ISOLATION AND CHARACTERIZATION OF *Vibrio* spp. FROM SURFACE WATER IN DHAKA CITY

This thesis is submitted to BRAC University in partial fulfilment of the requirements for the degree of Bachelor of Science in Biotechnology

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Declaration of Authenticity

I, the undersigned, declare that the research work embodying the results reported in this thesis entitled "Isolation and characterization of *Vibrio* spp. from surface water in Dhaka City" is my original work, gathered and utilized for the sole purpose of fulfilling the objectives of this study. I confirm that the work has not been previously submitted to any other institution, in whole or in part, for a higher degree or diploma. I further declare that the thesis has been composed entirely by me under the supervision of Mr. Mahbubul Hasan Siddiqee, Sr. Lecturer, Microbiology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, except where stated otherwise by reference or acknowledgement.

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Author

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List of Abbreviations

Abbreviation	In full
µl	Microlitres
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ETEC	Enterotoxigenic *Escherichia coli*
icddr, b	International Centre for Diarrhoeal Disease Research, Bangladesh
MAR	Multiple antibiotic resistance
MIU	Motility Indole Urease
ml	Millilitres
MNA	Modified nutrient agar
MNB	Modified nutrient broth
MRVP	Methyl red- Voges Proskauer
Nos.	Number of
PCR	Polymerase chain reaction
psi	Pounds per square inch
RNA	Ribonucleic acid
*rpo*A	RNA polymerase subunit Alpha
TAE	Tris base- Acetic acid- EDTA
TCBS	Thiosulfate-citrate-bile salts-sucrose
TSI	Triple sugar iron
UV	Ultraviolet

Abstract

Vibrio spp. is an estuarine bacterium prevalent in water bodies throughout the world. Twelve species of the Vibrioceae family are known to cause diseases in human. The aim of this study was to investigate the occurrence, biochemical characteristics, antibiotic resistance and haemolytic abilities of *Vibrio* spp. in water of Hatirjheel and the Buriganga River in the capital of Bangladesh. A total of 6 *Vibrio* isolates could be identified from Hatirjheel which were all confirmed using biochemical characterization. Water samples from the Buriganga river did not yield any *Vibrio*. Among the isolates collected during the entire study period, 3 of the isolates were found to be resistant to the antibiotic Ampicillin, 5 were resistant to Nalidixic Acid, 1 was resistant to Erythromycin and all 6 were resistant to Trimethoprim-sulfamethoxazole. However, all the isolates were susceptible to Impinem, Chloramphenicol, Amikacin, Gentamycin, Ciprofloxacin, Doxycycline and Tetracycline. The overall Multiple Antibiotic Resistance (MAR) index value varied from 0.02 and 0.04. Among the tested isolates, 2 of the isolates were found to be non-haemolytic and the remaining 4 showed complete haemolysis. These results are significant because of the role of these water bodies in the spread and transmission of *Vibrio* infections. This study highlights the presence of potential microbial hazard in the surface water bodies of Dhaka city.

Keywords: *Vibrio* spp., Antibiotic Resistance, Haemolysis

Chapter 1: Introduction

Bangladesh experiences at least 100,000 new cases of cholera and suspected 450 associated deaths each year (Islam, Clemens, & Qadri, 2018). Illness and death resulting from non-cholera *Vibrio* infections has become a global concern in recent years. *Vibrio* is a halophilic, motile, gram negative bacteria. At least 12 species of the *Vibrioceae* family have been directly linked to causing foodborne diseases in human (Pruzzo, Huq, Colwell, & Donelli, 2005).

Infection caused by *Vibrio* is broadly categorized into 2 groups- cholera and non-cholera infections with the latter being more prevalent in the developed world and the former in the developing and under-developed worlds. Majority of the non-cholera *Vibrio* infections are caused *by V. vulnificus* and *V. parahaemolyticus* and occasionally by *V. metschnikovii, V. damsela*, and *V. cincinnatiensis* etc. *Vibrio cholerae* is the solitary responsible agent for cholera infections and is mostly prevalent in areas surrounding the north and western coasts of the Indian Ocean and in the Latin Americas. The relatively poor sanitary conditions, lack of access to safe potable water, subordinate standard of living and weak infrastructure have contributed to the spread of cholera in these areas and cholera infections often take an epidemic form especially after natural disasters such as flood or hurricane (Huq et al, 2006). Diarrhoeal diseases resulting from *Vibrio* infections is a leading cause of infant mortality in South Asia and Central America (John, Tauwiwalo, Rabalino, & Frenk, 2004). Some of the worst epidemics of cholera in recent times have been reported in Yemen ("WHO launches second cholera vaccine drive", 2018), Haiti (Gladstone, 2016) and Zimbabwe (World Health Organization, 2018) with at least 2500, 9200, & 40 suspected deaths respectively.

Vibrio spp. is distributed in surface water all over the world as shown in Illustration 1.1. Most pathogenic species cause diseases of the gastrointestinal tract when ingested orally. However,

some species are known to utilise open wounds, skin and even blood as a path of entry into the human body (Janda, Powers, Bryant, & Abbott, 1988). *Vibrio* can also colonise the gut when ingested with contaminated water and raw or undercooked marine shellfish harbouring the pathogen. Exposure of broken skin to contaminated salt and brackish water may also cause illness. Illness caused by *Vibrio* encompasses a broad spectrum ranging from gastroenteritis to primary sepsis to necrotizing fasciitis and even a few respiratory infections (Elhadi, 2012).

Illustration 1. 1: Abundance of *Vibrio* spp. in different marine environments. Orange colour circles, by culture-based methods; green circles, by culture-independent methods. (Zhang, Wang, Lin, & Austin, 2018)

Hatirjheel is a lakefront in the centre Dhaka, the capital and largest city of Bangladesh. Inaugurated in 2013, it became one of the most notable places in the capital. Soon afterwards, it became a local favourite of the city dwellers, attracting thousands seeking to find a relief from the bustle of the city each day (Preetha, 2013). Authorities have plans to further develop the surrounding areas to attract more visitors. A water transportation system was introduced in 2016 and is estimated to ferry 1500 people across the capital each day (Salman, 2017).

Nonetheless, dumping of sewage waste and other garbage from nearby localities soon polluted the lake and reports published in the following years have warned about the alarming state of water pollution in the lake (Yusuf, 2013).

The Buriganga on the other hand, flows beside the southern part of the capital. The river has played a significant role in the establishment and development of the city since time immemorial. Unfortunately, the disproportionate and irresponsible dumping of raw waste from various industries have been a major concern for the river. Buriganga has often been termed as among the most polluted water bodies in the region (Kamal, Malmgren-Hansen, & Badruzzaman, 1999). Nonetheless, measures by the authorities and other organizations in recent years have seen hints of improvement in the water quality although experts have unanimously that there is still a very long way to go ("Buriganga water improving", 2017). Ingestion of water or merely coming in contact with it has been linked to various diseases (Bhowmik, 2008) although, data on the exact nature of microbial life in the water is scarce.

Infections resulting from *Vibrio* spp. and especially *V. cholerae* remains one of the major causes of disease related deaths in Bangladesh. While the majority of the death were in minors below five years of age, nonetheless, other people groups especially ones with a higher iron content in blood, for example the elderly, expecting mothers, malnourished individuals, individuals who consume antacids regularly and individuals with a history of tuberculosis, liver disease and habitual drinkers are also susceptible to *Vibrio* infections (Lund, & O'Brian, 2012). Diabetes mellitus and haematological disorders have also been identified as significant risk factors (Elhadi, 2012).

The history of large incidents of cholera in the country and emergence of the disease as an epidemic in the country has interested researchers to study the causative agent. The International Centre for Diarrhoeal Disease Research (icddr, b) was established in 1960 with the very aim of studying, treating and limiting diarrhoeal diseases in the country. Many researches have been conducted in the country over the years. For obvious reasons, the majority of this work is focused on *V. cholerae* only. Moreover, these researches have been aimed to study, prevent and treat cholera only. A research by Chowdhury et al (2011) studied the impact of rapid urbanization in Dhaka city on the rate of infection by *V. cholerae O1* and ETEC. Other studies have focused on the roles of plasma and memory B cell responses in protection against *V. cholerae O1* infections (Aktar et al, 2018; Patel et al, 2012). Other studies have focused on developing filtration methods to limit plankton related cholera infections (Huq, Xu, Chowdhury, Islam, Montilla, & Colwell, 1996) or on developing vaccines against V. cholerae and testing its feasibility (Qadri et al, 2015). With such a large number of vulnerable people groups it is however, disappointing to realize that very little studies have been conducted on the actual sources harbouring the pathogens, namely, the large water bodies in and around the heavily populated areas of the country.

Dhaka, with its colossal population of 18 million (World Population Review, 2018) is currently 11th largest and the fastest growing megacity in the world (Hossain, & Easson, 2015). The city has seen an unprecedented rate of economic and industrial growth within the past few decades. Large densities of population in compact areas make megacities on of the most vulnerable environments. Pollution and spread of diseases have a multi-faceted impact on these areas, especially the ones in developing countries most often because of the uneven growth rate and adaptation. Any environmental study considering Dhaka as a model will have far-reaching

implications surpassing regional boundaries to solve the challenges faced by similar areas all over the globe.

It is worth mentioning that Hatirjheel is the largest publicly accessible recreational body of water in the Capital and pollution of its water will have a negative impact on a large number of people living in proximity to or simply visiting the lake on a daily basis. In spite of the prevalence of a high incidence of *Vibrio* related infections, little is known about the environments of the water bodies in the region as research on the topic is rare. As Hatirjheel and the Buriganga are two of the most significant water bodies in the capital, the present study aims to isolate *Vibrio* spp. samples from different sites of them with the purpose of studying its biochemical nature, antibiotic sensitivity and haemolytic abilities.

Chapter 2: Methods and Materials

Place of work

The entirety of this research work was conducted in the life sciences laboratories of the Department of Mathematics and Natural Sciences of BRAC University.

Site Selection and Water Sample Collection

Two of the very common water bodies of the capital- the Hatirjheel Lake and the River Buriganga (Sadarghat) were selected as test subjects in this work. 10 sites were randomly chosen on the Hatirjheel Lake (Gulshan- Karwan Bazar) as shown in Illustration 2.1 to include all parts of the lake. Sites on the river were selected in a similar fashion and are also shown in Illustration 2.1. 50 samples from Hatirjheel and 20 from the Buriganga were collected from October, 2017 to January, 2018. Motorboats and local dinghy boats were used to arrive at the sites and samples were collected in autoclaved laboratory-grade plastic bottles from 1 metre below the water surface. The samples were processed within 120 minutes of collection.

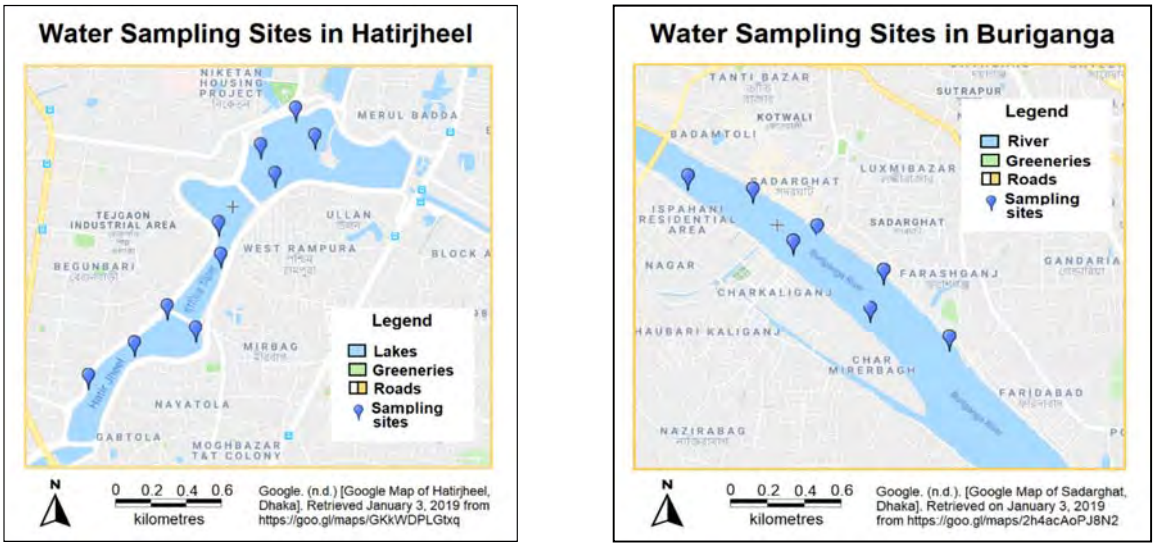

Illustration 2. 1: Sampling sites on the Hatirjheel (left) and the Buriganga (right) shown on map

Isolation

Pre-enrichment prior to culture is strongly advised for microbes isolated from environmental samples (Huq et al., 2012). Nonetheless, both pre-enriched and non-enriched samples were isolated for the purpose of this study. Water samples were enriched in autoclaved Alkaline Peptone Water. Equal volumes of water sample and the enrichment medium were mixed using an autoclaved graduated cylinder and it was incubated overnight at 37°C. On the following day, a sterile metal loop was used to collect the surface growth and inoculate it on TCBS agar. The non-enriched samples were directly inoculated on TCBS agar from the surface of the collected water samples. Both plates were incubated overnight at 37°C. Nextly, yellow, green and black-centred colonies were collected from the TCBS plates and separately inoculated on TCBS agar and allowed to grow overnight at 37°C. After that, loopsful of the samples were transferred to Modified Nutrient Broth medium stored in microcentrifuge tubes and incubated overnight at 37°C. Finally, the samples were stored at -20°C after adding 300 µl of autoclaved glycerol into each of the tubes.

Identification

Presumptive colonies of *Vibrio* spp. were subjected to a series of laboratory biochemical tests as mentioned in the following:

Oxidase Test

A filter paper was saturated with tetramethyl-p-phenylenediamine (oxidase reagent) on a clean and sterile petri plate. Nextly, a portion of the paper was heavily smeared with the sample using a sterile toothpick. Finally, the smear was checked for any change in colour. Organisms possessing the cytochrome c oxidase enzyme gave positive tests and their colonies turn a strong hue of pink whereas negative tests failed to show any change in colour.

Citrate

The surface of Simmons's citrate agar slants prepared in sterile glass vials were inoculated with the samples and incubated overnight at 37°C. The slants were then checked for bacterial growth and change in colour. Change of the medium to a blue colour indicated positive for utilization of citrate as a sole carbon source and unchanged colour indicated negative result.

TSI Agar

TSI Agar slants were inoculated with the samples by stabbing the medium with an inoculating needle. The slants were checked for sugar fermentation, gas production and H_2S production following an incubation period of 24 hours at 37°C.

Motile Indole Urea

MIU agar was prepared and sterilized in test tubes and cooled to approximately 50°C. After that, 5% (v/v) urea solution sterilized by syringe filtration was then added to each of the tubes and allowed to solidify completely. The tubes were inoculated by stabbing them with needles containing the samples. Following overnight incubation at 37°C, the tubes were checked for any change in colour and bacterial growth. Pink colour indicated positive for utilization of urea whereas motility was indicated by a spiralling growth away from the site of inoculation.

Methyl Red

A loopful of each of the samples was inoculated into separate test tubes containing 5 ml of MRVP broth and incubated overnight at 37°C. For observation, 5 drops of the methyl red reagent was added to the broth carefully without shaking the tube. Appearance of a cherry red colour indicated positive result, orange colour indicated inconclusive results and yellow indicated negative results. Results were noted down carefully with corresponding sample codes to be analysed later.

Vogues-Proskauer

Test tubes containing 5 ml of MRVP broths were inoculated with the samples and incubated at 37°C overnight. Afterwards, 6 drops of Barritt's reagent A was added and shaken. Nextly, 6 drops of Barritt's reagent B was added and the tubes were observed for 10 minutes. Appearance of a red ring indicated positive tests and negative tests were indicated by the absence of any rings.

Biochemical Characterization

The results obtained from the respective biochemical tests were analysed using the ABIS online tool for bacterial identification (Costin & Ionut, 2017). Samples showing the most biochemical similarity to *Vibrio* spp. were chosen to be worked with further.

DNA Extraction

Samples were grown in a shaking incubator at 37°C for 48 hours in alkaline peptone water. The protocol utilized for the extraction of DNA from these samples is stated as follows. 50 mL of the culture was centrifuged at 13000 rpm at room temperature for 10 minutes. The supernatant was discarded and 1.5 mL autoclaved distilled water stored at room temperature was added to the tube. The tube was then inverted gently to wash the pellet. The supernatant was discarded after centrifuging the tube at 13000 rpm for 5 minutes. The pellet was resuspended in 1.5 mL autoclaved distilled water and boiled in a water bath at 98°C for 7 minutes. The tube was immediately transferred into ice for 10 minutes after that. Afterwards, the tube was centrifuged at 13000 rpm for 5 minutes. Finally, the supernatant was collected in a sterile microcentrifuge tube and stored at -20°C until use.

Primers

The primers used for this research was designed against the rpoA gene sequence of *Vibrio* spp. In order to achieve that, firstly, all known rpoA gene sequences available in the GenBank (https://www.ncbi.nlm.nih.gov/genbank) were aligned using the ClustalX software (Higgins et al., 1992). The primers were then, chosen from the highly conserved region among all the sequences.

The chosen sequences for the primers were: 5'- AAATCAGGCTCGGGCCCT-3' (sense) and 5'-GCAATTTTGTCAGACCGG-3' (antisense) (Dalmasso et al., 2009). The primers were synthesized by Integrated DNA Technologies (Iowa, USA).

Polymerase Chain Reaction Protocol

PCR was performed in 0.2 ml microcentrifuge tubes. The reaction mix was prepared at room temperature following the guidelines provided by the manufacturer. The final volume of the reaction mix was 25 µl and it consisted of 12.5 µl of GoTaq®G2 Hot Start Colourless Master Mix(2X), 2.5 µl each of 1 µM upstream and downstream primers, 5 µl of the template DNA and 2.5 µl of nuclease-free water (Promega, Wisconsin, USA). The amplification condition used was 3 minutes at 94^{o}C for the initial denaturation of DNA and then 35 cycles, each consisting of 1 minute at 94^{o}C, 1 minute at 55^{o}C and 1 minute at 72^{o}C. A final extension was carried out at 72^{o}C for 5 minutes (Dalmasso et al., 2009). After amplification, 5µl of each of the reaction mixtures were separated on a 1.5% agarose gel with added ethidium bromide by electrophoresis at 80 volts for 40 minutes and finally, the amplified gene products were visualized under UV light. For the purpose of this thesis, 1x TAE Buffer was prepared and used as both the solvent to dissolve agarose powder and the running buffer in the electrophoresis chamber.

Kirby-Bauer Disc Diffusion

The *Vibrio* samples were grown in alkaline peptone water at 37°C for 48 hours in a shaking incubator. The Kirby- Bauer disc diffusion method was used to determine the antibiotic drug susceptibility of the samples as instructed by the Clinical Laboratory Standard Institute (CLSI, 2017). Firstly, *Vibrio* cultures were adjusted in 0.9% sterile saline to 1 McFarland standard. After that, a sterile cotton swab was dipped into the saline and then it was used to streak heavily on a Mueller- Hinton Agar plate. The streaking was performed 3 times rotating the plate by 90° each time to form a uniform bacterial lawn. The plate was allowed to absorb the inoculum for 5 minutes and then, the antibiotic discs were placed on it using sterile forceps. (Bauer et. Al, 1966). The plates were incubated at 37°C for 24 hours. Following their incubation period, the plates were checked for their zones of inhibition of bacterial growth. The clear zone around each disc was observed through the back of the petri plates and the diameter was noted down in millimetres.

For the purpose of this thesis, the following antibiotics impregnated discs were used: Ampicillin (25 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Gentamycin (10 µg), Streptomycin (10 µg) (Kumar, Patterson & Karpagam, 2008), Nalidixic acid (30 µg), Tetracycline (30 µg) (Lee, Najiah, Wendy, & Nadirah, 2009), Ciprofloxacin (5 µg), Doxycycline (30 µg) (Yu et al., 2012), Amikacin (30 µg), Imipenem (10 µg), Norfloxacine (10 µg), and Trimoxazole (25µg).

Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR index of the present isolates against the tested antibiotics was calculated utilising the following equation (Sarter, Nguyen, Hung, Lazard, & Montet, 2007).

$$Multiple\ Antibiotic\ Resistance\ (MAR)\ Index = \frac{X}{Yx\ Z}$$

Where, X= total of antibiotic resistance case, Y= total of antibiotics used in study and Z= total number of isolates.

A MAR index value of ≤0.2 was defined as an indication of seldom or never before introduction of the tested antibiotics to the water source. On the other hand, MAR index value of >0.2 was accepted as an indication of high risk of introduction of the tested antibiotics to the water source (Lee, Najiah, Wendy, & Nadirah, 2009).

Haemolysis

Blood agar plates containing 5% defibrinated sheep blood were streaked with pure cultures. The plates were allowed an incubation time of 24 hours at 37^{o}C after that. Finally, the plates were checked for a clearing of media indicating alpha haemolytic, greening of media indicating beta haemolytic or unchanged media indicating gamma haemolytic organisms.

Chapter 3: Findings

Biochemical Characterization

The results obtained from the biochemical tests were analysed using the ABIS online bacterial identification software which confirmed 21 *Vibrio* samples along with 34 non-*Vibrio* samples as shown in Table 3.1. Among the *Vibrio* samples, the vast majority were the 18 *V. metcshnikovii* samples (85.71%). Besides, 2 *V. parahaemolyticus* (9.5%) and 1 *V. gazogens* sample (4.76%) were also obtained as has been displayed in the Illustration 3.1.

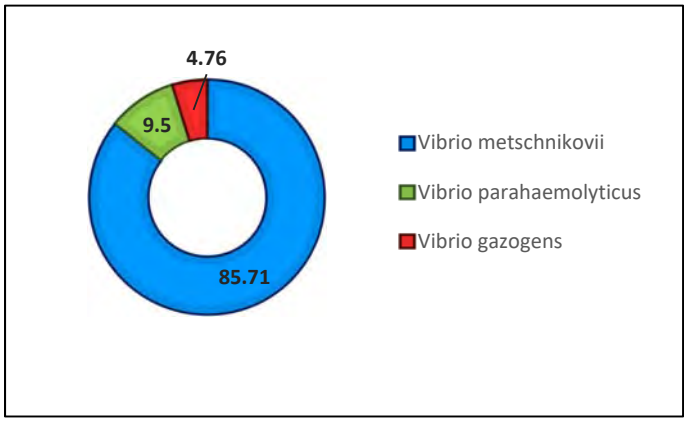

Illustration 3. 1: Doughnut showing percentage of different *Vibrio* species identified

Genus Specific PCR

PCR using primers designed against unique *rpo*A gene sequence of *Vibrio* spp. confirmed 6 samples with absolute certainty to be *Vibrio* spp. The sample ID for these samples were 32EVG, 33EVY, 34OVY, 30EVY, 55OVY & 55EVY. These samples had produced bands at both the 242 bp and 456 bp positions.

The result of PCR as viewed under UV light following gel electrophoresis has been shown in Illustration 3.2.

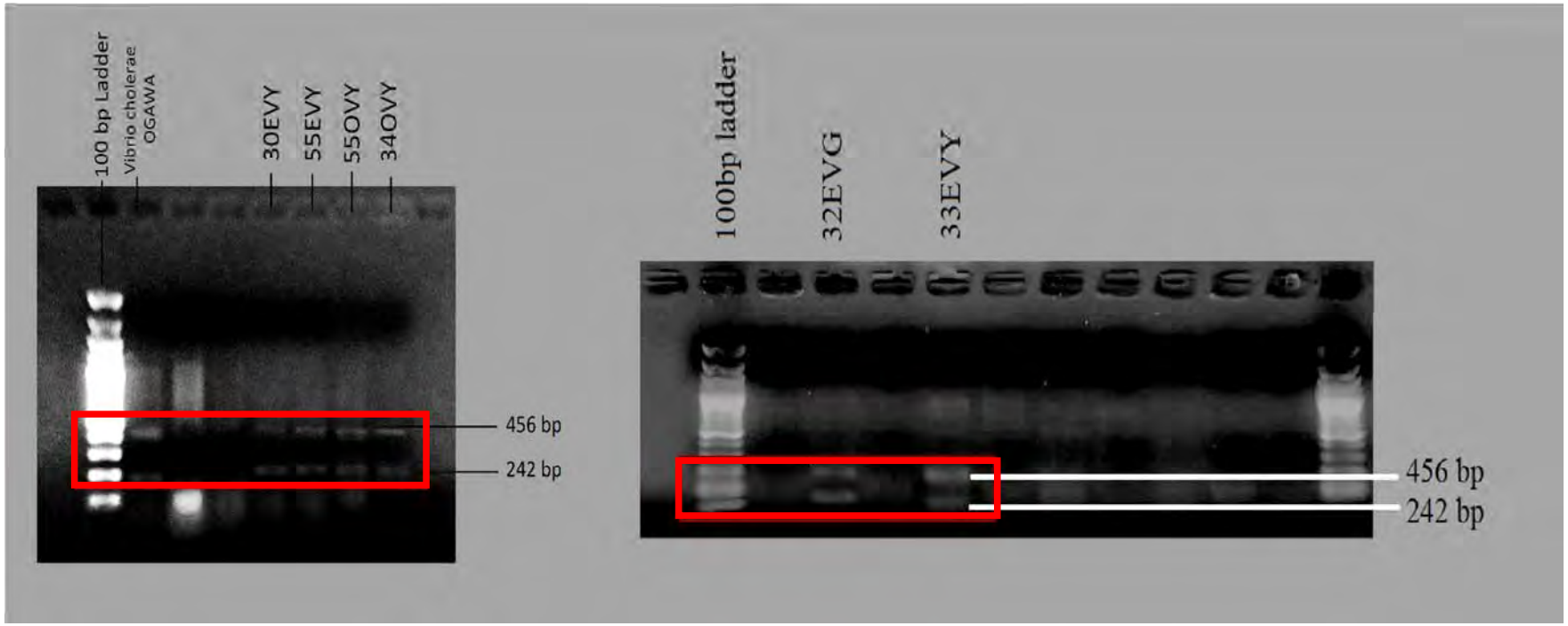

Illustration 3. 2: A. Vibrio samples identified in lanes 6, 7, 8 and 9 and B. Vibrio samples identified in lanes 4 and 6 by comparing with the positive control

Table 3. 1: Biochemical observations of the different isolates from the different water sources

Sample	Oxidase	Citrate	Glucose	Sucrose	Lactose	H2S	Gas	Urease	Motility	MR	VP	Microbe identified
11EVB	-	-	+	+	+	+	+	-	+	+	-	No matches
11EVG	-	-	+	+	+	+	-	-	+	+	-	*Vibrio metschnikovii*
11EVY	-	-	+	+	+	-	-	+	+	-	-	No matches
21EVG	-	-	+	+	+	+	+	-	+	-	-	No matches
21EVY	-	-	+	+	+	+	+	-	+	+	-	No matches
31EVG	-	-	+	+	+	+	+	-	+	+	-	No matches
32OVG	-	-	+	+	+	+	-	-	-	+	-	*Vibrio metschnikovii*
32OVY	-	-	+	+	+	+	+	-	+	+	-	No matches
32EVG	-	-	+	+	+	+	-	-	+	+	-	*Vibrio metschnikovii*
32EVY	-	-	+	+	+	-	-	-	+	+	-	*Vibrio metschnikovii*
33EVY	-	-	+	+	+	-	-	-	+	+	-	*Vibrio metschnikovii*
34OVG	-	-	+	+	+	-	+	+	+	-	-	No matches
34OVY	+	-	+	+	+	-	-	-	+	+	-	*Vibrio metschnikovii*

Sample	Oxidase	Citrate	Glucose	Sucrose	Lactose	H2S	Gas	Urease	Motility	MR	VP	Microbe identified
34EVB	-	-	+	+	+	+	+	-	-			*Vibrio gazogens*
34EVG	-	-	+	-	-	+	+	+	+	+	-	No matches
34EVY	-	-	+	+	+	+	+	+	+	+	-	No matches
35OVY	-	-	+	+	+	+	-	-	+	+	+	*Vibrio metschnikovii*
35EVG	-	-	+	+	+	+	-	-	+	+	-	*Vibrio metschnikovii*
35EVY	-	-	+	+	+	-	+	+	+	+	-	No matches
36OVG	-	-	+	+	+	+	-	-	-	+	-	No matches
36EVG	-	-	+	-	-	-	-	-	+	+	-	*Vibrio parahaemolyticus*
37OVY	-	-	+	+	+	-	+	+	+	-	-	No matches
37EVG	-	-	+	+	+	-	+	-	+	+	-	No matches
37EVY	-	-	+	+	+	+	+	-	+	+	-	No matches
38OVY	-	-	+	+	+	-	+	+	+	-	-	No matches
38EVY	-	-	+	+	+	+	+	-	+	+	-	No matches
39EVG	-	-	+	+	+	-	-	-	-	-	-	*Aeromonas salmonicida*
39EVY	-	-	+	+	+	-	-	-	+	+	-	*Vibrio metschnikovii*

Sample	Oxidase	Citrate	Glucose	Sucrose	Lactose	H2S	Gas	Urease	Motility	MR	VP	Microbe identified
30OVY	-	-	+	+	+	+	+	-	+	+	-	No matches
30EVG	-	-	+	+	+	+	+	-	+	+	-	No matches
30EVY	-	+	+	+	+	+	-	-	+	+	-	*Vibrio metschnikovii*
41OVY	-	-	+	-	-	-	-	-	+	+	+	*Photobacterium leiognathi*
41EVG	-	-	+	+		+	+	-	+	+	-	No matches
41EVY	+	-	+	+	+	+	+	-	+	-	-	No matches
42OVG	-	-	+	+	+	-	-	-	+	-	-	No matches
42EVY	-	-	+	+	+	+	+	-	+	+	-	No matches
51OVY	-	-	+	+	+	+	+			+	-	No matches
51EVY	-	-	+	+	+	-	-	-	+	+	-	*Vibrio metschnikovii*
52OVY	-	-	+	+	+	+	+	-	+	-	-	No matches
52EVY	-	-	+	+	+	+	+	-	+	+	-	No matches
53OVY	-	+	+	+	+	-	-	-	+	+	-	*Vibrio metschnikovii*
53EVY	-	+	+	+	+	+	+	-	+	+	-	No matches
54OVY	-	-	+	-	-	+	-	-	+	+	-	*Vibrio parahaemolyticus*

Sample	Oxidase	Citrate	Glucose	Sucrose	Lactose	H2S	Gas	Urease	Motility	MR	VP	Microbe identified
54EVY	-	-	+	+	+	-	-	-	+	+	-	*Vibrio metschnikovii*
55OVY	-	-	+	+	+	-	-	+	+	-	-	*Vibrio metschnikovii*
55EVY	-	-	+	+	+	+	+	-	+	+	-	*Vibrio metschnikovii*
56OVG	-	-	+	-	-	-	+	-	-	-	-	No matches
56OVY	-		+					-	-	+	-	No matches
56EVY	-	-	+	-	-	+	+	-	+	-	-	No matches
X1EVY	-	-	+	+	+	+	+	-	+	+	-	No matches
X2EVG	-	-	+	+	+	+	-	-	+	+	-	*Vibrio metschnikovii*
X3EVB	-	-	+	+	+	+	-	-	+	+	-	*Vibrio metschnikovii*
X5EVY	-							-	+	-	-	No matches
N8EVY	-	-	+	-	-	+	+	+	+	+	-	No matches
N9EVY	-		+	+	+	+	-	-	-	+	-	*Vibrio metschnikovii*

Table 3. 2: Results from genus specific PCR

Sample	242 bp segment	456 bp segment	Result
Positive control	+	+	***Vibrio* spp.**
11EVG	-	+	Non *Vibrio*
32OVG	-	+	Non *Vibrio*
32EVG	+	+	***Vibrio* spp.**
32EVY	-		Non *Vibrio*
33EVY	+	+	***Vibrio* spp.**
34OVY	+	+	***Vibrio* spp.**
34EVB	-	+	Non *Vibrio*
35OVY	-	+	Non *Vibrio*
35EVG	-	+	Non *Vibrio*
36EVG	-		Non *Vibrio*
39EVY	-	+	Non *Vibrio*
30EVY	+	+	***Vibrio* spp.**
51EVY	-	+	Non *Vibrio*
53OVY	-	+	Non *Vibrio*
54OVY	-	+	Non *Vibrio*
54EVY	+	+	***Vibrio* spp.**
55OVY	+	+	***Vibrio* spp.**
55EVY	-	+	Non *Vibrio*
X2EVG	-	+	Non *Vibrio*
X3EVB	-	+	Non *Vibrio*
N9EVY	-	+	Non *Vibrio*

Table 3. 3: Percentage of positive samples corresponding to the time of sampling

Date of sampling	Site	Nos. samples	Nos. positive samples
October, 2017	Hatirjheel	3	0
November, 2017	Hatirjheel	28	4
December, 2017	Hatirjheel	4	0
	Buriganga	4	0
January, 2018	Hatirjheel	14	2
	Buriganga	2	0

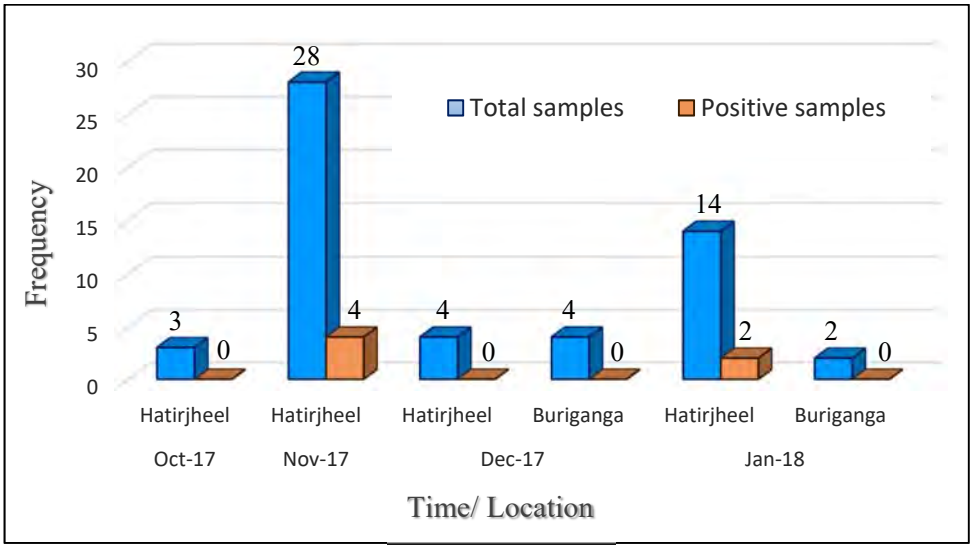

Illustration 3. 3: Bar diagram showing the number of positive samples with time and location

Table 3. 4: Interpretative criteria used to determine antibiotic susceptibility with the disk diffusion test in *Vibrio* spp. isolates

Antimicrobial class	Antimicrobial agent	Abbreviation	Disk content (µg)	Zone diameter interpretative criteria (mm)			Reference
				Susceptible	Intermediate	Resistant	
β lactams	Ampicillin	AM	10	≥17	14-16	≤13	CLSI, 2010a
	Impinem	IPM	10	≥23	20-22	≤19	CLSI, 2010a
Phenicols	Chloramphenicol	C	30	≥18	13-17	≤12	CLSI, 2010a
Aminoglycosides	Amikacin	AMK	30	≥17	15-16	≤14	CLSI, 2010a
	Gentamycin	GEN	10	≥15	13-14	≤12	CLSI, 2010a
	Streptomycin	STR	10	≥17	13-16	≤12	CLSI, 2016
Quinolones	Ciprofloxacin	CIP	5	≥21	16-20	≤15	CLSI,2010a
	Nalidixic Acid	NA	30	≥19	14-18	≤13	CLSI, 2016
	Norfloxacine	NOR	10	≥17	13-16	≤12	CLSI, 2016
Folate Pathway Inhibitors	Trimethoprim-sulfamethoxazole	SXT	25	≥16	Nov-15	≤10	
Tetracyclines	Tetracycline	TET	30	≥15	12 14	≤11	CLSI, 2010a
	Doxycycline	DO	30	-	-	-	
Macrolides	Erythromycin	ERY	15	≥17	13-16	≤12	CLSI 2016

Table 3. 5: Phenotypic antibiotic susceptibility profiles of the 6 *Vibrio* spp. isolates

Sample ID	Resistance Profile	AM (10)	IPM (10)	C (30)	AMK (30)	GEN (10)	STR (10)	CIP (5)	NA (30)	DO (30)	SXT (25)	TET (30)	ERY (15)
Positive control	SXT-NA	S	S	S	S	S	S	S	R	S	R	S	S
32EVG	SXT-NA	S	S	S	S	S	I	S	R	S	R	S	S
33EVY	SXT-NA-AM	R	S	S	S	S	I	S	R	S	R	S	S
34OVY	SXT-NA-ERY	S	S	S	S	S	S	S	R	S	R	S	R
30EVY	SXT-NA	S	S	S	S	S	S	S	R	S	R	S	S
55OVY	SXT-NA-AM	R	S	S	S	S	S	S	R	S	R	S	S
55EVY	SXT-AM	R	S	S	S	S	S	S	S	S	R	S	S

Antibiogram

All of the isolates in this test showed resistance against Trimethoprim-sulfamethoxazole (25) as is evident from the Table 3.6. In addition to that, 85.7% showed resistance against Nalidixic Acid (30) and 42.8% showed resistance against Ampicillin (10). Moreover, 28.5% of the isolates exhibited intermediate resistance to streptomycin (10). Conversely, Impinem (10), Chloramphenicol (30), Amikacin (30), Gentamycin (10), Ciprofloxacin (5), Doxycycline (30) and Tetracycline (30) were bactericidal against the *Vibrio* samples in 100% of the cases

Table 3. 6: Total percentage sensitivity of *Vibrio* spp. isolated from surface water to 12 antibiotics

Antibiotic Agent	Susceptibility	Intermediate response	Resistance
AM (10)	57.14%	0%	42.86%
IPM(10)	100%	0%	0%
C (30)	100%	0%	0%
AMK(30)	100%	0%	0%
GEN(10)	100%	0%	0%
STR(10)	71.43%	28.57%	0%
CIP(5)	100%	0%	0%
NA (30)	14.28%	0%	85.71%
DO (30)	100%	0%	0%
SXT(25)	0%	0%	100%
TET(30)	100%	0%	0%
ERY(15)	85.71%	0%	14.28%

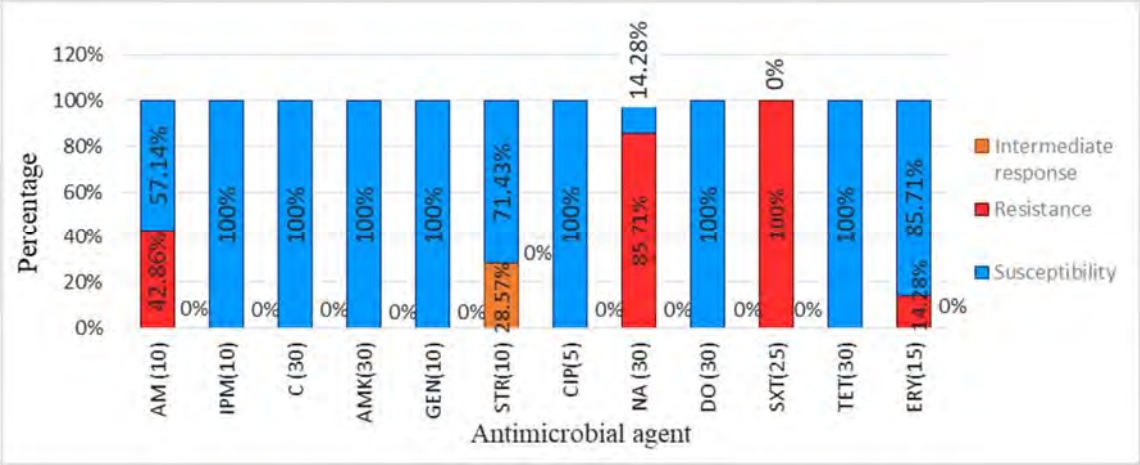

Illustration 3. 4: Bar diagram showing the total percentage sensitivity of *Vibrio* spp. isolated from surface water to 12 antibiotics

The Multiple Antibiotic Resistance (MAR) Index of the positive control (OGAWA) was determined to be 0.02. Among the isolates, 3 (50%) had MAR index equal to the positive control- 0.02 while the remaining had a MAR index of 0.04 as shown in Table 3.7. MAR index was calculated using the formula in Chapter 2.

Table 3. 7: The MAR indices of all *Vibrio* isolates against the tested Antibiotics

Sample ID	MAR Index
Positive control	0.02
32EVG	0.02
33EVY	0.04
34OVY	0.04
30EVY	0.02
55OVY	0.04
55EVY	0.02

Haemolysis

Of the 6 Vibrio isolates, 4 were β- haemolytic (66.67%) and 2 were γ- Haemolytic (33.33%). The laboratory standard *Vibrio cholerae* OGAWA sample used as a positive control for other parts of this thesis was also tested for haemolysis and the result came up as γ- haemolytic in both plates. The results from haemolysis are summarized in Table 3.8 and Illustration 3.5.

Table 3. 8: Haemolytic activity of 6 *Vibrio* isolates

Sample ID	Haemolytic activity
V. cholerae OGAWA	γ (non-haemolytic)
32EVG	β (haemolytic)
33EVY	γ (non-haemolytic)
34OVY	γ (non-haemolytic)
30EVY	β (haemolytic)
55OVY	β (haemolytic)
55EVY	β (haemolytic)

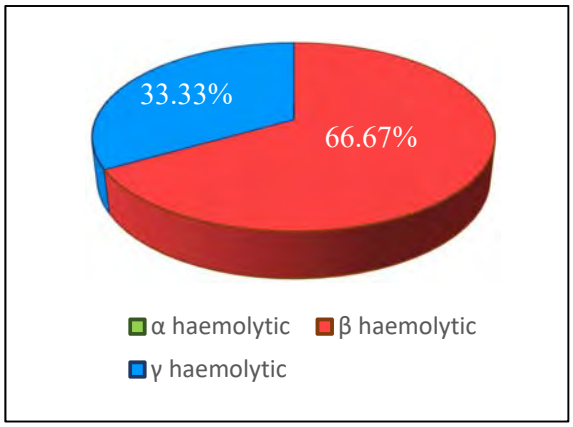

Illustration 3. 5: Pie-chart showing the percentage of different types of haemolytic activity observed within the *Vibrio* isolates

Chapter 4: Discussions and Conclusion

Discussion

The objective of this study was to isolate *Vibrio* species contaminating two prominent water bodies in and around the Capital and to study and possibly compare the biochemical nature, reaction to antimicrobial agents and behaviour on blood infused medium of isolates coming from the different sources. Several members of the genus *Vibrio* are well-known pathogens for humans, fish and some other mammals (Janda, Powers, Bryant, & Abbott, 1988). Nevertheless, it was realized throughout the course of this research that isolation and preservation of fastidious organisms such as *Vibrio* from environmental samples is not an easy task. Firstly, the microbes did not survive in the lab for a very long time. Among the 86 or so colonies suspected to be *Vibrio*, only 55 could be revived for biochemical analysis. Presumptive biochemical tests reduced the number to 21. Modern more specific PCR technique was finally used as a confirmatory test which eventually brought down the number to 6, which is only about 7% of what was initially suspected. In this test, 6 isolates were confirmed to be *Vibrio* through PCR. Biochemical test analysis using the ABIS online bacterial identification software identified all 6 of these isolated to be *V. metschnikovii*. Although fairly uncommon, there have been however, reported cases of *V. metschnikovii* causing abdominal pain and cholecystitis (Janda et al, 1988), wound infection (Linde et al, 2004), pneumonia and acute-respiratory failure (Wallet, Tachon, Nseir, Courcol, & Roussel-Delvallez, 2005) in human. A 2007 study about the virulence factors of *V. metschnikovii* in Brazil by Matté et al has therefore labelled the microbe as a potential pathogen that we need to be careful about.

Nevertheless, no matter how disappointing that number may look to be, a number of causes can be identified as contributing factors behind such numbers. To begin with, Buriganga is undoubtedly, among the most polluted water bodies in the country. Several reports including one by the Daily Star on January 6, 2018 for example, have pointed out to the presence of

industrial waste and heavy metal in its water. Conversely, the dumping of sewage by nearby residential areas, garbage by careless visitors and the recent water taxi services which run on fuel rich in heavy metals and other mutagens have introduced the Hatirjheel to a vast number of pollutants. The role of heavy metals such as chromium present in tannery waste and mercury and cadmium present in fuel leaking from faulty engines in causing mutation is well documented (Bronzetti et al, 2008). It can therefore, be hypothesized that exposure to such mutagens may have altered the biochemical characteristics of the microbes in these ecosystems.

It is possible that many *Vibrio* organisms present in the water body had mutated to show different result in biochemical tests and hence were not identified a *Vibrio* by the programme used to analyse the result. Nonetheless, The 6 organisms confirmed by PCR were all identified as *V. metschnikovii* in the ABIS online bacterial identification system. Interestingly, the results obtained in this thesis from biochemical characterization are very similar to the results obtained in previous works using the same organism. A 1978 paper by Lee, Donovan and Furniss on the characterization and description of *V. metschnikovii* was consulted in this regard. Comparison of the obtained result was identical for motility, glucose utilization, lactose utilization and sucrose utilization tests. Contrarily, 16.87% of the Hatirjheel isolates showed an opposite result in each of oxidase test, urease test and gas production from glucose while 33.33% showed a variation in haemolysis. Table 4.1 summarizes this result for better understanding.

Table 4. 1: Table showing expected results and percentage of isolates with variable results

Name of test	Expected result	Percentage of isolates showing variation
Oxidase	-	16.67%
Urease	-	16.67%
Motility	+	0%
Sucrose Fermentation	+	0%
Lactose Fermentation	+	0%
Glucose Fermentation	+	0%
Gas production	-	16.67%
Haemolysis	+	33.33%

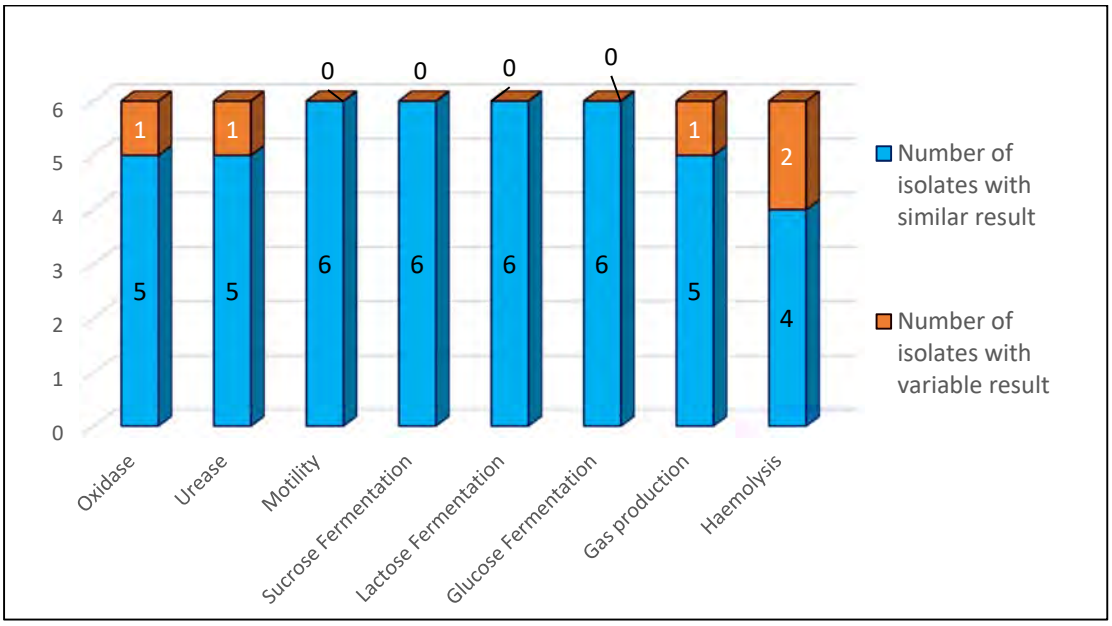

Illustration 4. 1: Bar diagram comparing biochemical characteristics of test isolates with result published in Lee, Donovan, & Furniss (1978).

In addition to that, incorrect preparation methods for media and its overall quality, accidental mishandling and failure to maintain a sterile work environment may be assumed to have impacted the test results. Moreover, biochemical tests are never a cent percent accurate and relying on it entirely is not usually recommended.

Furthermore, members of the genus *Vibrio* are known to go into a VBNC state in response to environmental stimulus (Halpern, Landsberg, Raats, & Rosenberg, 2007; Whitesides, & Oliver, 1997; Falcioni et al, 2008). Cold weather, falling level of water bodies, excessive rainfall, low temperature and shortage of nutrients are known to induce VBNC state in *Vibrio*. The sample collection for this thesis was carried out from late-fall to mid-winter (October – January). Hence, it is possible that there were *Vibrio* samples in the water but could not be isolated due to their VBNC state. An earlier study showed that *V. metschnikovii* is more tolerant to cold weather compared to other species of *Vibrio* (Kokashvili et al, 2015). Illustration 4.2 compares seasonal abundance of 5 *Vibrio* species determined by biochemical identification. This graph was accepted as a standard to compare the result obtained in this work. The 2 isolates obtained in January (55OVY & 55EVY) are in line with the curve in the graph. However, in this study, the maximum number of isolates were obtained in November when, according to the graph, the number should have been the least. It can be hypothesized that the stagnant nature of the lake which tends to accumulate the contaminants

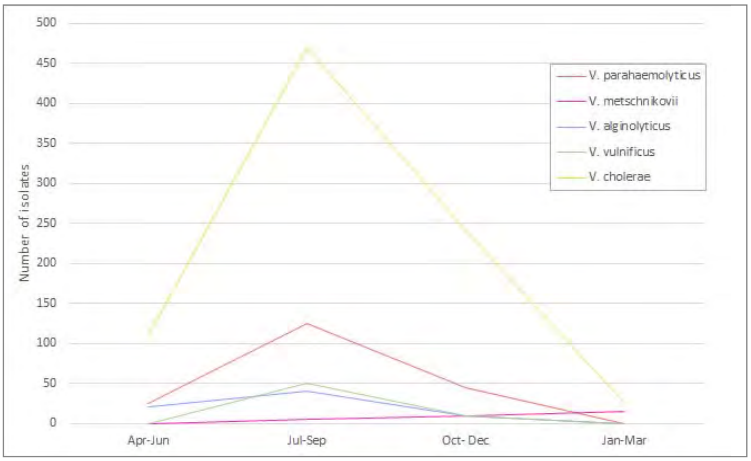

Illustration 4. 2: Seasonal abundance of 5 species of Vibrio determined by biochemical methods (Kokashvili et al, 2015)

rather than dilute them may have had an impact on the organisms to make them more tolerant to cold. For comparison, water collected from Buriganga around the same time yielded no *Vibrio* isolates.

Nextly, the antibiogram results also showed an interesting pattern. Admittedly, all the samples showed MAR index values on the safer side, but 50% of the environmental isolates showed MAR values more than the laboratory *Vibrio cholerae* OGAWA control. Noticeably, the stagnant nature of water in Hatirjheel is a barrier unlike in say, rivers, to dilution the amount of contaminants and mutagens in the water. The concentration of these accumulating contaminants increases even more during the dry winter seasons when the sampling for this thesis was conducted. The discrepancies in the MAR index values obtained in this work may suggest to an undergoing process of development of multidrug resistance in the Hatirjheel micro biota. However, the speed and impact of this change would require further studies to determine.

Finally, 66.67% of the test *Vibrio* isolates (samples 32EVG, 30EVY, 55OVY and 55EVY) displayed complete haemolytic nature whereas the remaining 33.33% samples (33EVY and 34OVY) were non-haemolytic. *Vibrio metschnikovii* is generally a haemolytic organism. However, the haemolytic process is dependent on temperature, can be inhibited by certain molecules and functions in a multihit mechanism (Miyake, Honda, & Miwatani, 1988). Considering all these factors, presence of a high number of erythrocytes in the media can be identified as haemolysis inhibitor in this case.

Conclusion

To sum up, the lack of sufficient published data was regarding *Vibrio* spp. And their phenotypic nature was evident throughout the term of this thesis. The culture method for isolation of *Vibrio* also proved to be fairly unreliable. The isolated in this research often times showed significant deviation from the traditional phenotypic features associated with *Vibrio*. In short, databases regarding the topic seem to have reached a point where updating the already available knowledge seems compulsory. Furthermore, a detailed study on the quality of the two water bodies utilized in this work and its impact is necessary to accurately interpret the results of this study. Finally, it can only be hoped for that many more works on this topic and its derivations will be carried on in the near future utilizing the more sophisticated and modern technologies made available recently for a better understanding of the aquatic microbial ecosystem in heavily populated settlements and its impact on human life.

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Appendices

Appendix A: Media Composition

All the media mentioned below except TCBS Agar was sterilized by autoclaving for 15 minutes at 121°C at 15 psi pressure.

Alkaline Peptone Water

Ingredients	Grams/litre
Bacteriological peptone	20.0
Sodium chloride	20.0
Final pH (at 25°C) 7.4 ± 0.2	

Blood Agar Base

Ingredients	Grams/litre
Proteose peptone	15.0
HML extract	2.5
Yeast Extract	5.0
Sodium chloride	5.0
Agar	15.0
Final pH (at 25°C) 7.4 ± 0.2	

Mueller-Hinton Agar

Ingredients	Grams/litre
Beef, hydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

Final pH (at 25°C) 7.3 ± 0.1

Motility Indole Urease (MIU) Agar

Ingredients	Grams/litre
Tryptone	10.0
Phenol red	0.1
Sodium chloride	2.0
Agar	5.0
Final pH (at 25°C) 7.0	

Modified Nutrient Agar

Ingredients	Grams/litre
Peptone	5.0
Beef extract	3.0
Sodium chloride	30.0
Agar	15.0
Final pH (at 25°C) 7.0	

Modified Nutrient Broth

Ingredients	Grams/litre
Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	30.0
Final pH (at 25°C) 7.4± 0.2	

MRVP Broth

Ingredients	Grams/litre

Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH (at 25°C) 7.0	

Saline

Ingredients	Grams/litre
Sodium chloride	9.0

Simmon's Citrate Agar

Ingredients	Grams/litre
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

TCBS Agar

Ingredients	Grams/litre
Yeast extract	5.0
Peptic digest of animal tissue	10.0
Sodium citrate	10.0
Sodium thiosulphate	10.0
Sodium cholate	3.0

Oxgall	5.0
Sucrose	20.0
Sodium chloride	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.0

Triple Sugar Iron Agar

Ingredients	**Grams/litre**
Beef extract	3.0
Peptone	20.0
Yeast extract	30
Lactose	10.0
Sucrose	10.0
Dextrose monohydrate	1.0
Ferrous sulphate	0.2
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

Appendix B: Reagents and Buffers

Barritt's Reagent

Solution A: 5 g alpha-naphthol was dissolved in 95% ethanol. The reagent was covered in aluminium foil and stored at 4°C.

Solution B: 40 g KOH was dissolved in distilled water. Once the mixture cooled, creatine was added. Final volume was adjusted with distilled water and the reagent covered with aluminium foil was stored at 4°C.

Methyl Red Reagent

0.1 g methyl red was dissolved in 300 mL of 95% ethyl alcohol. To this, distilled water was added to make up the final volume of 500 ml. The reagent was covered with aluminium foil and stored at 4°C.

Oxidase reagent

100 mg of N, N, N1, N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 mL distilled water. The solution was covered with aluminium foil and stored at 4°C.

EDTA (0.5 M)

In a sterile McCartney bottle, 1.861 grams of EDTA was dissolved in 10 ml of distilled water to prepare 10 ml of 0.5 M EDTA solution. The pH of the solution was adjusted at 8.0 and stored at room temperature for future use.

TAE Buffer (1x)

A 100 ml of 50 x stock solution was prepared by dissolving 24.2 grams of TrisHCl in 70 ml of autoclaved distilled water. Afterwards, 5.71 ml of 100% acetic acid and 10 ml of 0.5M EDTA

(pH 8.0) were added to it. The volume was brought to 100 ml by adding 14.3 ml of autoclaved distilled water and the pH was adjusted at 8.5 in room temperature. The solution was stored at room temperature. For the purpose of this work, TAE buffer was always used in 1x concentration. For example, 50 ml 1x TAE Buffer solution was prepared by diluting 1 ml of the stock solution with 49 ml of autoclaved distilled water.

TrisHCl (1 M)

In a sterile McCartney bottle, 1.576 grams of TrisHCl was dissolved in 10 ml of distilled water to prepare 10 ml 0f 1 M TrisHCl solution. The pH of the solution was adjusted at 8.0 and stored at 4^{o}C for future use.

Agarose gel (1.5%)

0.9 grams of pure agarose was added to 60 ml of prepared 1x TAE Buffer in a 250 ml flask. The combination was allowed to hydrate for about 1 minute at room temperature and then heated in the microwave until the agarose powder dissolved completely. Once the solution had cooled off to approximately 50^{o}C, 3 µl of Ethidium bromide was added and mixed evenly by carefully swirling the flask. The molten gel was then poured into the casting trey secured with dams and the comb. The comb and the dams were removed after the gel had cooled off and set completely.

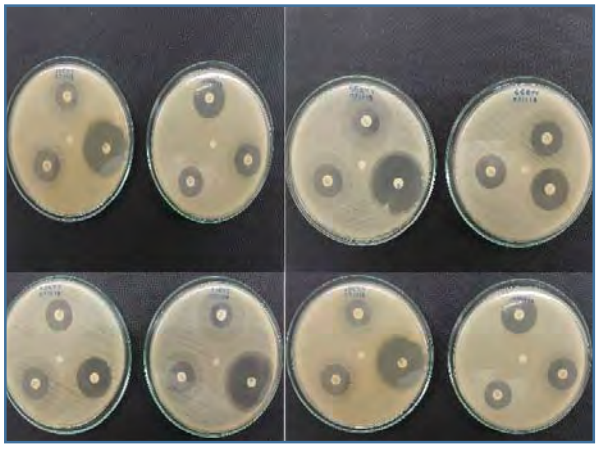
Appendix C: Instruments

Instruments	Company
Autoclave	SAARC
Cellulose filter paper(9.0 cm)	Whatman
Freeze(-20^{o}C)	Siemens
High Speed Refrigerated Micro Centrifuge, Model: MX- 307	Tomy Kogyo Co Ltd, Japan
Incubator	SAARC
Microcentrifuge tubes	Tarsons, India
Micropipette ()	Eppendorf, Germany
Micropipette (2-20 µl)	Eppendorf, Germany
Micropipette (100-1000 µl)	Eppendorf, Germany
PCR Tubes	Tarsons, India
pH meter, Model: E-201-C	Shaangai Ruosuaa Technology Company, China
Pipette (5 ml, 10ml)	Eppendorf, Germany
Power Supply, Model: ELITE 300 Plus	Wealtec Corp, USA
Refrigerator (4^{o}C), Model:0636	Samsung
Safety cabinet, Class II Microbiological	SAARC
Shaking Icubator, Model: JSSI-1000C	JS Research Inc, Rep. of Korea
Shaking Incubator, Model: WIS-20R	Daihan Scientific Co Ltd, Korea
Surgical Millipore syringe filter (0.22 µm)	Millex-GS
Thermal Cycler, 2720	Applied Biosystems, Singapore
UV Transilluminator, Model MO-20 312 nm	Wealtec Corp., USA
Vortex Mixer	VWR International
Water bath	Daihan Scientific Co Ltd, Korea
Weighing balance	ADAM Equipment TM, United Kingdom

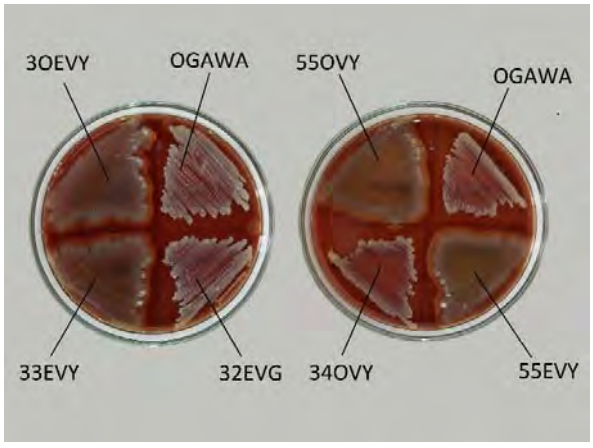
Appendix D: Photographs

A. Hatirjheel lakefront in the Capital

B. Water taxis that were used to reach sampling sites on the Hatirjheel



C. Sample *Vibrio* isolates on MHA plates after antibiogram



D. *Vibrio* isolates grown on Blood Agar