HORIZONTAL TRANSFER OF ANTIBIOTIC RESISTANCE IN THE SURFACE WATER OF DHAKA CITY

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 **"Horizontal Transfer of Antibiotic Resistance in Surface Water of Dhaka City"** has been written and submitted by me, JITU PAUL and has been carried out under the supervision of Mahbubul Hasan Siddiqee, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University & co-supervision of Romana Siddique, Senior Lecturer, Biotechnology 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

Horizontal gene transfer is one of the most widespread phenomenon by which bacteria evolve themselves in the environment. Such transfer is often reported to be responsible for spreading antibiotic resistance genes in nature. This study was designed to understand the significance of natural transformation among different bacterial species in the surface water of Dhaka city in the light of emergence of antibiotic resistance; *Salmonella* sp. and *Klebsiella* sp. were used as model donor and recipient respectively. A total 10 environmental *Salmonella* isolates and 5 potential recipients (ATCC strains) were collected from BRAC University Microbiology Lab. By using Kirby-Bauer Disk Diffusion method, donor and recipient strains were selected. Among the 10 *Salmonella* isolates, 3 (*Salmonella* GL6.2, *Salmonella* HJ & *Salmonella* HJ3.1) showed highest number of resistant against 9 different antibiotics and these isolates were considered as a highly resistant donor for this experiment. Whereas, among those 5 ATCC stains, *Klebsiella variicola* showed highest level of sensitivity against 9 different antibiotics and this strain was selected for this study. After the antibiotic susceptibility test was done, the chromosomal DNA (boiling method) and plasmid DNA (Kado-Liu method) of the donor *Salmonella* strains were extracted for bacterial natural transformation experiment. Obtained results indicate that, the rate of chromosomal DNA mediated transformation is higher than plasmid DNA mediated transformation between *Salmonella* spp. and *Klebsiella variicola*. Further, it has been found that among the two matrices tested in this study (waters from Hatirjheel and Gulshan lakes), bacterial transformation is higher in Gulshan Lake; 34 successful transformed-bacterial cell growth found on antibiotic containing LB plates out of 90 different cases as opposed to 23 successful transformed-bacterial cell growth in Hatirjheel surface water. Further assays indicate that transfer of antibiotic resistance mostly involves chromosomal DNA-mediated transformation. Overall, this study reveals that horizontal gene transfer in surface waters could be one of the significant factors behind the rise of antibiotic resistance in Dhaka.

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Chapter 1: Introduction

Water is an essential human need. Safe drinking water supply and sufficient ecological sanitation are the conditions for wellbeing and for accomplishment in the battle against poverty, appetite and child death(Jahangir, 2016). Contaminated water isn't simply messy, it's fatal. More than two billion individuals have no entrance to improved sanitation, and more than 1.5 million children die every year from waterborne diseases(Brooks et al., 2005). As indicated by the WHO, the mortality of water related illnesses surpasses 5 million individuals for each year(Crump & Mintz, 2009). In Bangladesh, individuals that utilize the focal water supply framework for everyday use represents just 2-3% while most of the country population for the most part rely upon vast water sources(Jahangir, 2016). 40 out of 50 predominant sicknesses in Bangladesh, for example, loose bowels, typhoid, parasitic worm disease, and so on are identified with the polluted water(Crump & Mintz, 2009). Enteric fever which incorporates typhoid fever brought about by *Salmonella typhi* and para-typhoid fever by *Salmonella paratyphi* (A B C) is a significant general medical issue in many nations(Maisnier-patin, Berg, Liljas, & Andersson, 2002). Around the world, typhoid fever influences more than 12.5 million individuals yearly. In South Asia, it is evaluated that yearly 7 million instances of typhoid happen with the development of enteric fever identified with multidrug-resistance (MDR) strains of S.*typhi* and S. *paratyphi* A(Carrasco, Morales-Rueda, & García-Gimeno, 2012). Dhaka city, the capital of Bangladesh. The surface water region of Dhaka city is around 10-15% of the all-out land region. Hatirjheel, Gulshan Lake, Banani Lake, Ramna Lake, and Dhanmondi Lake are the mainstream unwinding spots in the city, is being populated by slums and sewage, industries and factories working in the zone(Miah, Majumder, & Latifa, 2017). Hatirjheel Lake assumes an imperative job in keeping up the main seepage arrangement of those zones. Because of water contamination, the close-by nearby networks frequently experience the ill effects of the runs, skin maladies, gastric ulcers, respiratory disease, sickliness, hypertension and jaundice(Ahmed, 2013).

Salmonella is one of the main sources of intestinal illness everywhere throughout the world just as the etiological operator of typhoid and paratyphoid fevers(Crump & Mintz, 2009). While water is known to be a typical vehicle for the transmission of typhoidal *Salmonella* serovars, non-typhoidal salmonellae are primarily known as foodborne pathogens(Levantesi et al., 2012). In industrialized nations, *Salmonella* was once in a while announced in water-borne flare-ups notwithstanding it being habitually identified in surface waters including recreational waters and waters utilized for water system or as a drinking water source. Multi drug resistant Salmonella strains, that speak to an expanded danger for human wellbeing which is identified in surface water in developed nations(Spector & Kenyon, 2012). Surface overflow was appeared to assume a fundamental job as driver of *Salmonella* load in surface waters. Information identifying with *Salmonella* prevalence in surface and drinking water in developing nations are very uncommon(Maisnier-patin et al., 2002). Information on water-borne out breaks are just as case control considers exploring the hazard factors for endemic typhoid fever affirmed the significance of water as hotspot for the transmission of this disease(Carrasco et al., 2012). What's more epidemiological investigations and Salmonella studies, reliably gave an unquestionable proof of the relevance of MDR *Salmonella Typhi* strains in water-borne typhoid fever in developing nations(Levantesi et al., 2012).

Microbes can react to specific pressures and adjust to new situations by procuring new hereditary qualities because of transformation, a change of gene function inside a bacterium, and because of horizontal gene transfer, the acquisition of new genes from other microscopic organisms (Havarstein, 2011). The normal mutation rate in nature is in the scope of 10-6 to 10-9 for every nucleotide for each bacterial generation, in spite of the fact that when bacterial populations are under pressure or stress they can significantly build their transformation rate(Chen & Dubnau, 2004). Moreover, most transformations are harmful to the bacterium. Horizontal gene transfer, on the other hand, empowers microbes to react and adjust to their condition significantly more quickly by gaining enormous DNA sequences from another bacterium in a single exchange(Gogarten & Townsend, 2005).

Horizontal gene transfer, also known as lateral gene transfer, is a procedure where microbes' exchanges hereditary material to another that isn't its offspring's. The capacity of Bacteria and Archaea to adjust to new conditions as a piece of bacterial advancement and evolution most frequently results from the acquisition of new genes through horizontal gene transfer rather than by the alteration of gene functions through mutations(Thomas & Nielsen, 2005).

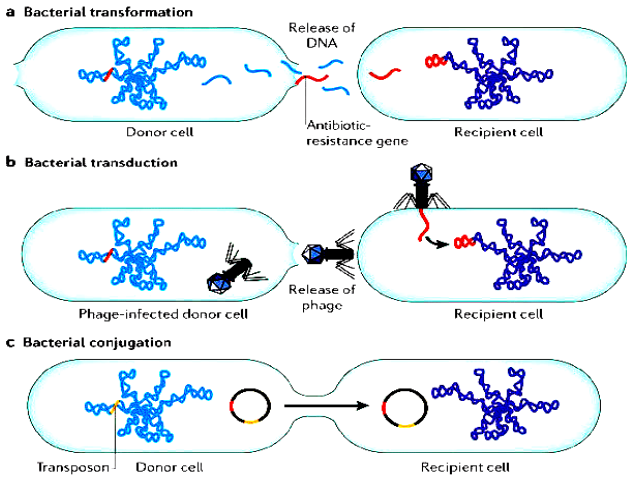


Figure 1: Types of Horizontal Gene Transfer (Furuya and Lowy, 2006)

Horizontal gene transfer is able to cause rather large-scale changes in a bacterial genome(Redfield, 1988). For instance, certain microscopic organisms contain different virulence genes considered pathogenicity islands that are situated on huge, unstable regions of the bacterial genome. These pathogenicity islands can be transmitted to other microorganisms by horizontal gene transfer. However, if these transferred genes provide no selective advantage to the bacteria that acquire them, they are usually lost by deletion. In this way the size of the bacterium's genome can remain approximately the same size over time.

There are three mechanisms of horizontal gene transfer in bacteria: transformation, transduction, and conjugation(Gogarten & Townsend, 2005). The most widely recognized mechanisms of gene transmission among microscopic organisms, especially from a donor bacterial species to different recipient species is called bacterial transformation. Although bacteria can acquire new genes through conjugation and transduction, this is usually a more rare transfer among bacteria of the same species or closely related species(Redfield, 1988).

Bacteria have no sexual reproduction in the sense that eukaryotes do. They have no alternation of diploid and haploid generations, no gametes, and no meiosis. However, the essence of sex is genetic recombination, and bacteria do have three mechanisms to accomplish that: transformation, conjugation and transduction (Redfield, 1988).

Many bacteria can acquire new genes by taking up DNA molecules (e.g., a plasmid) from their surroundings(Chen & Dubnau, 2004). The ability to deliberately transform the bacterium E. coli has made possible the cloning of many genes, including human genes, and the development of the biotechnology industry. The first demonstration of bacterial transformation was done with *Streptococcus pneumoniae* and led to the discovery that DNA is the substance of the genes(Méthot, 2016). The path leading to this epoch-making discovery began in 1928 with the work of an English bacteriologist, Fred Griffith.

The cells of S. *pneumoniae* (also known as the pneumococcus) are usually surrounded by a gummy capsule made of a polysaccharide. When grown on the surface of a solid culture medium, the capsule causes the colonies to have a glistening, smooth appearance. These cells are called "S" cells. However, after prolonged cultivation on artificial medium, some cells lose the ability to form the capsule, and the surface of their colonies is wrinkled and rough ("R"). With the loss of their capsule, the bacteria also lose their virulence. Injection of a single S pneumococcus into a mouse will kill the mouse in 24 hours or so. But an injection of over 100 million (100 x 106) R cells is he capsule prevents the pneumococci from being engulfed and destroyed by scavenging cells, neutrophils and macrophages, in the body. The R forms are completely at the mercy of phagocytes. Pneumococci also occur in over 90 different types: I, II, III and so on. The types differ in the chemistry of their polysaccharide capsule. Unlike the occasional shift of S -> R, the type of the organism is constant. Mice injected with a few S cells of, say, Type II pneumococci, will soon have their bodies teeming with descendant cells of the same type entirely harmless(Méthot, 2016).

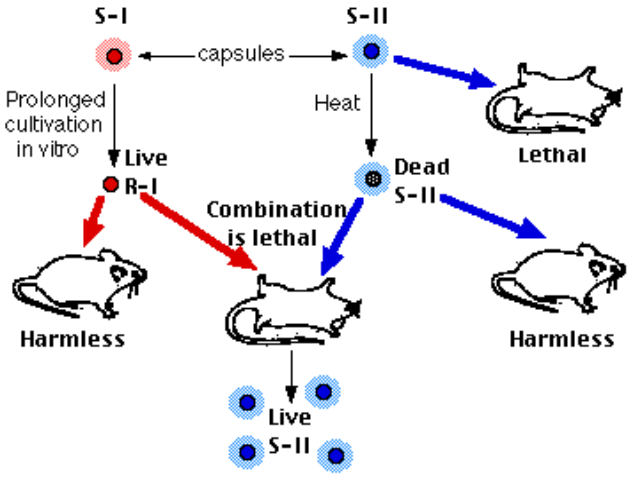


Figure 2: Fred Griffith Transformation Experiments (Source: bio.libretexts.org)

However, Griffith found that when living R cells and dead S cells were injected together, the mouse became ill and living S cells could be recovered from its body. Furthermore, the type of the cells recovered from the mouse's body was determined by the type of the dead S cells. In the experiment shown above, injection of living R-I cells and dead S-II cells produced a dying mouse with its body filled with living S-II pneumococci. The S-II cells remained true to their new type. Something in the dead S-II cells had made a permanent change in the phenotype of the R-I cells. The process was named transformation(Méthot, 2016).

Bacterial transformation is a process of horizontal gene transfer by which some bacteria take up foreign genetic material (naked DNA) from the environment(Juhas et al., 2009). The process of gene transfer by transformation does not require a living donor cell but only requires the presence of persistent DNA in the environment(Lederberg & Cohen, 1974). The prerequisite for bacteria to undergo transformation is its ability to take up free, extracellular genetic material. Such bacteria are termed as competent cells. The phenomenon of natural transformation has enabled bacterial populations to overcome great fluctuations in population dynamics and overcome the challenge of maintaining the population numbers during harsh and extreme environmental changes(Domingues et al., 2012). During such conditions some bacterial genera spontaneously release DNA from the cells into the environment free to be taken up by the competent cells(MacLachlan & Sanderson, 1985). The competent cells also respond to the changes in the environment and control the level of gene acquisition through natural transformation process.

The factors that regulate natural competence vary between various genera(Domingues et al., 2012). Once the transforming factor (DNA) enters the cytoplasm, it may be degraded by nucleases if it is different from the bacterial DNA. If the exogenous genetic material is similar to bacterial DNA, it may integrate into the chromosome. Sometimes the exogenous genetic material may co-exist as a plasmid with chromosomal DNA(Merrick, Gibbins, & Postgate, 2009).

A few bacteria, such as Neisseria gonorrhoeae, Neisseria meningitidis, Hemophilus influenzae, Legionella pneomophila, Streptococcus pneumoniae, and Helicobacter pylori tend to be naturally competent and transformable(Juhas et al., 2009). Competent bacteria are able to bind much more DNA than non-competent bacteria. Some of these genera also undergo autolysis that then provides DNA for homologous recombination. In addition, some competent bacteria kill non-competent cells to release DNA for transformation(Merrick et al., 2009).

During transformation, DNA fragments are released from a dead degraded bacterium and bind to DNA binding proteins on the surface of a competent living recipient bacterium(MacLachlan & Sanderson, 1985). Depending on the bacterium, either both strands of DNA penetrate the recipient, or a nuclease degrades one strand of the fragment and the remaining DNA strand enters the recipient(O'Callaghan & Charbit, 1990). This DNA fragment from the donor is then exchanged for a piece of the recipient's DNA by means of RecA proteins and other molecules and involves breakage and reunion of the paired DNA segments.

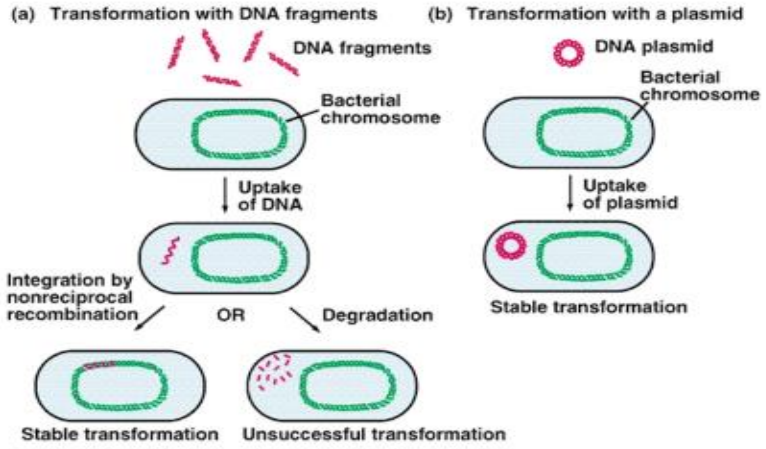


Figure 3: Schematic Representation of Bacterial Transformation (Source: bio.libretexts.org)

The completion of the sequence of the entire genome of a variety of different bacteria and archaea suggest that genes have in the past moved from one species to another. This phenomenon is called lateral gene transfer (LGT)(Chen & Dubnau, 2004). The remarkable spread of resistance to multiple antibiotics may have been aided by the transfer of resistance genes within populations and even between species. Many bacteria have enzymes that enable them to destroy foreign DNA that gets into their cells. It seem unlikely that these would be needed if that did not occur in nature(Thomas & Nielsen, 2005).

The discovery of the first antibiotic, penicillin, in the early 20th century was a landmark medical breakthrough that helped protect humans and their domesticated animals from bacterial agents(Neu, 1992). Many believed that this discovery would lead to the elimination of all illnesses and a society of essentially perfect health. However, none of these predictions were true and slowly the "miracle medicine penicillin" became less effective. With the discovery of DNA being the genetic code, scientists determined that some bacteria were resistant to particular antibiotics because of genes that rendered bacteria unaffected by the effects of some antibiotics(Threlfall, 2002). Populations contain genetic variation that allows the survival of some individuals of the population when faced with changing environmental factors.

Genetic variation is the result of mutations in coding regions of DNA. The introduction of antibiotics into bacterial colonies increased the biological fitness and the frequency of antibiotic resistant genes in bacterial populations. Horizontal gene transfer allows new variants to arise without a mutation in that variant. In addition to antibiotic resistance increasing from natural selection, bacteria can receive genetic material through the process of horizontal gene transfer. The genetic material is received in two forms: a DNA plasmid or a transposon(Thomas & Nielsen, 2005). A transposon is genetic material from one organism that becomes incorporated into the DNA of another organism, while plasmids do not become incorporated into the DNA of the host organism(Juhas et al., 2009). There are two main mechanisms in which bacteria receive extracellular genes. Transformation is the uptake of genetic material from a dead organism, while conjugation is the transfer of genetic material between two living organisms. Horizontal gene transfer contributes to the spread of antibiotic resistance through the exchange of genetic material across genera, which increases the potential for a harmful, antibiotic resistant bacteria to develop(Domingues et al., 2012).

The unnecessary use of antibiotics by humans acts as a selective pressure to increase the frequency of antibiotic resistance genes(Neu, 1992). When an illness is caused by a microbial, antibiotics are initially effective because the resistance to that antibiotic is either not present or has a low frequency in the population. Although the individual will most likely be healthy after their treatment, the microbes remaining have a higher likelihood of containing the resistance gene. The continuous use of an antibiotic will make it less effective over time because it will increase the frequency of a resistance gene(Knothe, Shah, Krcmery, Antal, & Mitsuhashi, 1983). Hospitals often rotate the use of different antibiotics to try and prevent the frequency of one antibiotic resistance gene from becoming too prevalent or standardized in a bacterial colony(Webb & Davies, 2009). Rotating antibiotics removes the selective pressure favoring one specific type of antibiotic resistance causing the frequency of the resistance gene to decrease. However, this leads to the selection for resistance to another type of antibiotic(Neu, 1992).

Transformation and conjugation contribute to increasing frequencies of antibiotic resistant genes because of genes transferring between different species(HÅVARSTEIN, 2011). Although natural selection plays a prominent role in increasing the frequency of alleles, transformation and conjugation make the gene available to a wide variety of microbes(Redfield, 1988). The over prescribing of antibiotics in agriculture and human illnesses has largely reduced the effectiveness of antibiotics. If society continues to abuse antibiotics, it will have difficulty treating potentially life- threatening illnesses because many antibiotics will become ineffective(Neu, 1992). The discovery of antibiotics was one of the greatest medical innovations in human history, while its abuse can lead to the development of one of society's worst epidemics.

Domingues, Sara Harms, Klaus Fricke, W. Florian, Johnson, Pal J, (2012) examined on the variables of characteristic change. DNA substances like versatile hereditary components engaged with change between bacterial species. Normally transformable cells of Acinetobacter baylyi were presented to DNA conveying strains of Escherichia, Pseudomonas, Salmonella to decide the nature and the occasion of change. Introduction to the different DNA sources brought about securing of anti-toxin obstruction characteristics just as whole integrons and transposons, over a 24 h presentation period.

DNA consolidation was not exclusively subject to integrase capacities or the hereditary relatedness between species. DNA succession examinations uncovered that few instruments encouraged stable combination in the beneficiary genome relying upon the idea of the giver DNA; homologous or heterologous recombination and different kinds of transposition (Tn21-like and IS26-like). Both benefactor strains and changed secludes were widely portrayed by antimicrobial defenselessness testing, integron-and tape explicit PCRs, DNA sequencing, beat field gel electrophoreses (PFGE), Southern smear hybridizations, and by re-change measures. Two transformant strains were additionally genome-sequenced. Their information exhibit that common change encourages interspecies exchange of hereditary components, recommending that the transient nearness of DNA in the cytoplasm might be adequate for genomic incorporation to happen. Their investigation gives a conceivable clarification to why grouping moderated transposons, IS components and integrons can be found spread among bacterial species. In addition, characteristic change of integron harboring populaces of equipped microscopic organisms uncovered that interspecies trade of quality tapes can be exceptionally productive, and free on hereditary relatedness among benefactor and beneficiary (Domingues et al., 2012)

Threlfall (2002) reviewed that strains of *Salmonella* spp. with resistance to antimicrobial drugs are now extensive in both developed and developing countries. In developed countries it is now increasingly accepted that for the most part such strains are zoonotic in origin and acquire their resistance in the food-animal host before onward transmission to humans through the food chain. Of particular importance since the early 1990s has been a multiresistant strain of Salmonella typhimurium definitive phage type (DT) 104, displaying resistance to up to six commonly used antimicrobials, with about 15% of isolates also exhibiting decreased susceptibility to ciprofloxacin. Mutations in the gyrA gene in such isolates have been characterized by a PCR Light Cycler-based gyrA mutation assay, and at least four different mutations have been identified. Multiple resistance (to four or more antimicrobials) is also common in the poultry-associated pathogens Salmonella Virchow and Salmonella hadar, with an increasing number of strains of these serotypes exhibiting decreased susceptibility to ciprofloxacin. Multiple resistance is also being found in other serotypes in several other European countries, and has been associated with treatment failures. For Salmonella typhi, multiple drug resistance is now the norm in strains originating in the Indian subcontinent and south-east Asia. Such multiresistant strains have been responsible for several epidemics and some of these have been associated with contaminated water supplies. Furthermore, an increasing number of multiresistant strains of S. typhi are now exhibiting decreased susceptibility to ciprofloxacin, with concomitant treatment failures. In developed countries antimicrobial resistance in zoonotic salmonellas has been attributed to the injudicious use of antimicrobials in food-producing animals. It is hoped that the application of Codes of Practice for the use of such agents, which have been prepared by the pharmaceutical industry in response to widespread international concern about the development of drug resistance in bacterial pathogens, will now result in a widespread reduction in the incidence of drug-resistant salmonellas in food production animals and humans on an international scale (Threlfall, 2002)

Threlfall (2002) surveyed that strains of Salmonella spp. with protection from antimicrobial medications are currently broad in both created and creating nations. In created nations it is currently progressively acknowledged that generally such strains are zoonotic in beginning and gain their opposition in the nourishment creature have before ahead transmission to people through the natural way of life. Of specific significance since the mid-1990s has been a multiresistant strain of *Salmonella typhimurium* conclusive phage type (DT) 104, showing protection from up to six generally utilized antimicrobials, with about 15% of confines additionally displaying diminished powerlessness to ciprofloxacin. Changes in the *gyrA* quality in such segregates have been described by a PCR Light Cycler-based *gyrA* transformation test, and at any rate four distinct changes have been recognized. Various opposition (to at least four antimicrobials) is additionally regular in the poultry-related pathogens *Salmonella Virchow* and *Salmonella hadar*, with an expanding number of strains of these serotypes displaying diminished helplessness to ciprofloxacin. Various obstruction is likewise being found in different serotypes in a few other European nations, and has been related with treatment disappointments. For *Salmonella typhi*, various medication obstruction is presently the standard in strains beginning in the Indian subcontinent and south-east Asia. Such multiresistant strains have been in charge of a few scourges and a portion of these have been related with polluted water supplies. Moreover, an expanding number of multiresistant strains of S. *typhi* are presently showing diminished helplessness to ciprofloxacin, with associative treatment disappointments. In created nations antimicrobial obstruction in zoonotic salmonellas has been credited to the imprudent utilization of antimicrobials in sustenance delivering creatures. It is trusted that the use of Codes of Practice for the utilization of such specialists, which have been set up by the pharmaceutical business because of boundless worldwide worry about the advancement of medication obstruction in bacterial pathogens, will presently result in an across the board decrease in the frequency of medication safe salmonellas in nourishment creation creatures and people on a universal scale (Okamoto, Andreatti Filho, Rocha, Menconi, & Marietto-Gonçalves, 2009).

Tsai et al. (2009) led stable limitation inadequate, change capable galE (JR501) and F'galE+ (JR502) strains of *Salmonella typhimurium* and the impacts of confinement on change by plasmid pBR322 were tried. A few variables which influence change productivity were methodically analyzed to decide ideal change conditions and a disentangled technique is introduced(Tsai, Hartin, & Ryu, 2009).

An examination led by MacLachlan (1985) researched lipopolysaccharide-flawed freaks of Salmonella typhimurium and they were changed by plasmid DNA with a Ca2+ treatment strategy. Just those freaks with a Rc or Rd2 chemotype, because of galE or rfaF changes, separately, gave efficiencies more noteworthy than 10(5) transformants per microgram of DNA, frequencies 8-to 630-overlap higher than with smooth strains or other harsh freaks(MacLachlan & Sanderson, 1985).

Chapter 2: Materials and Methods

2.1 Study Area:

The study was conducted at BRAC University in Dhaka, Bangladesh. The laboratory processing, analysis of data and the overall experimental work were done in the Microbial & Environmental Biology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

Period of Study: The research work was carried out from November 2018 till May 2019.

2.2 Microbial Sample:

A total of 10 isolates of *Salmonella* and 5 different ATCC samples were collected and from the Microbial & Environmental Biology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

Organism	**ID**	**Collection Source**
Salmonella	HJ3	BRAC University Lab
Salmonella	GL6	BRAC University Lab
Salmonella	BR1	BRAC University Lab
Salmonella	GL7	BRAC University Lab
Salmonella	GL4	BRAC University Lab
Salmonella	BR11	BRAC University Lab
Salmonella	GL1	BRAC University Lab
Salmonella	BR6	BRAC University Lab
Salmonella	HJ5	BRAC University Lab
Salmonella	HJ3	BRAC University Lab
ATCC Sample	**ID**	**Collection Source**
S. Flexini	Sg	BRAC University Lab
E.Coli	E	BRAC University Lab
E. Coli (PGLO)	Ep	BRAC University Lab
K. Variicola	P	BRAC University Lab
S.Typhi	St	BRAC University Lab

Table 1: List of microbial sample collected from Laboratory

2.3 Microbial Culture of Sample:

The samples were initially cultured in nutrient agar and kept on incubator O/N at 37°C. Nutrient Agar media is the general purpose medium for the growth of a wide variety of non-fastidious microorganisms consisting of appropriate amount of necessary nutrients for growth and replication of the organisms. The sample to be cultured was taken from the stock and streaked on the nutrient agar medium. After incubation, the plates had growth of the desired organism and individual single colonies were taken for further procedures, ensuring a pure culture of the desired organism.

2.4 Experimental Design:

1. Sample collection from BRAC University MEB Lab
2. Identified isolates from glycerol stock revived on NA.
3. Antibiotic susceptibility testing using the Kirby Bauer Disk Diffusion Method.
4. Recipient and Donor Organism selection by Kirby Bauer Disk Diffusion method.
5. Chromosomal DNA Extraction of Donor sample and Gel Electrophoresis.
6. Plasmid DNA Extraction of Recipient sample & Gel Electrophoresis.
7. Natural Transformation Assay(Hatirjheel and Gulshan Lake water)
8. Screening of transformed sample (Over Agar Plating method)
9. Plasmid curing of the Donor sample with EtBr and SDS and Gel Electrophoresis.

2.5 Antibiotic susceptibility testing using the Kirby Bauer Disk Diffusion Method:

An antibiotic is an anti-microbial chemical that inhibits the growth or destroys the microorganism. These antibiotics are used to treat infectious diseases caused by microorganisms. Kirby Bauer Disk Diffusion technique is normally the standard procedure to check for the antibiotic sensitivity of these organisms. This technique allows in determination of whether the organism is resistant or sensitive to the antibiotic. The organisms to be tested for antibiotic sensitivity is cultured and then put away for incubation. After incubation, the organisms are inoculated in separate tubes containing 0.9% Saline. Saline (Sodium Chloride) used for the general cultivation and enumeration of less fastidious micro-organisms. The organisms are incubated for 1 hours. Afterwards a sterilized cotton swab is dipped into saline and that swab is used to inoculate the surface of the fresh plate containing Mueller Hinton Agar. Consequently, the MHA plates were kept at 37 °C for incubation for 24 – 48 hours. After incubation, the zone of inhibition were measured and compared against the CLSI reference chart for the antibiotic susceptibility, as either resistant (R), intermediate (I) or sensitive (S).

2.6 Water Sample collection from GLUSHAN AND HATIRJHEEL LAKE:

The aim of this research is the observation of the ability of Salmonella strain natural transformation in water. Therefore, we have collected each 1 litter of surface water of two different location namely Gulshan lake and Hatirjheel. These water sample was autoclaved so that no other organism can obstruct the transformation process when it will be used as a transformation medium.

Figure 4: Maps of water sample location and surface water sample in bottle

2.7 Chromosomal DNA Extraction of Donor sample (Salmonella) by Boiling Method:

Chromosomal Deoxyribonucleic Acid (C-DNA) extraction is the process by which DNA is separated from proteins, membranes, and other cellular material contained in the cell from which it is recovered. This extraction can be one of the most labor-intensive parts of DNA analysis. This particular method of Chromosomal DNA extraction is considered as one of the fastest techniques to isolate bacterial DNA. For D isolation, the cells are treated with PBS (Phosphate buffer saline) a typical buffering substance, which is responsible for the inhibition of the nuclease activity. This procedure requires fresh culture of organism which is inoculated in LB broth, which allows the cells to multiply. After overnight incubation, 700µl of the culture is taken in a micro-centrifuge tube and is rotated at 3000 rpm for 10 minutes. The supernatant was discarded and PBS was added and mixed gently via pipetting. Again mixture is rotated at 14000 rpm for 5 minutes and the supernatant was discarded and 200µl TE buffer was added to that micro centrifuge tube. The tube was put away in a water bath at 100°C for 15 minutes. After that, tube was chilled for 10 minutes and rotated again at 14000 rpm for 5 minutes. Cell debris were precipitated at the bottom. Lastly, supernatant (containing chromosomal DNA) was collected in new micro centrifuged tube and stored DNA samples at -20°C freezer(Englen & Kelley, 2000).

2.8 Plasmid DNA Extraction of Donor sample (Salmonella) by Kado-Liu Method:

This particular method of plasmid extraction is considered as one of the fastest techniques to isolate plasmids. For plasmid isolation, the cells are treated with Tris -Buffer, a typical buffering substance for DNA with buffering capacity in the slightly alkaline range (pH 7.5 – 8.2) and EDTA (Ethylene-diamine tetra-acetic acid) which is responsible for the inhibition of the nuclease activity. This procedure requires fresh culture of organism which is inoculated in nutrient broth, which allows the cells to proliferate. After overnight incubation, 1.5 ml of the culture is taken in a micro-centrifuge tube and is spun at 14000 rpm for 5 minutes. The supernatant was discarded and first solution Kado-I was added and mixed gently via pipetting. Kado-I is a solution that comprises of Tris- HCl and EDTA. Consequently, the second solution was added, Kado-II, which essentially contains Tris Buffer, SDS and 2N Sodium Hydroxide and was homogenized by rolling the tube. Then, the mixture was put away in a water bath at 55°C for an hour. After the hour long incubation, 400µl Phenol-Chloroform was added and the mixture was hand-mixed by lateral inversion for 30 minutes. Finally, the mixture was centrifuged again and the top layer consisting of plasmid is separated out in a fresh micro-centrifuged tube and stored at -20°C freezer(Fujita, Ike, & Suzuki, 1993).

2.9 Agarose-gel Electrophoresis:

Agarose Gel Electrophoresis is a technique that separates DNA fragments on the basis of molecular weight and the charge applied by the electric fields in the apparatus.

Electrophoresis 1 - After the successful extraction of bacterial chromosomal DNA (Salmonella), the next procedure that is carried out is, gel electrophoresis, to test the presence of C-DNA in the desired organisms. Therefore, in order to analyze the chromosomal DNA that have been extracted earlier, at first, 1% gel is prepared separately using 50ml TBE buffer solution and 0.57g of Agarose powder. 2.8µl of Ethidium Bromide, an intercalating agent used as a fluorescent tag, is also added to the gel. Then, the electrophoresis apparatus is set up using the power supply, gel is set onto the apparatus and the samples are loaded along with the dye (Bromophenol Blue). At the voltage of 80 and time of about 1 hours, the gel run is complete and the bands are seen under the Ultra-violet (UV) radiation.

Electrophoresis 2 - On the other hand, after the successful extraction of bacterial plasmid DNA (Salmonella), the next procedure that is carried out is gel electrophoresis, to test the presence of plasmid DNA in the desired organisms. Therefore, in order to analyze the plasmid DNA that have been extracted earlier, at first, 0.8% gel is prepared separately using 50ml TBE buffer solution and 0.41g of Agarose powder. 2.7µl of Ethidium Bromide, an intercalating agent used as a fluorescent tag, is also added to the gel. Then, the electrophoresis apparatus is set up using the power supply, gel is set onto the apparatus and the samples are loaded along with the dye (Bromophenol Blue). At the voltage of 70 and time of about 2 hours, the gel run is complete and the bands are seen under the Ultra-violet (UV) radiation.

2.10 Bacterial Natural Transformation Assay:

For this bacterial natural transformation assay, *Klebsiella variicola* used as a recipient strain that have been determined and selected by Kirby-Bauer disk diffusion antibiotic (Azithromycin, Cefixime) susceptibility test. On the other hand, three *Salmonella* samples used as a donor strains that also have been determined and selected by Kirby-Bauer disk diffusion antibiotic (Azithromycin, Cefixime) susceptibility test.

Moreover, this bacterial natural transformation experiment was slightly modified from the traditional bacterial transformation process that usually done by preparing competent cell (recipient) using $CaCl_2$, providing LB broth as a nutrient source for bacterial growth and antibiotic selection marker for artificial selection(Lederberg & Cohen, 1974). As the aim and objective of this study was to just observe bacterial natural transformation between *Klebsiella variicola* and *Salmonella* in surface water sample. For that reason, the transformation process was modified in such a way that it is free from any chemical and artificial interruption. In this experiment, water sample will serve as a nutrient source for bacterial growth instead of LB broth and *Klebsiella variicola* will not be treated with $CaCl_2$ to make it chemically competence and no antibiotic selection marker will not be used.

a) Bacterial Natural Transformation Assay on Hatirjheel Lake Water sample:

In order to perform transformation an autoclaved vial was taken and filled each vial with 2ml autoclaved Hatirjheel water. Then *Klebsiella variicola* (recipient strain) was inoculated using sterile loop into the vial containing autoclaved Hatirjheel water sample. Then the vial was placed on incubator for 5 hours. (In between 5 hrs. recipient bacteria move from lag phase to stationary phase).After incubation 50µl Plasmid DNA/Chromosomal DNA of Donor (*Salmonella*) was added to that vial and placed the vial containing recipient strain (*Klebsiella variicola*) and donor DNA(*Salmonella*) components on incubator for O/N at 37°C.

b) Bacterial Natural Transformation Assay on Gulshan Lake Water sample:

In order to perform transformation an autoclaved vial was taken and filled each vial with 2ml autoclaved Hatirjheel water. Then *Klebsiella variicola* (recipient strain) was inoculated using sterile loop into the vial containing autoclaved Gulshan water sample. Then the vial was placed on incubator for 5 hours. (In between 5 hrs. recipient bacteria move from lag phase to stationary phase).After incubation 50µl Plasmid DNA/Chromosomal DNA of Donor (*Salmonella*) was added to that vial and placed the vial containing recipient strain (*Klebsiella variicola*) and donor DNA(*Salmonella*) components on incubator for O/N at 37°C.

2.11 LB-Agar antibiotic Plating:

Plasmid or Chromosomal DNA can carry one or more antibiotic resistance genes, which confer resistance to a specific antibiotic to the bacteria carrying them. The presence of an antibiotic resistance gene on a plasmid or in chromosomal DNA allows researchers to easily isolate bacteria containing that plasmid or chromosomal DNA from bacteria that do not contain it by artificial selection i.e. growing the bacteria in the presence of the antibiotic(LEDERBERG & LEDERBERG, 1952).

Luria broth (LB) is a nutrient-rich media commonly used to culture bacteria in the lab. The addition of agar to LB results in the formation of a gel that bacteria can grow on, as they are unable to digest the agar but can gather nutrition from the LB within(LEDERBERG & LEDERBERG, 1952). The addition of an antibiotic to this gel allows for the selection of only those bacteria with resistance to that antibiotic (in our case azithromycin and Cefixime) usually conferred by a plasmid carrying the antibiotic resistance gene. Plating antibiotic over-agar for the selection of transformed cells. Over-agar spreading of antibiotic makes it easy for an investigator to suitably plate and select transformed cells containing plasmids differing in their resistance genes. This experiment will focus specifically on selection of transformed *Klebsiella variicola* with Azithromycin and Cefixime concentrated LB Agar plates.

Table 2: Concentration of Antibiotics used during over-agar plating method

SL No.	Concentration of Azithromycin (mg/ml)	Concentration of Cefixime (mg/ml)
1.	15	5
2.	16	10
3.	17	15
4.	18	20
5.	19	25

Total 20 LB-Agar plates with specific concentration antibiotic were prepared with following table. To observe transformed cells of *Klebsiella variicola* (from the bacterial transformation experiment) an autoclaved cotton swap was taken and dipped on vial (containing *Klebsiella variicola* and Donor DNA mixture). Later, an antibiotic selective LB-agar plate was taken and drawn a short linear line on plate with same cotton swap containing transformed cells (from vial). Then, an autoclaved cotton swap was taken and dipped in to culture broth of *salmonella* and drawn a short linear line on the same LB plate. Once again an autoclaved cotton swap was taken and dipped in to single culture broth of Klebsiella variicola and drawn a short linear line on the same LB plate. These process was frequently performed for all the antibiotic concentrated plates and transformation samples. Then all the plates were placed on incubator for 24 hours at 37°C.

2.12 Plasmid Curing:

Bacterial plasmids are known to harbor genes for resistances to antibiotics. In many cases the characteristics of the host organism conferred by the plasmids remain elusive, and such mysterious plasmids are abundant in nature. Curing of this mysterious plasmid from a bacterial strain is a method to substantiate the relationship between a genetic trait and carriage of that specific trait in the plasmid(Kahl, 2015).

In the instances where the plasmid is stable or the loss of property difficult to determine, the bacteria can be treated with curing agents. These include chemical agents interfere specifically with its replication or affect particular structural components or enzymes of the bacterial cell. Protocols for curing plasmids consist frequently of exposure of a culture to sub-inhibitory concentrations of some chemical agents, e.g. Acridine orange, Ethidium bromide (EtBr), and Sodium dodecyl sulfate (SDS) or to a super-optimal temperature followed by selection of cured derivatives. The DNA intercalating agents such as Acridine orange and Ethidium bromide are the most commonly used because they are found to be effective against plasmids in a wide variety of genera. The efficiency of curing can also vary widely depending on the plasmid and the particular bacterial host carrying it. In most instances, the underlying mechanism of curing is not known.

Concentration of EtBr (µg/ml)	Amount of EtBr (µl)	Concentration of SDS%	Amount of SDS (g)
50	45	5	0.5
100	90	10	1
150	135	15	1.5

Table 3: Concentrations of curing agents used in plasmid curing of salmonella

The protocol of curing plasmids goes as such, first the organisms to be cured is cultured and incubated overnight at 37°C. The 1ml fresh culture has been inoculated in 9ml nutrient broth containing different concentrations of the curing agents, like Ethidium bromide (EtBr) and Sodium dodecyl sulfate (SDS). The concentrations of SDS and EtBr used are listed in the table. After overnight incubation of the cultures in EtBr and SDS were plated on LB agar containing plates with different concentrations of antibiotics. Same as mentioned in table above. Presence or absence of growth in the antibiotic containing plates enables us to determine whether the resistance gene is present in the chromosomal DNA or in the plasmid DNA(Kahl, 2015).

Chapter 3: Results

3.1 Microbiological culture of the organisms

The isolates that were collected from the BRAC University Microbiology Laboratory were cultured. Three *Salmonella* samples and *Klebsiella Variicola* were cultured from the stock in nutrient agar and incubated for 24 hours at 37°C.

Figure 5: Culture Plates of *Salmonella* & *Klebsiella variicola*

3.2 Antibiotic susceptibility test:

Antibiogram was performed on chosen isolates for the study to observe the antibiotic resistance pattern.

Table 4: Antibiogram of ATCC stains

Drugs	**AZM 15 (mm)**		**VA 30 (mm)**		**CFM 5 (mm)**		**LE 5 (mm)**		**CIP 5 (mm)**		**CMX 30 (mm)**		**AMP 25 (mm)**		**TE 30 (mm)**		**CL 10 (mm)**		**Total result**		
Strains	Z	S	Z	S	Z	S	Z	S	Z	S	Z	S	Z	S	Z	S	Z	S	**R**	I	S
S. Flexneri	25	**s**	Nil	**R**	30	**s**	15	**s**	16	**s**	22	**I**	Nil	**R**	Nil	**R**	16	**s**	**3**	**1**	**5**
E.Coli	20	**s**	Nil	**R**	30	**s**	36	**s**	40	**s**	29	**s**	27	**S**	21	**S**	20	**s**	**1**	**-**	**8**
E.Coli PGLO	30	**s**	Nil	**R**	37	**s**	38	**s**	39	**s**	27	**s**	Nil	**R**	10	**R**	21	**s**	**3**	**-**	**6**
K. Variicola	15	**s**	Nil	**R**	29	**s**	30	**s**	32	**s**	24	**s**	15	**I**	20	**S**	17	**s**	**1**	**1**	**7**
S.Typhi	22	**s**	Nil	**R**	36	**s**	30	**s**	36	**s**	29	**s**	35	**s**	25	**S**	20	**s**	**1**	**-**	**8**

Key: S – Sensitive or Susceptible, I – Intermediate, R – Resistant, Z – Zone of Diameter

From the above table, *Klebsiella variicola* shows sensitivity against 7 antibiotics out of 9 antibiotics whereas *S. Typhi* shows sensitivity against 8 antibiotics out of 9 antibiotics.

Antibiogram was performed on chosen isolates for the study to observe the antibiotic resistance pattern.

Table 5: Antibiogram of 10 *Salmonella* isolates

Drugs	**AZM 15 (mm)**		**VA 30 (mm)**		**CFM 5 (mm)**		**LE 5 (mm)**		**CIP 5 (mm)**		**CMX 30 (mm)**		**AMP 25 (mm)**		**TE 30 (mm)**		**CL 10 (mm)**		**Total result**		
Strains ID	Z	S	Z	S	Z	S	Z	S	Z	S	Z	S	Z	S	Z	S	Z	S	**R**	I	S
HJ3.2	12	**R**	Nil	**R**	33	**s**	35	**s**	35	**s**	20	**I**	20	**s**	Nil	**R**	nil	**R**	**4**	**1**	**4**
GL6	nil	**R**	Nil	**R**	28	**s**	21	**s**	24	**s**	20	**I**	12	**R**	nil	**R**	20	**s**	**4**	**-**	**4**
BR1	12	**R**	Nil	**R**	36	**s**	21	**s**	25	**s**	26	**s**	nil	**R**	nil	**R**	nil	**R**	**5**	**-**	**4**
GL6.2*	nil	**R**	Nil	**R**	Nil	**R**	25	**s**	20	**I**	23	**s**	nil	**R**	nil	**R**	nil	**R**	**6**	**1**	**3**
GL4	10	**R**	Nil	**R**	36	**s**	29	**s**	22	**s**	28	**s**	nil	**R**	nil	**R**	nil	**R**	**5**	**-**	**4**
BR11	9	**R**	Nil	**R**	30	**s**	30	**s**	29	**s**	23	**s**	nil	**R**	nil	**R**	nil	**R**	**5**	**-**	**4**
GL1	7	**R**	Nil	**R**	24	**s**	28	**s**	22	**s**	28	**s**	nil	**R**	nil	**R**	nil	**R**	**5**	**-**	**4**
BR6	11	**R**	Nil	**R**	26	**s**	29	**s**	30	**s**	28	**s**	28	**s**	nil	**R**	nil	**R**	**4**	**-**	**5**
HJ2 *	nil	**R**	Nil	**R**	Nil	**R**	30	**s**	31	**s**	nil	**R**	nil	**R**	nil	**R**	nil	**R**	**7**	**-**	**2**
HJ3.1*	nil	**R**	Nil	**R**	nil	**R**	22	**s**	26	**s**	nil	**R**	nil	**R**	nil	**R**	nil	**R**	**7**	**-**	**3**

Key: S – Sensitive or Susceptible, I – Intermediate, R – Resistant, Z – Zone of Diameter

From the above table it can be state that among 10 isolates of salmonella the stains namely GL6.2, HJ2, and HJ3.1 showed maximum resistant against nine antibiotics. Furthermore, GL6.2 shows resistant towards 6 antibiotics while both HJ2, HJ3.1 shows resistant towards 7 antibiotics out of 9 antibiotics.

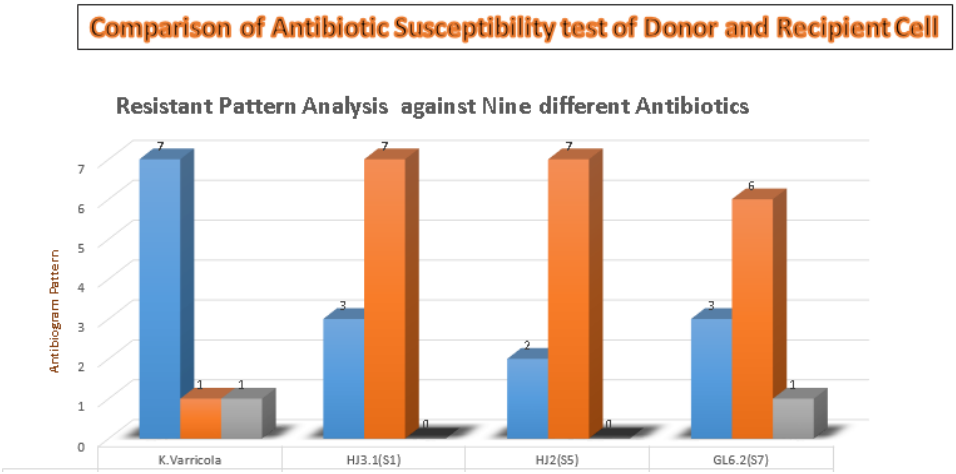


Figure 6: Graphical representation of Antibiogram of donor strains and recipient strain

This graphical representation of Klebsiella stain and 3 salmonella strains (GL6.2, HJ2, and HJ3.1) indicates the resistant pattern of 4 strains against nine antibiotics. From this graph it can be state that, recipient strain *Klebsiella* has maximum sensitivity against nine antibiotics whereas three salmonella strains have maximum resistance against nine antibiotics.

Antibiogram of Klebsiella Variicola (Recipient) and Three Salmonella Strains (Donor)

This antibiogram was performed to ensure re-conformation of the recipient strain and donor strain selection and to study their resistant pattern.

Strain Name	Strain ID	AZM 15 (mm)	CFM 5 (mm)
K.Variicola	**K**	**16(S)**	**28(S)**
Salmonella(GL6.2)	**7**	**nil(R)**	**16(R)**
Salmonella(HJ2)	**5**	**nil(R)**	**19(R)**
Salmonella(HJ3.1)	**1**	**nil(R)**	**16(R)**

The table above describes the results obtained from the antibiotic susceptibility testing using the Kirby-Bauer method. Given the results and analysis of all the isolates it can be said that *K.Variicola* again *(K)* shows susceptibility or sensitivity against Azithromycin and Cefixime. Which indicates that it lacks resistance genes on its chromosomal DNA or plasmid DNA against these β-lactam and macrolide antibiotics. For this reason we will use this *Klebsiella* strain as our recipient, which will receive resistance genes via natural transformation from *Salmonella* strain as our donor. Salmonella strains are repetitively showing resistant against these β-lactam and macrolide antibiotics.

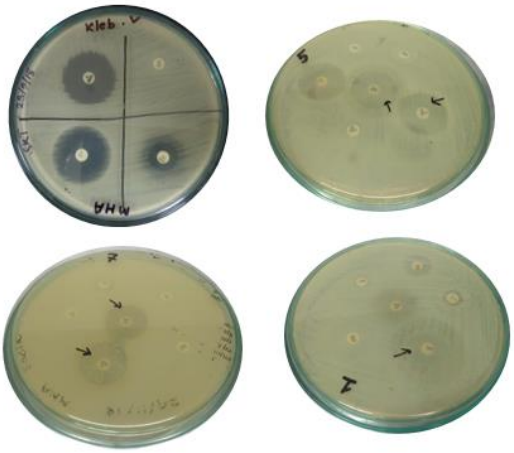


Figure 7: MHA plates with Azithromycin and Cefixime antibiotic disk and the zone of inhibition. [1st plate – K.Variicola, 2nd, 3rd & 4th plates – Salmonella (5, 7, 1) isolates]

3.3 Chromosomal DNA Isolation and Gel Electrophoresis

After performing antibiogram of the selected strains, the chromosomal DNA of those strains were extracted using boiling extraction method. After extracting the chromosomal DNA carefully, gel electrophoresis was performed to observe the separated bands of chromosomal DNA.

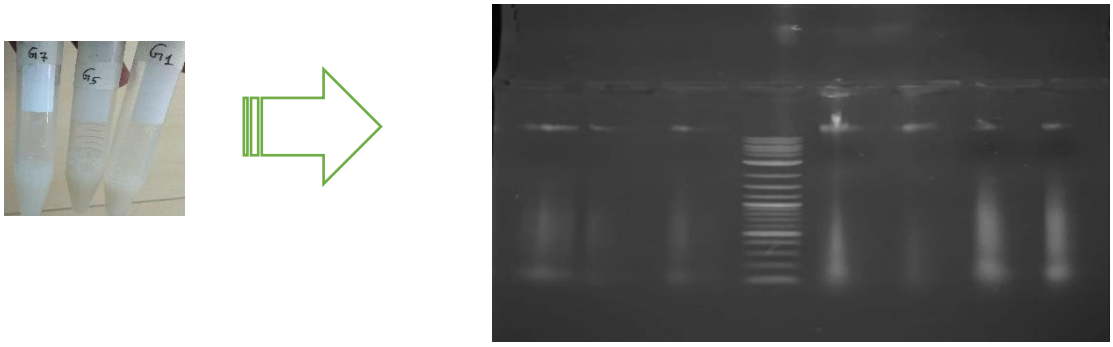


Figure 8: Isolation of Chromosomal DNA and bands on gel electrophoresis

3.4 Plasmid DNA Isolation and Gel Electrophoresis

After performing antibiogram the plasmids of those isolates were extracted using the Kado-Liu method. After extracting the plasmids carefully, gel electrophoresis was performed to observe the separated bands of plasmids.

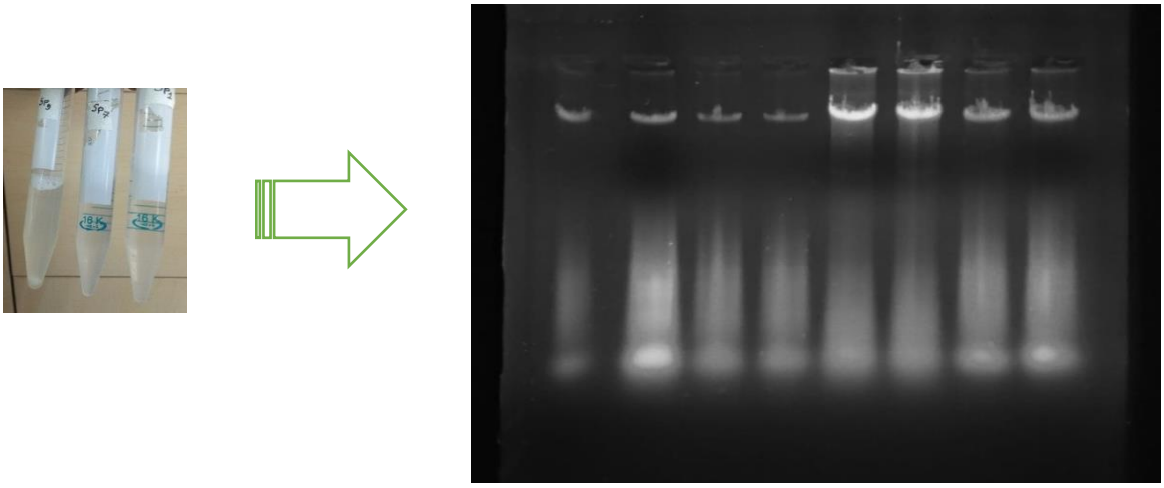


Figure 9: Isolation of Plasmid DNA and bands on gel electrophoresis

3.5 Transformation assay and LB Plate Screening

After the gel electrophoresis experiment and the conformation of chromosomal DNA and plasmid DNA the bacterial natural transformation assay was performed on vial and kept on incubator. After a successful incubation the transformation mixture was swapped on antibiotic specific LB-Agar plates to observe the transformed cell growth.

Table 6: List of Recipient sample and Donor Sample for the transformation experiment

Strain Name	Strain ID	Transformation Materials	Experimental Role
K.Variicola	K	Live organism	Recipient
Salmonella(GL6.2)	7	Chromosomal/Plasmid DNA	Donor
Salmonella(HJ2)	5	Chromosomal/Plasmid DNA	Donor
Salmonella(HJ3.1)	1	Chromosomal/Plasmid DNA	Donor

Results of natural transformation experiment on Gulshan Lake water:

After observing all the antibiotic specific LB-agar plates the growth pattern was jotted down on the table below.

Cefixime Plates	K + C_1	K + C_5	K + C_7	K + P_1	K + P_5	K + P_7
5 mg/ml	**G**	**G**	**G**	**NG**	**G**	**NG**
10 mg/ml	**G**	**G**	**G**	**G**	**G**	**G**
15 mg/ml	**G**	**G**	**G**	**NG**	**G**	**NG**
20 mg/ml	**G**	**G**	**G**	**NG**	**G**	**NG**
25 mg/ml	**G**	**G**	**G**	**NG**	**G**	**NG**

Table: [Key- Growth on Plate (G) & No growth on plate (NG), C (Chromosomal DNA), P (Plasmid DNA]

Azithromycin Plates	K + C_1	K + C_5	K + C_7	K + P_1	K + P_5	K + P_7
15 mg/ml	**G**	**G**	**G**	**NG**	**NG**	**NG**
16 mg/ml	**G**	**G**	**G**	**NG**	**NG**	**NG**
17 mg/ml	**NG**	**G**	**NG**	**G**	**NG**	**NG**
18 mg/ml	**NG**	**G**	**G**	**NG**	**NG**	**NG**
19 mg/ml	**NG**	**G**	**G**	**NG**	**NG**	**NG**

Table: [Growth on Plate (G) & No growth on plate (NG), C (Chromosomal DNA), P (Plasmid DNA

Cefixime Plates	*K.Variicola(K)*	*Salmonella(1)*	*Salmonella(5)*	*Salmonella(7)*
5 mg/ml	NG	G	G	G
10 mg/ml	NG	G	G	G
15 mg/ml	NG	G	G	G
20 mg/ml	NG	G	G	G
25 mg/ml	NG	G	G	G

Table: Key -Growth on Plate (G) & No growth on plate (NG)

Azithromycin Plates	*K.Variicola(K)*	*Salmonella(1)*	*Salmonella(5)*	*Salmonella(7)*
15 mg/ml	NG	NG	NG	NG
16 mg/ml	NG	G	G	G
17 mg/ml	NG	G	NG	NG
18 mg/ml	NG	NG	G	G
19 mg/ml	NG	NG	NG	NG

Table: Key -Growth on Plate (G) & No growth on plate (NG)

Results of natural transformation experiment on Hatirjheel Lake water:

After observing all the antibiotic specific LB-agar plates the growth pattern was jotted down on the table below.

Cefixime Plates	K + C_1	K + C_5	K + C_7	K + P_1	K + P_5	K + P_7
5 mg/ml	**G**	**G**	**G**	**NG**	**NG**	**G**
10 mg/ml	**G**	**G**	**G**	**NG**	**NG**	**G**
15 mg/ml	**G**	**G**	**G**	**NG**	**NG**	**G**
20 mg/ml	**G**	**G**	**G**	**NG**	**NG**	**G**
25 mg/ml	**G**	**G**	**G**	**NG**	**NG**	**G**

Key -Growth on Plate (G) & No growth on plate (NG), C (Chromosomal DNA), P (Plasmid DNA)

Azithromycin Plates	K + C_1	K + C_5	K + C_7	K + P_1	K + P_5	K + P_7
15 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
16 mg/ml	**NG**	**G**	**G**	**NG**	**NG**	**NG**
17 mg/ml	**NG**	**NG**	**G**	**NG**	**NG**	**NG**
18 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
19 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**

Key- Growth on Plate (G) & No growth on plate (NG), C (Chromosomal DNA), P (Plasmid DNA)

Cefixime Plates	*K.Variicola(K)*	*Salmonella(1)*	*Salmonella(5)*	*Salmonella(7)*
5 mg/ml	NG	G	G	G
10 mg/ml	NG	G	G	G
15 mg/ml	NG	G	G	G
20 mg/ml	NG	G	G	G
25 mg/ml	NG	G	G	G

Key -Growth on Plate (G) & No growth on plate (NG)

Azithromycin Plates	*K.Variicola(K)*	*Salmonella(1)*	*Salmonella(5)*	*Salmonella(7)*
15 mg/ml	NG	G	G	G
16 mg/ml	NG	G	G	G
17 mg/ml	NG	G	G	G
18 mg/ml	NG	NG	NG	G
19 mg/ml	NG	NG	NG	G

Key -Growth on Plate (G) & No growth on plate (NG)

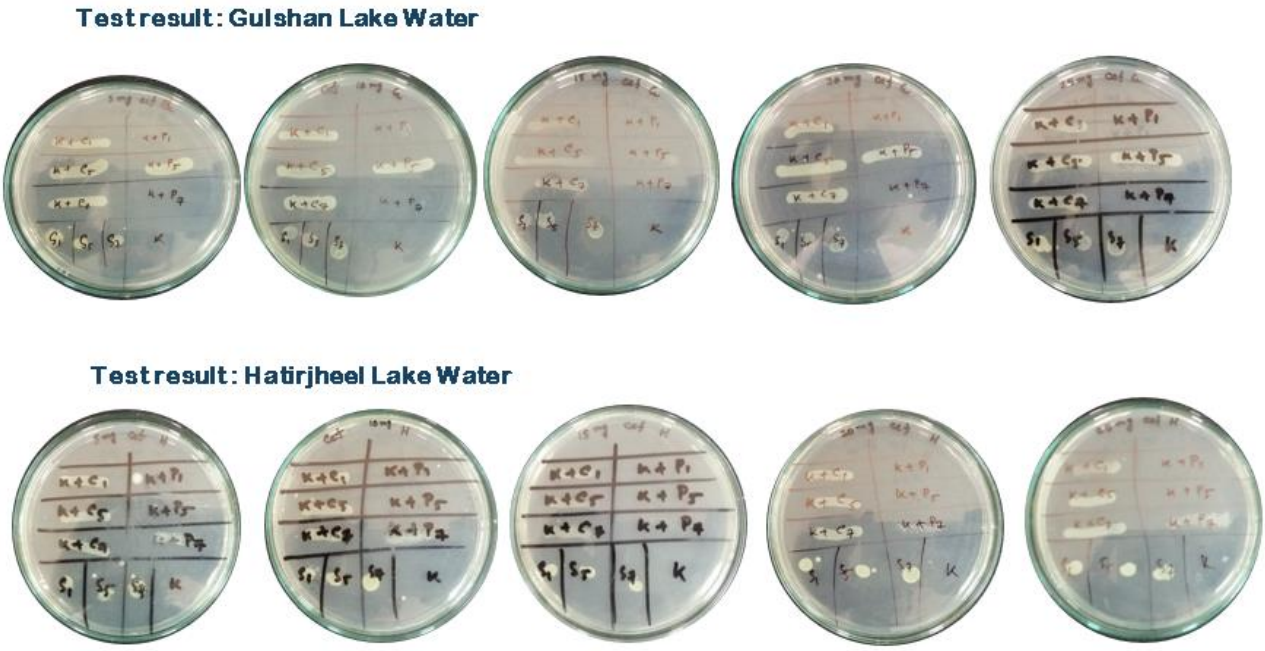


Figure 10: Screening of Bacterial Transformation results on Cefixime containing LB Plates

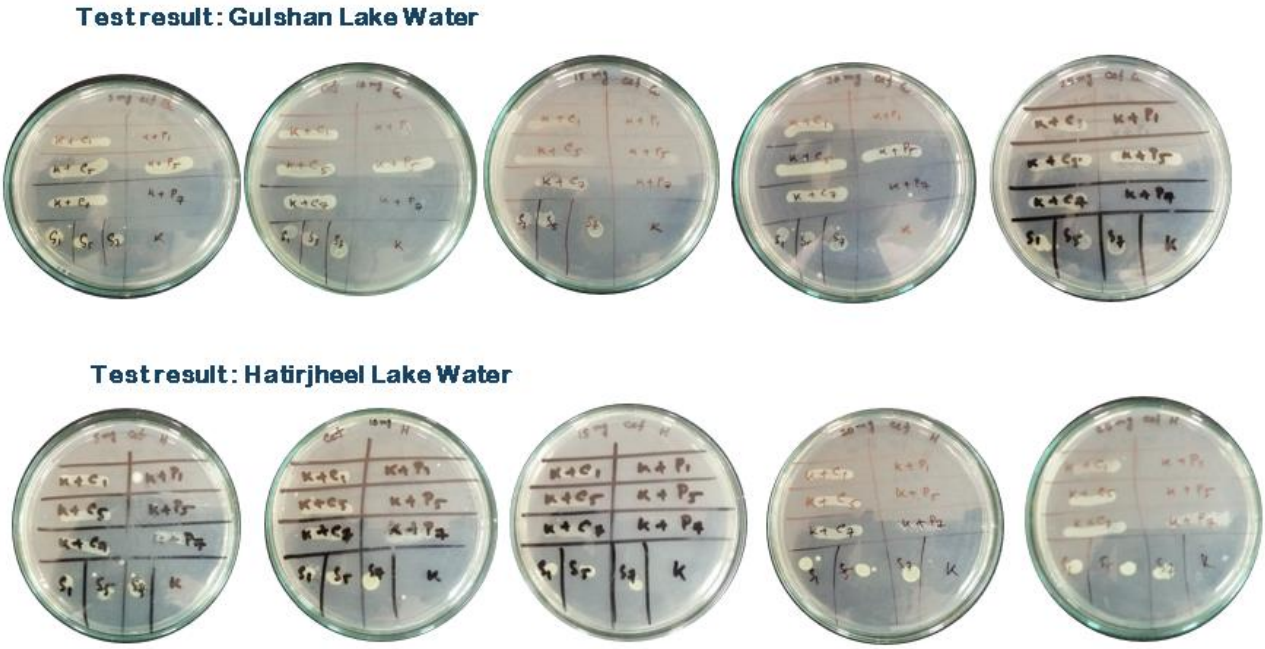


Figure 11: Screening of Bacterial Transformation results on Azithromycin containing LB Plates

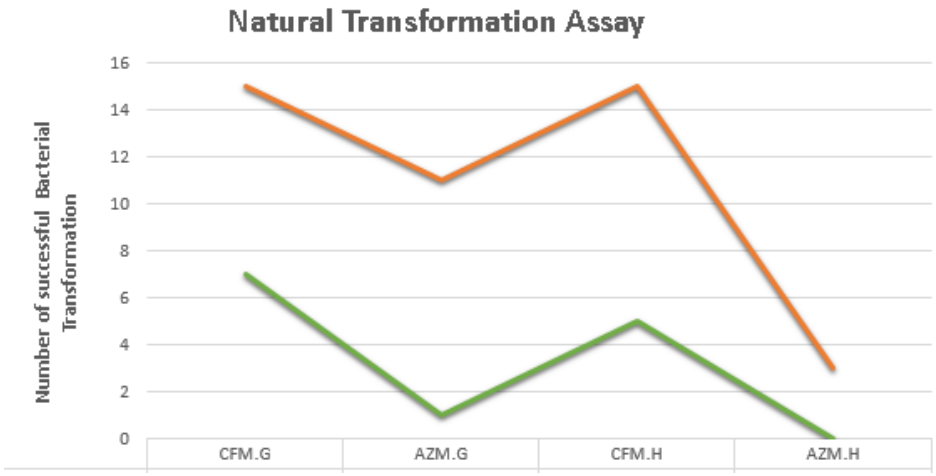


Figure 12: Graphical representation of the rate of bacterial transformation [red curve- chromosome mediated transformation, green curve- plasmid mediated transformation]

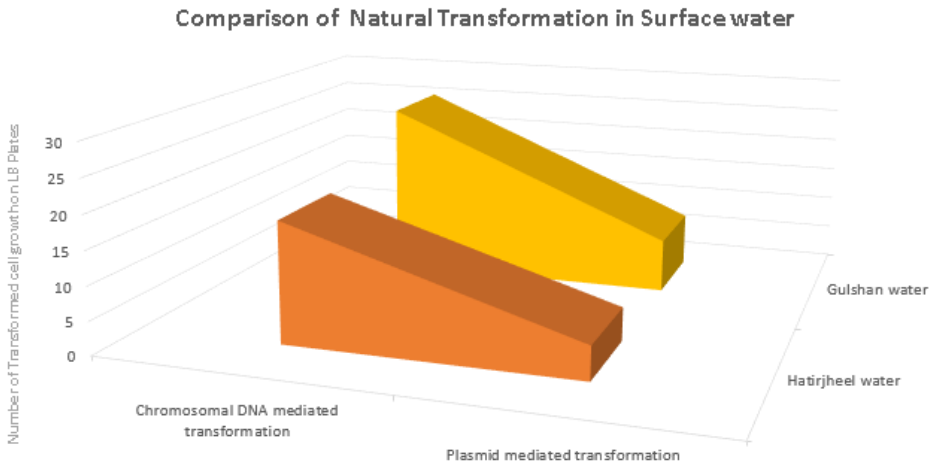


Figure 13: Representation of bacterial transformation on different surface water bodies of DHAKA city

From figure 12 & 13 it can be summarized that, the rate of chromosomal mediated bacterial transformation is higher than plasmid mediated bacterial transformation between Klebsiella and Salmonella. The amount of successful bacterial transformation is higher in Gulshan surface water. The quantity of successful bacterial transformation is lower in Hatirjheel surface water. Transformed cells growth appeared mostly on Cefixime-LB plates rather than Azithromycin LB plates in Gulshan surface water. Transformed cells growth appeared mostly on Cefixime-LB plates rather than Azithromycin-LB plates in Hatirjheel surface water.

3.6 Plasmid Curing results:

As mentioned in the methods section plasmid curing was conducted using EtBr and SDS. After overnight incubation of the cultures in EtBr and SDS were plated on LB agar containing plates with different concentrations of antibiotics. Presence or absence of growth in the antibiotic containing plates enables us to determine whether the resistance gene is present in the chromosomal DNA or in the plasmid DNA.

5 mg/ml Cefixime plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	G	G	G	G	NG	NG
Salmonella(HJ2)	5	G	NG	G	G	NG	NG
Salmonella(GL6.2)	7	G	NG	G	G	NG	G

Key -Growth on Plate (G) & No growth on plate (NG)

10 mg/ml Cefixime plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	G	G	G	G	NG	NG
Salmonella(HJ2)	5	G	G	G	G	NG	G
Salmonella(GL6.2)	7	G	G	G	G	NG	G

Key -Growth on Plate (G) & No growth on plate (NG)

15 mg/ml Cefixime plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	G	G	G	G	NG	G
Salmonella(HJ2)	5	G	G	G	G	NG	G
Salmonella(GL6.2)	7	G	G	G	NG	NG	NG

Key -Growth on Plate (G) & No growth on plate (NG)

20 mg/ml Cefixime plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	G	G	G	NG	NG	NG
Salmonella(HJ2)	5	G	G	G	NG	NG	NG
Salmonella(GL6.2)	7	G	G	G	G	NG	NG

Key -Growth on Plate (G) & No growth on plate (NG)

25 mg/ml Cefixime plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	G	G	G	NG	NG	NG
Salmonella(HJ2)	5	G	G	G	G	NG	NG
Salmonella(GL6.2)	7	G	G	G	G	NG	NG

Key -Growth on Plate (G) & No growth on plate (NG)

15 mg/ml Azithromycin plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	NG	NG	NG	G	NG	G
Salmonella(HJ2)	5	G	NG	G	G	NG	NG
Salmonella(GL6.2)	7	G	G	NG	G	G	G

Key -Growth on Plate (G) & No growth on plate (NG)

16 mg/ml Azithromycin plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	NG	NG	NG	G	NG	G
Salmonella(HJ2)	5	G	NG	G	G	NG	NG
Salmonella(GL6.2)	7	G	G	NG	G	G	G

Key -Growth on Plate (G) & No growth on plate (NG)

17 mg/ml Azithromycin plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	G	NG	NG	NG	G	G
Salmonella(HJ2)	5	G	NG	NG	NG	NG	NG
Salmonella(GL6.2)	7	G	NG	NG	G	G	G

Key -Growth on Plate (G) & No growth on plate (NG)

18 mg/ml Azithromycin plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	NG	NG	NG	NG	NG	NG
Salmonella(HJ2)	5	NG	NG	NG	NG	NG	NG
Salmonella(GL6.2)	7	G	NG	NG	NG	NG	G

Key -Growth on Plate (G) & No growth on plate (NG)

19 mg/ml Azithromycin plate	Organism ID	EtBr 50µg/ml	EtBr 100µg/ml	EtBr 150µg/ml	SDS 5%	SDS 10%	SDS 15%
Salmonella(HJ3.1)	1	NG	NG	NG	NG	NG	NG
Salmonella(HJ2)	5	G	NG	NG	NG	G	NG
Salmonella(GL6.2)	7	G	G	NG	G	G	G

Key -Growth on Plate (G) & No growth on plate (NG)

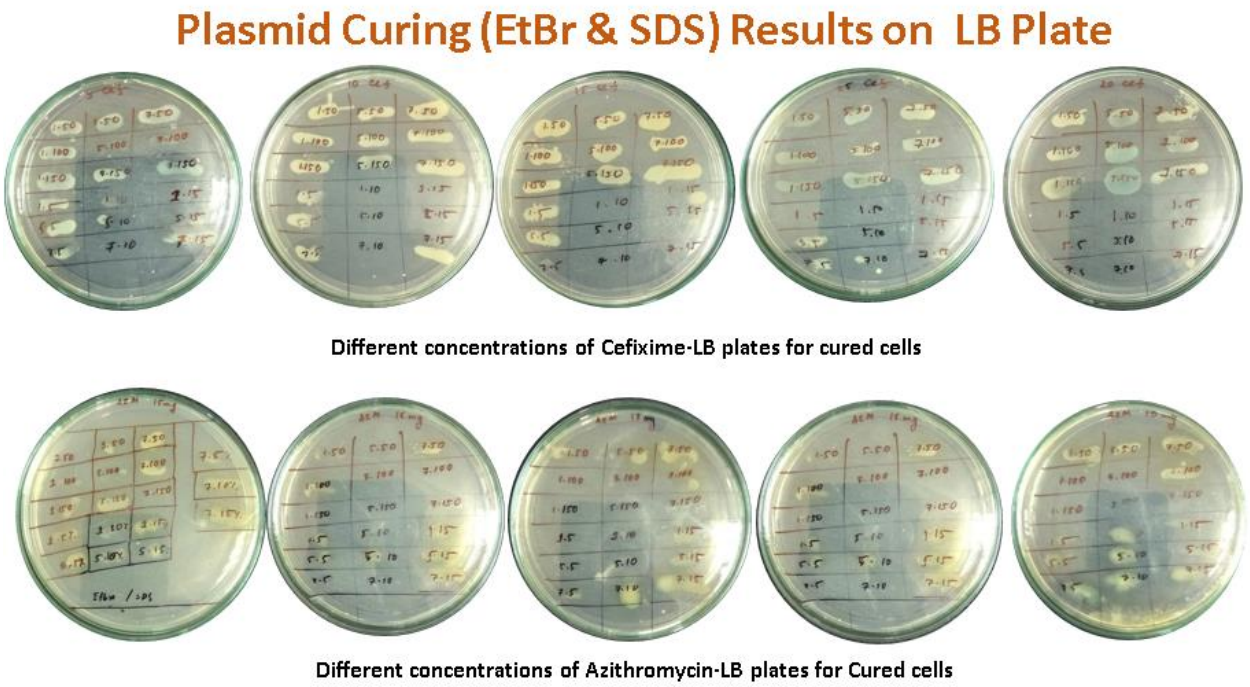


Figure 14: Screening of Plasmid Cured samples growth on LB plates

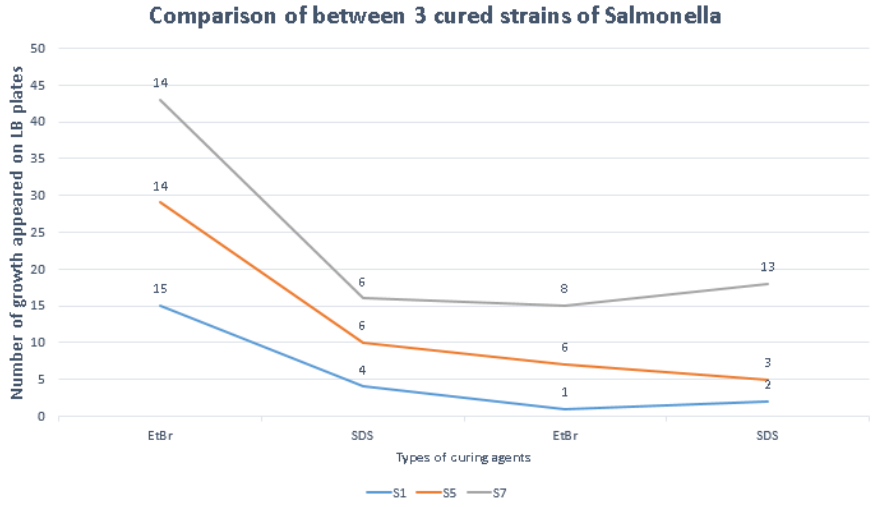
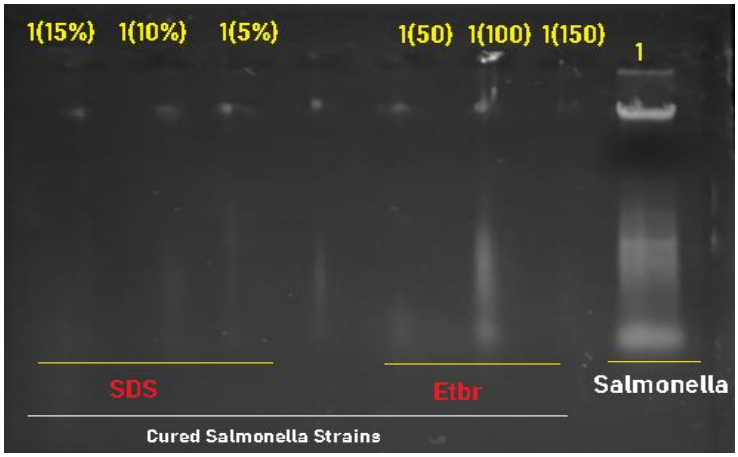


Figure 15: Representation of cured salmonella samples growth on LB Plates against curing agents [grey curve- Salmonella 7, red curve- salmonella 5, and blue curve- salmonella 1]

From the above graph it can be states that, Cured-Salmonella-7(GL6.2) exhibited highest bacterial growth rather than Cured-Salmonella-1(HJ3.1) & Salmonella-5(HJ2) in different states of curing.

Gel Electrophoresis of Cured Isolates of *Salmonella*:



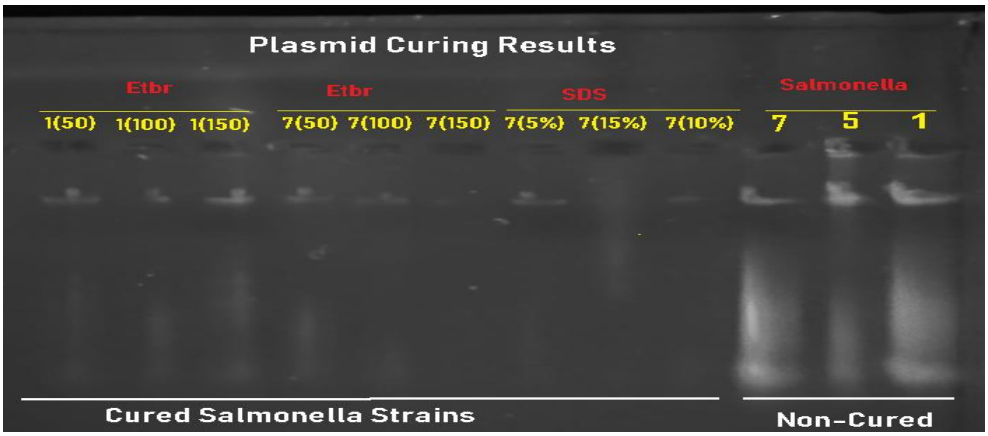


Figure: The result of gel electrophoresis showing the loss of plasmid bands

The extracted plasmids of the Salmonella isolates shows several bands under UV light. It was found that the well contains different types of SDS (5%, 15%, 15%) treated plasmids of all three salmonella strains (1, 5, and 7), the band is missing in 8 different wells in other words 8 strains were possibly cured namely 1(5%,10%,15%), 5(5%,15%), 7(5%,10%, 15%) . Which indicates that, due to the SDS exposure the plasmid is eliminated from the cured salmonella strains. Whereas, the well contains different types of EtBr (50mg/ml, 100mg/ml, 150mg/ml) treated plasmids, of all three salmonella train (1, 5, 7) very few wells retained its band similar to non-cured salmonella strains and some of the wells band is missing. That is why 6 strains were possibly cured namely 1(50mg/ml,150mg/ml), 5(150mg/ml), 7(100mg/ml, 150mg/ml) .Which indicates that, due to the EtBr exposure the plasmid is not that much eliminated from the cured salmonella. Therefore, SDS was more effective curing agent than EtBr during this experiment.

Chapter 4: Discussion

The purpose of this study was to observe natural transformation between *klebsiella* and Salmonella on water bodies of the Dhaka city; Hatirjheel Lake and Gulshan Lake. Salmonella the causative organism for typhoid is both food-borne as well as a waterborne organism(Levantesi et al., 2012). Due to rapid unplanned urbanization and industrialization, the water bodies are getting polluted and typhoid is becoming endemic in Bangladesh(Brooks et al., 2005). This is why it is of utmost importance for us to know whether the water bodies are contaminated with *Salmonella* chromosomal DNA or plasmid DNA which promotes other organism to cause same disease or transform them to a virulent strain like salmonella. To begin with, the ATCC Strains result obtained from the antibiotic susceptibility testing using the Kirby-Bauer method. Given the results and analysis of all the isolates it can be said that three ATCC strains namely *E.Coli* , *K.Variicola* , *S.Typhi* showed lowest number of resistant and highest number of susceptibility against 9 different antibiotics. As our aim and objective was to observe the natural transformation of *Klebsiella variicola* and *Salmonella* therefore we will going to consider *Klebsiella* antibiogram result and targeted it as an experimental recipient strain (*K.Variicola)*. Moreover, *K.Variicola* shows susceptibility against seven antibiotics namely Azithromycin, Cefixime, Levofloxacin, Ciprofloxacin, Cefuroxime, Tetracycline and Colistin and shows resistant against only Vancomycin. In Addition to that, *K.Variicola* shows sensitivity against Azithromycin and Cefixime antibiotics which indicates that this strain lacks resistant genes against β-lactam or macrolide class antibiotics in their chromosomal DNA or Plasmid DNA.

In addition to that, the results obtained from the antibiotic susceptibility testing using the Kirby-Bauer method. Given the results and analysis of all the isolates it can be said that three isolates namely GL6.2, HJ2, and HJ3.1 showed maximum number of resistance against nine antibiotics. These three strains of *salmonella* shows resistant against Azithromycin, Vancomycin, Ampicillin, Tetracycline, Colistin, and Cefixime antibiotics. Among them GL6.2 strain of *salmonella* shows resistant against 6 antibiotics namely Azithromycin, Vancomycin, Ampicillin, Tetracycline, Colistin, and Cefixime. Whereas, both HJ3, 1 HJ2 strains of *salmonella* showed resistant against 7 antibiotics namely Azithromycin, Vancomycin, Ampicillin, Tetracycline, Colistin, Cefixime, Cefuroxime. In addition to that these three strains of salmonella (GL6.2, HJ2, and HJ3.1) showed lowest number of sensitivity against remaining antibiotics. By observing disk diffusion result it can be demonstrated that these three strains of *salmonella* have high resistant number than other salmonella strains. This type of high resistant number also indicates that they have some genetic components or resistant genes in their chromosomal DNA or plasmid DNA which are responsible for their high number of resistant pattern against many antibiotics. As our aim and objective is to observe the natural transformation of *Klebsiella variicola* and *Salmonella* therefore we will going to consider these three *salmonella* (GL6.2, HJ2, and HJ3.1) antibiogram result and used it as an experimental donor strains. Interestingly, these three strains of salmonella showed resistant against Azithromycin and Cefixime which specifies that these strains have some resistant genes in their intra-cellular environment which are responsible for their resistance against these two β-lactam and macrolide class antibiotics.

In the same time, the results of bacterial natural transformation on Gulshan Lake water sample. From the above results it can be analyzed that in different concentration of Cefixime plates maximum growth of transformed *Klebsiella Variicola* appeared when they usually mixed with the donor (Salmonella*)* chromosomal DNA. Moreover, 15 transformed cells showed growth on 5 different concentrated Cefixime plates. For that reason it indicates that K.Variicola receives some genetic material from donor chromosomal DNA by which they were able to grow against β-lactam class antibiotic. On the other hand, in different concentration of Cefixime plate lowest number growth of transformed K.Variicola appeared when they are mixed with donor plasmid DNA. In addition to that, out of 15 different cases 7 transformed K.Variicola cells showed growth on plates and 8 cells showed no growth on plates. Those 7 transformed Klebsiella cells growth on Cefixime plates indicates that K.Variicola receives some genetic material such as resistant genes from donor plasmid by which they were able to grow against β-lactam class antibiotic. It can also demonstrate that, the rate of bacterial transformation via chromosomal DNA of donor is higher than the rate of bacterial transformation via plasmid DNA of donor cell against β-lactam antibiotic.

Moreover, in different concentration of Azithromycin plates maximum growth of transformed *Klebsiella Variicola* appeared when they usually mixed with the donor (Salmonella*)* chromosomal DNA. Moreover, 11 transformed cells showed growth on 5 different concentrated azithromycin plates. For that reason it indicates that K.Variicola receives some genetic material from donor chromosomal DNA by which they were able to grow against macrolide class antibiotic. On the other hand, in different concentration of azithromycin plate lowest number growth of transformed K.Variicola appeared when they are mixed with donor plasmid DNA. In addition to that, out of 15 different cases 1 transformed K.Variicola cells showed growth on plates and 14 cells showed no growth on plates. Only one transformed Klebsiella cells growth on azithromycin plates indicates that K.Variicola receives some genetic material such as resistant genes from donor plasmid by which they were able to grow against macrolide class antibiotic. It can also demonstrate that, the rate of bacterial transformation via chromosomal DNA of donor is higher than the rate of bacterial transformation via plasmid DNA of donor cell against macrolide antibiotic.

Eventually, the single Klebsiella strain (ATCC strain) was unable to grow on different concentration of Cefixime and Azithromycin plates. Which specifies that, lack resistant genes in their cellular component and due to no transformation or no receive of any foreign DNA material from other donor cell such as salmonella they were unable to grow against β-lactam and macrolide class antibiotic. On the other hand, three different donor salmonella samples were also subjected to Cefixime and azithromycin plates. Interestingly, most of them showed growth on plates. Which specifies that our donor strains have some resistant gene components by which they were able to grow against β-lactam and macrolide class antibiotic. Along with that, if their DNA is present in water it can transform other recipient cell into a resistant one like it.

Furthermore, in different concentration of Cefixime plates maximum growth of transformed *Klebsiella Variicola* appeared when they usually mixed with the donor (Salmonella*)* chromosomal DNA. Moreover, 15 transformed cells showed growth on 5 different concentrated Cefixime plates. For that reason it indicates that K.Variicola receives some genetic material from donor chromosomal DNA by which they were able to grow against β-lactam class antibiotic.

On the other hand, in different concentration of Cefixime plate lowest number growth of transformed K.Variicola appeared when they are mixed with donor plasmid DNA. In addition to that, out of 15 different cases 5 transformed K.Variicola cells showed growth on plates and 10 cells showed no growth on plates. Those 5 transformed Klebsiella cells growth on Cefixime plates indicates that K.Variicola receives some genetic material such as resistant genes from donor plasmid by which they were able to grow against β-lactam class antibiotic. It can also demonstrate that, the rate of bacterial transformation via chromosomal DNA of donor is higher than the rate of bacterial transformation via plasmid DNA of donor cell against β-lactam antibiotic. Moreover, in different concentration of Azithromycin plates maximum growth of transformed *Klebsiella Variicola* appeared when they usually mixed with the donor (Salmonella*)* chromosomal DNA. Moreover, 3 transformed cells show growth and other 12 cells do not show any growth on 5 different concentrated azithromycin plates. For that reason, those 3 transformed cells of Klebsiella indicates that K.Variicola receives some genetic material from donor chromosomal DNA by which they were able to grow against macrolide class antibiotic.

While in different concentration of azithromycin plate lowest number growth of transformed K.*Variicola* appeared when they are mixed with donor plasmid DNA. In addition to that, out of 15 different cases of plasmid mediated transformation in azithromycin plates K.Variicola cells do not show any growth on plates. This type of result indicates that K.Variicola didn't receive any genetic material such as resistant genes from donor plasmid by which they can able to grow against macrolide class antibiotic. It can also demonstrate that, the rate of bacterial transformation via chromosomal DNA of donor is higher than the rate of bacterial transformation via plasmid DNA of donor cell against macrolide antibiotic.

Conversely, the single Klebsiella strain (ATCC strain) was unable to grow on different concentration of Cefixime and Azithromycin plates. Which specifies that, lack resistant genes in their cellular component and due to no transformation or no receive of any foreign DNA material from other donor cell such as salmonella they were unable to grow against β-lactam and macrolide class antibiotic. On the other hand, three different donor salmonella samples were also subjected to Cefixime and azithromycin plates. Interestingly, most of them showed growth on plates. Which specifies that our donor strains have some resistant gene components by which they were able to grow against β-lactam and macrolide class antibiotic. Along with that, if their DNA is present in water it can transform other recipient cell into a resistant one like it.

Additionally, the plasmid curing experiment was done to observe the persistence or the reservoir of resistant genes in the intracellular compartment of donor cells (Salmonella strains). As three Salmonella strains are used as a donor cell therefore it is mandatory to know from which part of (Chromosomal DNA / Plasmid DNA) of donor cells transfer foreign DNA or resistant genes. From the above plasmid curing results it can be summarized that *Salmonella HJ3.1* shows growth on 18 LB plates concentrated with Cefixime antibiotic. These 18 bacterial growth appeared against three different concentration of EtBr (50µg, 100µg, 150µg). On the other hand, *Salmonella HJ3.1* shows growth on 4 LB plates concentrated with Cefixime antibiotic. These 4 bacterial growth appeared against three different concentration of SDS (5%, 10%, and 15%). Moreover, *Salmonella HJ3.1* shows growth only on 1 LB plate concentrated with Azithromycin antibiotic. This one bacterial growth appeared against 50µg/ml EtBr. On the other hand, *Salmonella HJ3.1* shows growth on 6

LB plates concentrated with azithromycin antibiotic. These 6 bacterial growth appeared against three different concentration of SDS (5%, 10%, and 15%). Secondly, *Salmonella HJ2* shows growth on 14 LB plates concentrated with Cefixime antibiotic. These 14 bacterial growth appeared against three different concentration of EtBr (50µg, 100µg, 150µg). Besides, *Salmonella HJ2* shows growth on 6 LB plates concentrated with Cefixime antibiotic. These 6 bacterial growth appeared against three different concentration of SDS (5%, 10%, and 15%). Moreover, *Salmonella HJ2* shows growth only on 7 LB plate concentrated with Azithromycin antibiotic. This one bacterial growth appeared against (50µg, 100µg, 150 µg) EtBr. On the other hand, *Salmonella HJ2* shows growth on 3 LB plates concentrated with azithromycin antibiotic. These 3 bacterial growth appeared against two different concentration of SDS (5%, 10%). Meanwhile, *Salmonella GL6.2* shows growth on 14 LB plates concentrated with Cefixime antibiotic. These 14 bacterial growth appeared against three different concentration of EtBr (50µg, 100µg, 150µg). On the other hand, *Salmonella GL6.2* shows growth on 6 LB plates concentrated with Cefixime antibiotic. These 6 bacterial growth appeared against three different concentration of SDS (5%, 10%, and 15%). Moreover, *Salmonella GL6.2* shows growth only on 9 LB plate concentrated with Azithromycin antibiotic. This one bacterial growth appeared against (50µg, 100µg, 150 µg) EtBr. On the other hand, *Salmonella GL6.2* shows growth on 13 LB plates concentrated with azithromycin antibiotic. These 3 bacterial growth appeared against two different concentration of SDS (5%, 10%, and 15%).

In fact, among three strains of donor, *Salmonella (HJ3.1)* shows highest number (22) of growth on Cefixime antibiotic plates of LB after the exposure of curing agents like EtBr and SDS. This result indicates that curing agents was unable to eliminate the plasmid DNA from *Salmonella (HJ3.1)* sample and this strain showing resistance against β-lactam class antibiotic via expressing resistant genes from plasmid DNA. Whereas, *Salmonella (HJ3.1)* shows lowest number (7) of growth on azithromycin antibiotic plates of LB after the exposure of curing agents like EtBr and SDS. This result indicates that curing agents was able to eliminate vast amount of the plasmid DNA from *Salmonella (HJ3.1)* sample and this strain is unable showing resistance against macrolide class antibiotic as it lacks plasmid DNA. In addition to that, *Salmonella (HJ2)* shows highest number (20) of growth on Cefixime antibiotic plates of LB after the exposure of curing agents like EtBr and SDS. This result indicates that curing agents was unable to eliminate the plasmid DNA from *Salmonella (HJ2)* sample and this strain showing resistance against β-lactam class antibiotic via expressing resistant genes from plasmid DNA. Whereas, *Salmonella (HJ2)* shows lowest number (10) of growth on azithromycin antibiotic plates of LB after the exposure of curing agents like EtBr and SDS. This result indicates that curing agents was able to eliminate vast amount of the plasmid DNA from *Salmonella (HJ2)* sample and this strain is unable showing resistance against macrolide class antibiotic as it lacks plasmid DNA. Likewise, Salmonella *(GL6.2)* shows highest number (20) of growth on Cefixime antibiotic plates of LB after the exposure of curing agents like EtBr and SDS. This result indicates that curing agents was unable to eliminate the plasmid DNA from *Salmonella (GL6.2)* sample and this strain showing resistance against β-lactam class antibiotic via expressing resistant genes from plasmid DNA. Whereas, *Salmonella (GL6.2)* shows highest number (22) of growth on azithromycin antibiotic plates of LB after the exposure of curing agents like EtBr and SDS. This result indicates that curing agents was unable to eliminate vast amount of the plasmid DNA from *Salmonella (GL6.2)* sample and this strain is able showing resistance against macrolide class antibiotic as the curing agents have no effect on the plasmid of this donor strain.

Chapter 5: Conclusion

More than 10 million people in the year 2000 and annually causes approximately 100000 deaths due to typhoid fever. Previously several studies have proven the fact that surface water of lakes inside Dhaka city are polluted and contains various disease causative microscopic organisms. However, the existence of pathogenic organisms DNA or genetic materials or mobile genetic elements such as *Salmonella* virulent genes in these water bodies is a serious headache issue for public health. For this very reason, this study was done which focused on the horizontal gene transfer among bacterial species especially *Salmonella and Klebsiella* and most importantly; their gene transformation factor. Their growth pattern on LB plates has showed that under favorable condition the bacterial species are able to transfer genes among them. The isolates of salmonella from these water bodies contained the virulent genes in their plasmid or chromosomal DNA, which makes it safe to say that they are pathogenic and are capable of causing waterborne diseases. In addition, this paper speculated on the fact that the antibiotic resistance shown by these organisms might be transferring horizontally via chromosome which opens a new paradigm to the public health sector.

In the future, this work can be extended by performing the PCR and sequence analysis for all isolates. To sum up, more work should be done on the molecular level to figure out the modes of resistance transfer within these environmental isolates especially if chromosomal or plasmid mediated transfer of gene occurs within the genomic island of *Salmonella* via various mobile genetic elements such as integrons, transposons or plasmid.

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Appendix

Media Composition:

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final 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: 7.4±0.2 at 25ºC	

Muller Hinton Agar

Component	Amount (g/L)
Beef, dehydrated infusion form	300
Casein hydro lysate	17.5
Starch	1.5
Agar	17.0
Final pH: 7.3± 0.1 at 25°C	

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Luria Bertani Broth (LB Broth)

Component	Amount (g/L)
Yeast Extract	5.0
Sodium Chloride	10.0
Tryptone	10.0
Final pH: 6.9-7.1	

Luria Bertani Agar (LB Agar)

Component	Amount (g/L)
Casein enzymic hydrolyte	10.0
Yeast Extract	5.0
Sodium Chloride	15.0
Agar	10.0
Final pH: 7.5±0.2 at 25°C	

Appendix- II

Reagents and Buffers:

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.

2N NaOH:

In a small Durham bottle 4 g NaOH was added. Then 50 ml distilled water was added to prepare 50 ml of 2N NaOH. Then it was stored at room temperature.

Kado-I Buffer:

In a Durham bottle, 4 ml of 1M Tris Hcl and 400µL of 0.5M EDTA were added. Then 96 ml distilled water was added to prepare 100ml Kado-I Buffer. Then it was stored at room temperature.

Kado-II Buffer:

In a Durham bottle, 0.6 g of Tris base, 3 g of SDS, 6.4 ml of 2N NaOH were added. Then 94ml distilled water was added to prepare 100ml Kado-II Buffer. 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.

Appendix- III

Equipment:

Autoclave	Model: WIS 20R Daihan Scientific Co. ltd, Korea
Sterilizer	Model no: NDS-600D, Japan
Balance machine: Adam	UK
Centrifuge, Model No: Code: 5433000.011	Eppendorf, Germany
Freezer (-20o C)	Siemens Germany
Incubator	Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan
Laminar Airflow Cabinet	Model-SLF-V, vertical, SAARC group Bangladesh
Micropipettes	Eppendorf, Germany
Oven (Universal drying oven)	Model: LDO-060E, Labtech, Singapore
Refrigerator	Samsung
Vortex mixture	Digi system Taiwan, VM-2000