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, Al Imran Ahmed Ovi 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

Unplanned urbanization, defective sewerage system and lack of public concern can result in contamination of surface water bodies. As these water bodies are a major part of diurnal and recreational activities, contamination may possess a huge health risk. The purpose 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. To carry out the study, two of the largest water bodies of Dhaka city (Gulshan lake and Buriganga river) were chosen. A total of 40 water samples were aseptically collected from Gulshan Lake and 40 samples from Buriganga River from November 2017 to February 2018. Then the samples were processed for isolation of culturable *Salmonella*, followed by checking for *Salmonella* specific virulence gene *invA*. After that, ten randomly selected strains from each sources were checked for their susceptibility to an array of eight antibiotics. After that, three strains based on their antibiogram pattern from each sources were tested for survival potential under starvation stress. During the study period, 75% of the sample collected from the lake and 82.5% of the sample collected from the river yielded culturable *Salmonella* of which 76.6% isolates from the lake and 60.6% isolates from the river contained *invA* gene. The antibiogram revealed that all the isolates from both Buriganga and Gulshan showed complete resistance to amoxicillin, 90% isolates from Buriganga showed resistance to chlorpheniramine while 80% of the isolates from Gulshan showed complete resistance to chlorpheniramine. Around 60% isolates from Buriganga showed an intermediate result against co-trimoxazole. The survival patterns between the lake and river showed slight difference but both showed a gradual decrease in culturable count over the time. Finally, an observation of static biofilm formation showed that isolates from both of the sources form biofilm in adverse environment but biofilm formed by the isolates from Gulshan Lake remain more viable over time. The study reveals that both Buriganga River and Gulshan Lake can be endemically contaminated with potentially pathogenic *Salmonella.*

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Chapter 1

Introduction

British poet W. H. Auden once noted, –Thousands have lived while not love, not one while not water." nonetheless whereas we have a tendency to all understand water is crucial for keeps, we have a tendency to trash it anyway. Some 80 percent of the world's wastewater is dumped—largely untreated—back into the environment, polluting rivers, lakes, and oceans (Denchak, 2018).

This widespread water pollution problem puts our health at risk. Every year unsafe water kills more people than war and combines all other forms of violence. In the meantime, our drinkable water sources are finite: we actually have access to less than 1 percent of the fresh water on earth. Without action, the challenges will only rise by 2050, when global freshwater demand is expected.

The United Nation, in collaboration with individual nations, regularly monitors access to water and sanitation. The most recently completed assessment, revealed in 2000 by the globe Health Organization (WHO, 2000), is that the most comprehensive up to now, providing info for eighty nine p.c of the world's population. According to this assessment, 1.1 billion individuals round the world lacked access to –improved water supply" and quite a pair of.4 billion lacked access to –improved sanitation." Previous assessments were released in 1994, 1990, and during the International Safe Drinking Water Supply and Sanitation Decade of the 1980s (Gleick, 2002).

Typhoid fever may be a critical infection caused by the bacteria *Salmonella* Typhi. It is typically unfold through contaminated food or water. A calculable 11–20 million individuals get sick from typhoid fever and between 128 000 and 161 000 individuals die from it once a year. Symptoms include prolonged fever, fatigue, headache, nausea, abdominal pain, and constipation or diarrhoea. Some patients may have a rash. Severe cases might result in serious complications or perhaps death. Typhoid fever can be treated with antibiotics although increasing resistance to different types of antibiotics is making treatment more complicated (WHO, 2018).

Typhoid fever spreads through contaminated food and water or through shut contact with somebody who's infected. Signs and symptoms usually include a high fever, headache, abdominal pain, and either constipation or diarrhea (Wain et al., 2014).

Although water-related diseases have for the most part been eliminated in wealthier nations, they remain a major concern in much of the developing world. While knowledge square measure incomplete, the World Health Organization estimated in the 2000 assessment that

there are four billion cases of diarrhea each year in addition to millions of other cases of sickness related to the dearth of access to scrub water. Since several diseases square measure unknown and unreported, the true extent of these diseases is unknown. Water-related diseases square measure usually placed in four classes: waterborne, water-washed, water-based, and water-related insect vectors. The first three are most clearly associated with lack of improved domestic water supply (Gleick, 2002).

Water sources are susceptible to contamination from several origins, to incorporate humans and animals (Dechesne and Soyeux, 2007). several serovars of enterobacteria are isolated in natural environments, contaminated by human and animal ordure, significantly in stream waters, water waters and seawaters (Ahmed et al. 2009; Touron et al. 2005). The prevalence of those organisms depends on the character of the supply and also the water, excretion and alternative waste disposal processes, and environmental and environmental condition factors (WHO, 2004) Surface water quality is subject to frequent, dramatic changes in microorganism quality as a results of a spread of activities, as a result of discharges of municipal raw (untreated) water, treated effluents from process facilities, storm water runoff, or alternative non-point supply runoff all have an effect on surface waters (Anderson and Davidson, 1997).

The growth of Salmonella in water provides is additionally thought of doable, because of its ability to colonize surfaces and replicate in biofilms (Levantesi et al., 2005) and because of its ability to survive for weeks outside the host bowel (Access). The presence of morbific enteric microorganisms in surface waters, as well as Salmonella, constitutes a possible threat to human health each for his or her drinking or recreational use (Bonetta et al., 2011).

Dhaka city, the capital of Bangladesh, lies beside one in all the most important rivers named Buriganga. As a results of industrialization, most of the factories and industries, preponderantly tanneries and textiles, are set on its banks and discharges untreated industrial effluents that is increasing pollution and influencing human health further because the surroundings (Moniruzzaman et al., 2009; Rabbani et al., 2016).

Apart from the most streams, urbanization inside Dhaka city has led to the event of various lakes and ponds within the cosmopolitan. The surface water space of Dhaka town is regarding 10-15% of the whole acreage (Miah et al., 2017). Hatirjheel, Gulshan Lake, Banani Lake, Ramna Lake and Dhanmondi Lake are the popular relaxation spots within the town, is being contaminated by slums and sewages, the business corporations and industries in

operation within the area (Miah et al., 2017) Gulshan Lake plays an important role in maintaining the sole system of these areas (Miah et al., 2017). As results of pollution, the near native communities typically suffer from diarrhoea, skin diseases, viscus ulcers, respiratory disease, anaemia, high pressure and jaundice (Ullah et al., 2006).

Due to the distinction in location and therefore the sources of pollution, the water quality of Hatirjheel Lake, Gulshan Lake and Buriganga stream are totally different. Vital sources of pollution for Buriganga are from the tanneries that use robust chemicals that contain atomic number 24 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., 2014).

Gulshan-Baridhara Lake was declared associate Ecologically vital space (ECA) in 2001. Continuation of all forms of illegal activities within the ECA has turned it into associate ecologically dead lake. This has been highlighted recurrently through the death of fishes, receiving media coverage vigorously each year. Pollution load (Biological element Demand/BOD) measured within the lake has been abundant bigger than the vital level for fisheries. Also, there is spatial variation in BOD load in different parts of the lake. In the middle points of each part of the lake, BOD load is smaller compared to the end-points (in longitudinal direction) or close to culverts.

A similar trend in Dissolved Oxygen (DO) levels is observed throughout the lake. These negative impacts might have originated due to fragmentation of lake habitat that hinders lake water movement and ultimately affects load accumulation capacity of the lake. Temporal variations in DO level in lake water (considering a 24-hour cycle) are ascertained. The deep green colour of water indicates the presence of an algal boom in the lake, owing to high pollution load. After dawning, because the sun is high, the DO level in lake water reaches its most around high noon, thanks to protoctist chemical change by that element is discharged into lake water. The DO level begins to decline as the sun goes down. At night, through too early in the morning, high respirational demand for oxygen causes exhaustion of oxygen in the water, leading to a DO level near zero. Such a temporary anaerobic condition might have been responsible for the death of fishes in the lake.

Since the amount and sources of pollution in Gulshan Lake and Buriganga River are completely different, it's presumably to have an effect on the characteristics and prevalence of microorganisms within the waterbodies. While these waters don't seem to be used as sources of drinkable, individuals are exposed to the harmful contents on an everyday basis. Buriganga River is densely inhabited with ferries and boats liable for the transport of humans similarly as numerous food things like fruits and vegetables. The water from the watercourse is employed by several to clean their hands or to spray it over vegetables to stay then contemporary or employed by native food venders like chotpoti and jhalmuri venders to clean their utensils. These incidences produce to a public health side to work out however safe the waters are for such usage and if it'll lead to the unfold of enteric diseases like Asiatic cholera, enteric fever and infectious disease. one in all the earliest steps within the infective cycle of *Salmonella* spp. is that the invasion of the cells of the internal organ animal tissue. A genetic locus, invA, permits Salmonella spp. to enter cultivated somatic cell and invA may be a member of this locus (Galan et al., 1992).

There's growing concern about metal contamination functioning as a selective agent that can initiate the proliferation of antibiotic resistance. Cellular inclusion sequencing in several studies has shown that antibiotic resistance arises because of the presence of business pollutants. Genomic sequencing of the infectious agent *Salmonella enterica* serovar Typhi CT18 from a dump web site wealthy in significant metals discovered the presence of a conjugative cellular inclusion that confers resistance to trimethoprim, bactericide, antibiotic drug ,

Principen and antibiotic drug and contains a mercury-resistance deoxyribonucleic acid (Baker-austin et al., 2006). The annual incidence rate of enteric fever in capital of Bangladesh town varied from eight (in 2006) to eleven (in 2007/8) per one hundred,000 individuals and also the average range of enteric fever occurrences in annually was 871 (Islam et al., 2015). Examination of the monthly distribution of enteric fever reveals that the very best cases have fluctuated over the years, July-October being the very best, followed by April-June. Most of the enteric fever cases occurred within the proximity of enormous water bodies like rivers and lakes (Islam et al., 2015). This can be why it's vital to work out if the Hatirjheel Lake, Gulshan Lake and Buriganga River are contaminated with enteric spp. If so, what are the characteristics of those species in terms of antimicrobial resistances or die-off rate and whether or not these strains are capable of biofilm formation. Moreover, because of the

variations in location of those waterbodies, rate of pollution varies wide that is a vital think about poignant the characteristics of the microorganisms.

Buriganga River and Gulshan lake are contaminated by numerous pollutants and their microorganism analysis indicated the amount of Coliform microorganism gift as an indicator organism. However, the presence of actual infectious agent to blame for inflicting diseases isn't wide studied additionally to the affects the pollutants are having on these organisms. As a result, this study primarily focuses on the presence of enteric bacteria within the waterbodies that is to blame for inflicting enteric diseases and compares the characteristics of the salmonella strains between the Gulshan Lake and Buriganga River. While antibiogram may be a common parameter checked in clinical strains, it's seldom finished the strains that are environmentally gift as there are often multiple aspects wherever this resistance will get transferred and indirectly have an effect on humans. Therefore shedding light on however the pollutants are affecting the pathogenicity of the organisms between the lakes and river.

Our aim of the study is to sight the amount of pollution within the Gulshan Lake and Buriganga river and whether or not their incidence varies or not. What is more, to access the pathogenicity via virulent gene *inv*A and static biofilm formation in the waters contaminated with *Salmonella* spp and get into a conclusion if these water bodies are safe or not.

Chapter 2

Materials and Methods

2.1 Sample collection

Water samples were collected in autoclaved plastic bottles from different points of the Buriganga River and Gulshan Lake. In order to reach the various points of sampling, small boats and water ferries were used. The sample collection procedure started from the mid November 2017 and lasted till the end of February 2018. Total of 80 samples were collected, 40 from the Gulshan 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. Year]. 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 Alkaline peptone water and incubated at 42ºC for 24 hours in a shaker incubator. After the 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 37ºC 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 T1N1 media. After 24 hours incubation at 37ºC, paraffin oil was added to the broth and stored at -20ºC.

2.3 Description of sampling sites

Gulshan Lake is a lake in Dhaka, Bangladesh, that borders Gulshan Thana, Shahjadpur, and Baridhara Diplomatic Zone. In the context of very scarce recreational opportunity in Dhaka city, this lake could be an important recreational point for city dwellers. Despite being a source of ground water recharge, the lake has a very important cooling effect on the city environment. Also this lake is a source of drinking water for wild fowl and other animals in the area.The lake is elongated in a north-south direction and surrounded mainly by residential areas and some industrial units as well. The lake is fragmented into four parts by culvert type structures. Openings of such structures appear to be very insufficient for free flow of water among different parts of the lake. This badly affects the dilution of pollution loads, dissolution of oxygen into lake water and as a result, the localised effect of pollution

sometimes becomes so severe that it causes death of aquatic organisms, such as fish. 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 1: Gulshan Lake sampling site

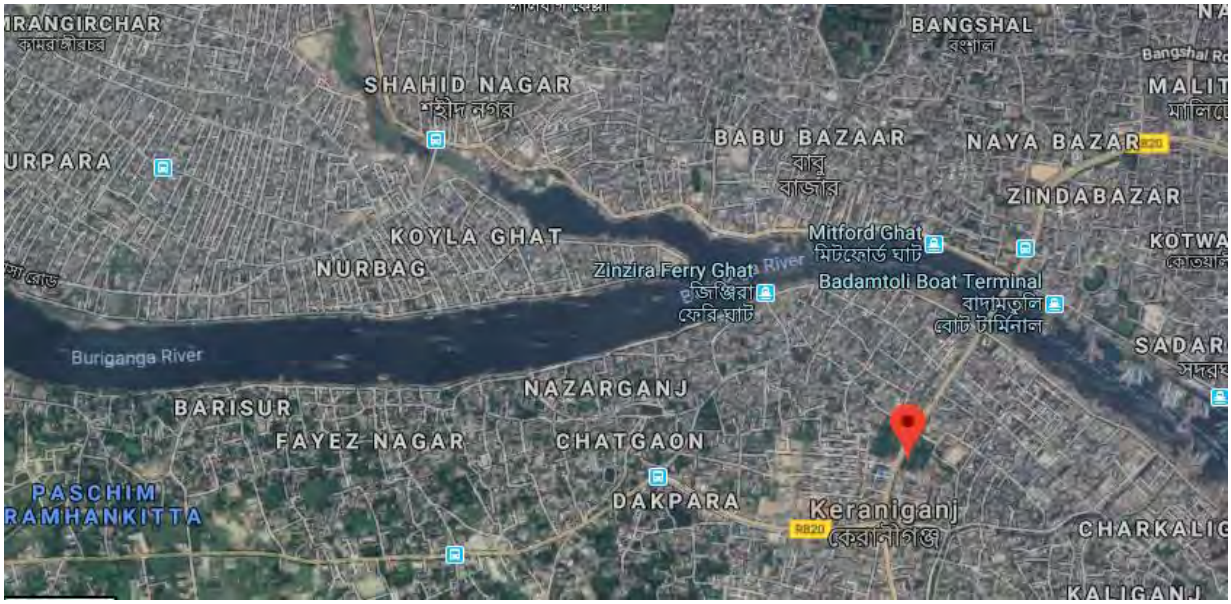

Figure 2: Buriganga River sampling site

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 121ºC. 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 37ºC (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 121ºC. 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 37ºC. 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 121ºC. 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 121ºC. 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 37ºC (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 121ºC. the media was cooled to about 50-55ºC 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 37ºC (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% 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 37ºC 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 biologygrade 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 further 3 to 10 minutes 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 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 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.)

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 95ºC for 2 min, followed by 35 cycles of 95ºC for 30 s, 64ºC for 30 s, and 72ºC for 30 s and a final extension of 5 min at 72ºC was employed. (Kumar et al., 2008)

2.5.3 Gel conditions

2% 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 dH_2O) were used separately along with 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 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.

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 commercial15 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., 2011). 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 108 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)

2.7 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 37ºC for 24 hours. Then two colonies were taken from each sample and inoculated in 10ml LB (Luria Bertani Broth) and incubated at 37ºC 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, 2003).

2.7.2 Preparation of microcosm

Tap water was taken in plastic bottles and autoclaved at 121ºC 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 3: 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.

2.8 Biofilm Formation

A biofilm is a population of microbic cells growing on a surface and encircled in an amorphous animate thing matrix (Donlan 2002). Bacteria in biofilms exhibit increased resistance to improvement and sanitation (Bower and Daeschel 1999; Joseph et al. 2001). Enteric bacteria *Salmonella* Typhi and enteric bacteria *Salmonella* Typhimurium are among the foremost unremarkably encountered foodborne and waterborne pathogens worldwide. Though their several malady states disagree, each organism are capable of forming microorganism biofilms in

class and/or environmental niches. Biofilm aids bacterium to endure severe conditions and facilitate microorganism persistence by increasing antimicrobial resistance and meddlesome with the host response in order that they'll become acquainted with their extreme surroundings. Biofilms is also of one species or of a different gathering of microorganisms [Tortora et al., 2011]. Assemblage sensing or chemical correspondence inside cells of this biological system known as biofilm permits the bacteria to prepare their movement and type communities. Within a biofilm, the bacterium will share supplements and are protected from touch-and-go environmental factors, as an example, parching, antibiotics and also the system of the body. The near neighborhood of microorganisms within a biofilm might likewise have the advantage of exchanging the hereditary knowledge by, as an example, conjugation. Biofilms are unremarkably connected to a surface, as an example, a stone during a lake, human tooth, rest room sinks, bathtubs, medical devices, cooling towers or may be accessible as a flock in waste treatment and during a filamentlike streamer structure in fast flowing streams [(Tortora et al., 2011)]. Following the antibiotic susceptibity 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 afterwards 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 static biofilm.

- Each of the *S.* Typhi isolated from different environmental samples and the clinical strain was inoculated in separate test tubes of 5ml of Luria-Bertani (LB) media and grown to stationary phase respectively.

- Each of the culture was diluted in fresh Luria-Bertani (LB) media to 1:10, 1:100 and 1:200 in three different eppendorfs.

- A fresh 96-well microtitre plate was taken that has not been tissue culture treated. For each isolate, 100µl of the raw culture was placed in the first two wells, then 100 µl of the 1:10 dilution was pippeted in the next two wells, then 100 µl of the 1:100 dilution was pippeted in the next two wells and lastly, 100 µl of the 1:200 was pippeted in the next

two wells. The same thing was repeated for all the cultures and the clinical stain of *P.aeruginosa*.

		B3	**B2**	**B1**	**WB**	**WT**	**-ve**	**+ve**	**WG**	**G3**	**G2**	**G1**
A		1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10
B		1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10
C		1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100
D		1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100
E		1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200
F		1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200	1:200
G												
H												

[Keywords: B1, B2, B3 = Buriganga isolates

G1, G2, G3 = Gulshan isolates

+ve = Positive control (P. aeruginosa)

-ve = Negative control

WB = Buriganga river water

WT = Tap Water

WG = Gulshan lake water]

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

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).

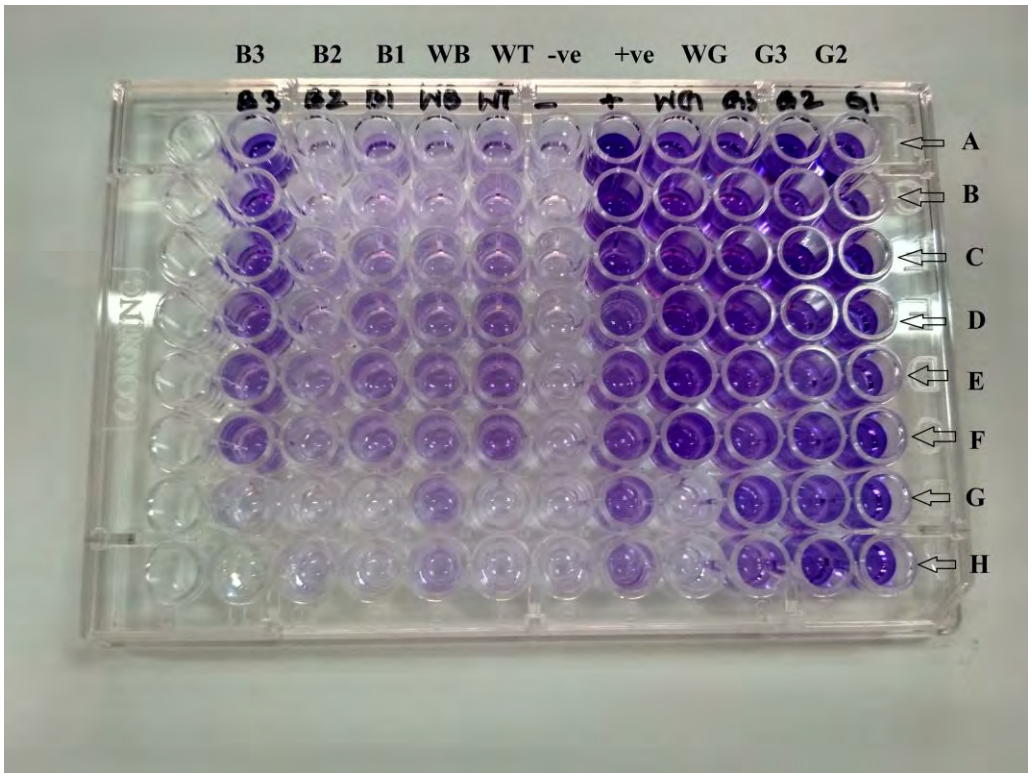

Figure 4: Formation of static biofilm in 96 well microtitre plate

Chapter 3

Results

3.1 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.

Here,

(+) = Sample is positive for *Salmonella* (-) = Sample is negative for *Salmonella*

R= Red, B= Black, Y= Yellow

Table 2: 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	GL4	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
2	GL6	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
3	GL9	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
4	GL11	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
5	GL13	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
6	GL15	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
7	GL16	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
8	GL18	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)

9	GL21	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
10	GL25	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
11	BR2	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
12	BR3	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
13	BR5	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
14	BR7	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
15	BR9	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
16	BR10	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
17	BR11	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
18	BR13	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
19	BR17	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)
20	BR19	(-)	R	B	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)	(+)

A total of 40 samples were collected from Gulshan Lake out of which 30 isolates were presumptively positive for *Salmonella* which means that 75% of the samples were positive. On the other hand, 40 samples were collected from Buriganga River from which 33 isolates were presumptively positive. This accounts for 82.5% positive samples collected from the River.

3.2 Antibiogram

Randomly selected 20 isolates, 10 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 Gulshan Lake and Buriganga River.

Table 3: Antibiotic resistance pattern in Gulshan Lake and Buriganga River

Here, S= Susceptible; R = Resistant; I = Intermediate

Sample No	Sample Name	*Norfloxacin*	*Amikacin*	*Cefepime*	*Amoxicillin*	*Ceftriaxone*	*Gentamycin*	*Chloramphenicol*	*Cotrimoxazole*
1.	**GL4**	S	S	S	R	S	S	S	R
2.	**GL6**	S	S	R	R	S	S	S	R
3.	**GL9**	S	S	R	R	S	S	S	I
4.	**GL11**	S	S	R	R	S	S	S	S
5.	**GL13**	S	S	R	R	S	S	S	S
6.	**GL15**	S	S	S	R	S	S	S	S
7.	**GL16**	S	S	R	R	S	S	S	S
8.	**GL18**	S	S	R	R	S	S	I	S
9.	**GL21**	S	S	R	R	S	S	S	S
10.	**GL25**	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

The isolates from Gulshan lake showed complete resistance to Amoxicillin (100%) while 80% of the isolates showed resistance to Cefepime. Also 20% of the samples showed resistance to Cotrimixazole. Other than that, 100% susceptibility was seen against antibiotics Norfloxacin, Amikacin, Ceftriaxone and Gentamycin whereas against Chloramphenicol the susceptibility was 90% and 70% for Cotrimoxazole. Some intermediate zone were also seen for Chloramphenicol and Cotrimoxazole which accounts for 10% of the isolates.

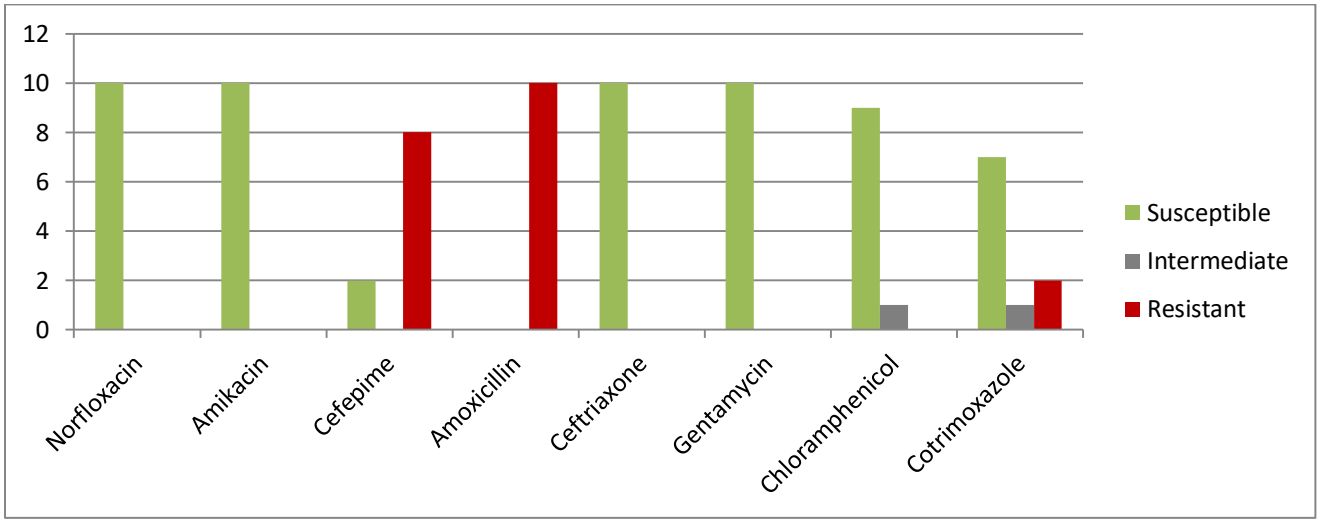

Figure 5: Graphical translation of the susceptibility and resistance pattern of the Gulshan isolates to different antibiotics

The isolates from Buriganga river showed kind of similar results to the Gulshan isolates. 100% isolates showed complete resitance to Amoxicillin and 90% showed resistance to Cefepime. Susceptible results were seen against antibiotics Norfloxacin (90%), Ceftriaxone (80%), Gentamycin (90%), Cefepime (60%) and Cotrimoxazole (30%). Comparing to Gulshan isolates quite a few intermediate zones were seen for Buriganga isolates against antibiotics Cotrimoxazole (60%), 40% for Chloramphenicol, 20% for Ceftriaxone and 10% for Norfloxacin, Cefepime and Gentamycin.

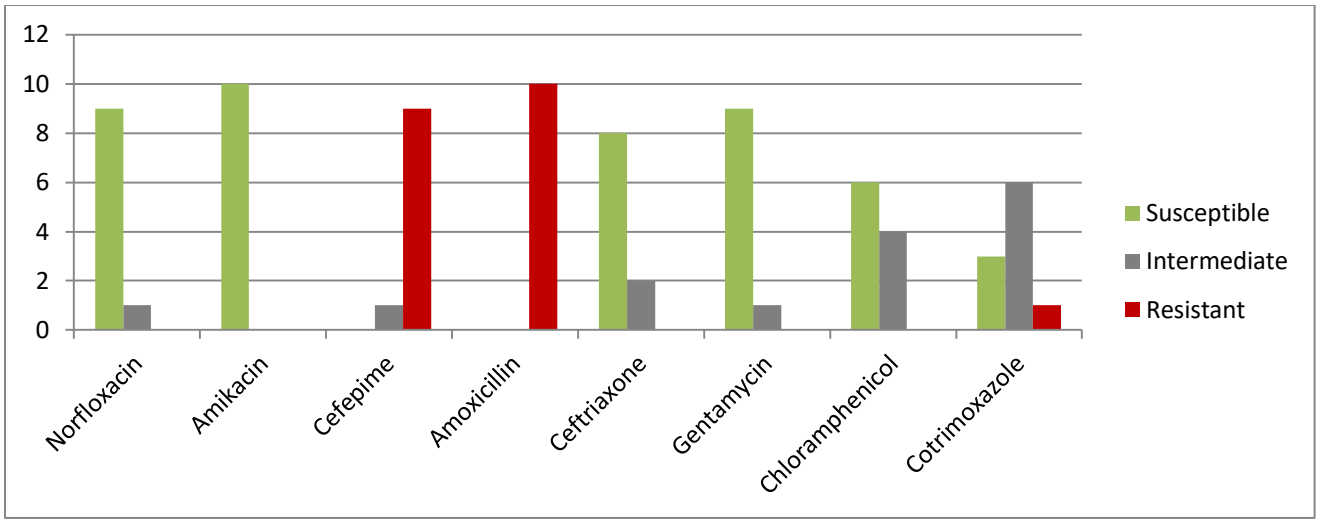

Figure 6: Graphical translation of the susceptibility and resistance pattern of the Buriganga isolates to different antibiotics

From the antibiogram test we found that a significant amount of samples were showing multidrug resistance means they were resistant to at least two or more antibiotics. Among the Gulshan lake isolates 44% were resistant to at least 2 or more antibiotics while 56% of the isolates were resistant to atleast 1 antibiotic.

The isolates from Buriganga river did not show much different result from the Gulshan isolates. For the Buriganga isolates 47% were showing resistance to atleast 2 or more antibiotics while 57% were resistant to 1 antibiotic.

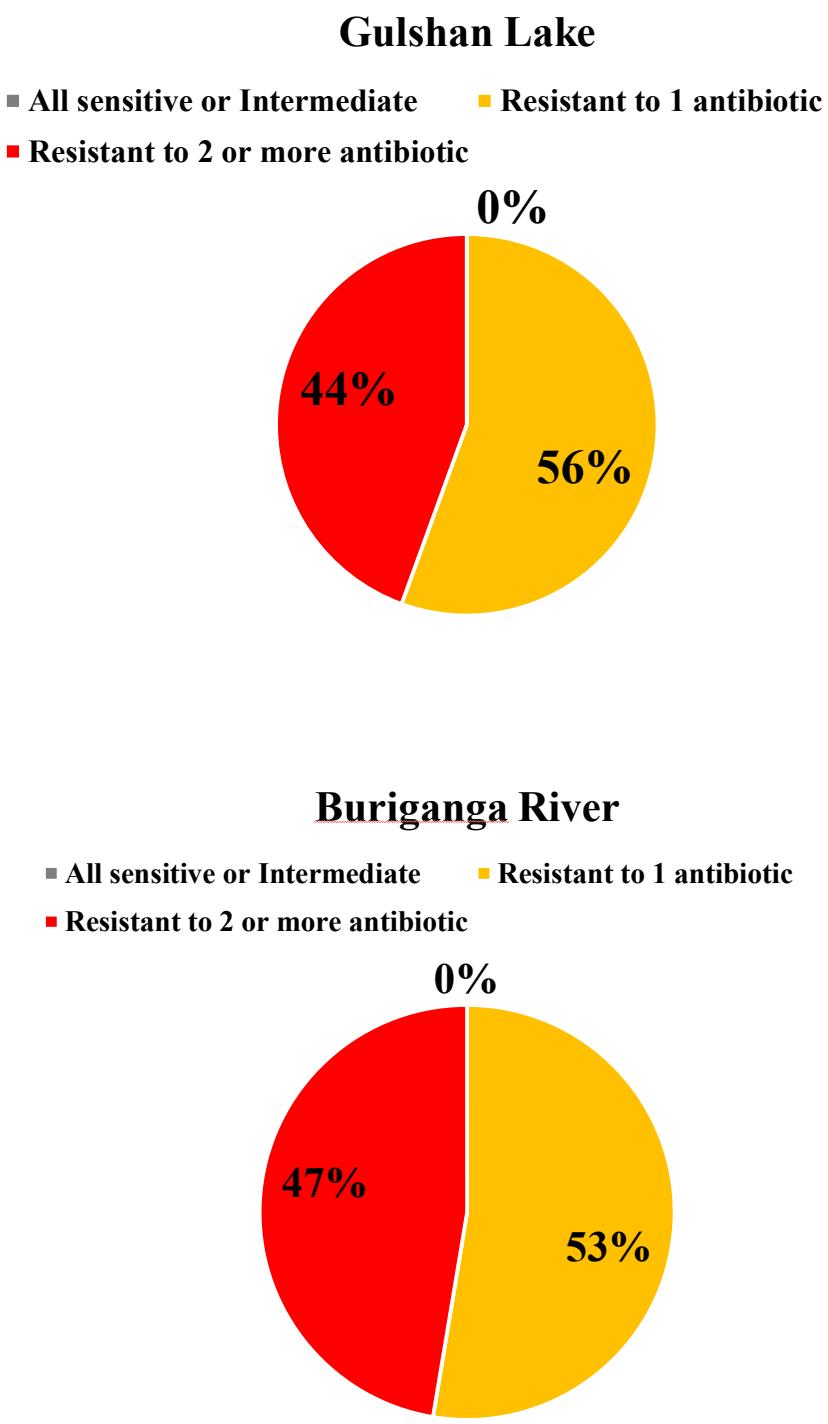

Figure 7: Graphical presentation of Multidrug resistant isolates from Gulshan Lake and Buriganga River

3.3 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 *inv*A which was then run in 1.5% agarose gel. The bands are usually approximately ~290 bp long DNA.

57.5% of the Buriganga isolates gave positive bands for the specific gene which means that the isolates present the surface of the waterbodies might be pathogenic Salmonella spp.

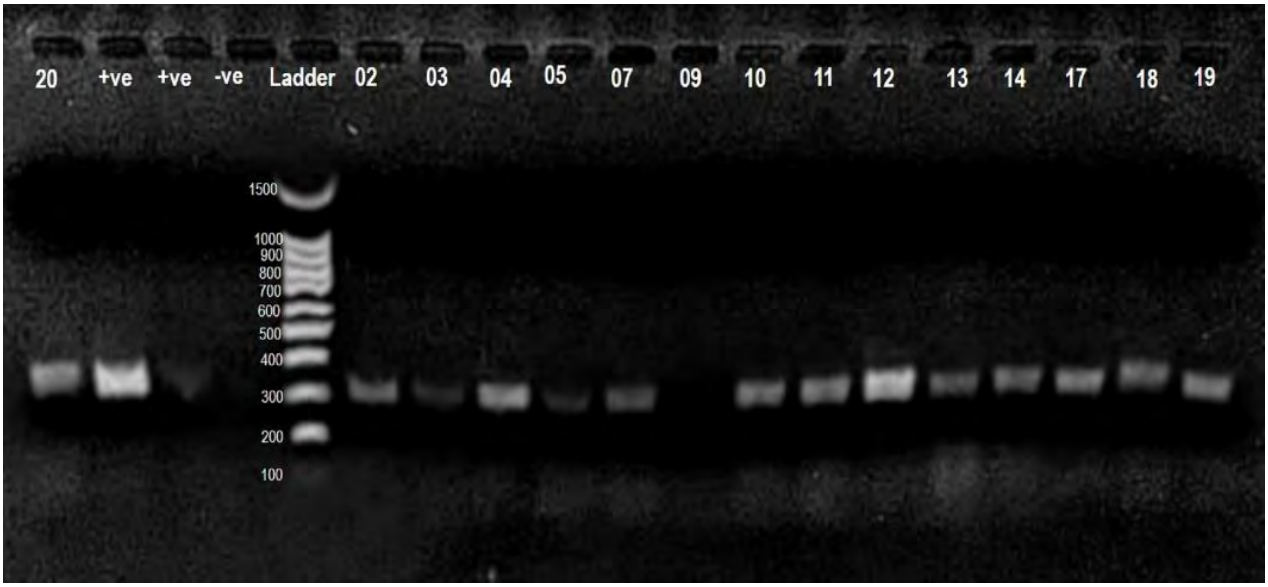

Figure 8: Agarose gel electrophoresis of isolates from Buriganga river showing positive bands for primer specific *inv*A gene

50% isolates from Gulshan lake gave positive bands for *invA* gene hence proves the presence of pathogenic *Salmonella* spp.

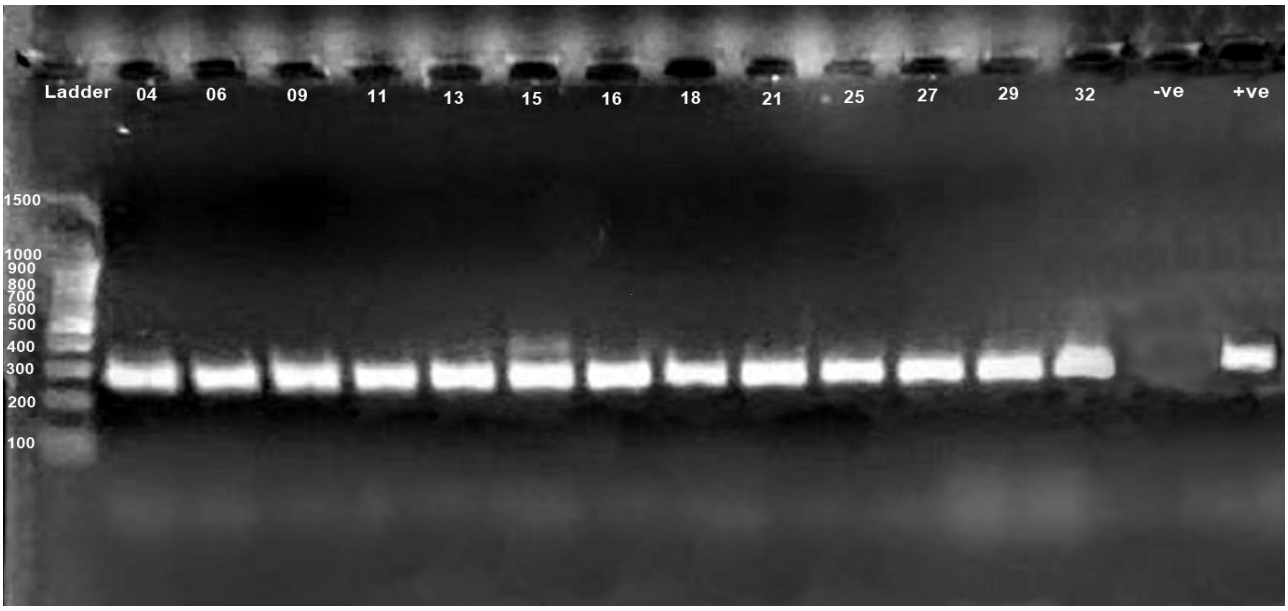

Figure 9: Agarose gel electrophoresis of isolates from Gulshan Lake showing positive bands for primer specific *inv*A gene

3.3 Survival Assay

The survival pattern of *Salmonella* spp. between Gulshan 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 Buriganga isolates were slightly faster than the Gulshan 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 of the isolates were inoculated in the autoclaved tap water.

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

A rapid decrease to 3.31 log CFU/ml of the Gulshan isolates was seen compared to 3.27 log CFU/ml of the Buriganga isolates on day 3 which is quite similar. On the same day, the positive

control *S.*Typhi showed a slightly slower decrease in growth at 3.83 log CFU/ml. At day 5 the number of viable cells decreased to almost 0 and at day 7 no sign of viable cell was seen. 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 the positive control.

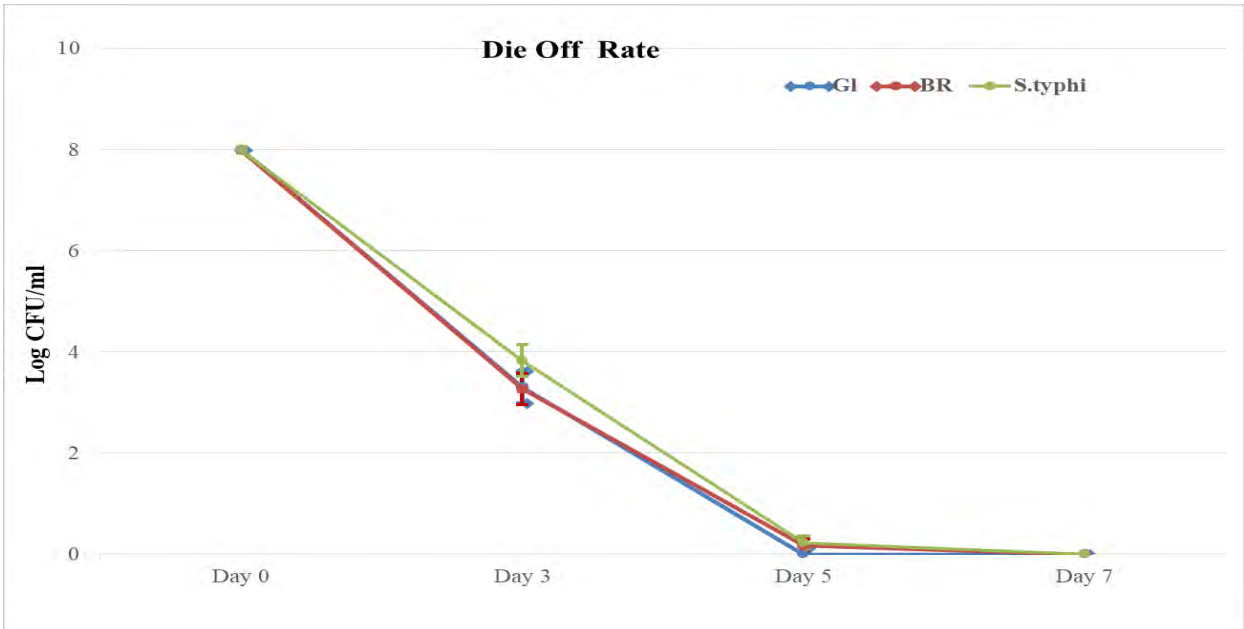

Figure 10: Die-off rate comparison between the isolates of Gulshan Lake and Buriganga Rivver and positive control which shows a gradual decrease in growth

3.5 Static Biofilm Formation

Using ELISA reader optical density was measured to observe presence of static biofilm. After one week the biofilms were stained crystal violet and their optical density was measured usinf an ELISA machine at 620nm for the quantification of biofilm. Significant amount of biofilm formation was observed.

At raw concentration Buriganga isolate (B1) forms maximum amount of biofilm (0.73nm). On the other hand at max dilution (1:200) Gulshan isolate (G2) forms maximum amount of biofilm. From raw concentration to diluted concentration a steady decrease in biofilm formation is observed.

Table 4: Absorbance reading of 1 week biofilm screening by ELISA machine (Multiskan, Finland) at 620nm

Source	Raw	1:10 dilution	1:100 dilution	1:200 dilution
B1	0.73	0.355	0.209	0.188
B2	0.65	0.206	0.108	0.051
B3	0.266	0.228	0.119	0.043
-ve	0.061	0.06	0.059	0.008
+ve	0.395	0.275	0.199	0.077
G1	0.453	0.355	0.285	0.107
G2	0.634	0.499	0.321	0.284
G3	0.456	0.377	0.281	0.141

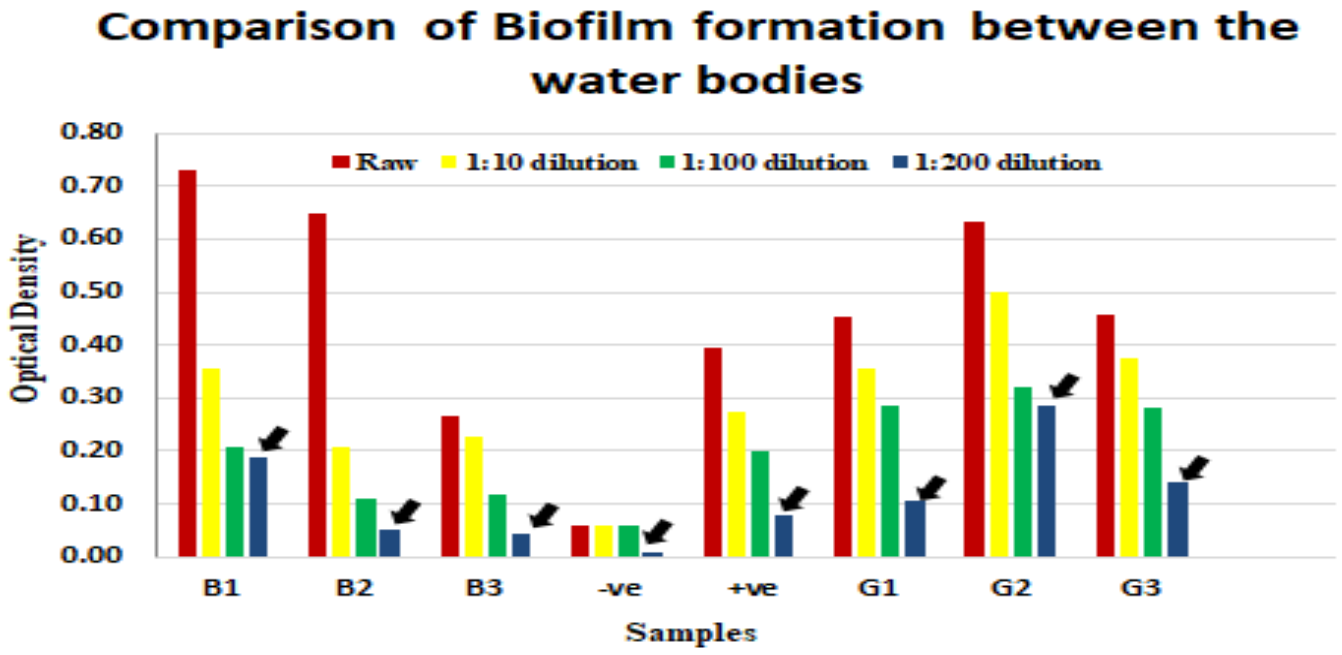

Figure 11: Bar chart illustrating the extent of biofilm formation according to dilution, by environmental strains and clinical strain

Chapter 4

Discussion

The purpose of this study is to evaluate the safety issues of Gulshan lake and Buriganga river for water based recreation by identifying and characterizing the presence of pathogenic Salmonella spp. by chemical and molecular analysis, evaluate the severity of virulence by observing the resistance pattern by an antibiogram test, run a survival assay to observe how the isolates survive in a different environment other than their sources and finally observing formation of static biofilm and discuss if these biofilm carry any meaning in their survival and pathogenicity.

Typhoid fever may be a major explanation for malady in most developing countries, including Bangladesh. A recent estimate found that twenty two million new enteric fever cases occur annually within the world, with some 200,000 of these resulting in death. Bangladesh is located in a region where typhoid is highly endemic (Abdullah, 2013).

From the 80 samples isolated from Gulshan lake and Buriganga river (40+40) a tolal of 63 isolets (30+33) were found presumptively positive for Salmonella spp. through certain biochemical tests. These isolates were subjected to molecular analysis for a definitive confirmation.

In molecular analysis the isolates were examined for the presence of virulence gene invA by gene specific PCR using the primer invA. invA 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.). invA gene enables the gene carrying organism to penetrate the wall of small intestine of human and initiate infection. Out of 63 presumptively positive isolates 43 isolates (23+20) gave a positive band for the specific pathogenic gene. This means that the organisms which were 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 like typhoid, diarrhea, salmonellosis etc.

Salmonella gastroenteritis is usually a self-limiting disease. Fluid and solution replacement is also indicated in severe cases. Because antibiotics do not appear to shorten the duration of symptoms and may actually prolong the duration of convalescent carriage, they are not routinely used to treat uncomplicated nontyphoidal Salmonella gastroenteritis. Current recommendations are that antibiotics be reserved for patients with severe disease or patients who are at a high risk for invasive disease (klochko,2018).

In things during which antibiotics area unit required, trimethoprim/sulfamethoxazole, ampicillin, or amoxicillin, are the best choices. Ceftriaxone, cefotaxime, or flouroquinolones are effective options for antimicrobial-resistant strains, although fluoroquinolones are not approved for persons less than 18 years of age. For persons with AN infection in an exceedingly specific organ or tissue (invasive disease), treatment with an expanded-spectrum cephalosporin is recommended, until it is known if the bacteria is susceptible to one of the additional usually used antibiotics listed on top of. For these rare things, treatment with antibiotics for four weeks is usually suggested. For enteric fever, including S. Typhi infections, treatment for 14 days is recommended. The specific antibiotic chosen depends on the susceptibility of the bacteria and the response to treatment (Clark, 2019).

In our study we found out that, all the samples of Gulshan and Buriganga were resistant to amoxicillin which is one of the most preferable antibiotics. 20% of the Buriganga isolates also showed intermediate result against another preferable option ceftriaxone. This means that in case of potential infection from the waters of Gulshan and 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 resistances (Baker-austin 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.). Mutation could be another possible explanation for multidrug resistance. 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.).

After initial characterization a survival assay was done with some potential isolates to see the die-off rate. The isolates were placed into autoclaved tape water to give them a nutrient free environment. This starvation stress was given to create a similar environment of their sources

where different pollutants work as the stress factors. Plate count was taken on certain intervals to observe the die-off rate. A decrease in growth was observed over days for all the isolates along with the clinical control. The difference between die-off rate of the lake and river isolates were insignificant and faster than the positive control. This could happen because of the difference between the stress factors they harbor into hence prove that the pollutants might affect the survival of the organisms and spreading of these pollutants into different sources can also trigger contamination.

The same isolates that were used for survival assay were observed for a static biofilm formation. According to National Institute of Health, 80% of all infections are associated with biofilms (Sanders et al., 2012). *Pseudomonas aeruginosa* was used as the positive control as it is increasingly exhibiting resistance to multiple antibiotics verified in present studies. Both Gulshan and Buriganga isolates showed significantly higher amount of biofilm production comparing to the positive control. This could be a result of the sources being continuously exposed to pollutants that made the isolates immunosuppressed and the evolutionary benefits of bacteria to mutate and adapt to antibacterial threats in their environment made them sharing theirs metabolic component to fight the threat by forming static biofilm.). 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 water bodies nearby.

Conclusion

Typhoid fever is a life-threatening infection caused by the bacterium *Salmonella* Typhi from which an estimated 11–20 million people get sick from typhoid and between 128,000 and 161,000 people die every year. Urbanization and temperature change have the potential to extend the worldwide burden of enteric fever. Dhaka city, being named as one of the most uninhabitable city in the world due to its over population, unplanned urbanization is at vast risk of having an outbreak of disease like typhoid fever. The polluted water bodies which are potential sources of Salmonella are also source of drinking and usable water for many people living around those sources. Despite these risk factors sufficient amount of credible research have not been conducted on this area. Our study focused on evaluating potential risk factor of two major water bodies of Dhaka city, Gulshan lake and Buriganga river presented by pathogenic Salmonella spp. After initial identification and characterization molecular analysis showed that 50%-60% samples were containing pathogenic gene giving it a potential to cause disease. The antibiogram test showed that a significant number of samples have established a resistance against multiple antibiotics. Antibiotic resistance shown by these organisms can be transferring horizontally via chromosome that opens a brand new paradigm to public health sector. To add that increasing resistance to antibiotic treatment is making it easier for typhoid fever to breakout through overcrowded populations in cities via flooded water and sanitation systems.

Due to limitation of time and resources we had to conduct our experiments on limited number of randomly selected samples. In future this work could be extended to performing antibiogram and molecular analysis to as many samples as possible. The molecular analysis could be also extended to a more profound level. Works could be done to understand the resistance pattern and how these isolates pass the genetic information. A vast study on mobile genetic element such as integron, transposon and plasmid could open up new era to invent more efficient vaccine and limit the potential health risk.

Chapter 5

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Appendix

Media compositions

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

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:

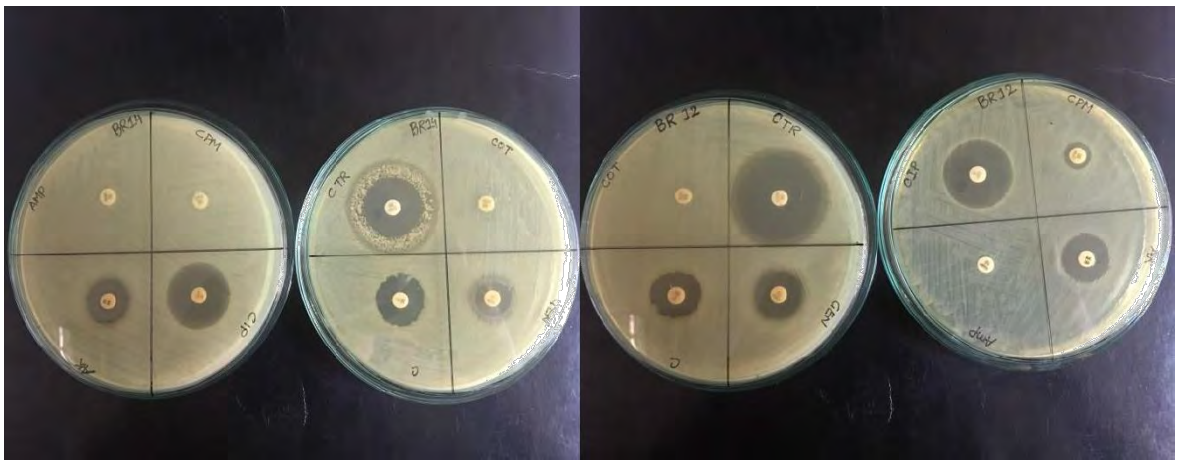
In a McCartney bottle, 1.861 g EDTA was added. Then 10 ml distilled water was added to prepare 10 ml 0.5M EDTA. After that pH was adjusted to 8. Then it was stored at room temperature.

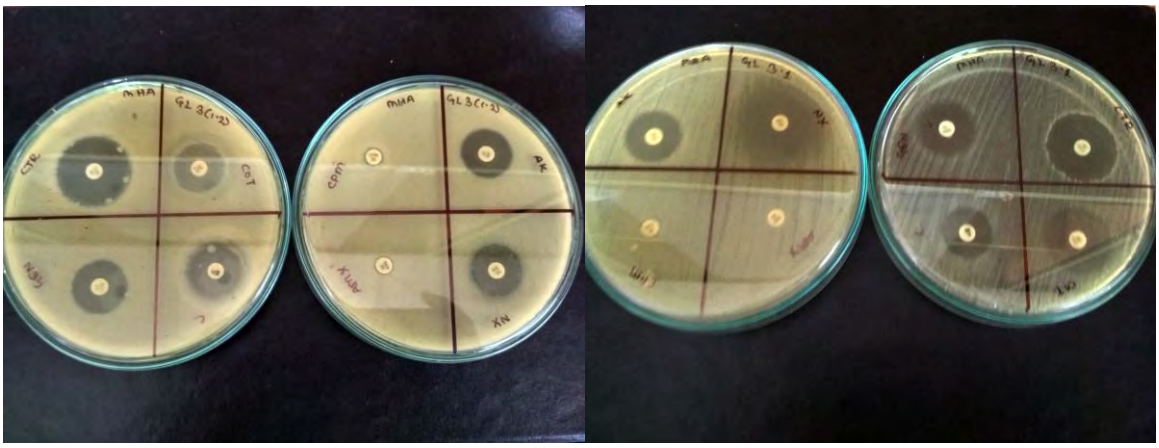
1X TBE Buffer:

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

Antibiogram test





Survival assay