gulshan lake isolates

Antibiotics	Resistant	Sensitive	Intermadiate
AMP 25	57.00%	43.00%	0
COT 25	29.00%	42.00%	29%
NA 30	86.00%	14.00%	0
DO 30	0.00%	57.00%	43%
E 15	57.00%	29.00%	14%
C 30	0.00%	86.00%	14%
TE 30	14.00%	57.00%	29%
GEN 10	0%	57%	43%
NX 10	29%	29%	42%
S10	57%	29%	14%

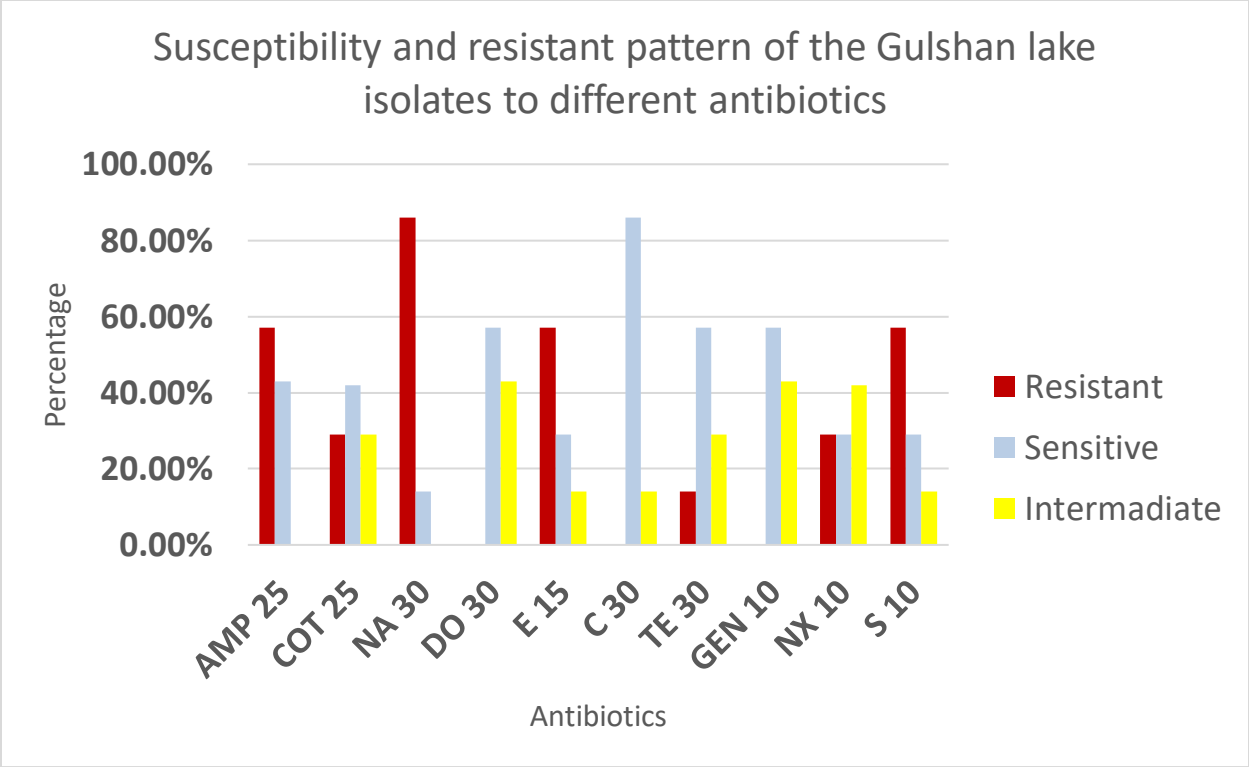

Figure 3.3 : Graphical translation of the susceptibility and resistance pattern of the Gulshan lake isolates to different antibiotics.

Table 3.7: Comparison of antibiotic resistant pattern of *Vibrio* spp. against 5 antibiotics

Antibiotic	**Percentage in my study**	**Percentage in another study(Raissy M et al., 2012)**
Ampicillin	57%	97.20%
Gentamycin	0%	83.30%
Streptomycin	57%	25.00%
Erythromycin	57%	6.90%
Tetracycline	14%	18.10%

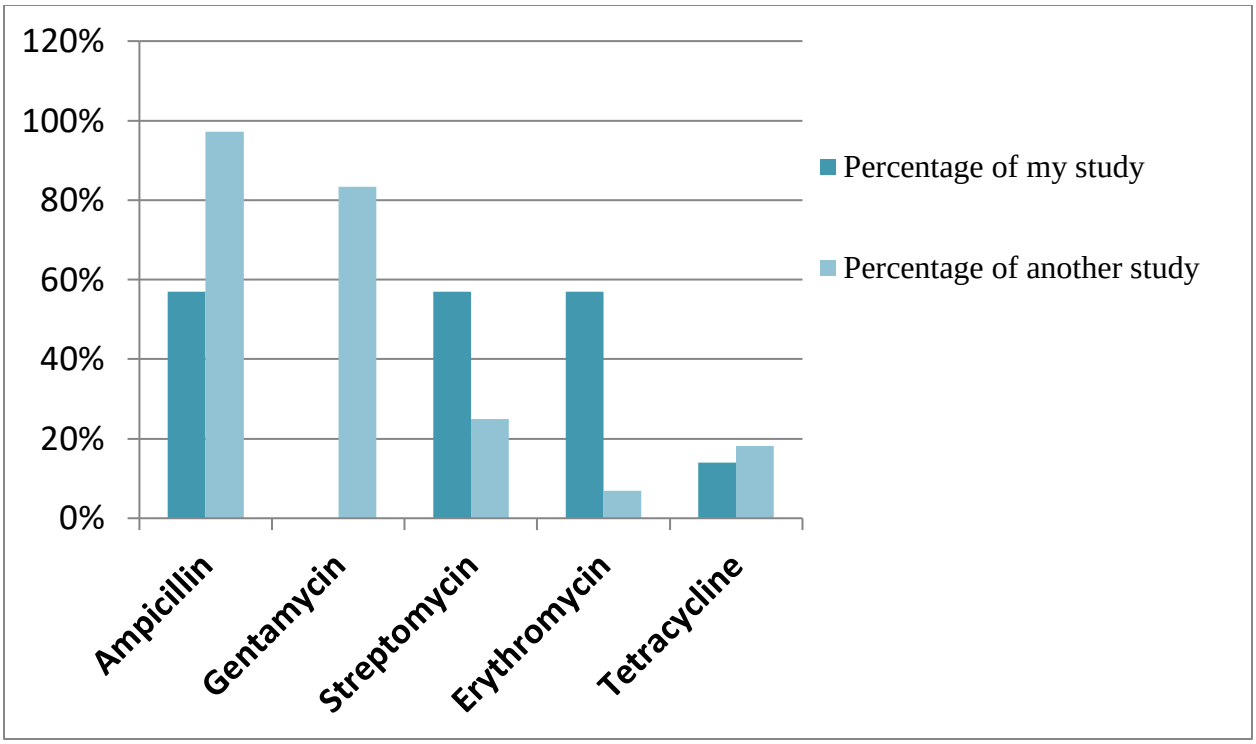

Figure 3.4: Graphical representation of Comparison of antibiotic resistant pattern of *Vibrio* spp. against 5 antibiotics.

3.4 Hemolysis test

The 6 isolates (Gulshan lake) which gave PCR positive result were taken for Hemolysis test. Only Gamma and Beta hemolysis were obtained.

Table 3.8: Gamma and Beta hemolysis percentage in Gulshan lake isolates

Total=7	
Beta hemolysis	**Gamma hemolysis**
86%	14%
6	1

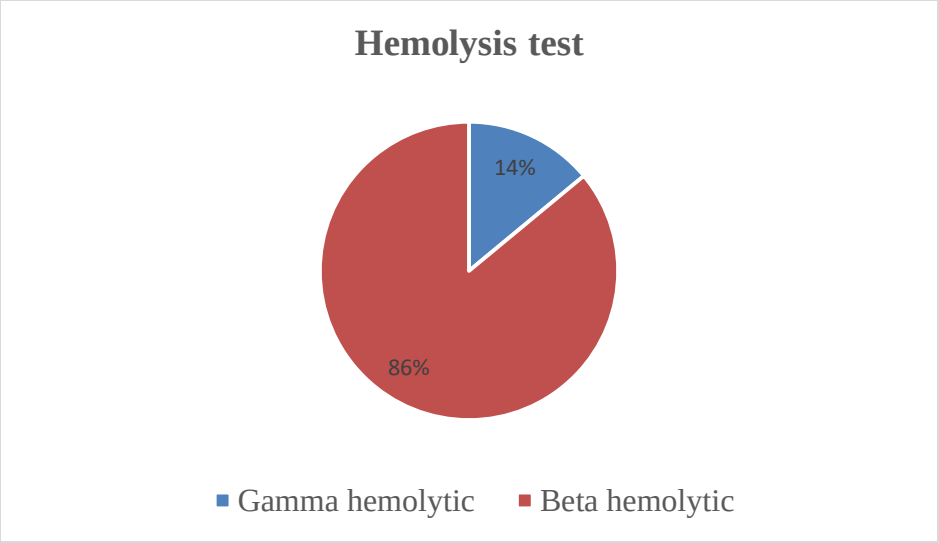

Figure 3.5 : Gamma and Beta hemolysis percentage in Gulshan lake isolates

In a study, blood of seven species tested was hemolyzed by most (> 71 %) strains of group I *V. parahaemolyticus*. Hemolytic activity was predominantly beta upon monkey, hamster, chicken, and goose blood; primarily alpha on sheep and shark; and mixed alpha, beta, and gamma on ox blood (Twedt,Novelli,Spaulding & Hall,1970)

CHAPTER 4

DISCUSSION

4.0 Discussion

The purpose of this study was to isolate and identify the presence of *Vibrio* from two of the most prominent water bodies in the Dhaka city ,Gulshan 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* spp. was done. Working with *Vibrio* spp. is difficult because it's very uncertain that they will survive till the last or not. At first, 80 samples are collected and lastly, 21 of them are *Vibrio* according to ABIS online software and 6 of them gave the Genus specific PCR positive result. There might be errors in culturing methods or in using the media (TCBS) for *Vibrio*. Enrichment is done by using Alkaline peptone water. pH of APW should be 8.4. It must be measured properly.

Before the Genus specific PCR, DNA extraction by boiling method for 21 *Vibrio* spp. was performed. In lower concentration of DNA (3-5ml), PCR results were all negative. Microcentrifuge tubes were used for centrigugation. Later, to increase the concentration of DNA, 50ml of inoculated APW was used. Then,15ml falcon tubes were used for centrifugation of 8ml of inoculated APW and the process was repeated continuosly until the whole 50ml was finished which is really a time consuming process. Folowing this,6 isolates gave the Genus specific positive PCR result. The rpoA primers correctly amplified all the *Vibrio* species considered.

Accurate phenotypic identification of *Vibrio* species is problematic, largely because of the great variability in their biochemical characteristics. A common example of misidentification involves *Aeromonas caviae*, often identified as *V. fluvialis* (Abbott et al. 1998). In addition, *Aeromonas hydrophila* and *Plesiomonas shigelloides* could also be mistaken for *Vibrio* when a complete battery of screening tests is not performed (Kwok et al. 2002). For these reasons, more specific,

rapid, and sensitive molecular methods for Vibrio species identification are needed (Dalmasso et al., 2009).

On the other hand, many strains that do not contain known virulence markers have been recognized as causative agents of a large number of diarrheal cases (Singh et al. 2002). For instance, the majority of V. cholerae O1 or O139 pathogenic strains do not present virulence markers, thus making their identification difficult (Nandi et al. 2000). To address these difficulties, in the last few years, new polymerase chain reaction (PCR) methods have been explored. These methods target housekeeping genes, like the collagenase or RNA polymerase subunits (rpoA and rpoB) genes. Such approaches can reliably identify *Vibrio* strains, irrespective of their phenotypes, serotypes, and virulence status needed (Dalmasso et al., 2009).

Among 67% of Vibrio spp, 33% of them are *Vibrio parahaemolyticus*. It is the major food-borne pathogen. Consumption of foods contaminated with high concentration of total *V. parahaemolyticus* and/or pathogenic *V. parahaemolyticus* can cause gastrointestinal infections. An open wound in skin comes in contact with *V. parahaemolyticus* is suggested as an infection pathway as well. Major syndromes caused by *V. parahaemolyticus* include gastroenteritis, wound infection, and septicaemia. Typical symptoms of the gastroenteritis may include abdominal pain, diarrhea, nausea, and fever (Tan et al. 2017).

From the antibiogram result of *Vibrio* spp., Nalidixic acid has shown the highest percentage (86%) of resistance and Tetracycline has shown the lowest percentage (14%) of resistance where, Doxycycline, Chloramphenicol & Gentamycin has shown 0% of resistance. This Nalidixic acid has also shown the lowest percentage (14%) of susceptibility. So, this antibiotic is showing the higest percentage of resistancy and the lowest percentage of susceptibility at the same time. *Vibrio* can retain their virulent characteristics during the VBNC state and it shows more resistance against Nalidixic acid. In another study, the percentage of resistancy is 97.2% against Ampicillin and I have got 57% against it. Again, Erythromycin has showed 6.9% of resistancy on the same study where in my study,it showed 57% of resistancy (Raissy M et al. 2012).

From the hemolysis test of *Vibrio* spp. I have got 86% of beta hemolytic result using the Blood agar media that contains 5% sheep blood. But in another study, the percentage of beta hemolysis was 71% and the media did not contain sheep blood, rather their BAP contained with monkey, chicken, goose or hamster blood. I did not get any alpha hemolysis result from *Vibrio* isolates but they got is from the BAP contained with the blood of Sheep and Shark. They have also got alpha, beta and gamma or mixed result by using ox blood in Blood agar media; where I have got 14% gamma hemolysis result from sheep blood containg BAP (Twedt, Novelli, Spaulding& Hall,1970).

Conclusion and Future Direction:

The purpose of the study was to identify the presence of *Vibrio* spp. in two main water bodies of Dhaka city (Gulshan Lake and Buriganga River) as well as determine 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 hemolysis test was done with the isolates from Dhaka city surface water bodies and compared to another study and difference was found in using different mammal blood in BAP. In future, these differences 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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Appendix

Media compositions

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

All the media used were from Himedia

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 Extract	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

Muller 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

Motility, 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

Simmon's 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

TSI agar

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

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.

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.

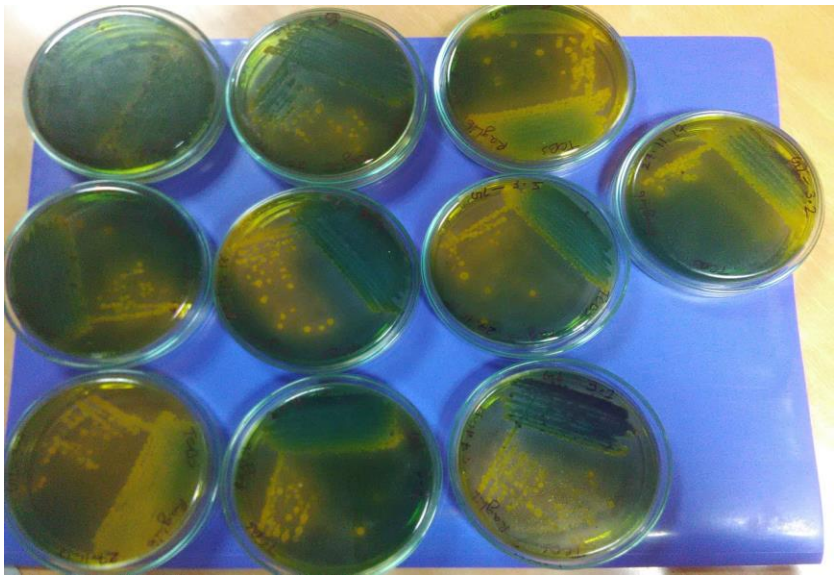
0.5M EDTA:

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.

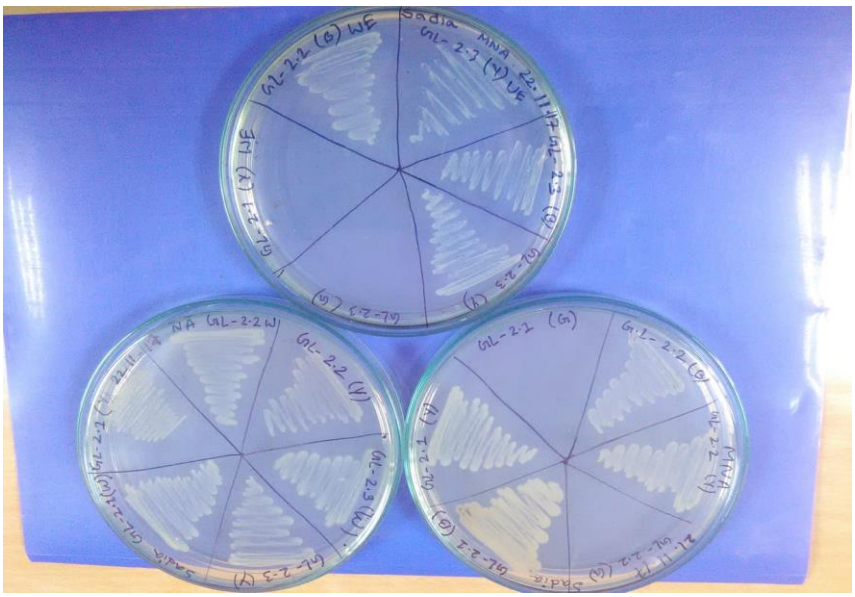
1X TBE Buffer:

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.

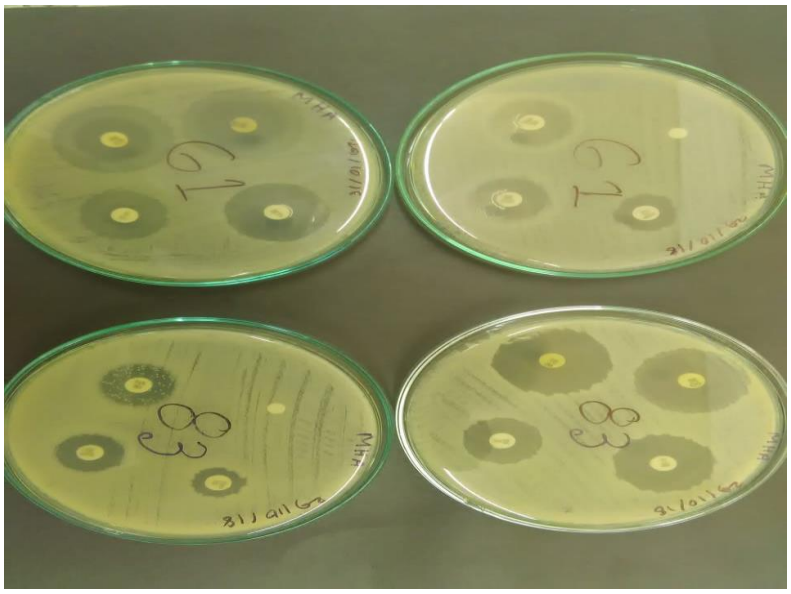
Supplementary



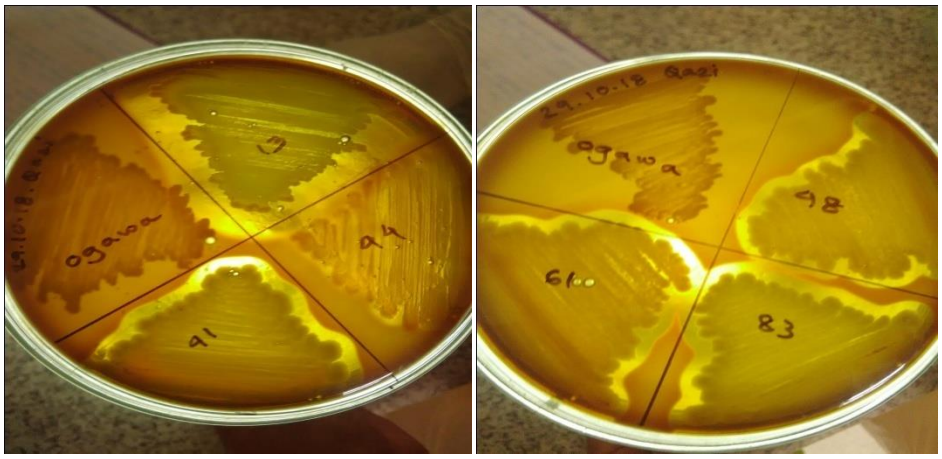
TCBS plates



MNA plates



Antibiogram plates



Blood Agar plates