ARE THE LAKES AND RIVERS OF DHAKA CITY SAFE FOR WATER-BASED RECREATION? A CASE STUDY OF *SALMONELLA* SPP

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 **"Are the Lakes and Rivers of Dhaka City Safe for Water-Based Recreation? A case study of *Salmonella* spp."** has been written and submitted by me, Tanmoy Chakma and has been carried out under the supervision of Mahbubul Hasan Siddiqee, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

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

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Abstract

Over the past years, surface water bodies have been contaminated due to unplanned urbanization, industrialization and defective sewerage systems. As these water bodies are a major part of diurnal and recreational activities, contamination may possess a huge health risk. The aim of this particular case study is to observe the presence and responses of *Salmonella* spp. in such water bodies contaminated with industrial and household pollutants. In this study, two of the most significant water bodies of Dhaka city (Hatirjheel Lake and Buriganga River) were chosen. A total of 80 water samples were collected; 40 from Hatirjheel Lake and 40 from Buriganga River respectively. Samples were collected from the month of November to February. Membrane filtration method was used to process the samples, then isolates were enriched and cultured in suitable selective media. A total of 63 presumptively Salmonella isolates was isolated from the 80 samples. The cultured isolates were then identified using molecular methods in which *Salmonella* specific virulence gene *invA* was targeted. 27 out of 30 isolates were *invA* positive for Hatirjheel and 20 out of 33 were *invA* positive for Buriganga. Following identification, antibiotic susceptibility test of the identified isolates was done using an array of 8 antibiotics of different classes. All the isolates of both Buriganga and Hatirjheel Lake were completely resistant to amoxicillin, 90% of the Buriganga isolates were resistant to cefepime while 80% of the Hatirjheel isolates were resistant to cefepime. Isolates of both water bodies were completely sensitive to Amikacin with a few intermediate sensitive seen against Norfloxacin and Gentamycin. Survival assay of the isolates resistant to most antibiotics was conducted for a period of 7 days. The survival patterns between the lake and river showed slight difference but both showed a gradual decrease in culturable count over the time. Finally, static biofilm formation was observed. Isolates from both sources form biofilm in adverse condition but biofilm formed by Buriganga river isolates at raw concentration remain more viable over time. At lower concentration (1:200) Hatirjheel isolates biofilm remain more viable. The study reveals that both Buriganga River and Hatirjheel Lake can be endemically contaminated with potentially pathogenic *Salmonella* and that there is an indication that the pollution level might have some influence on biofilm formation, survival rate and antibiotic resistance of *Salmonella*.

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Chapter 1

Introduction

Water is a basic human need. Safe drinking water supply and adequate environmental sanitation are the stipulations for health and for success in the fight against poverty, hunger and child deaths (Parveen, Ahmed, & Nasreen, 2008). Polluted water isn't just dirty, it's deadly. In 1995, about 3 million deaths were caused by contaminated water worldwide where more than 80% were among children under five years of age (WHO 1996). According to a report by Faechem (1980), it states that at least one and a half thousand million people worldwide used polluted water.

In Bangladesh, people that use the central water supply system for daily use accounts for only 2-3% while the majority of the rural population mostly depend on open water sources. 40 out of 50 prevalent diseases in Bangladesh such as dysentery, typhoid, parasitic worm infection, etc. are related to the contaminated water (Acharjee et al., 2011). Enteric fever which includes typhoid fever caused by *Salmonella typhi* and paratyphoid fever by *Salmonella paratyphi* A B C is an important public health problem in many developing countries. Worldwide, typhoid fever affects more than 12.5 million people annually (Edelman, R., & Levine, M. M., 1986). In South Asia, it is estimated that annually 7 million cases of typhoid occur with the emergence of enteric fever related to multidrug resistant (MDR) strains of *S.typhi* and *S. paratyphi A* (Crump et al. 2003; WHO 2003; Ochiai et al. 2005).

Despite the use of antibiotics and the development of newer antibacterial drugs, enteric fever continues to be a major health problem. Enteric fever carries a high mortality rate of 30% if treated improperly but with appropriate antimicrobial treatment, the mortality rate is as low as 0.5% (Ali & Sultana, 2016).

Water sources are vulnerable to contamination from many origins, to include humans and animals (Dechesne and Soyeux, 2007). Many serovars of Salmonella have been isolated from natural environments, contaminated by human and animal feces, particularly in river waters, estuarine waters and seawaters (Ahmed et al. 2009; Touron et al. 2005). Environmental and climatic factors along with nature and source of water supply such as excreta and other waste disposal processes influence the prevalence of these organisms (WHO, 2004). Variety of activities like discharges of municipal raw (untreated) water and treated effluents from processing facilities, storm water runoff, or other non-point source runoff all affect surface waters and thus the quality of surface water is on a state of constant change in terms of microbial quality (Anderson and Davidson, 1997). The growth of Salmonella in water supplies is also considered possible, due to its ability to colonize surfaces and replicate in

biofilms (Levantesi et al., 1998) and due to its ability to survive for weeks outside the host intestine (Cabral, 2010).

Dhaka city, the capital of Bangladesh, lies beside one of the major rivers named Buriganga. As a result of industrialization, most of the factories and industries, predominantly tanneries and textiles, are located on its banks and discharges untreated industrial effluents which are increasing water pollution and influencing human health as well as the environment (Rabbani et al., 2008).

Apart from the main river, urbanization within Dhaka city has led to the development of numerous lakes and ponds within the cosmopolitan. The surface water area of Dhaka city is about 10-15% of the total land area (Miah et al., 2016). Hatirjheel, Gulshan Lake, Banani Lake, Ramna Lake, and Dhanmondi Lake are the popular relaxation spots in the city, is being polluted by slums and sewage, the business firms and industries operating in the area (Miah et al., 2016). Hatirjheel Lake plays a vital role in maintaining the only drainage system of those areas (Miah et al., 2016). As a result of water pollution, the nearby local communities often suffer from diarrhea, skin diseases, gastric ulcers, respiratory illness, anemia, high blood pressure and jaundice (Ullah et al., 2006).

Due to the difference in location and the sources of pollution, the water quality of Hatirjheel Lake and Buriganga River are different. Significant sources of pollution for Buriganga are from the tanneries which use strong chemicals which contain chromium and wash them out into the rivers. As a result, it has been found that the water of Buriganga contains 2.6 to 28.0 mg/L of hexavalent chromium where 0.1 mg/L is the permissible limit. There is a mixture of other heavy metals such as arsenic, lead, mercury, cadmium, chromium, nitrates, nitrites, etc. in the polluted waters some of which are believed to be cancer-causing agents (Huq et al., 2013).

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

Figure 1: Compliance of Buriganga River water quality parameters with DOE guidelines (Rahman and Bakri).

Figure 1 demonstrates a high level of DO (Dissolved Oxygen), BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), ammonia and chromium. High BOD level clearly indicates that the River Buriganga is polluted with the organic chemical as well as bacterial pollutants and is unsuitable for fisheries (Saifullah et al., 2012).Low DO is probably due to the easily oxidized industrial and municipal organic wastes which are directly linked to the high turbidity of the water. This results in low photosynthesis and a negligible replacement of oxygen (Rahman & Al Bakri, 2010). COD is an important parameter that measures the total quantity of oxygen required to oxidize all organic material into carbon dioxide and water (Masters & Hall). High COD and ammonia values are a result of severe pollution from the chemical and sewage discharges in the area.

Hatirjheel Lake was mainly designed to catch sewage and the runoff storm water of the nearby areas. Due to the lack of proper design and improvement but with an increasing population the pollution is increasing. The lake is situated beside the housing estates and often large pipes are seen on the banks of the lake discarding the sewage water from these buildings. In addition, the growths of slums nearby further deteriorate the pollution in the lake due to unplanned sanitation. The water quality degrades more during the wet season when the rainwater-sewage flows into the lake. This results in high BOD and COD values of 90 mg/l and 175 mg/l, respectively which improves a bit in the dry seasons. Unlike BOD and COD, ammonia concentration continues to increase. The free ammonia concentration throughout Hatirjheel was more than the standard value with 2.5 mg/l being the highest recorded. Nitrate concentration increases after wet season when the organic compounds are degraded into ammonia which oxidizes to nitrate. On the other hand, sulfide concentration remains relatively high throughout the seasons increasing more during the wet seasons. The average

concentration of DO varies from 1.6 to 3.6ppm when the standard is 5ppm. This 5 parameter is required for the survival and decomposition of compounds by microorganisms (Islam et al., 2015).

Since the level and sources of pollution in Hatirjheel Lake and Buriganga River are different, it is most likely to affect the characteristics and occurrence of microorganisms in the water bodies. Even though these waters are not used as sources of drinking water, people are exposed to the toxic contents on a regular basis. Hatirjheel lake area is a recreation spot for the city dwellers where they go for boat rides and sit by the banks for entertainment. Many children from nearby slums or the homeless children swim and bathe on this lake as well. The banks of Buriganga River is densely populated with ferries and boats responsible for the transport of humans as well as various food items such as fruits and vegetables. The water from the river is used by many to wash their hands or to spray it over vegetables to keep then fresh or used by local food vendors such as chotpoti and jhalmuri vendors to wash their utensils. These incidences give rise to a public health aspect to determine how safe the waters are for such usage and if it will result in the spread of enteric diseases such as cholera, typhoid, and dysentery.

One of the earliest steps in the pathogenic cycle of Salmonella spp. is the invasion of the cells of the intestinal epithelium. A genetic locus, inv, allows Salmonella spp. to enter cultured epithelial cell and invA is a member of this locus, (Galan et al., 1992). There is growing concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance. Plasmid sequencing in many studies has shown that antibiotic resistance arises due to the presence of industrial pollutants. Genomic sequencing of the pathogen Salmonella enterica serovar Typhi CT18 from a dump site rich in heavy metals revealed the presence of a conjugative plasmid that confers resistance to trimethoprim, sulfonamide, chloramphenicol, ampicillin and streptomycin and contains a mercury-resistance operon (Baker-Austin et al., 2006).

The annual incidence rate of typhoid in Dhaka city varied from 8 (in 2006) to 11 (in 2007/8) per 100,000 people and the average number of typhoid occurrences in each year was 871 (Islam et al., 2015). Examination of the monthly distribution of typhoid reveals that the highest cases have fluctuated over the years, July-October being the highest, followed by April-June. Most of the typhoid cases occurred in the proximity of large water bodies such as rivers and lakes (Islam et al., 2015). This is why it is important to determine if the Hatirjheel Lake and Buriganga River are contaminated with Salmonella spp. If so, what are the

characteristics of these species in terms of antimicrobial resistances or die-off rate and whether these strains are capable of Biofilm formation? Furthermore, due to the differences in location of these water bodies, the rate of pollution varies widely which is an important factor in affecting the characteristics of the microorganisms.

In accordance with the literature review, it has been shown that the water bodies; Buriganga, and Hatirjheel are polluted by various pollutants and their microbial analysis indicated the level of Coliform bacteria present as an indicator organism. However, the presence of actual pathogen responsible for causing diseases is not widely studied in addition to the effects the pollutants are having on these organisms. As a result, this study mainly focuses on the presence of Salmonella in the water bodies which is responsible for causing enteric diseases and compares the characteristics of the Salmonella strains between the Hatirjheel Lake and Buriganga River. Even though antibiogram is a common parameter checked in clinical strains, it is rarely done with the strains that are environmentally present as there can be multiple aspects where this resistance can get transferred and indirectly affect humans. Thus shedding light on how the pollutants are affecting the pathogenicity of the organisms between the lakes and river.

Our aim of the study is to detect the level of pollution in Hatirjheel Lake and Buriganga River and whether their occurrence varies or not. Furthermore, to access the pathogenicity via virulent gene *invA* and static biofilm formation. According to our hypothesis, the waters will be contaminated with *Salmonella* spp. and the contaminants will have different characteristics between the Lakes and River.

Chapter 2

Materials and Methods

2.1 Sample collection

Water samples were collected in autoclaved plastic bottles from different points of the Hatirjheel Lake, and Buriganga River. In order to reach the various points of sampling, small boats and water ferries were used. The sample collection procedure started from mid-November 2017 and lasted until the end of February 2018. Total of 120 samples was collected, 40 from the Hatirjheel Lake while a total of 40 samples were collected from the Buriganga River. The collection took place weekly and the water samples were brought back to the lab within 2 hours for further processing.

2.2 Processing of Water Sample

The water samples were processed for isolation of culturable Salmonella following a previously reported method [Borch et al., 2008]. In brief, a total of 50ml of the collected water samples were filtered using a single 0.45µm membrane. The filter papers were then placed in 50ml of Buffered peptone water and incubated at 37° C for 24 hours. Afterward, 5ml of the buffered peptone was added to 50ml Rappaport Vassiliadis broth (RVB) for selective enrichment of Salmonella. The broth was then incubated at 42° C for 24 hours. After the selective enrichment, loopful of the broth was streaked onto the selective media Xylose Lysine Deoxycholate (XLD) agar plates for the selective growth of Salmonella and then incubated at 37° C for 24 hours. The suspected colonies appeared to have dark centers with translucent surroundings. These colonies were then sub-cultured three times subsequently in XLD media for pure isolated colonies and then transferred to Luria-Bertani agar. In order to store the samples, colonies from LB agar was taken and inoculated in T1N1 media. After 24 hours incubation at 37° C, paraffin oil was added to the broth and stored at -20° C.

2.3 Description of sampling sites

Haitrjheel Lake is a well-known waterbody present in Dhaka city. The Lake is about 302 acres of Tejgaon, Moghbazar, Rampura region and has been playing a vital role in maintaining the only drainage system of those areas (Miah et al., 2016).

Buriganga River is one of the prime rivers located just outskirt of the city which has been turned into a dumping site of untreated effluents for the many industries which are located on its bank as a result of unplanned urbanization. These two polluted water bodies have been targeted as the sampling site for this study.

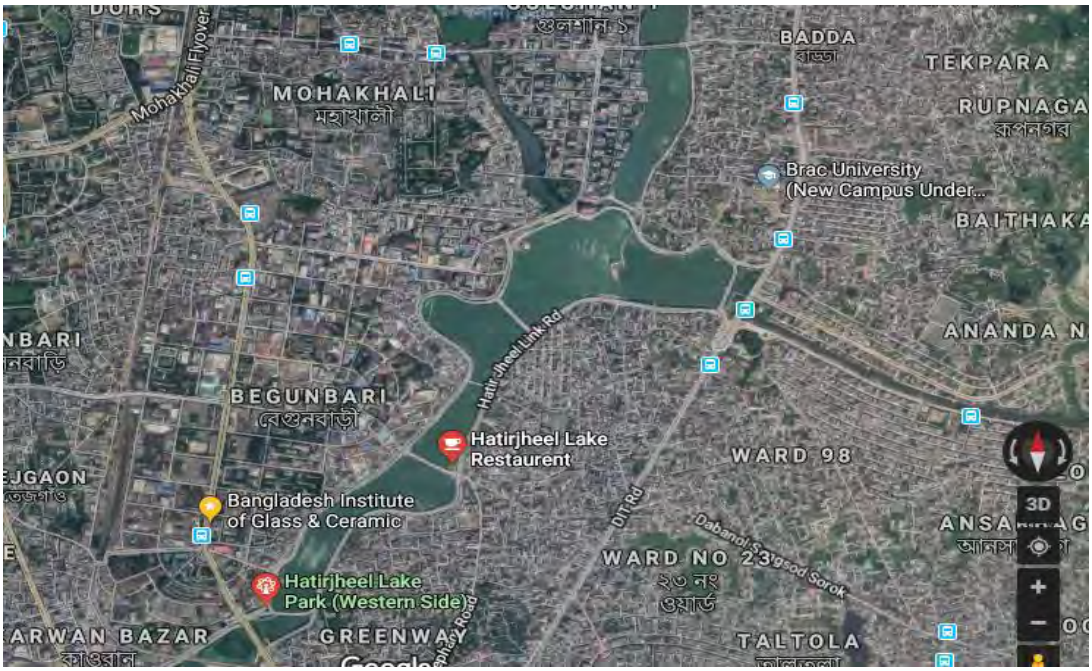

Figure 2: Hatirjheel Lake sampling site.

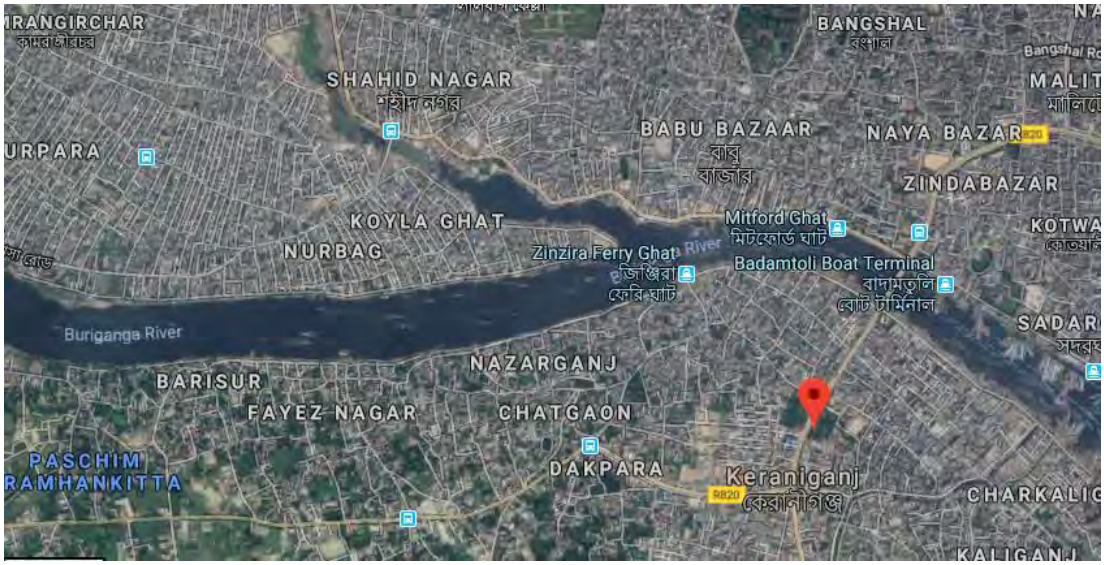

Figure 3: Buriganga River sampling site.

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

2.4 Identification

The suspected colonies were then tested for the following biochemical tests to presumably conclude whether they are Salmonella species.

Gram stain

Gram staining was done to differentiate between two principal groups of bacteria: gram positive and gram negative.

Biochemical characterization of the bacteria

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino & Sherman, 2005). The biochemical tests performed were Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, and Citrate utilization test), MIU test (Motility test, Indole test, and Urease test), and Catalase test.

Triple Sugar Iron Agar test

Triple sugar iron test was done to differentiate among the different groups or genera of the Enterobacteriaceae based on the ability to reduce sulfur and ferment carbohydrates. Triple sugar iron slants were prepared in the test tubes by autoclaving at 15 psi 121° C. Using sterile technique; 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).

Indole Production test

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

Methyl red test

Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products. MR-VP broth of 7 ml in each test tubes was prepared by autoclaving at 15 psi 121° C. Using sterile technique,

small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 hours at 37◦C. After 24 hours 3.5 ml from the culture tubes were transferred to clean test tubes for Voges- Proskauer test and the remaining broth were re-incubated for additional 24 hours. After 48-hour incubation 5 drops of methyl red indicator was added directly into the remaining aliquot of the culture tubes to observe the immediate development of a red colour. (Cappuccino & Sherman, 2005)

Voges Proskauer test

Voges Proskauer test was done to determine the capability of the organism to produce non-acidic or neutral end products such as acetylmethylcarbinol. To the aliquot of MR-VP broth after 24-hour incubation, 0.6 ml (12 drops) of 5% alpha-naphthol (reagent A) was added followed by 0.2 ml (4 drops) of 40% KOH (reagent B). The tube was gently moved to expose the medium to atmospheric oxygen (30 seconds-1 minute) and the medium was allowed to remain undisturbed for 10-15 minutes. The test was read, but not beyond, one hour following the addition of the reagents (McDevitt, 2009).

Citrate utilization test

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrate permease. Simmons citrate agar slants of 2 ml in each vial were prepared by autoclaving at 15 psi 121º 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 48 hours at 37º C (Cappuccino & Sherman, 2005).

MIU (Motility- Indole- 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 by12 autoclaving at 15 psi 121º C. The media was cooled to about 50-55º 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, a 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 37º C (Acharya, 2015).

Catalase test

Catalase test was done to determine the ability of the bacteria to degrade hydrogen peroxide by producing the enzyme catalase. A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small number of bacteria from 24-hour pure culture was placed onto the microscopic slide. 1 drop of 3% H_2O_2 was placed onto the organism on the microscopic slide using a dropper and observed for immediate bubble formation (Reiner, 2010).

2.5 Virulence Determination

One of the earliest steps in the pathogenic cycle of the facultative intracellular pathogen Salmonella spp. is the invasion of the cells of the intestinal epithelium. A genetic locus, inv, allows Salmonella spp. to enter cultured epithelial cells. *invA* is a member of this locus, and it is the first gene of an operon consisting of at least two additional invasion genes (Galan et al., 1992).

2.5.1 DNA extraction by boiling method:

The selected isolates were inoculated in nutrient broth and incubated at 37⁰ C for 24h. The next day, 1.5ml of the broth was transferred to Eppendorf and centrifuged at 15,000g for 15 min. The supernatant was eliminated, and the pellet was resuspended in molecular biology grade water (Eppendorf, Hamburg, Germany) and centrifuged at 15,000g for 10 min. The supernatant was discarded and the pellet was resuspended in 40µl of autoclaved distilled water, subjected to boiling at 100°C in a water bath for 10min, cooled on ice foe further13 10minutes and centrifuged at 15,000g for 10s before it was stored at -20°C. Aliquots of 2 µl of template DNA were used for PCR. (Queipo-Ortuño et al., 2008)

2.5.2 PCR

Polymerase Chain Reaction (PCR) is an in vitro technique based on the principle of DNA polymerization reaction by which a particular DNA sequence can be amplified and made into multiple copies. It relies on thermal cycling consisting of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using thermo stable DNA polymerase, primer sequence (complementary to target region) and dNTPs. Aliquots of extracted DNA by boiling method were used as a template for the PCR encoding virulent gene invA.

Salmonella-specific *invA* primers (5'GTGAAATTATCGCCACGTTCGGGCAA-3'and 5'TCATCGCACCGTCAAAGGAACC-3') was used for the detection of Salmonella in this study (Kumar et al., 2008).

Table 1: Primer used for gene-specific PCR.

Target Gene	Primers	Sequence (5'-3')	*Amplicon Size (bp)*	*Annealing Temp (°C)*	Reference
*inv*A	Primer F Primer R	5'-GTGAAATTATCGCCACGTTCGGGCAA -3' 5'- TCATCGCACCGTCAAAGGAACC -3'	284	64	(Kumar et al., 2008)

25µl of PCR mixture contained 12.5µl of Master Mix, 2µl of both primers, 6µl of nuclease-free water and finally 2.5µl of the template DNA. The amplification was carried out with the reaction condition: initial denaturation at 95° C for 2 min, followed by 35 cycles of 95° C for 30 s, 64° C for 30 s, and 72° C for 30 s and a final extension of 5 min at 72° C was employed. (Kumar et al., 2008)

2.5.3 Gel conditions

1.5% agarose gel was made using 1X TE Buffer and run with 1X TBE buffer. For staining Ethidium bromide was added to the gel after it cooled down slightly. 2µl dye and 6µl of the PCR product were mixed well and then added to each well. The gel was run for 40 minutes at 80V. The product size (284 bp) was determined with a 1000 bp DNA molecular weight ladder. Finally, the gel image was viewed under UV light. For each PCR test, a positive (*Salmonella typhimurium*) and negative control (sterile dH_2O) were used separately along with the water sample. (Kumar et al., 2008)

2.6 Determination of the Antibiotic Resistance Pattern of the Target

After the identification of suspected colonies, randomly selected five isolates (replicative measure) from each lake and river were used to compare the antibiotic susceptibilities as well as resistance via the Kirby-Bauer disc diffusion method. The antibiogram was performed using antibiotics that are usually tested against Salmonella for the treatment of enteric

diseases and some general first generation antibiotics. The mode of transmission for these antibiotics is shown below:

Table 2: Antibiotic Resistance mode of transmission.

Antibiotic	Plasmid mediated	Reference	Chromosome mediated
Ampicillin	Yes	(Kehrenberg et al.)	Yes
Chloramphenicol	Yes	(Winokur et al.)	Yes
Co-trimoxazole	Yes	(Winokur et al.)	Yes
Gentamycin	Yes	(Aitmhand et al.)	Yes
Amikacin	Yes	(Casin, Hanau-berc, et al.)	No
Ceftriaxone	Yes	(Report)	No
Ciprofloxacin	Yes	(Gay et al.)	Yes
Cefepime	Yes	(Izumiya et al.)	Yes

2.6.1 Kirby-Bauer Disc diffusion:

The isolates were subjected to antimicrobial susceptibility testing by disk diffusion method as recommended by the Clinical Laboratory Standard Institute (CLSI) using commercial15 antimicrobial disks. The antibiotic disks used in this study were: Norfloxacin (10µg), Amikacin (30µg), Cefepime (30µg), Amoxicillin (30µg), Ceftriaxone (30µg), Gentamycin (10µg), Chloramphenicol (30µg), Cotrimoxazole (sulfamethoxazole 23.75 µg + trimethoprim 1.75 µg) (Bhattacharya et al., 2011). The method described by Bauer and Kirby (1969) was followed. An inoculating needle was touched to a freshly grown, well isolated colony on a plate and then inoculated into 1 ml of Muller-Hinton Broth (MHB). The culture was then incubated in a shaker at 37°C for 4 hours to obtain the actively growing culture, equivalent to 0.5 McFarland standard (1.5x 10^8 CFU/mL). A sterile cotton swab was dipped into the

standard suspension, the excess broth was purged by pressing and rotating the swab firmly against the inside wall of the tube above the fluid. The swab was then streaked evenly in three directions over the entire surface of the agar plate to obtain a uniform inoculum. A final sweep was made of the agar rim with the cotton swab. This plate was then allowed to dry for three to five minutes before the disks were applied. Antibiotic-impregnated disks were then applied to the surface of the inoculated plates with a sterile syringe needle. All disks were gently pressed down onto the agar with sterile forceps to ensure complete contact with the agar surface. Within 15 minutes after the disks were applied, the plates were inverted and placed in an incubator at 37°C. After overnight incubation, the plates were examined for a 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)

2.7 Survival Assay

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

2.7.1 Salmonella culture and Preparation of Inoculum

The Salmonella species were revived from the stock culture in XLD media and incubated at 37º C for 24 hours. Then two colonies were taken from each sample and inoculated in 10ml LB (Luria Bertani Broth) and incubated at 37º C for 3 hours in a shaking incubator. The optical density of the broths was measured until it reached 0.1 OD at 600nm wavelength using a spectrophotometer. The cultures were then centrifuged at 5000 rpm for 15 minutes to harvest the cells. The cells were then washed with sterile saline twice and resuspended in it to get a final concentration of approximately 10ˆ8 CFU/ml which was then inoculated in the microcosms (Sugumar & Mariappan, 2003).

2.7.2 Preparation of microcosm

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

Figure 4: Microcosm prepared for isolates.

2.7.3 Enumeration of survivors

Plate count in order to determine the colony forming units was taken starting from day 0 the day of inoculation followed by day 2, day 3 and day 5 on selective media XLD. On day 0, the samples were diluted up to 6 times and then 100µl of it was spread plated. On the next count, the samples were diluted up to 4 times while on the 3rd day one fold dilution of the sample was made and then spread plated on XLD.

2.8 Biofilm Formation

A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous extracellular matrix (Donlan 2002). Bacteria in biofilms exhibit enhanced resistance to cleaning and sanitation (Bower & Daeschel, 1999; Joseph et al., 2001). *Salmonellat yphi* and *Salmonella typhimurium* are among the most commonly encountered foodborne and waterborne pathogens worldwide. Although their respective disease states differ, both organisms are capable of forming bacterial biofilms in mammalian and/or environmental niches. Biofilm aids bacteria to endure severe conditions and facilitate bacterial persistence by increasing antimicrobial resistance and interfering with the host immune response so that they can become accustomed to their extreme surroundings. Biofilms may be of a single species or of an assorted gathering of microorganisms [Tortora et

al., 2011]. Quorum sensing or chemical correspondence within cells of this biological system called biofilm permits the bacteria to organize their movement and form communities. Inside of a biofilm, the bacteria can share supplements and are shielded from perilous environmental factors, for example, parching, antibiotics and the immune system of the body. The nearby vicinity of microorganisms inside of a biofilm may likewise have the benefit of exchanging the hereditary data by, for instance, conjugation. Biofilms are normally connected to a surface, for example, a stone in a lake, human tooth, bathroom sinks, bathtubs, medical devices, cooling towers or might be available as a flock in sewage treatment and in a filamentous streamer structure in quick flowing streams (Tortora et al., 2011).

Following the antibiotic susceptibility test, the environmental isolates that showed a considerable amount of resistance against most of the antibiotics were checked for biofilm formation. The protocol was modified and followed from [Merritt, J. H., Kadouri, D. E. and O'Toole, G. A. (2005). Growing and Analyzing Static Biofilms]. The optical density was measured afterward to assess the bacterial attachment by measuring the staining of the adherent biomass. This experimental system is a simple high-throughput method used to monitor microbial attachment to an abiotic surface and forming a static biofilm.

- Each of the Salmonella identified isolates collected from different environmental samples and the clinical positive strain *Pseudomonas aeruginosa* was inoculated in separate test tubes of 5ml of Luria-Bertani (LB) media and grown to stationary phase respectively.
- Each of the cultures was diluted in fresh Luria-Bertani (LB) media to 1:10, 1:100 and 1:200 in three different micro-centrifuge tubes.
- A fresh 96-well microtitre plate was taken that has not been tissue culture treated.

A	B3	B2	B1	WB	WT	-ve	+ve	WH	H3	H2	H1
B	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10
C	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10
D	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100
E	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100
F	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200
G	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200

- After that, to grow and analyze static biofilms the plates were covered and incubated at 37°C for 7days.

After one week the biofilm was stained before measuring the optical density:

- Following the incubation, for one week the biofilms formed in the plate were observed.
- Four small trays were arranged in a series and 1 to 2 inches of distilled water was added to the last three. The first tray was used to collect waste, while the others are used to wash the assay plates.
- To remove the planktonic bacteria from each microtiter dish shake the dish out over the waste tray.
- To wash the wells, the plates were first submerged in the first water tray and then the water was vigorously shaken over the waste tray. The water was replaced when it becomes cloudy.
- 125µl of 0.1% crystal violet solution to each well and stained for 10 minutes at room temperature.
- After that, the microtitre dish was shaken over the waste tray to remove the crystal violet solution. The plate was washed successively in each of the next two water trays and shaken thoroughly. This step removed any crystal violet that did not specifically stain the adherent bacteria.
- The microtiter dish was inverted and vigorously tapped on a paper towel to remove any excess liquid. The plates were allowed air-dry.
- After the plates were completely air dried, 200µl of 30% acetic acid solvent was added to each stained well and allowed solubilize by covering the plates and incubating 10 to 15 min at room temperature.
- The contents of each well were mixed briefly by pipetting, and then 125 µl of the crystal violet/acetic acid solution from each well was transferred to a separate well in an optically clear flat-bottom 96-well plate.
- Lastly, the optical density (OD) of each of these 125-µl samples was measured at a wavelength of 620 nm using an ELISA machine (Finland).

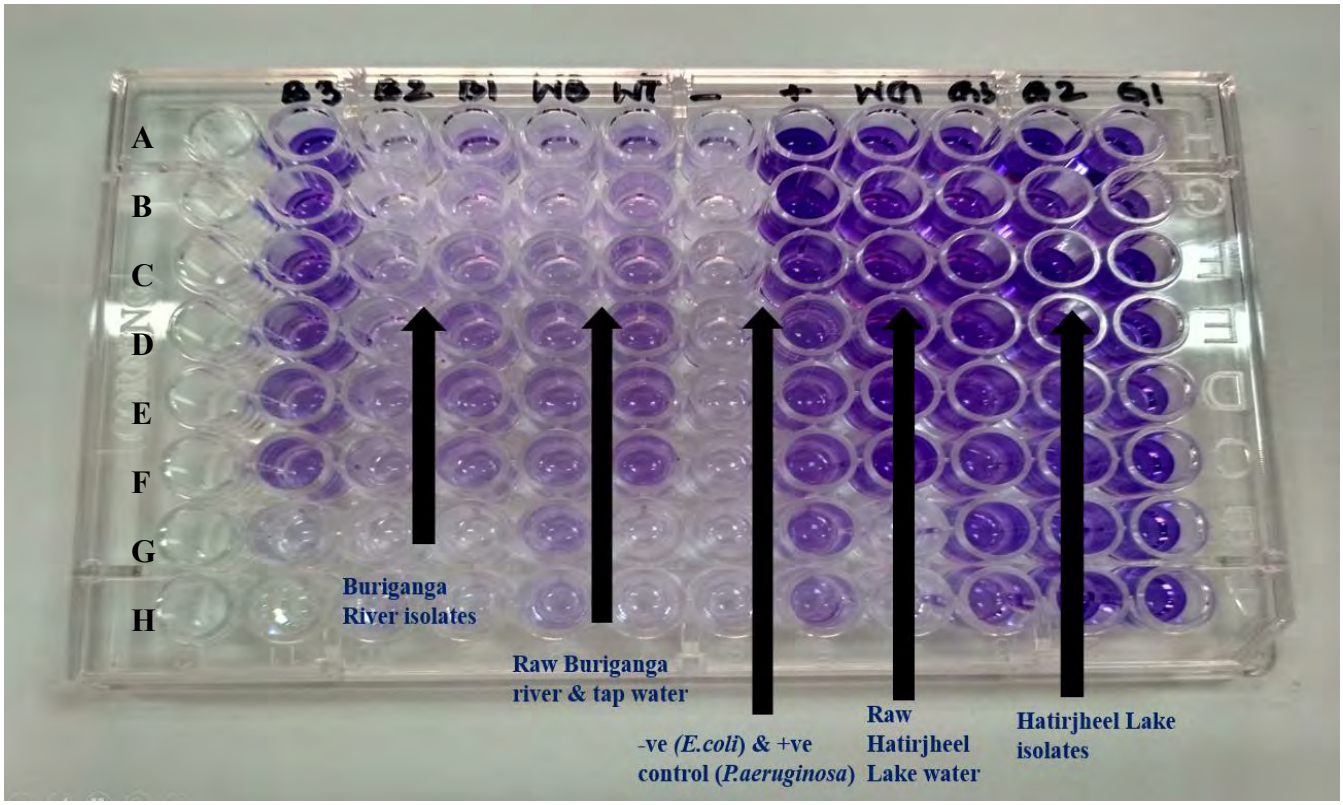

Figure 5: Static Biofilm formation in a 96 well microtitre plate.

Chapter 3

Results

3.1 Qualitative analysis:

The highest temperature recorded was from Hatirjheel Lake at 29.4^{0}C while the lowest was 20.8^{0}C. On the other hand, the temperature in the Buriganga River remained almost constant at 25^{0}C. The pH in both water bodies remained with a range of 6 to 7.

3.2 Identification:

The table below is the Biochemical test chart. More than 80% of the total isolates showed the positive result which concludes that the positive isolates are presumptive Salmonella. A total of 40 samples were collected from Hatirjheel Lake out of which 30(75%) isolates were presumptively positive for Salmonella. On the other hand, 40 samples were collected from Buringanga river where 33(82.5%) were presumptively positive for Salmonella.

Table 3: Biochemical test chart. (R = red, B = black)

Sample No.	*Sample*	*Gram Staining*	**TSI**				**MIU**			*Citrate*	*Methyl Red*	*Voges Proskauer*	*Catalase*
			Slant	*Butt*	*Gas*	*H_2S*	*Motility*	*Indole*	*Urease*				
1	HL 8	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
2	HL 9	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
3	HL 10	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
4	HL 13	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
5	HL 17	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
6	HL 18	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
7	HJ20	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
8	HJ21	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
9	HJ22	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
10	HJ24	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve

11	BR2	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
12	BR3	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
13	BR5	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
14	BR7	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
15	BR9	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
16	BR10	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
17	BR11	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
18	BR13	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
19	BR17	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve
20	BR19	-ve	R	B	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve

Table 4: Sampling month, number of samples and percentage of the positive sample.

Month of collection	Sampling site	Number of analyzed samples	Positive sample (%)
November 2017	Hatirjheel Lake	10	30%
December 2017	Hatirjheel Lake	10	40%
January 2018	Hatirjheel Lake	10	60%
	Buriganga River	20	45%
February 2018	Hatirjheel Lake	10	40%
	Buriganga River	20	55%

3.3 Antibiogram

Randomly selected 20 isolates, 10 from lake and river were tested for antibiotic susceptibility against 8 antibiotics. The zones of inhibition were measured and compared with

the standard CLSI chart to determine if they are resistant, intermediate or sensitive. Patterns of antibiogram showed a slight difference between Hatirjheel Lake and Buriganga River. Out of the 8 antibiotics, Norfloxacin, Amikacin, and Gentamycin were the most effective and Cefepime along with Amoxycillin being the least effective for the selected isolates.

Table 5: Antibiotic Resistance pattern in Hatirjheel Lake and Buriganga River. (S = sensitive, I = intermediate, R = resistant)

Sample No	Sample Name	*Norfloxacin*	*Amikacin*	*Cefepime*	*Amoxicillin*	*Ceftriaxone*	*Gentamycin*	*Chloramphenicol*	*Cotrimoxazole*
1.	HJ8	S	S	R	R	R	S	S	S
2.	HJ9	S	S	R	R	S	S	S	I
3.	HJ10	S	S	S	R	S	S	I	R
4.	HJ13	S	S	R	R	S	S	S	R
5.	HJ17	S	S	S	R	S	S	S	S
6.	HJ18	S	S	R	R	S	S	S	S
7.	HJ20	S	S	R	R	S	S	S	S
8.	HJ21	S	S	R	R	S	S	S	S
9.	HJ22	S	S	R	R	S	S	S	S
10.	HJ24	S	S	R	R	S	S	S	S
11.	BR2	S	S	R	R	I	S	I	I
12.	BR3	S	S	I	R	I	S	I	I
13.	BR5	I	S	R	R	S	I	I	S
14	BR7	S	S	R	R	S	S	I	S
15.	BR9	S	S	R	R	S	S	S	S
16.	BR10	S	S	R	R	S	S	S	R
17.	BR11	S	S	R	R	S	S	S	I
18.	BR13	S	S	R	R	S	S	S	I
19.	BR17	S	S	R	R	S	S	S	I
20.	BR19	S	S	R	R	S	S	S	I

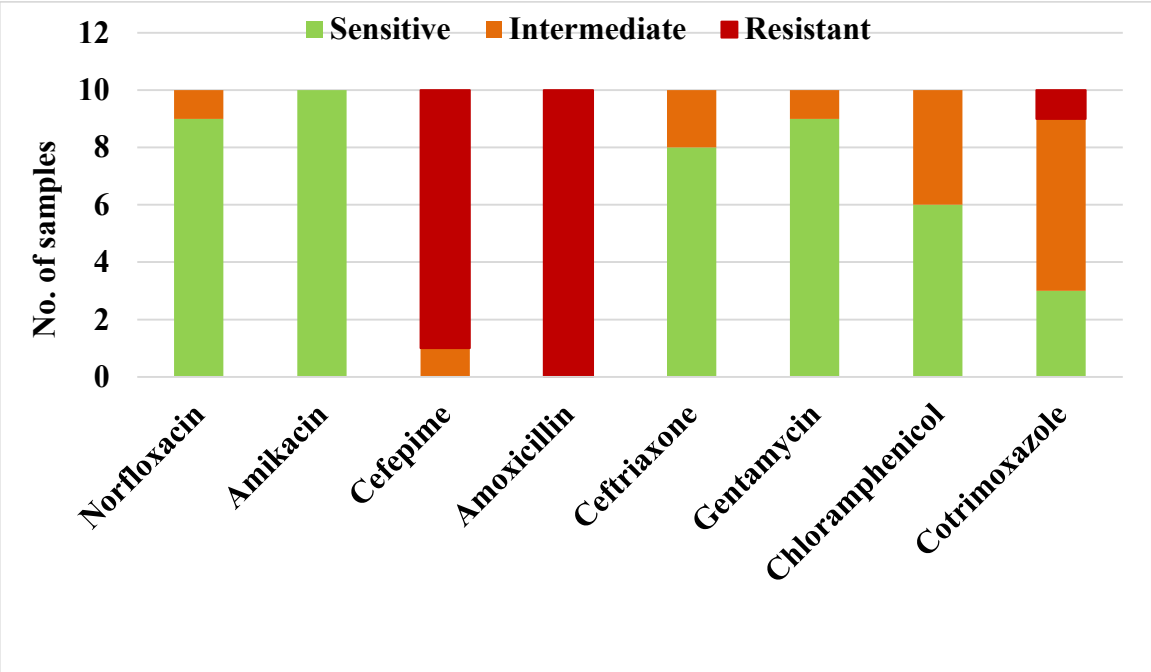

Figure 6: Graphical representation of antibiogram (Buriganga isolates).

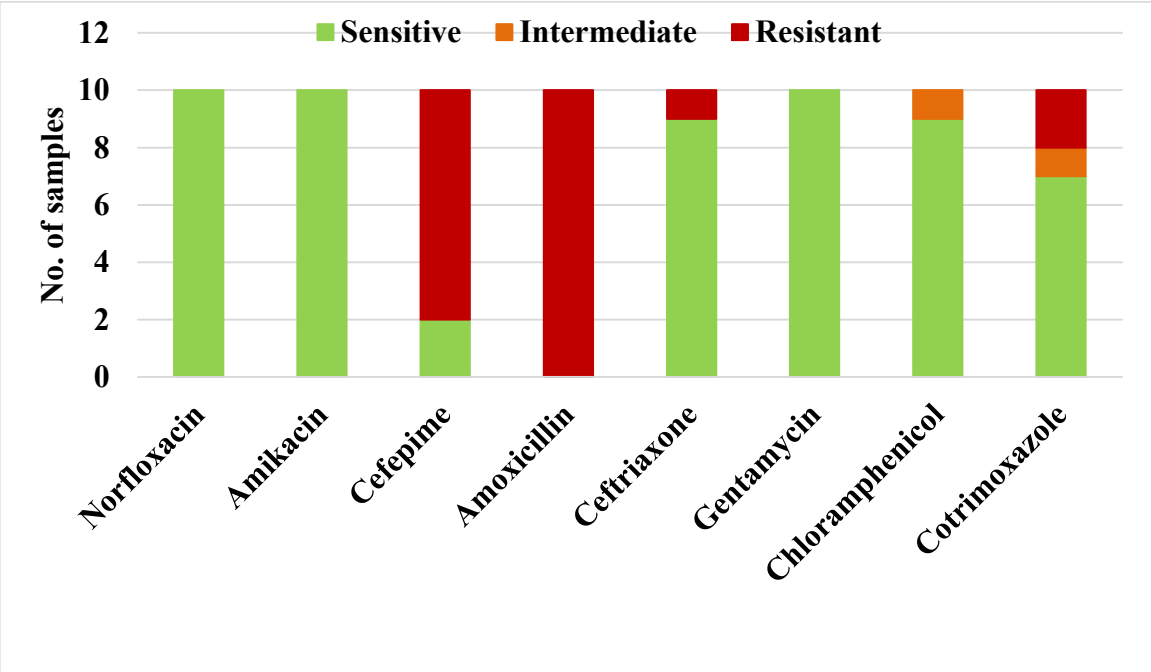

Figure 7: Graphical representation of antibiogram (Hatirjheel isolates).

3.4 Survival Assay

The survival pattern of *Salmonella spp* between Hatirjheel Lake and Buriganga River showed a slight difference in their die-off rate. Both of the isolates decreased in cell number rapidly

within the days observed however, the rate of death for the Hatirjheel isolates was faster than that of Buriganga isolates as shown in the graph below. As a positive control, clinical *S.typhi* strain was used which also showed a similar die-off rate as the other isolates. Cell count was taken by direct plating on selective media XLD.

Colony forming unit/ml = Number of colonies × Dilution Factor/ Volume Plated

A rapid decrease of 2.18 log CFU/ml of the Hatirjheel isolates was seen compared to 3.27 log CFU/ml of the Buriganga isolates on day 3. On the same day, the positive control S. typhi showed a much slower decrease in growth at 3.83 log CFU/ml. From these values, we can say that the overall die-off rate difference is not that significant between the river and lake as well as river and positive control. However, there is a significant difference between the Lake Isolate and positive control.

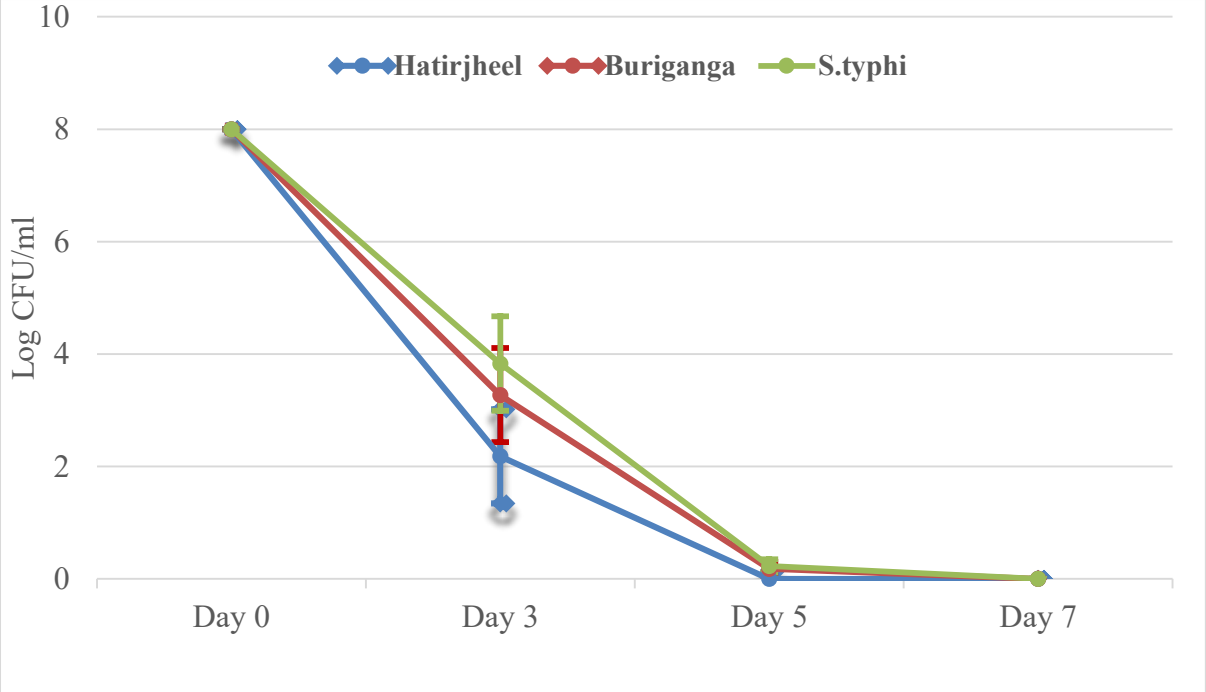

Figure 8: Die-off rate comparison between the isolates of Hatirjheel as well as Buriganga and positive control which shows a gradual decrease in growth.

3.5 Virulence Determination (Molecular Analysis)

After the presumptive identification of the samples, genetic amplification of the pathogenic gene invA was performed using PCR in order to determine whether the isolates present in the environment are carrying this gene and are capable of causing diseases if ingested. The primer used in the PCR mixture was specific for invA which was then run in 1.5% agarose gel. The bands are usually approximately ~290 bp long DNA. Majority of the isolates gave

positive bands for the specific gene which means that the isolates present the surface of the water bodies might be pathogenic *Salmonella* spp.

Figure 9: Agarose gel electrophoresis of isolates from Hatirjheel Lake showing positive bands for primer specific *inv*A gene.

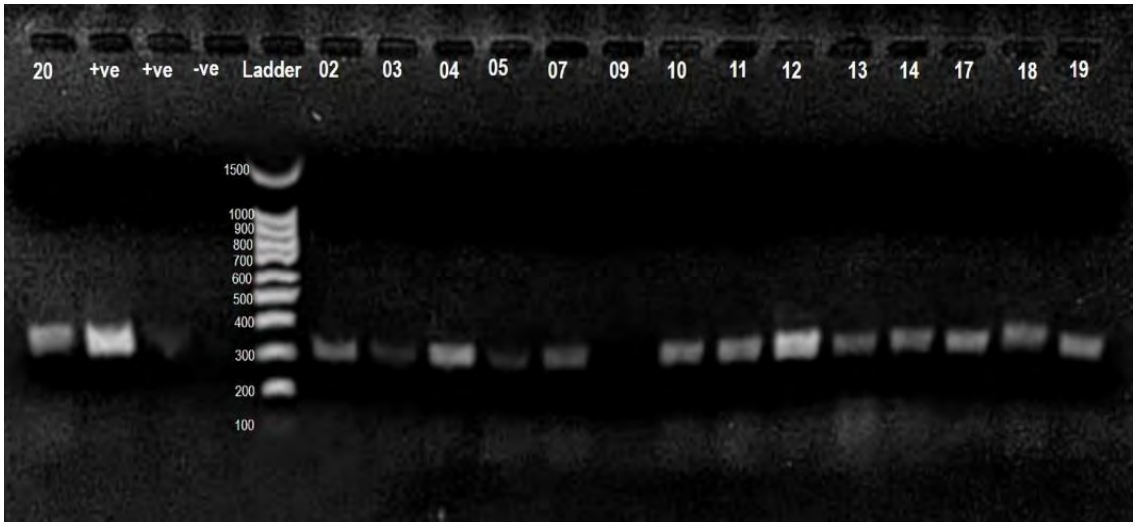

Figure 10: Agarose gel electrophoresis of isolates from the Buriganga River showing positive bands for primer specific *inv*A gene.

3.6 Biofilm Formation

The Buriganga river isolates were more capable of forming static biofilm compared to that of Hatirjheel Lake isolates. On a raw concentration (equivalent to 1 Mcfarland standard), isolates B1 gave an absorbance of 0.73 nm; B2 an absorbance of 0.65 nm and B3 an absorbance of 0.266 nm respectively. On the other side, on raw concentration Hatirjheel isolates H1 showed an absorbance of 0.129 nm; H2 absorbance 0.31 nm and H3 absorbance 0.259 nm respectively. However, it was observed that at lower concentrations .ie. 1:200

dilution, Hatirjheel isolates were more capable of static biofilm formation than that of Buriganga. This indicates that Hatirjheel isolates are likely to survive longer and thrive at lower concentrations. Nonetheless, the ability of these isolates to form biofilm confirms that these environmental isolates are pathogenic and likely to cause diseases.

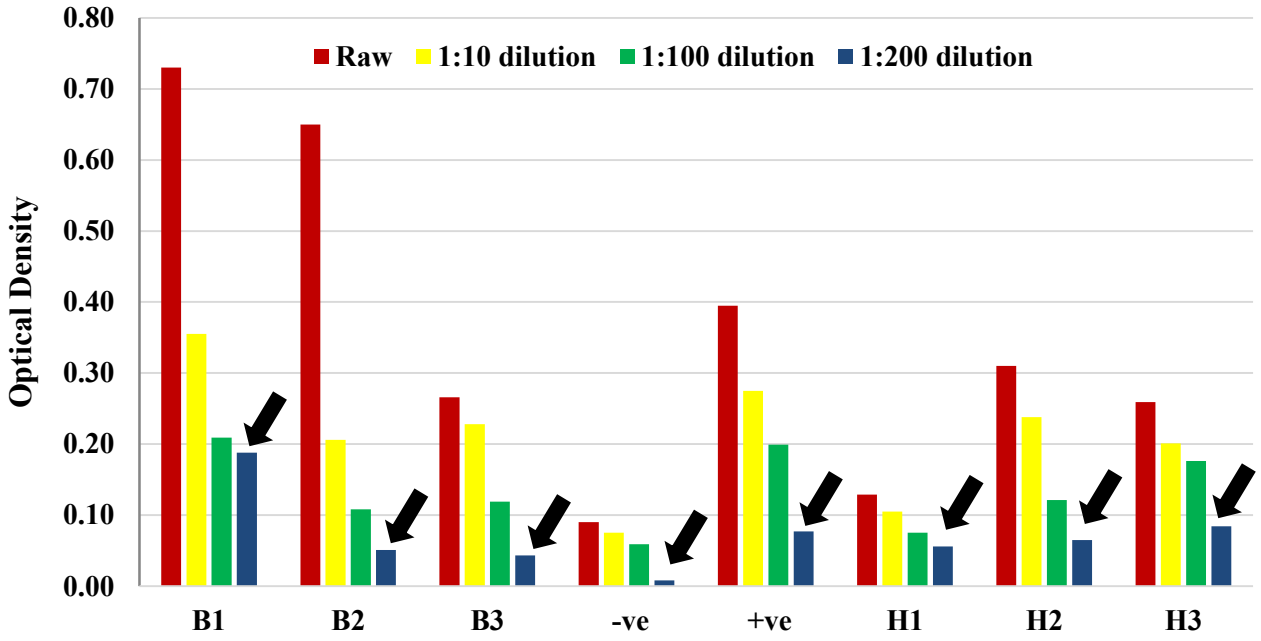

Figure 11: Graphical representation of Biofilm formation.

Chapter 4

Discussion

The purpose of this study was to isolate and identify the presence of *Salmonella* spp from prominent water bodies in the Dhaka city; Hatirjheel Lake and Buriganga River. Salmonella the causative organism for typhoid is both food-borne as well as a waterborne organism. Due to rapid unplanned urbanization and industrialization, the water bodies are getting polluted and typhoid is becoming endemic in Bangladesh (Shumy et al., 2015). This is why it is of utmost importance for us to know whether the water bodies are contaminated with *Salmonella* or not. The overall incidence of typhoid in Bangladesh was 3.9/1000 persons/year and the rate was higher in preschool children aged between 0 and 4 years according to a community-based study (Dewan et al., 2013).

Following the isolation of presumptive *Salmonella*, the isolates were further examined for the presence of *Salmonella* specific virulence gene by performing gene-specific PCR using the primer *inv*A. *inv*A is a member of genetic locus *inv*, and it is the first gene of an operon consisting of at least two additional invasion genes (Galan et al., 1992). 37 out of 63 isolates gave a positive band for the specific pathogenic gene in which 17 was for Hatirjheel lake and 20 for Buriganga river respectively. This means that the organism which was found from the lakes and rivers are pathogenic and has the ability to infect humans. If these waters are used and somehow enters our body it has the potential to cause enteric diseases.

Physiochemical parameters of the water such as pH and temperature were measured. The average temperature for Hatirjheel Lake was 25.8^0C while for Buriganga River it was 25.0^0C. The average pH for lake water was 7.0 while the pH for river water was 6.74. According to the standard chart (Islam et al., 2015) both values fall within the range.

A recent study revealed that typhoid fever was endemic in urban areas in Bangladesh with a high incidence of multi-drug resistant strains (Dewan et al., 2013). After identification of the organisms, their antibiogram was performed to determine their resistance pattern.

Norfloxacin, Amikacin, and Gentamycin are the drug of choice for typhoid, according to the antibiogram, all the isolates in the river are showing sensitive zones for Amikacin while Norflaxacin and Gentamycin show both sensitive as well as intermediate zones. Furthermore, these antibiotics show sensitivity (>80%) in the lake isolates as well**.** This means that in case of potential infection from the water bodies these drugs are likely to be effective in the treatment of the disease. However, >80% of the isolates were resistant against Cefepime and Amoxicillin which marks them ineffective against treatment should waterborne Salmonella infection happen. This can be a result of the increased exposure to various stresses in river

water from the various sources. The effluents from industrial wastes contain metals such as mercury and chromium which can trigger the development of metal resistance along with various antibiotic resistance (Baker-Austin et al., 2006).

For further analysis of the antibiotic resistance, the ability of these isolates to form static biofilm was observed. Bacterial biofilms were first reported in 1978 when they were observed both in the environment and under conditions associated with chronic human infections. Decades of research have now showed that these biofilms can greatly improve bacterial resistance to antimicrobials and host immune molecules. Biofilms are a unique and dynamic bacterial growth environment associated with altered gene expression profiles and increased horizontal transmission of resistance elements, facilitating adaptation to hostile environments. Such functions enhance bacterial growth in difficult host niches and aid the establishment of chronic infections. Biofilm also allows bacteria to survive in hostile conditions such as exposure to UV light, metal toxicity, acid exposure, dehydration and salinity, phagocytes, and several antibiotics and antimicrobial agents.

It was observed that at raw concentration Buriganga isolates were more capable of forming biofilm than that of Hatirjheel Lake. However, at lower concentration (1:200) Hatirjheel isolates formed more biofilm than that of Buringanga. This points out an important fact that though Buriganga isolates form more biofilm at raw concentration but at lower concentration Hatirjheel isolates are likely to thrive and survive for a longer period of time. Putting aside little differences, all these results point out to the ultimate fact that the ability to these isolates to form biofilm alone makes them a pathogenic posing a threat to human health. Many studies have proved connections between biofilm formation by nontyphoidal *Salmonella* strains, antimicrobial resistance, and its persistence in food animals and throughout the food chain.

Inexpensive and readily available antibiotics including ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, and streptomycin are ineffective which led to the emergence of multidrug-resistant (MDR) strains of *S.typhi*. Although resistance to ciprofloxacin, a second-generation fluoroquinolone, is increasing, it is still recommended as first-line therapy for children and adults. Complete antibiotic resistance was seen against the antibiotics: amoxicillin and cefepime. Previous reports on clinical strains (please refer to table 2) have shown that this resistance can be carried in the main genome of *Salmonella* SGI.

Genomic islands are relatively large DNA segments, acquired via horizontal gene transfer, carrying a suite of genes to confer unique functions such as antibiotic resistance, fitness, new

metabolic pathways, pathogenicity, and symbiosis that are beneficial to the survival of bacteria in the environment. *Salmonella* genomic islands SGI1 to SGI10 have been designated with respect to the DNA segments associated with antibiotic resistance. In the past decades, several chromosome-mediated multidrug resistances have been identified in *S. enterica*. (Chiou et al., 2015).

Salmonella genomic island 1 (SGI1) harbors an antibiotic resistance gene cluster and was previously identified in the multidrug-resistant *Salmonella typhimurium DT104, Agona, Paratyphi B,* and *Albany*. This antibiotic resistance gene cluster is a complex class 1 integron and most often confers resistance to ampicillin (Ap), chloramphenicol (Cm)/florfenicol (Ff), streptomycin (Sm)/spectinomycin (Sp), sulfonamides(Su), and tetracycline(Tc) (ApCmFfSmSpSuTc profile). The occurrence of SGI1 in different *S. enterica* serovars, now including serovar *Newport,* strengthens the hypothesis of horizontal transfer of SGI1. (Fabre et al. 2004).

In a study using MDR *S. typhi* isolates from Bangladesh showed that the strains contain designated *Salmonella* genomic island 11 (SGI11) which consists of transposons flanked by perfect 9-bp direct repeats in the insertion site and comprised 27 open reading frames (ORFs), including genes involved in antimicrobial resistance beta-lactamases, chloramphenicol, streptomycin A,B, sulphonamides and trimethoprim (*bla*TEM-1, *catA1*, *strA*, *strB*, *sul1*, *sul2*, and *dfrA7*) and mercury resistance (*merD*, *merA*, *merC*, *merP*, *merT*, and *merR*) genes (Chiou et al., 2015).

Presence of antibiotic resistance gene in chromosomal DNA is not uncommon even though, in most cases it is carried on and passed by plasmids. A study on multi-drug resistant *S.typhi* was performed in Asia where all the isolates were resistant to five widely used antibiotics in this region: ampicillin, co-trimoxazole, chloramphenicol, tetracycline, and streptomycin. This resistance in each case was associated with self-transferable 98- MDa plasmids (Mirza et al., 2000).

Integrons are mobile genetic elements that can integrate into chromosomes and plasmids, by site-specific recombination, thereby providing antibiotic resistance. For instance, a recent study showed that in *S.typhi* resistance to ampicillin, chloramphenicol, tetracycline, trimethoprim, and sulfonamides was transferred from each of the strains harboring the integron to *E. coli*. All the transconjugants contained the class 1 integron and plasmid which was detected by PCR, indicating that the integron was borne on this element (Chainier et al.,

2003). Several cases were observed in which the chromosomal DNA contained the integrase gene along with the resistance gene as found in (Casin, Breuil, et al., 2018).

Mutation might be another possible reason for resistance to antibiotics. A project in China showed that *Salmonella typhimurium* has acquired resistance to antibiotic ciprofloxacin which occurred due to a point mutation in GyrA (Cui et al., 2008).

Thus, chances are that the antibiotic resistance genes of the isolates might be present in the chromosome of the organisms. This phenomenon can happen due to reasons discussed above but are yet to be proven to be present in environmental strains. However, a lot of MDR clinical strains have shown these patterns over the past few years. *Salmonella* strains harboring an MDR genomic island may be more virulent and have a tendency to rapidly disseminate (Chiou et al., 2015). Should this be the case, then these virulent organisms in the environment pose a great threat to public health issues and should be considered for remedy immediately. Awareness and prevention of pollution in nearby water bodies should be spread among the people.

It was hypothesized that the characteristics of the organisms will be different due to the fact that the rate of pollution and contaminants vary between the water bodies. The organisms are constantly under pressure from the pollutants present in the water. In regard to the fact that the rate of pollution and contaminants vary between the water bodies, these pollutants are likely to affect the adaptability of the organisms in a different way. This difference might be in the form of genetic mutation or natural selection for surviving in diverse conditions. The antibiogram results demonstrated the initial characterization of the isolates and further characterization was done by observing the die-off rate of the selected isolates. The die-off rate was tested by placing them in autoclaved tap water and taking their viable count by spread plate method. The autoclaved tap water provides a nutrient-free media that acts as a source of starvation stress for the organisms. As depicted the organisms showed a rapid decline in growth over 7 days observed. The rate of death was faster for lakes isolates than the river isolates by a significant amount. Comparing the isolates of the lakes with positive control shows a large difference between them which is insignificant between the isolates of the river and the positive control. This shows that the pollutants might be affecting the survival rate of *Salmonella* and should these pollutants spread and contaminate other water bodies it could also affect the organisms present there. Therefore, our next target should be to determine what pollutants are causing this change of survival pattern in water so that its spread can be prevented.

Conclusion and Future direction

Typhoid fever occurred in more than 20 million people in the year 2000 and annually causes approximately 200000 deaths. Previous studies have established the fact that surface water of lakes and rivers inside Dhaka city are polluted. However, studies on the presence of pathogenic organisms such as *Salmonella* in these waterbodies is lacking. For this very reason, this study was done which focused on identifying the presence of *Salmonella* in Hatirjheel Lake and Buriganga River and characterizing if the pollutants are actually affecting their survival, the ability to form static biofilm and most importantly; their virulence. Their survival pattern showed that under stress organisms from both lake and water die within a week and the ability to form static biofilm confirmed that even at lower concentrations, they are quite persistent. The isolates from these waterbodies contained the virulent gene invA, which makes it safe to say that they are pathogenic and are capable of causing waterborne diseases. In addition, this paper speculated on the fact that the antibiotic resistance shown by these organisms might be transferring horizontally via chromosome which opens a new paradigm to the public health sector.

In the future, this work can be extended by performing the antibiogram and survival assay for all isolates. More controls can be included when observing biofilm formation with more isolates. Finally, more work should be done on the molecular level to figure out the modes of resistance transfer within these environmental isolates especially if chromosomal transfer of gene occurs within the genomic island of *Salmonella* via various mobile genetic elements such as integrons, transposons or plasmid.

Chapter 5

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Apendix

Media compositions

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

All the media used were from Himedia

XLD Agar:

Composition	**Amount (g/L)**
Yeast Extract	3.0
L-Lysine	5.0
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.0
Sodium deoxycholate	2.5
Sodium thiosulfate	6.8
Ferric ammonium citrate	0.80
Phenol red	0.80
Agar	15.0
Final pH at 25^0C	7.4±0.2

SS Agar:

Composition	**Amount (g/L)**
Proteose Peptone	5.0
Lactose	10.0
Bile salts mixture	8.5
Sodium citrate	8.5
Sodium thiosulfate	8.5
Ferric citrate	1.0
Brilliant green	0.00033
Neutral red	0.025
Agar	13.5
Final pH at 25^0C	7.0±0.2

Nutrient Agar:

Composition	Amount (g/L)
Peptone	5.0
Sodium Chloride	5.0
Beef extract	3.0
Agar	15.0
pH	7.0

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

Luria Bertani Agar Miller:

Component	Amount (g/L)
Casein enzymic hydrolysate	10.0
Yeast extract	5.0
Sodium chloride	10.0
Agar	15.0
Final pH at 25^0C	7.5±0.2

Luria Bertani Broth:

Component	Amount (g/L)
Casein enzymic hydrolysate	10.0
Yeast extract	5.0

Sodium chloride	10.0
Final pH at 25^0C	7.5±0.2

Mueller- Hinton Agar:

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

Saline:

Component	**Amount (g/L)**
Sodium chloride	9.0

Motility, Indole, Urease Agar:

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

Simmons Citrate Agar:

Component	**Amount (g/L)**
Magnesium Sulfate	0.02%
Sodium chloride	0.5%

Sodium Citrate	0.2%
Di potassium Phosphate	0.1%
Mono potassium phosphate	0.1%
Bromothymol Blue	0.008%
Agar	2%
pH	7

Triple Sugar Iron (TSI):

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

Indole Broth:

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

Methyl Red and Voges Proskauer Media (MR-VP):

Component	**Amount (g/L)**
Peptone	7.0
Dextrose	5.0

Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Reagents and Buffer

Gram's iodine (300 ml)

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

Crystal Violet (100 ml)

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

Safranin (100ml)

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

Kovac's Reagent (150 ml)

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

Methyl Red (200 ml)

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

Barrit's Reagent A (100 ml)

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

Barrit's Reagent B (100 ml)

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

Catalase Reagent (20 ml 3% hydrogen peroxide)

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

Urease Reagent (50 ml 40% urea solution)

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

1M Tris HCl:

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

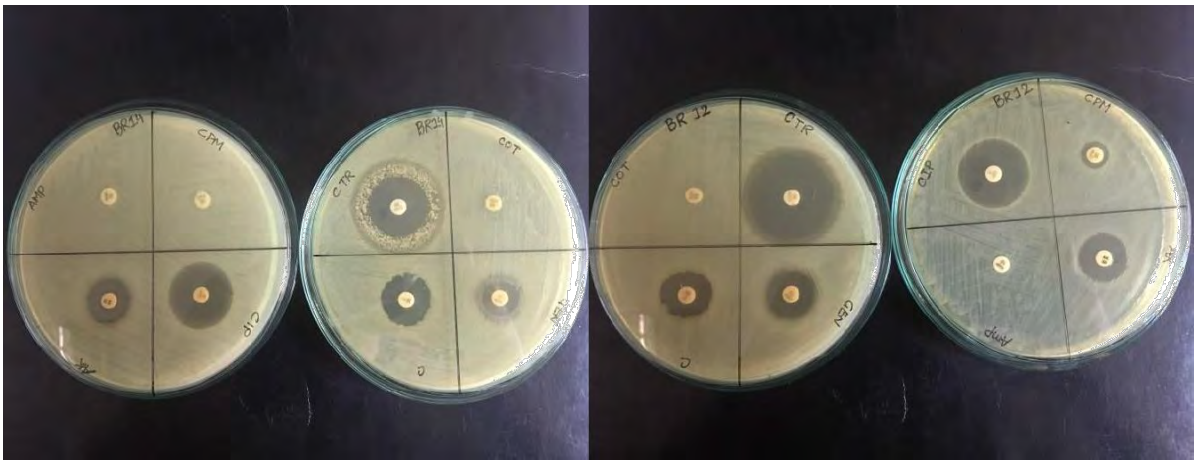
0.5M EDTA:

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 Materials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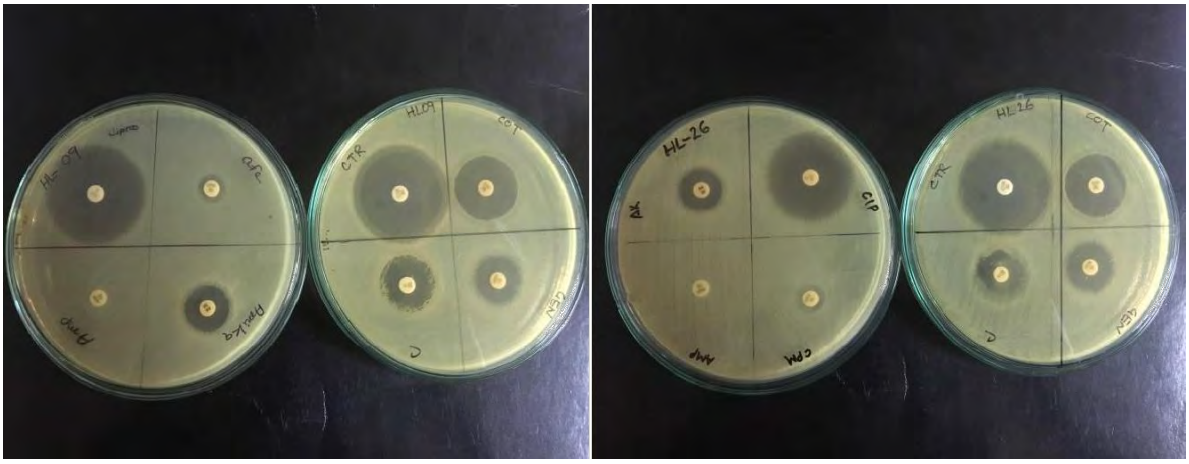
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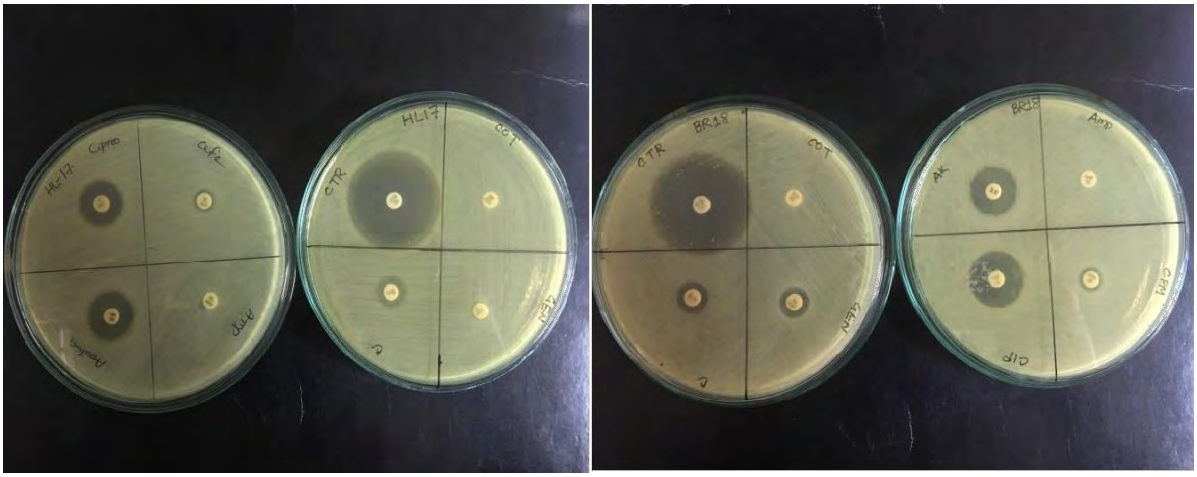
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