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

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Declaration

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

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

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Abstract

Unplanned urbanization often results in the surface waters getting contaminated with industrial and faecal pollutants. The wide spectrum of industrial pollutants put pressure on the faecal organisms during their survival in these polluted waterbodies. Although such pressure is commonly known to result in shorter survival of these organisms which can be pathogenic to humans, its effect on the virulence indicators is not fully understood. This study attempts to detect the presence and responses of *Salmonella* in waterbodies contaminated with industrial pollutants. A lake (Hatirjheel) receiving low level of industrial pollutants and a river (Buriganga) receivng high level of industrial pollutants within Dhaka city were selected for sampling. A total of 40 water samples were aseptically collected from Hatirjheel Lake and 20 samples from Buriganga River within a duration of three months. Then they were processed for isolation of cultureable *Salmonella*, before checking for *Salmonella* specific virulence gene *invA*. After that, randomly selected 5 strains from each were checked for their susceptibility to an array of eight antibiotics. Further, randomly selected three strains from each lake were tested for survival potential under starvation stress. Across the study period, 40% of the sample collected from the lake and 70% of the sample collected from the river yielded culturable *Salmonella* all of which contained *invA* gene. The antibiogram revealed that isolates from Buriganga showed complete resistance to ampicillin, co-trimoxazole and cefepime while the Hatirjheel isolates showed complete resistance to only ampicillin and cefepime. The survival patterns between the lake and river showed slight difference but both showed a gradual decrease in culturable count over the time. 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 antibiotic resistance of *Salmonella*. To the best of our knowledge, this is the first study on focusing on *Salmonella* in the surface waters of Dhaka city.

Table of Contents

Chapter	Title	Page No.
Chapter 1	**Introduction**	1
Chapter 2	**Materials and Methods**	**7**
2.1	Water sampling	8
2.2	Processing of Water Sample	8
2.3	Description of Sampling Sites	8
2.4	Identification	10
2.5	Virulence determination	12
2.5.1	DNA extraction by boiling method	12
2.5.2	Polymerase Chain Reaction (PCR)	13
2.5.3	Gel conditions	13
2.6	Determination of the Antibiotic Resistance Pattern of the Target	14
2.6.1	Kirby-Bauer Disc diffusion	14
2.6.2	Plasmid profiling	15
2.7	Survival Assay	16
2.7.1	*Salmonella* culture and preparation of inoculum	17
2.7.2	Preparation of microcosm	17
2.7.3	Enumeration of survivors	17
Chapter 3	**Results**	18
3.1	Qualitative analysis	18
3.2	Identification	18
3.3	Virulence determination	22
3.4	Antibiogram	23
3.5	Plasmid profiling	25
3.6	Survival assay	26
Chapter 4	**Discussion**	27
Chapter 5	**References**	33

List of Figures:

Figure No.	Heading	Page No.
Figure 1	Compliance of Buriganga River water quality parameters with DOE guidelines (Rahman and Bakri)	4
Figure 2	Hatirjheel Lake sampling site.	9
Figure 3	Buriganga River sampling site	9
Figure 4	Microcosm prepared for isolates	17
Figure 5	Agarose Gel Electrophoresis of isolates from Hatirjheel Lake showing positive bands for primer specific *inv*A gene	22
Figure 6	Agarose Gel Electrophoresis of isolates from Buriganga River showing positive bands for primer specific *inv*A gene	22
Figure 7	Graphical translation of the susceptibility and resistance pattern of the Buriganga isolates to different antibiotics	24
Figure 8	Graphical translation of the susceptibility and resistance pattern of the Hatirjheel isolates to different antibiotics	24
Figure 9	Agarose gel electrophoresis of isolates showing respective band patterns for plasmids.	25
Figure 10	Die-off rate comparison between the isolates of Hatirjheel as well as Buriganga and positive control which shows a gradual decrease in growth	26

List of Tables

Table No.	Heading	Page No.
Table 1	Primer used for gene-specific PCR	13
Table 2	Antibiotic Resistance mode of transmission	14
Table 3	Biochemical Test Chart	19
Table 4	Sampling Month, number of samples and percentage of positive samples	21
Table 5	Antibiotic Resistrance Pattern in Hatirjheel Lake and Buriganga River	23

Chapter 1

Introduction

1.0 Introduction

Water is essential for survival. An adequate, safe and accessible water supply must be available to every individual in order to be healthy (Edition and First). In developing countries, the lack of access to clean water as well as proper sanitation system leads to various waterborne diseases; acute microbial diarrheal diseases being the major public health problem (Access). The people affected are those with the lowest financial resources and poorest hygienic facilities (Access).

Two and a half billion people have no access to improved sanitation, and more than 1.5 million children die each year from diarrheal diseases. According to the WHO, the mortality of water associated diseases exceeds 5 million people per year. From these, more that 50% are microbial intestinal infections. Worldwide it is seen that, 88% percent of cases of diarrhoea are attributable to unsafe water, inadequate sanitation or insufficient hygiene (Bos, Gore, & Bartram, n.d.).

Diarrhoea is cause by the ingestion of pathogens and involves some serious diseases such as cholera, typhoid and dysentery (Bos et al.). Enteric fevers, typhoid and paratyphoid fever are severe, contagious systemic diseases caused by the infection of the serovars *Salmonella* Typhi and Paratyphi (Levantesi et al.). Salmonellae are constantly found in environmental samples. Their presence in natural water is mainly due to municipal sewage, agriculture pollution, and storm water runoff (Access). Enteric fever is estimated to affect over 37 million people every year.

Water contaminated with faeces of human cases and carriers is one of the main vehicles of typhoid fever infections. 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.) and due to its ability to survive for weeks outside the host intestine (Access).

Surface water refers to river, streams, lakes, ponds and reservoirs. During rainfall or storm the runoff water flows downhill and carries a diverse mix of pollutants along with it into the waterbodies.

If pollution occurs from a single location such as discharge pipe attached to a factory or industrial discharges it is called point source while non-point sources are diffusive sources of pollutants such as runoff water from nearby area into lakes. The construction of housing,

commercial buildings, roads and other infrastructure, reduces the permeability of the surrounding land, and so alters the hydrological characteristics of storm water flowing to those lakes and rivers. As a result, runoff volumes increase carrying more discharges into the waterbodies (Unless et al.). Overall, urbanization and industrialization exert pressure on the quality of water resources (Rabbani et al.).

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

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.). Hatirjheel, Gulshan Lake, Banani Lake, Ramna Lake and Dhanmondi Lake are the popular relaxation spots in the city, is being polluted by slums and sewages, the business firms and industries operating in the area.(Miah et al.) Hatirjheel lake plays a vital role in maintaining the only drainage system of those areas (Miah et al.). As a result of water pollution, the nearby local communities often suffer from diarrhea, skin diseases, gastric ulcers, respiratory illness, anaemia, high blood pressure and jaundice (Ullah et al.).

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.).

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).

The figure demonstrates 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.). 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 and Bakri). COD is an important parameter that measures the total quantity of oxygen required to oxidise all organic material into carbon dioxide and water (Masters and 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 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 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 sulphide concentration remains relatively high throughout the seasons increasing more during the wet seasons. The average concentration of DO vary from 1.6 to 3.6ppm when the standard is 5ppm. This

parameter is required for the survival and decomposition of compounds by microorganisms (Islam et al.).

Since the level and sources of pollution in both Hatirjheel Lake and Buriganga River are different, it is most likely to affect the characteristics and occurrence of microorganisms in the waterbodies. 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 venders such as chotpoti and jhalmuri venders 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 *inv*A is a member of this locus, (Galan et al.). There is growing concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance. Plasmid sequencing in many studies have 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.).

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.). 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.). 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 causing typhoid if ingested to a certain limit? Furthermore, due to the differences in location of these waterbodies rate of pollution varies widely which is an important factor in affecting the characteristics of the microorganisms.

According to the literature review it has been established that the waters of Buriganga plus 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 affects the pollutants are having on these organisms. As a result this study focuses on the presence of *Salmonella* in the waterbodies 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 when there can be multiple aspects where this resistance can get transferred and indirectly affect humans. In order to address this issue, antibiogram of the strains are observed in addition to their plasmid profile to determine their mode of resistance. Thus shedding light on how the pollutants are affecting the pathogenicity of the organisms between the lake and river.

Our aim of the study is to detect the level of pollution in the Hatirjheel Lake as well as Buriganga River and whether their occurrence varies or not. According to our hypothesis, the waters will be contaminated with *Salmonella spp* and the contaminants will have different characteristics between the Lake and River.

Chapter 2

Materials and Methods

2.0 Methods and Materials

2.1 Water Sampling:

Water samples were collected aseptically plastic bottles from different points of the Hatirjheel Lake as well as Buriganga River. In order to reach the various points of sampling, small boats and water ferries were used. The sample collection procedure started from the mid October of 2017 and lasted till the end of January 2018. Total of 40 samples were collected from the Hatirjheel Lake while a total of 20 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.). 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 37^0C for 24 hours. Afterwards, 5ml of the buffered peptone was added to 50ml Rappaport Vassiliadis broth (RVB) for selective enrichment of *Salmonella*. The broth was then incubated at 42^0C 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 37^0C for 24 hours. The suspected colonies appeared to have dark centres 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 nutrient broth. After 24 hours incubation at 37°C, glycerol 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.). 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 urbanisation. These two polluted waterbodies have been targeted as the sampling site for this study.

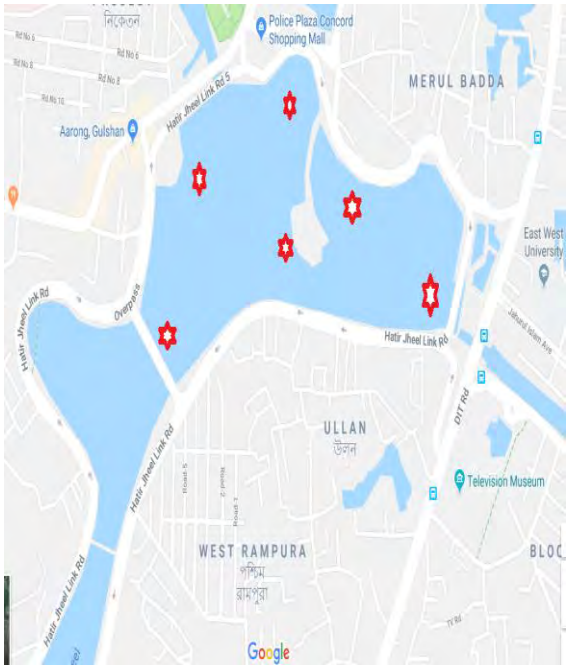

Figure 2: Hatirjheel Lake sampling site.

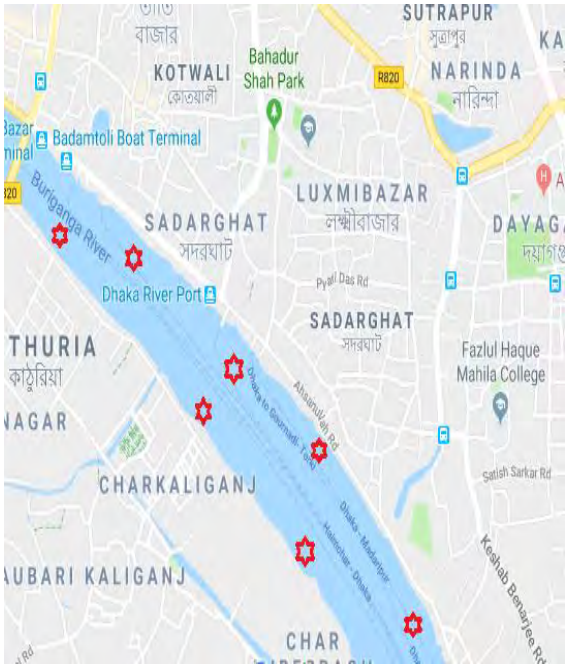

Figure 3: Buriganga River sampling site.

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; 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

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

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 were 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 vials were 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 vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37°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 by

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

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 amount of bacteria from 24-hour pure culture was placed onto the microscopic slide. 1 drop of 3% H2O2 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. *inv*A 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.).

2.5.1 DNA extraction by Boiling method:

The selected isolates were inoculated in nutrient broth and incubated at 37°C for 24h. The next day, 1.5ml of the broth was transferred to Eppendorf and centrifuged at 15,000*g* for 15 min. The supernatant was eliminated, and the pellet was resuspended in molecular biology-grade water (Eppendorf, Hamburg, Germany) and centrifuged at 15,000*g* 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 further

10minutes and centrifuged at 15,000*g* for 10s before it was stored at -20°C. Aliquots of 2 µl of template DNA were used for PCR. (Queipo-Ortuño et al.)

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 thermostable DNA polymerase, primer sequence (complementary to target region) and dNTPs. Aliquots of extracted DNA by boiling method were used as template for the PCR encoding virulent gene *inv*A. Salmonella-specific *inv*A primers (5'GTGAAATTATCGCCACGTTCGGGCAA-3'and 5'-TCATCGCACCGTCAAAGGAACC-3') was used for the detection of Salmonella in this study (Kumar et al.).

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.)

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^{0}C for 2 min, followed by 35 cycles of 95^{0}C for 30 s, 64^{0}C for 30 s, and 72^{0}C for 30 s and a final extension of 5 min at 72^{0}C was employed. (Kumar et al.)

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 1000 bp DNA molecular weight ladder.

Finally, the gel image was viewed under UV light. For each PCR test, a positive (Salmonella enterica serovar Typhimurium) and negative control (sterile dH2O) were used separately along with water sample. (Kumar et al.)

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

2.6.1 Kirby-Bauer Disc diffusion:

The isolates were subjected to antimicrobial susceptibility testing by disk diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI) using commercial

antimicrobial disks. The antibiotic disks used in this study were: Ampicillin (10µg), Chloramphenicol (30µg), Gentamycin (10µg), Ciprofloxacin (5µg), Ceftriaxone (30 µg), Amikacin (30µg), Cefepime (30µg), Chloramphenicol (30µg), Cotrimoxazole (sulfamethoxazole 23.75 µg + trimethoprim 1.75 µg) (Bhattacharya et al.).

The method described by Bauer and Kirby (1969) was followed. An inoculating needle was touched to a freshly grown, well isolated colony on plate and then inoculated into 1 ml of Muller-Hinton Broth (MHB). The culture were then incubated in a shaker at 37°C for 4 hours to obtain the actively growing culture, equivalent to 0.5 McFarland standard ($1.5x\ 10^8$ CFU/mL). A sterile cotton swab was dipped into the standard suspension, excess broth was purged by pressing and rotating the swab firmly against the inside wall of the tube above the fluid. The swab was then streaked evenly in three directions over the entire surface of the agar plate to obtain a uniform inoculum. A final sweep was made of the agar rim with the cotton swab. This plate was then allowed to dry for three to five minutes before the disks were applied. Antibiotic impregnated disks were then applied to the surface of the inoculated plates with sterile syringe needle. All disks were gently pressed down onto the agar with sterile forceps to ensure complete contact with the agar surface. Within 15 minutes after the disks were applied, the plates were inverted and placed in an incubator at 37°C. After overnight incubation, the plates were examined for zone of inhibition and the diameter of the zone of inhibition was measured to the nearest whole millimeter by a ruler. The zone diameters for individual antimicrobial agents were then translated into susceptible, intermediate, or resistant categories according to the CLSI guidelines (2017).

2.6.2 Plasmid Profiling

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

Procedure:

1. The bacterial cells were grown overnight in LB broth overnight.
2. 1.5ml of the broth was transferred to an Eppendorf in order to harvest by centrifugation at 10000 rpm for 2 min (repeated this step 3 times).

3. The pellet was resuspended in Solution 1 (200 µl) and then freshly prepared solution 2 (400 µl) was added; mixed by gentle inversion.
4. The cells were incubated at room temperature for 5 mins for cell lysis.
5. To this, ice cold solution 3 (300 µl) was added and mixed by inversion and incubated on ice for 10 mins.
6. The mixture was then centrifuged at 12000 rpm for 15 mins.
7. The supernatant was transferred in a fresh tube.
8. To this, equal volume of phenol: chloroform: IAA (25:24:1) was added, mixed by vortexing and centrifuged at 12000 rpm for 2 min.
9. The supernatant was carefully collected an equal volume of choloroform : IAA (24:1) was added to it, followed by mixed by vortexing and centrifuged at 12000 rpm for 5 mins.
10. The supernatant was collected and then 0.6 volume of isopropanol was added to this, then mixed by inversion and centrifuged at 12000 rpm for 15 minutes.
11. The DNA pellet was washed in 70% ethanol, air-dried and resuspend in 20 µl of TE buffer.

Separation of plasmid DNA by Gel Electrophoresis

Plasmid DNA was separated by horizontal electrophoresis in 0.7% agarose slab gels in TBE buffer at room temperature at 70 volts for 60 minutes. 8µl of plasmid DNA solution was mixed with 2µl of tracking dye and was loaded into individual well of the gel. The DNA bands were seen under UV light. A positive control was used as known size marker and 10 kbp DNA ladder was used in order to determine the size of the unknown plasmid.

2.7 Survival Assay

In order to carry this assay out, three isolates were randomly selected form each river and lake as 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 waterbodies. 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 37°C for 24 hours. Then two colonies were taken from each sample and inoculated in 10ml LB (Luria Bertani Broth) and incubated at 37°C for 3 hours in a shaking incubator. The optical density of the broths was measured till it reached 0.1 OD at 600nm wavelength using a spectrophotometer. The cultures were then centrifuged at 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 10^8 cfu/ml which was then inoculated in the microcosms (Sugumar and Mariappan).

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 and then 3 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.

Chapter 3

Results

3.0 Results

3.1 Qualitative analysis

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

3.2 Identification:

The following table describes the results of the samples tested for the biochemical tests. Few of the isolates did not show the desired result for all of the 12 tests. However if 80% of the tests gave the desired result the samples were regarded as presumptively positive. All of the isolates gave >80% of desired results thus were concluded to be presumptively *Salmonella* positive.

Table 3: Biochemical test chart.

Sample No.	Sample	Gram Staining	TSI				MIU			Citrate	Methyl Red	Voges Proskaeur	Catalase
			Slant	Butt	Gas	H_2S	Motility	Indole	Urease				
1	**HL 17**	-	R	B	-	+	+	-	-	-	+	-	+
2	**HL 18**	-	R	B	-	+	+	-	-	-	+	-	+
3	**HL 26**	-	R	B	-	+	+	-	-	-	+	-	+
4	**HL 22**	-	R	B	-	+	+	-	-	+	+	-	+
5	**HL 10**	-	R	B	-	+	+	-	-	-	+	-	+
6	**HL 13**	-	R	B	-	+	+	-	-	-	+	-	+

Sample No.	Sample	Gram Staining	TSI				MIU			Citrate	Methyl Red	Voges Proskaeur	Catalase
			Slant	Butt	Gas	H_2S	Motility	Indole	Urease				
7	**HL 21**	-	R	B	-	+	+	-	-	-	+	-	+
8	**HL 09**	-	R	B	-	+	+	-	-	-	+	-	+
9	**HL 31**	-	B	B	-	+	+	-	-	-	+	-	+
10	**HL 30**	-	R	B	-	+	+	-	-	-	+	-	+
11	**HL 24**	-	R	B	-	+	+	-	-	-	+	-	+
12	**HL 39**	-	Y	B	-	+	+	-	-	-	+	-	+
13	**HL 27**	-	R	B	-	+	+	-	-	+	+	-	+
14	**HL 33**	-	R	B	-	+	+	-	-	-	+	-	+
15	**HL 29**	-	B	B	-	+	+	-	-	-	+	-	+
16	**HL 08**	-	R	B	-	+	+	-	-	-	+	-	+
17	**HL 20**	-	R	B	-	+	+	-	-	+	+	-	+
18	**BR 20**	-	R	B	-	+	+	-	-	+	+	-	+
19	**BR 10**	-	Y	B	-	+	+	-	-	+	+	-	+
20	**BR 11**	-	B	B	-	+	+	-	-	-	+	-	+
21	**BR 03**	-	R	B	-	+	+	-	-	-	+	-	+
22	**BR 04**	-	Y	B	-	+	+	-	-	-	+	-	+
23	**BR 17**	-	R	B	-	+	+	-	-	-	+	-	+
24	**BR 14**	-	R	B	-	+	+	-	-	-	+	-	+

25	**BR 05**	-	R	B	-	+	+	-	-	-	+	-	+
26	**BR 09**	-	B	B	-	+	+	-	-	-	+	-	+
27	**BR 19**	-	R	B	-	+	+	-	-	-	+	-	+
28	**BR 13**	-	R	B	-	+	+	-	-	-	+	-	+
29	**BR 12**	-	R	B	-	+	+	-	-	-	+	-	+
30	**BR 02**	-	R	B	-	+	+	-	-	-	+	-	+
31	**BR 07**	-	R	B	-	+	+	-	-	+	+	-	+
32	**BR 18**	-	R	B	-	+	+	-	-	-	+	-	+

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

Month of collection	Sampling site	Number of analysed samples	Positive sample (%)
October 2017	Hatirjheel Lake	5	0
November 2017	Hatirjheel Lake	17	53%
December 2017	Hatirjheel Lake	6	50%
	Buriganga River	10	70%
January 2018	Hatirjheel Lake	12	42%
	Buriganga River	10	80%

A total of 40 samples were collected from Hatirjheel Lake out of which 17 isolates were presumptively positive for Salmonella which means that 43% of the samples were positive. On the other hand, 20 samples were collected from Buriganga River from which 15 isolates were presumptively positive. This accounts for 75% positive samples collected from the River.

3.3 Virulence Determination

After the presumptive identification of the samples, genetic amplification of the pathogenic gene *inv*A 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 *inv*A 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 waterbodies might be pathogenic *Salmonella* spp.

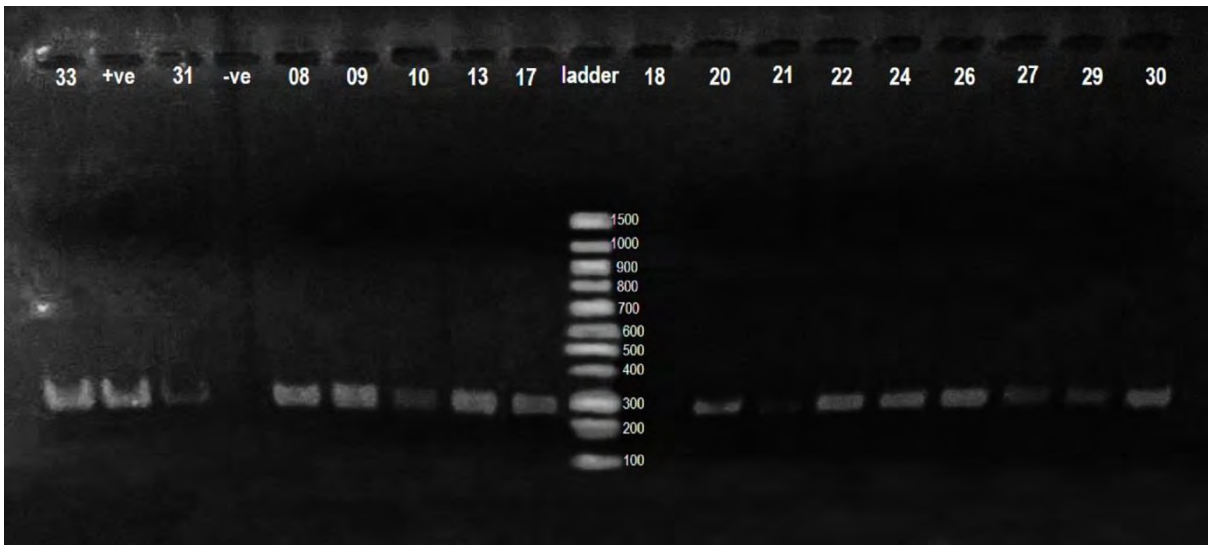

Figure 5: Agarose gel electrophoresis of isolates from Hatirjheel Lake showing positive bands for primer specific *inv*A gene.

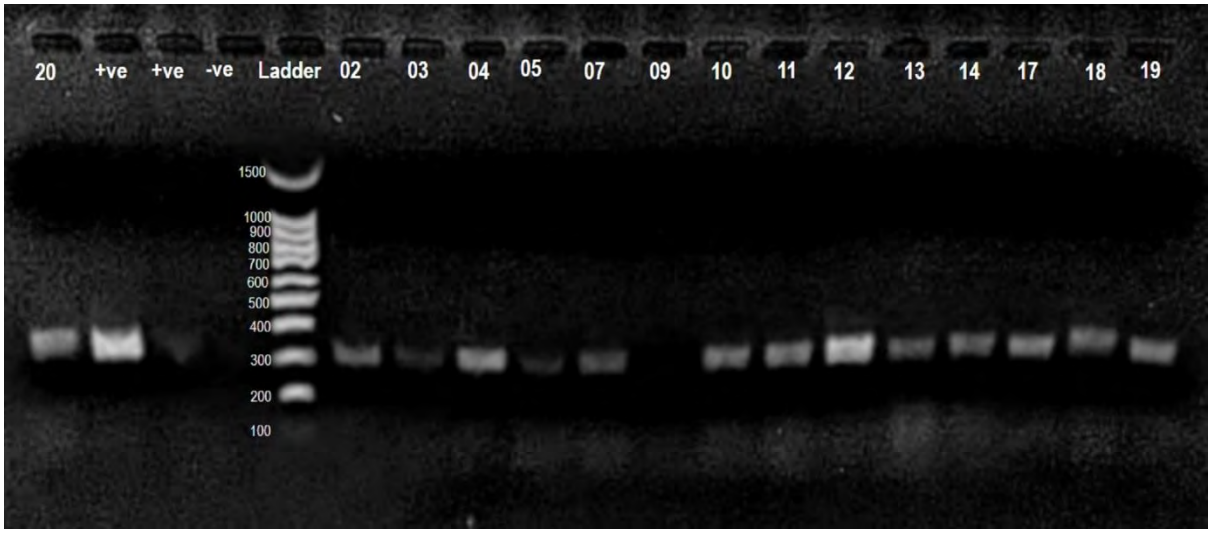

Figure 6: Agarose gel electrophoresis of isolates from Buriganga River showing positive bands for primer specific *inv*A gene.

3.4 Antibiogram

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

Table 5: Antibiotic Resistance pattern in Hatirjheel Lake and Buriganga River.

	Ampicillin	Chloramphenicol	Co-trimoxazole	Gentamycin	Ciprofloxacin	Ceftriaxone	Amikacin	Cefepime		Ampicillin	Chloramphenicol	Co-trimoxazole	Gentamycin	Ciprofloxacin	Ceftriaxone	Amikacin	Cefepime
HL 30	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**BR 18**	**R**	**R**	**R**	**R**	**I**	**S**	**S**	**R**
HL 17	**R**	**R**	**R**	**R**	**R**	**S**	**S**		**BR 17**	**R**	**R**	**R**	**I**	**I**	**I**	**R**	**R**
HL 26	**R**	**R**	**S**	**S**	**S**	**S**	**I**	**R**	**BR 12**	**R**	**S**	**R**	**S**	**I**	**S**	**S**	**R**
HL 09	**R**	**S**	**S**	**S**	**S**	**S**	**I**	**R**	**BR 14**	**R**	**S**	**R**	**I**	**I**	**I**	**I**	**R**
HL 13	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**BR 02**	**R**	**S**	**R**	**S**	**I**	**I**	**S**	**R**

The isolates from Buriganga showed complete resistance against the antibiotics: ampicillin, cotrimoxazole and cefepime. Chloramphenicol, gentamycin and amikacin showed resistance of 25%, 12.5% and 12.5% respectively while ciprofloxacin and ceftriaxone showed no resistance pattern. The isolates were most sensitive to amikacin and chloramphenicol at 75% followed by gentamycin and ceftriaxone at 62.5%. The antibiotic ciprofloxacin gave an intermediate zone for all the isolates while amikacin, gentamycin and ceftriaxone showed 12.5%, 25% and 37.5% of intermediate zone.

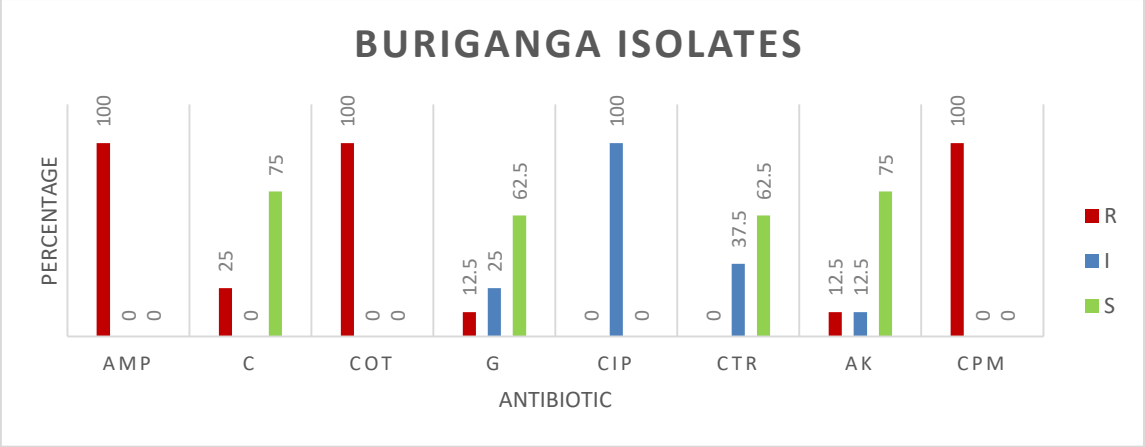

Figure 7: Graphical translation of the susceptibility and resistance pattern of the Buriganga isolates to different antibiotics.

The isolates from Hatirjheel showed complete resistance against ampicillin and cefepime. In Chloramphenicol (25%), cotrimoxazole (12.5%), gentamycin (12.5%) and ciprofloxacin (12.5%) resistance was seen. Amongst all the antibiotics ceftriaxone showed 100% sensitivity followed by cotrimoxazole, gentamycin and ciprofloxacin at 87.5% amikacin and chloramphenicol at 75%. Intermediate zone was seen only against amikacin at 25% of the isolates.

Overall, there was not much difference between the antibiograms however, isolates from Buriganga showed 100% resistance towards co-trimoxazole which was 87% sensitive for the Hatirjheel isolates.

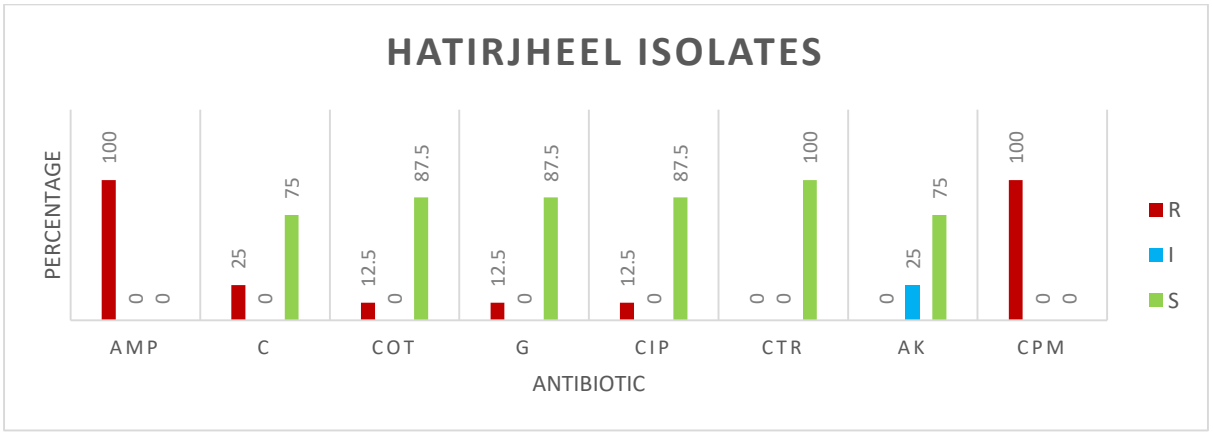

Figure 8: Graphical translation of the susceptibility and resistance pattern of the Hatirjheel Lake isolates to different antibiotics.

3.5 Plasmid Profiling

The isolates showed no bands for plasmid DNA but the positive control showed band. This ensures that the extraction was performed properly which is further ensured by the presence of the chromosomal DNA bands on the gel. Thus, it can be confirmed that the isolates do not carry a plasmid and whatever characteristics they show are present in their chromosomal gene.

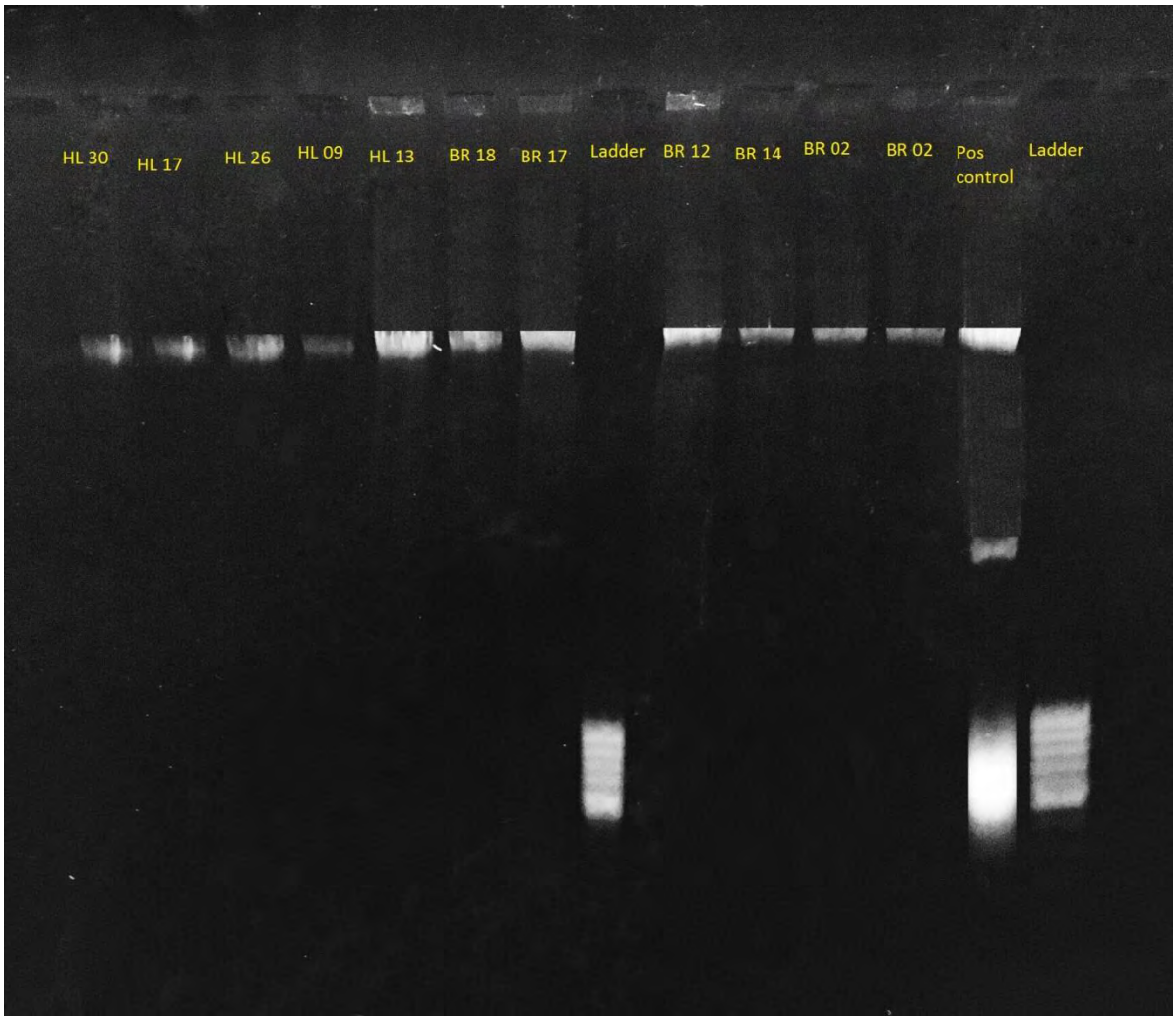

Figure 9: Agarose gel electrophoresis band for plasmid extracted from both Buriganga River and Hatirjheel Lake.

3.6 Survival Assay

The survival pattern of *Salmonella spp* between Hatirjheel Lake and Buriganga River showed 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 were faster than the Buriganga isolates as shown in the graphs below. As a positive control, clinical *S.typhi* strain was used which also showed similar die-off rate as the other isolates. Cell count was taken by direct plating on selective media XLD. Initially 10^8 cfu/ml if the isolates were inoculated in the autoclaved tap water which decreased to approximately 10^3 cfu/ml in Hatirjheel isolates and 10^4 cfu/ml in Buriganga isolates on day 3.

$$\text{Colony forming unit/ml} = \frac{\text{Number of colonies X Dilution Factor}}{\text{Volume Plated}}$$

A rapid decrease to 2 log CFU/ml of the Hatirjheel isolates was seen compared to 3.41 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 3.91 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.

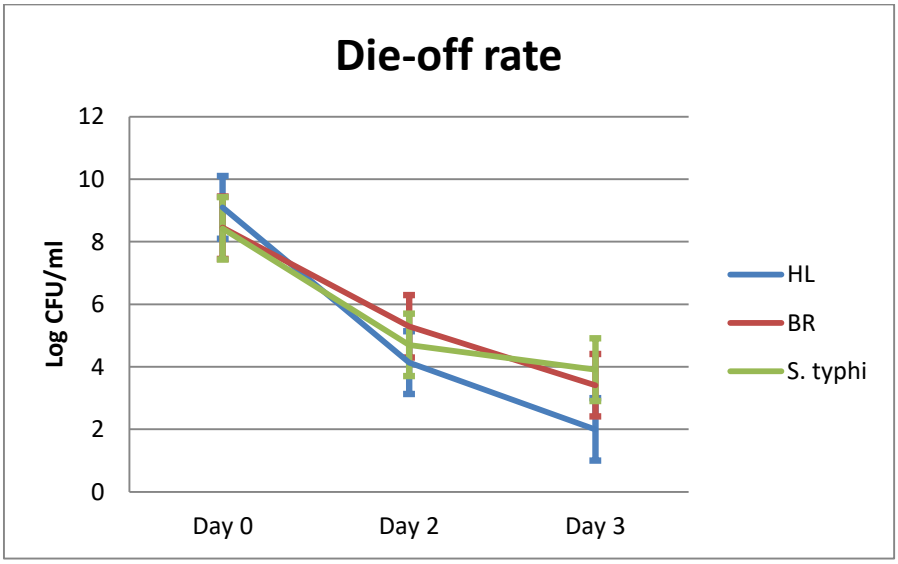

Figure 10: Die-off rate comparison between the isolates of Hatirjheel as well as Buriganga and positive control which shows a gradual decrease in growth.

Chapter 4

Discussion

4.0 Discussion

The purpose of this study was to isolate and identify the presence of *Salmonella spp* from two of the most 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 water-borne disease. As a result of unplanned urbanization and industrialization the waterbodies are getting polluted and typhoid is becoming endemic in Bangladesh (Shumy et al.). This is why it is important for us to know if the waterbodies 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.).

After 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.). Out of 32 isolates 30 isolates gave a positive band for the specific pathogenic gene. This means that the organism which was found from the lake 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 for both water bodies. The average temperature for Hatirjheel Lake was 25.8ºC while for Buriganga River it was 25.0ºC. 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.) 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.). After the completion of identification of the organisms, their antibiogram was performed in order to determine their resistance pattern.

Ciprofloxacin and ceftriaxone are drug of choice for typhoid, according to the antibiogram, all the isolates in river are showing intermediate zones for ciprofloxacin

while ceftriaxone shows both sensitive as well as intermediate zones. Both of these antibiotics show sensitivity (>80%) in the lake isolates. This means that in case of potential infection from the waters of Buriganga these drugs are less likely to be effective in the treatment of the disease. This trend can be a result of the increased exposure to various stresses in river water from the industries. The effluents from industrial wastes contain metals such as mercury and chromium which can trigger the development of metal resistance along with various antibiotic resistance (Baker-austin et al.).

For further analysis of the antibiotic resistance, plasmid extraction of the isolates was performed to see if the resistance gene was plasmid mediated or not. However, after performing gel electrophoresis it was seen that the isolates do not contain any plasmid which concludes that the resistance gene is present in the chromosomes of the isolates and not the plasmid. Complete antibiotic resistance was seen against the antibiotics: ampicillin, co-trmixazole and cefepime. Previous reports on clinical strains (please refer to table 2) have shown that these 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 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* (Typhi).

Salmonella genomic island 1 (SGI1) harbors an antibiotic resistance gene cluster and was previously identified in the multidrug-resistant *Salmonella enterica* serovars *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.).

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 (Typhi).

Another study has been done on the resistance against extended spectrum cephalosporin to determine how it is borne using Southern Hybridization of chromosomal DNA and plasmid DNA. The results showed that most isolates had the resistance *bla*CTX-M-15 gene located on chromosomal DNA. Analysis of the flanking regions of the chromosomes revealed that the resistance genes were derived from plasmids suggesting chromosomal integration (Espie et al.).

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 *Salmonella* serovar *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.).

Similar to plasmid, 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. enterica* serovar *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.). Several cases were observed in which the chromosomal DNA contained the integrase gene along with resistance gene as found in (Casin, Breuil, et al.).

Another possible reason for resistance might be mutation. A project in China showed that *Salmonella enterica* serotype *Typhimuriam* has aquired resistance to antibiotic ciprofloxacin which occurred due to point mutation in GyrA (Cui et al.).

Thus we can say that there can be chances that the antibiotic resistance genes of the isolates might be present in the chromosome of the organisms. This phenomenon can happen due to the horizontal transfer of genes into the SGI via the addition of insertion sequence by integrase, transposase sequence or integration of plasmid gene. These kinds of phenomenon 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 (Typhi). If this is the case then these virulent organisms present in the environment pose a great threat to the public health issues regarding heath issues and should be taken under immediate consideration for remedy. Awareness and prevention should be spread among the mass people regarding the pollution in the waterbodies nearby.

Due to the difference in the rate of pollution and contaminants between the two water bodies it was hypothesized that the characteristics of the organisms will be different. The reason for this hypothesis is that the organisms are constantly under pressure from the pollutants present in the water. These pollutants vary between the lake and river which can affect the adaptability of the organisms in different way. This difference might be the result of genetic mutation or natural selection for surviving in diverse conditions. The initial characterization of the isolates were demonstrated by the antibiogram results and for further characterisation, the die-off rate of the selected isolates were 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.

The stress is partially mimicking the lake and river water where the pollutants act as stress factor. As depicted the organisms showed rapid decline in growth over the three days observed. The limited numbers of observing days were just to view the trend of death rate which was achieved within these few days.

The rate of death was faster for lake isolates than the river isolates by a significant amount. Comparing the isolates of the lake with positive control shows a large difference between them which is insignificant between the isolates of river and the positive control. This shows that the pollutants might be affecting the survival rate of *Salmonella* and if these pollutants spread and contaminate other water bodies it might 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 causes approximately 200 000 deaths annually. Although we know that the surface water of lakes and rivers inside Dhaka city are polluted there is lack of research on the presence of pathogenic organisms such as *Salmonella* in these waterbodies. This study focused on identifying the presence of *Salmonella* in Hatirjheel Lake and Buriganga River and characterized if the pollutants are affecting their survival and virulence. Their survival pattern showed that under stress organisms from both lake and water die rapidly. As we saw the isolates from these waterbodies all contained the pathogenic gene giving it the potential to cause 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 public health sector.

In the future, this work can be extended by performing the antibiogram for all the isolates and checking their survival rates for a longer period of time. 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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Appendix

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

PCR mixture preparation

Components	**Amount (µl) for a 25 µl PCR reaction volume**
Eva Green Super Mix (X2) Bio Rad	12.5
Nuclease free water	6
Primer F (Forward) 100 pmol	2
Primer R (Reverse) 100 pmol	2
DNA template	2.5
MQ total volume	25

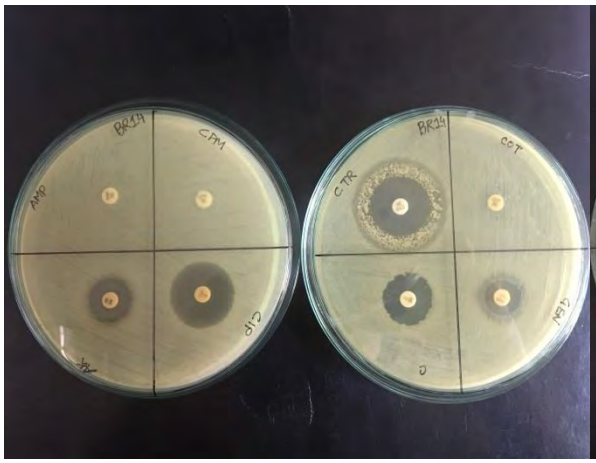
PCR Programs used for target DNA amplification

Temp (°C)	95	95	64	72	72
Time (min)	2	0.5	0.5	0.5	5
Cycles	45				

Recipe of solutions used for Plasmid Extraction

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

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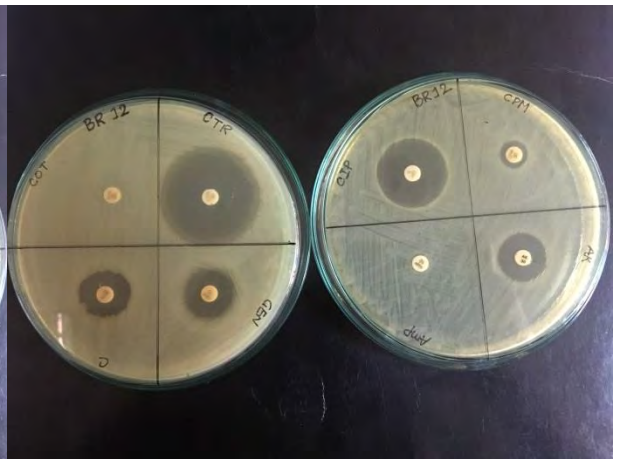
CTR
COT
GEN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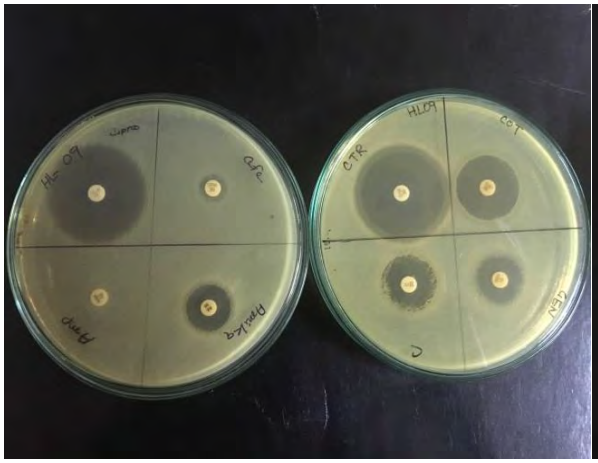
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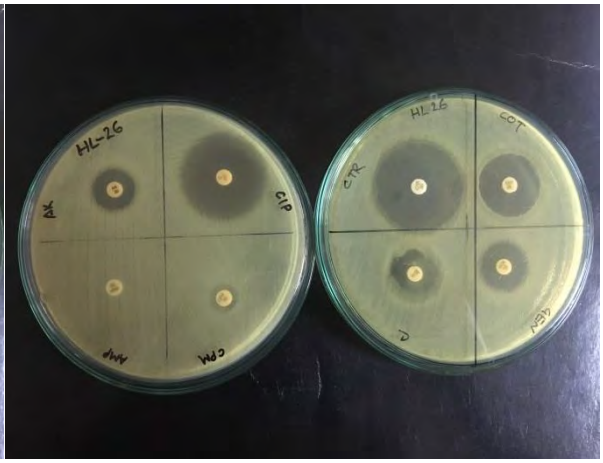
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COT
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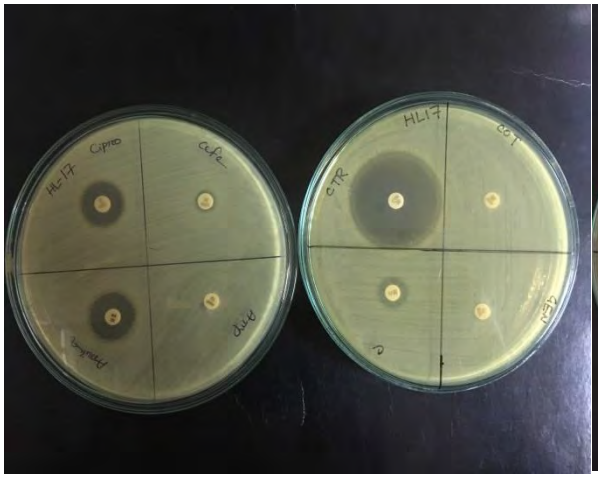
CPM
GEN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CTR
COT
GEN
AMP

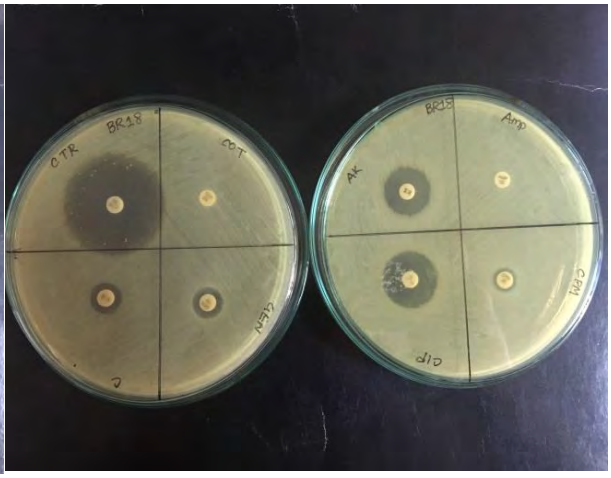
BR18
BR13
CTR
COT
GEN
AK
Amp
CIP

Fig: Antibiogram plates for Buriganga River and Hatirjheel Lake.

Table: Survival assay cfu/ml count.

DAY 0

Sample	**Dilution factor**	**Number of colonies**	**Cfu/ml**
HL 17	10^6	500	5.0×10^9
HL 26	10^6	60	6.0×10^8
HL 13	10^6	66	6.6×10^8
BR 03	10^6	42	4.2×10^8
BR 04	10^6	17	1.7×10^8
BR 12	10^6	32	3.2×10^8
S.typhi (control)	10^6	27	2.7×10^8

DAY 2

Sample	**Dilution factor**	**Number of colonies**	**Cfu/ml**
HL 17	10^4	0.5	5.0×10^4
HL 26	10^4	0.5	5.0×10^4
HL 13	10^4	01	1.0×10^5
BR 03	10^4	03	3.0×10^5
BR 04	10^4	05	5.0×10^5
BR 12	10^4	0.5	5.0×10^4
S.typhi (control)	10^4	0.5	5.0×10^4

DAY 3

Sample	**Dilution factor**	**Number of colonies**	**Cfu/ml**
HL 17	10^1	02	2.0×10^2
HL 26	10^1	0.5	5.0×10
HL 13	10^1	01	1.0×10^2
BR 03	10^1	231	2.31×10^4
BR 04	10^1	150	1.50×10^4
BR 12	10^1	0.5	5.0×10

S.typhi (control)	10^1	81	8.10×10^3