HORIZONTAL TRANSFER OF ANTIBIOTIC RESISTANCE BETWEEN TRANS-GRAM BACTERIA IN SURFACE WATER OF DHAKA CITY.

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology

Mathematics and Natural Sciences
BRAC University
October, 2019

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis titled **"Horizontal Transfer of Antibiotic Resistance Between Trans-Gram Bacteria in Surface Water of Dhaka City."** submitted by
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of Summer, 2014 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on 03 October, 2019.

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Ethics Statement

No human or animal subjects were studied in this experiment. Also no harms of any environmental substances were done by this experiment.

Abstract

A global medical crisis has already started as antibiotics have started losing their effectiveness against the growing number of bacterial pathogens. Horizontal Gene Transfer (HGT) system has a great impact on this spreading of antibiotic resistance. However, the transformation dynamic is poorly understood. To find out the condition of surface water of Dhaka city that, how favorable it is for transformation of antibiotic resistance gene, Gulshan Lake and Hatirjheel Lake were chosen for this experiment, where natural transformation was performed between trans-gram bacteria *Staphylococcus aureus* and *Salmonella typhi*. From the 10 isolates, that were collected from BRAC university laboratory, 3 highest resistance showing isolates of *Salmonella* (*Salmonella* GL6.2, *Salmonella* HJ & *Salmonella* HJ3.1) were chosen as donor and one ATCC strain of *Staphylococcus aureus* was confirmed as recipient by the Kirby-Buer disk diffusion method. Finally, transformation was performed in Gulshan lake water and in hatirjheel lake water mixing the chromosomal DNA (Boiling method) and plasmid DNA (Kado-Liu method) of donor with recipient. Further, the confirmation test of transformation showed that, no DNA was uptake in any sample of the Gulshan and Hatirjheel lake water against any of the two specific antibiotics (Azithromycin & Tetracycline). The findings from this experiment indicates that, though horizontal gene transfer has a lot of impact in the spread of antibiotic resistance, spreading through natural transformation between gram positive and gram-negative organism might not be a very common phenomenon. At this time of rapid spreading of antibiotic resistance, this finding will help us to think in different ways of possible trans-gram transformations.

Dedicated to

All My Beloved Ones

Acknowledgement

I would like to start by thanking the Almighty Allah for granting me to live this long and also fulfilling me with the strength and enough patience to finish my thesis work and accomplish all the objectives successfully.

Secondly, I would like to express my utmost gratitude to Professor **A.F.M. Yusuf Haider**, Chairperson of the Department of Mathematics and Natural Sciences, BRAC University, Professor **Dr. M. Mahboob Hossain** & Assistant Professor **Dr. Iftekhar Bin Naser** for allowing and providing me with all the opportunities to conduct my thesis work.

I was really honored and also grateful to my supervisor **Mr. Mahbubul Hasan Siddiqee**, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University. I will forever be obliged and grateful to him for his continuous support and useful knowledge throughout my work. He was also available whenever I needed some help or assistance regarding my work and paper. He also supported me morally and gave me much strength throughout the thesis to accomplish all the objectives of this work.

I would also like to give respect and show my appreciation towards Asma Binte Afzal and Md. Nazrul Islam for assisting me throughout my thesis work which would not have been possible without them. Most importantly, my gratitude knows no bounds to my mother and my father, who always believed in me no matter what the situation is and what the tasks I needed to accomplish. They are the reason of my sole existence and nothing can be compared to that. Special thanks also goes to Jitu Paul for his continues support help throughout my work.

Lastly, my special thanks and gratitude goes to Asif Iqbal for his constant moral support from behind, which made all the works seem easy and doable.

Fariha Binte Mostafa
October, 2019

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Chapter 1: Introduction

1.1 Introduction

The prevalence of antibiotic resistance has nowadays increased a lot (Chokshi *et al,* 2019). Global Antimicrobial Surveillance System (GLASS) of World Health Organization reveals widespread occurrence of antibiotic resistance among 5,00,000 people with suspected bacterial infections across 22 countries (W.H.O., 2018). Apart from that, antibiotic resistance reveals high levels of resistance to a number of serious bacterial infections in both high- and low-income countries (W.H.O., 2018). So as a developing country, Bangladesh cannot also ignore or pass away the problems or sufferings of antibiotic resistance which is an alarming problem of present world. However, Bangladesh has already started to suffer due to this problem and there is enough evidence to support this fact.

Saidur Rahman, associate professor of Bangladesh Medical College Hospital said that in recent years' typhoid, urinary infection and diarrhea are becoming resistant to most of the medicines at this time that are available in the market (Hasan, 2019). Professor Dr Sayedur Rahman of Pharmacology Department of Bangabandhu Sheikh Mujib Medical University (BSMMU) said that two antibiotics were discovered in 1994 and 2000, but before releasing these two, authorities found that some microbes have already developed resistance towards them and for that no major new class of antibiotics has been discovered since 1987, and too few antibacterial agents are in development to meet the challenge of multidrug resistance (Hasan, 2019). Poor healthcare standards, along with the misuse and overuse of antibiotics are mainly responsible for this condition in Bangladesh (Ahmed & Rabbi, 2018).

Resistant bacteria can spread in any way as bacteria has no boundaries to follow. It can spread from person to person, animal to person, from travelling, trading, food or even from water. But how a bacterium becomes resistant to an antibiotic is the matter of concern. Bacterial cells have the ability to acquire resistance and basically in two ways they can do this. Firstly, there is mutation, which occurs in the DNA of the cell when they go through replication process. And secondly, they become resistant through horizontal gene transfer (*How Bacteria Build Resistance at The Cellular Level,* 2017). Rapid use of antibiotics may help a bacterium to alter its genetic information and leads to mutation. But where there is no chance of it, horizontal gene transfer works as the main way.

Horizontal gene transfer (HGT) contributes significantly to the rapid spread of resistance by its multiple mechanisms that liberate genes from normal vertical inheritance. Conjugation by plasmids, transduction by bacteriophages, and natural transformation by extracellular DNA each allows

genetic material to jump between strains and species. In this way through horizontal gene transfer infectious diseases acquire an extra ability. The newly produced antibiotic resistant gene (ARG) contributes to the outbreak of any specific infectious disease and during these events they also transfer their resistance ability to other unrelated pathogens (Lerminiaux & Cameron, 2018).

Bacteria have the ability to respond to selective pressures and they can also adopt in the new environment. All these have been made possible due to the gene transfer, through which they acquired new genetic traits. Mutation process also leads them to have these new traits. Generally, mutation occurs relatively slowly. In nature, the normal mutation rate per bacterial generation is in the range of 10^{-6} to 10^{-9} per nucleotide. But when bacterial populations are under stress or they have to survive through tough environment, they can greatly increase their mutation rate (Kaiser, 2019). Furthermore, most mutations are harmful to the bacterium. Horizontal gene transfer, on the other hand, enables bacteria to respond and adapt to their environment much more rapidly by acquiring large DNA sequences from another bacterium in a single transfer (Kaiser, 2019). HGT is even more widespread in the human-associated microorganisms due to the close physical proximity and increased cell-to-cell contact within the human body.

The phylogenetic structure of microbial communities can further increase HGT likelihood since closely-related microorganisms that share similar mobiles are expected to colonize similar habitats. Proximity provides opportunities for genetic exchange occurring via plasmid (conjugation) and phage mediated gene transfer mechanisms (transduction) and uptake of exogenous DNA (transformation) from the environment (Jeong, 2019). The frequency of antibiotic resistant gene transfer rate via natural transformation is higher between the homologous chromosomal DNA rather than plasmid DNA where the rate between heterologous organisms is lower than homolog in both cases of chromosomal and plasmid DNA transfer (Ying, 1989). The test that has been conducted in this regard mainly involved the heterologous bacterium to be ensured about the transformation rate between the heterologous bacteria in the natural environment. As the target was to see the transformation that occur naturally all the factors were tried to keep in natural conditions.

1.2. Background:

Among the total surface of the earth, water contain the very large part. For safe drinking, sanitation and also for household courses and running industries we, the human being is highly dependent on water. Sources of this water are mainly different lake or river or ponds. To be safe and healthy in

life it is necessary to use purified water. But the fact is that 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 per year (Crump *et at,* 2009). In Bangladesh, for climate and physiography, surface and ground water are exceptionally available. The capital of Bangladesh is Dhaka which is mostly surrounded by the water zone, among them two important lakes are Gulshan and Hatirjheel lake. These two lakes are very important as they join different parts of the city and also many of the urban people are dependent on the water of these two lakes. The surface water of the country including these two lakes is unprotected from industrial waste, agricultural pesticides, and municipal wastewater. According to the report of World Bank, seventy-five million Bangladeshis are at risk of contracting the most serious diseases for drinking unsafe water (Hashim, 2018). According to US Environmental Protection Agency (EPA), there are 500 waterborne pathogens of potential concern in drinking waters (Ashbolt, 2015). Among them the most important are *E. coli, Shigella, Salmonella typhi*, *V. cholera.* 40 out of 50 predominant sicknesses in Bangladesh, for example, loose bowels, typhoid, parasitic worm disease, and so on are identified caused by these pathogens with the polluted water (Crump *et al,* 2009). This rate is not only alarming for human health but also a sign of environmental pollution and deterioration. According to The World Health Organization, improving water quality can reduce the global disease burden by approximately 4% (Ramírez-Castillo *et al*, 2015). Improvement of quality of water includes not only the waste minimization but also finding the solution of minimizing the rate of pathogens. In Bangladesh maximum people those are suffering from water borne disease are suffered by typhoid. Yearly 17 million people are being affected by typhoid worldwide and approximately 600,000 died (*Salmonella Typhi (typhoid Fever)*, 2019). In Bangladesh, during the test period of May 2014 to December 2015, almost 27% TP Test report were found positive and among them the highest number of enteric fever positive cases was found in Dhaka (TP Test 57.17%) (Mahmood *et al*, 2016).

Salmonella typhi is a highly contagious intestinal pathogen which is mainly responsible for food or water borne typhoid. More than 2500 Salmonella serotypes are found in environmental samples where they can persist for long periods. It has also been found that genotypically related strains can be isolated from water and wildlife samples, which suggests that they could be reservoirs for the dissemination of Salmonella in the environment (Kovacic, Huljev & Susic, 2017). Where *Salmonella* itself is a great threat for human there the news of Multi drug resistant Salmonella strains has expanded danger for human wellbeing which is identified in surface water in developed nations (Spector & Kenyon, 2012). It is reported that most countries in the Middle East have detected *S*

typhi with decreased ciprofloxacin susceptibility where several countries have reported the detection of multi drug resistant *S typhi* (Wain *et al,* 2014). When a bacterium becomes resistant to more than one antibiotic then it is considered as a multidrug resistant. A bacterium can achieve multidrug resistance by altering its own genomic code which is mutation or by acquiring foreign DNA via Horizontal gene transfer (W. H. O., 2018).

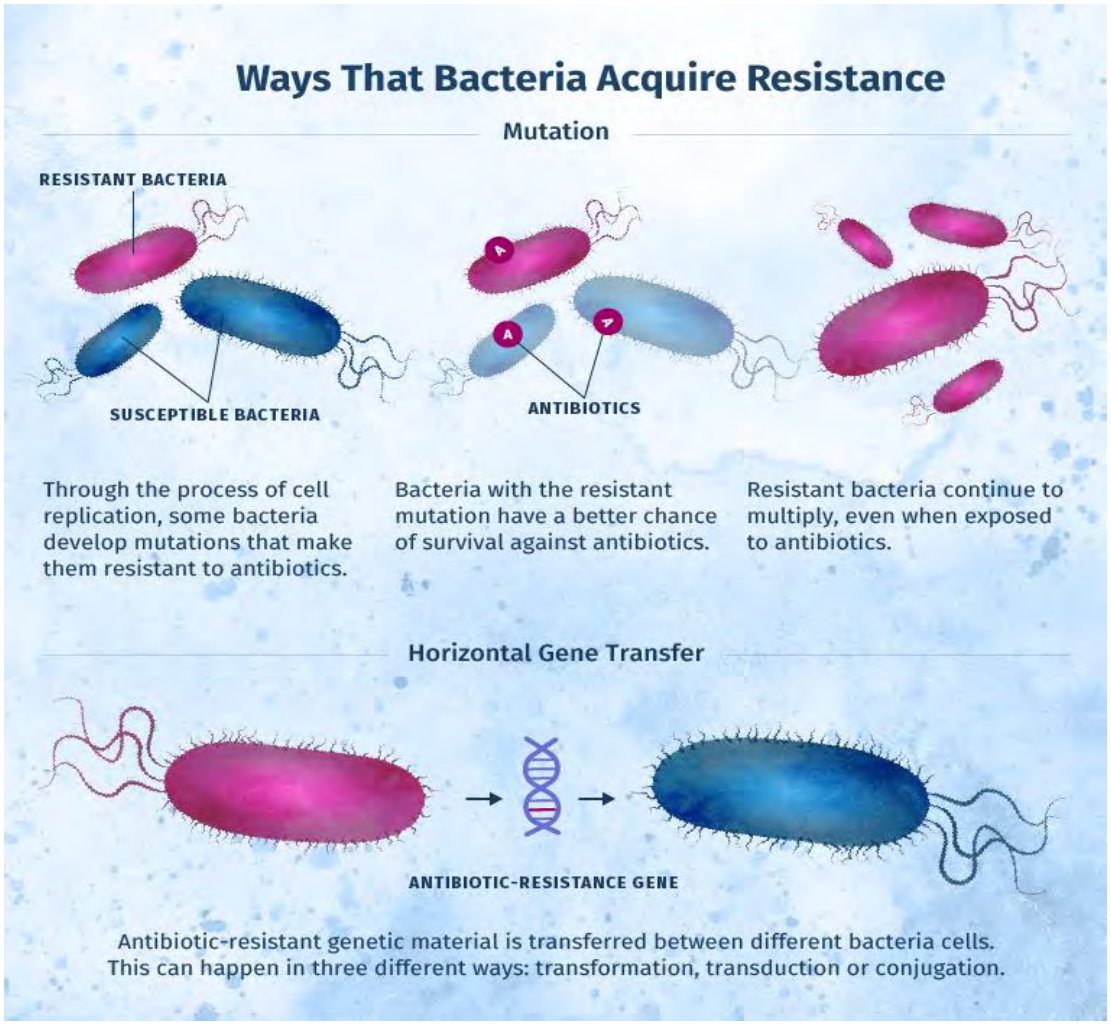


Figure-1: Two different ways of bacterium to acquire resistant gene.

The process through which an organism transfers its genetic material to other organisms, but not to its offspring, is called horizontal gene transfer. It is also known as lateral gene transfer. Bacterial evolution is a long lasting and proven phenomenon, which states that *Bacteria* and *Archaea* have the ability to adopt to new conditions and environment. Because of their ability to adopt to new environment easily, they are able to acquire new genes through horizontal gene transfer. And rate of this event is far greater than acquiring new genes through mutations (Kaiser, 2019). Horizontal

gene transfer is responsible for the huge changes in the bacterial genomes. For example, certain bacteria contain multiple virulence genes called pathogenicity that are located on large, unstable regions of the bacterial genome. This pathogenicity can be transmitted to other bacteria by horizontal gene transfer. But, if no selective advantage is gained through the transferred genes, the organism or bacteria automatically remove those genes through deletion process. And through this mechanism the overall size of the bacterial genome stays almost the same size for long a period of time. Bacteria transfer genes through three types of horizontal gene transfer mechanisms, which are- transformation, transduction, and conjugation. Among them, the most common one is the conjugation process. Especially for conducting the transfer process from a donor bacterial species to a different recipient species the most common type is conjugation process. Although bacteria can acquire new genes through transformation and transduction, this is usually rarer transfer mechanism among bacteria of the same species or closely related species (Kaiser, 2019).

Plasmids are extrachromosomal genetic elements that replicate independently of chromosomes. Persistence of these selfish genetic elements is improved when they carry genes that are useful to the host cell, such as Antibiotic Resistant Genes in the presence of antibiotics. Consequently, many different Antibiotic Resistant Genes circulate on plasmids (Blair *et al,* 2015). Plasmids disseminate through bacterial populations primarily through the process of conjugation. Conjugation requires physical contact between two cells in the same environment, followed by the formation of a bridge that enables the transfer of a plasmid from a donor to a recipient cell (Sørensen *et al,* 2005). Transduction is acknowledged as a potential contributor to the spread of Antibiotic Resistant Genes, especially between members of the same species (Brown-Jaque *et al,* 2015). Transduction occurs when viral particles transfer bacterial genes. After infection with a bacteriophage, bacterial DNA is sometimes accidentally packaged in a bacteriophage capsid. A capsid containing bacterial DNA is fully capable of binding to a recipient cell and injecting the foreign DNA. If the transferred bacterial DNA is recombined into the genome of the recipient cell, transduction has occurred (Lerminiaux & Cameron, 2018). Transduction was first seen in research on gene exchange between *Salmonella* bacteria. In later investigations it became clear that quite a few exceptional types of bacterial viruses can also occasionally serve as natural vectors for genes of their host bacteria (Arber, 2014).

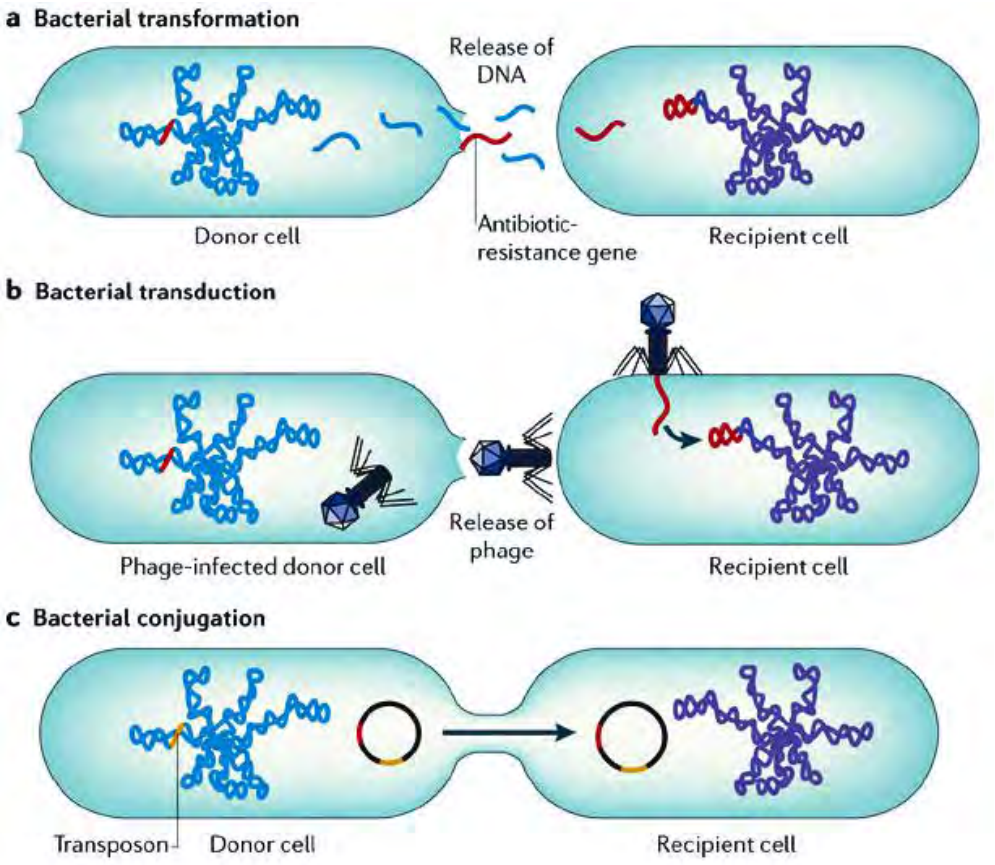


Figure-2: Classification of Horizontal gene transfer

When naturally competent bacteria take up extracellular DNA and the imported gene is recombined into the host genome then the process is called natural transformation (Johnston *et al.* 2014). Several clinically relevant antibiotic-resistant pathogens are capable of DNA uptake and natural transformation, including *Acinetobacter*, *Haemophilus*, *Neisseria*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* (Johnston *et al.* 2014). The molecular mechanism of natural competence and natural transformation has been studied for several decades but in nature, the frequency of it poorly understood (Lerminiaux & Cameron, 2018). Among these three types of HGT, natural transformation is still in mystery. Frederick Griffith famously discovered that nonvirulent *Streptococcus pneumoniae* strains can be transformed into virulent pathogens inside infected mice (Griffith, 1928). Similarly, *Helicobacter pylori* has been observed to acquire genes by natural competence and transformation in a colonized human and in mouse infection models (Kersulyte *et al,* 1999; Kennemann *et al,* 2011; Dorer *et al*, 2012), and resistance to the antibiotic metronidazole has been positively correlated with capacity for natural transformation in clinical isolates (Yeh *et al,* 2002). Despite evidence of natural transformation events during infection, currently there is no

direct evidence that natural transformation contributes to Antibiotic Resistant Gene transmission between bacteria in clinical environments. However, this lack of evidence may be due to the difficulty of detecting uptake and recombination of exogenous DNA (Lerminiaux & Cameron, 2018). 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). Basically, depending on the type of bacterium two different events can take place inside them. Either both strands of DNA of the bacteria penetrate the recipient, or a nuclease degrades one strand of the fragment and the other 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.

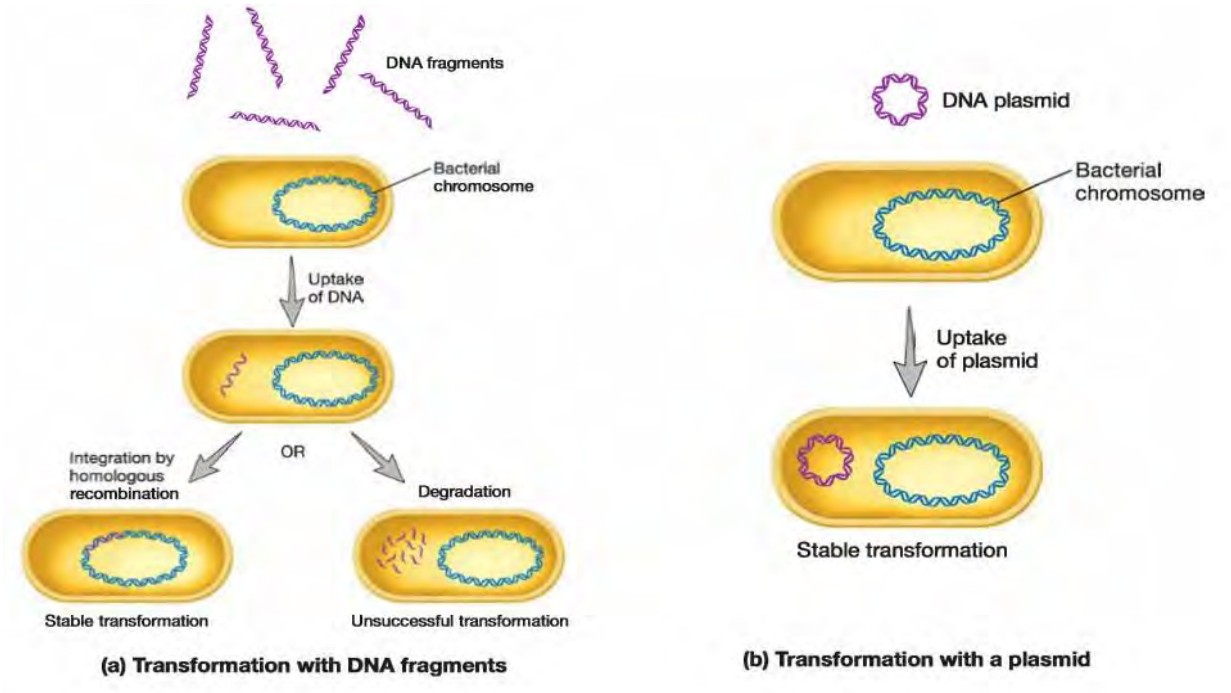


Figure-3: overview of bacterial natural transformation

However, little about natural transformation has been revealed till now but these information support strongly the homologous natural transformation. Homologous recombination involves mainly the reciprocal swapping of blocks of contiguous nucleotides between two or more very similar parental nucleic acids. On the other hand, heterologous recombination involves the unequal

swapping of blocks of contiguous nucleotides between two or more parental nucleic acids that need not to be similar in sequence or in length. In both cases the crossover points are denoted by small dashed lines (Lehman *et al,* 2005). Heterogeneity of microorganisms may include both the phenotypic and genotypic variations. Some internal and external factors are responsible for the heterogeneity of the microbial cells. Variation of the different physiological and biochemical characteristics of the microbial cells are also responsible for the heterogeneity. Bacterial population find difficulty in surviving through heterogeneous and different environmental conditions, but through cell heterogeneity their ability to survive in these conditions greatly improves (Magdanova, 2013). This phenomenon has taken into consideration for this research work where unrevealed natural transformation has been performed among two heterogeneous microorganisms.

Staphylococcus aureus is a gram-positive bacterium that are mostly available in the normal flora of human and animals. Occasionally this simple normal flora organism can make life-taking threats of its host by being resistant to one or more than one antibiotic. An antibiotic susceptibility test that was performed over 1200 dairy samples showed that 134 samples were positive for *S. aureus* where the resistance range was 71.6% for penicillin G and 39.2% for tetracycline. Also 14.9% samples were identified with resistance gene (blaZ) and 52.5% with MRSA (Pekana, 2018). So, all the statistics shows that there is the potential dissemination of multidrug-resistant *S. aureus* strains in the dairy farms. If we look beyond dairy industries, we can see in hospital region where *S. aureus* works as an opportunistic pathogen which even leading to death in few cases. Burns and surgical wound infections are generally invaded by *S. aureus*, where the production of toxins by *S. aureus* can give rise to toxic shock syndrome that can lead to fever, sickness or even to death (Stapleton & Taylor, 2007). The first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in the UK and the US in 1962 and 1968, respectively, the same decade when new antibiotics were applied (Aslam *et al,* 2018). This methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most successful modern pathogens. New proteins in *S. aureus* was invented during the investigation of methicillin resistant gene that involves in cell wall synthesis which might act like targets and compounds that lead the methicillin resistance. Later on, vancomycin resistance in *S. aureus* was expressed from other bacterial group that makes it sure that *S. aureus* may work as a good recipient for uptake antibiotic resistant gene when it is in need of it for survival (Stapleton & Taylor, 2007).

Considering all the factors about antibiotic resistance, in this research work *Salmonella typhi* was selected as a donor of resistant gene and *Staphylococcus aureus* was used as recipient to observe

the natural transformation in the natural resources which is water, to test whether they permit the heterogeneous horizontal gene transfer or not. However, each and every bacterium has its own virulence factors and working mechanism but here the main target was to observe what actually they do when they are in unfavorable condition to survive.

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 January 2019 to September 2019.

2.2 Microbial Sample:

A total of 10 isolates of *Salmonella* and 1 *Staphylococus* ATCC sample 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. aureus	S	BRAC University Lab

Table 1: List of microbial samples 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)

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 hour. 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 was measured and compared against the CLSI (Clinical and Laboratory Standards Instituter) 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 study is to observe the natural transformability of *Salmonella* strain in surface water of Dhaka city. Therefore, we have collected each 1 litter of surface water of two different locations namely Gulshan and Hatirjheel Lake. These two water samples were 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 was 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 com prises 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 hour, 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, *Staphylococcus aureus* used as a recipient strain that have been determined and selected by Kirby-Bauer disk diffusion antibiotic (Azithromycin, Tetracycline) 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, Tetracycline) 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 *Staphylococcus aureus* 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 were served as a nutrient source for bacterial growth instead of LB broth and *Staphylococcus aureus* was not be treated with $CaCl_2$ to make it chemically competence and no antibiotic selection marker was 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 *Staphylococcus aureus* (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 (*Staphylococcus aureus*) 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 *Staphylococcus aureus* (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 (*Staphylococcus aureus*) 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 tetracycline) usually conferred by a plasmid carrying the antibiotic resistance gene. For the selection of transformed cells, antibiotic was plated over agar. Over-agar spreading of antibiotic makes it easy for an investigator to suitably plate and selects transformed cells containing plasmids differing in their resistance genes. This experiment will focus specifically on selection of transformed *Staphylococcus aureus* with Azithromycin and Tetracycline concentrated LB Agar plates.

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

SL No.	Concentration of Azithromycin (mg/ml)	Concentration of Tetracycline (mg/ml)
1.	15	30
2.	16	31
3.	17	32
4.	18	33
5.	19	34

Total 20 LB-Agar plates with specific concentration antibiotic were prepared with following table. To observe transformed cells of *Staphylococcus aureus* (from the bacterial transformation experiment) an autoclaved cotton swap was taken and dipped on vial (containing *Staphylococcus aureus* 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 *Staphylococcus aureus* and drawn a short linear line on the same LB plate. This 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 Antibiotic susceptibility testing on MHA

For further reconfirmation, test on MHA plate was also done along with LB-agar antibiotic plating. After incubation, the transformed organisms from each vial were 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 were incubated for 1 hour. Afterwards sterilized cotton swab was dipped into saline and that swab is used to inoculate the surface of the fresh plate containing Mueller Hinton Agar. The disk of Tetracycline and Azithromycin were placed on the plates and consequently, the MHA plates were kept at 37 °C for incubation for 24 – 48 hours. After incubation, the zone of inhibition was observed.

Chapter 3: Results

3.1 Microbiological or Microbial culture of the organisms

From the BRAC University Microbiology Laboratory, the isolates were assembled and cultured. *Staphylococcus aureus* and three *Salmonella* samples were grown in nutrient agar from the stock culture and incubated at 37◦C for overnight growth.

Figure 5: Culture plates of *Salmonella* & *Staphylococcus aureus*

3.2 Antibiotic sensitivity test: Antibiotic sensitivity test was done on the selected isolates to accurately detect the antibiotic resistance.

Table 3: Antimicrobial susceptibility test of *Staphylococcus aureus*

Drugs	**AZM 15 (mm)**		**VA 30 (mm)**		**CFM 5 (mm)**		**LE 5 (mm)**		**CIP 5 (mm)**		**CXM 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. Aureus	19	**s**	18	**s**	Nil	**R**	23	**s**	27	**s**	22	**s**	20	**s**	25	**s**	Nil	**R**	**2**	**-**	**7**

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

After analyzing the table, *Staphylococcus aureus* exhibited sensitivity against 7 antibiotics out of 9 antibiotics. Antibiotic sensitivity test was done on the selected isolates to accurately detect the antibiotic resistance.

Table 4: Antimicrobial susceptibility test of 10 collected *Salmonella* isolates

Drugs	**AZM 15 (mm)**		**VA 30 (mm)**		**CFM 5 (mm)**		**LE 5 (mm)**		**CIP 5 (mm)**		**CXM 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**	14	**I**	15	**I**	13	**R**	19	**s**	nil	**R**	nil	**R**	**6**	**2**	**1**
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**	15	**R**	24	**s**	21	**s**	26	**s**	12	**R**	nil	**R**	nil	**R**	15	**R**	**6**	**-**	**3**
HJ3.1*	nil	**R**	Nil	**R**	nil	**R**	22	**s**	26	**s**	13	**R**	nil	**R**	nil	**R**	nil	**R**	**7**	**-**	**2**

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

After analyzing the table, it can be said that out of 10 *Salmonella* isolates the strains named as GL6.2, HJ2, and HJ3.1 exhibited maximum resistance against 9 antibiotics. Additionally, GL6.2 and HJ2 displayed resistance against 6 antibiotics out of 9 antibiotics. Furthermore, HJ3.1 showed resistance against 7 antibiotics out of 9 antibiotics.

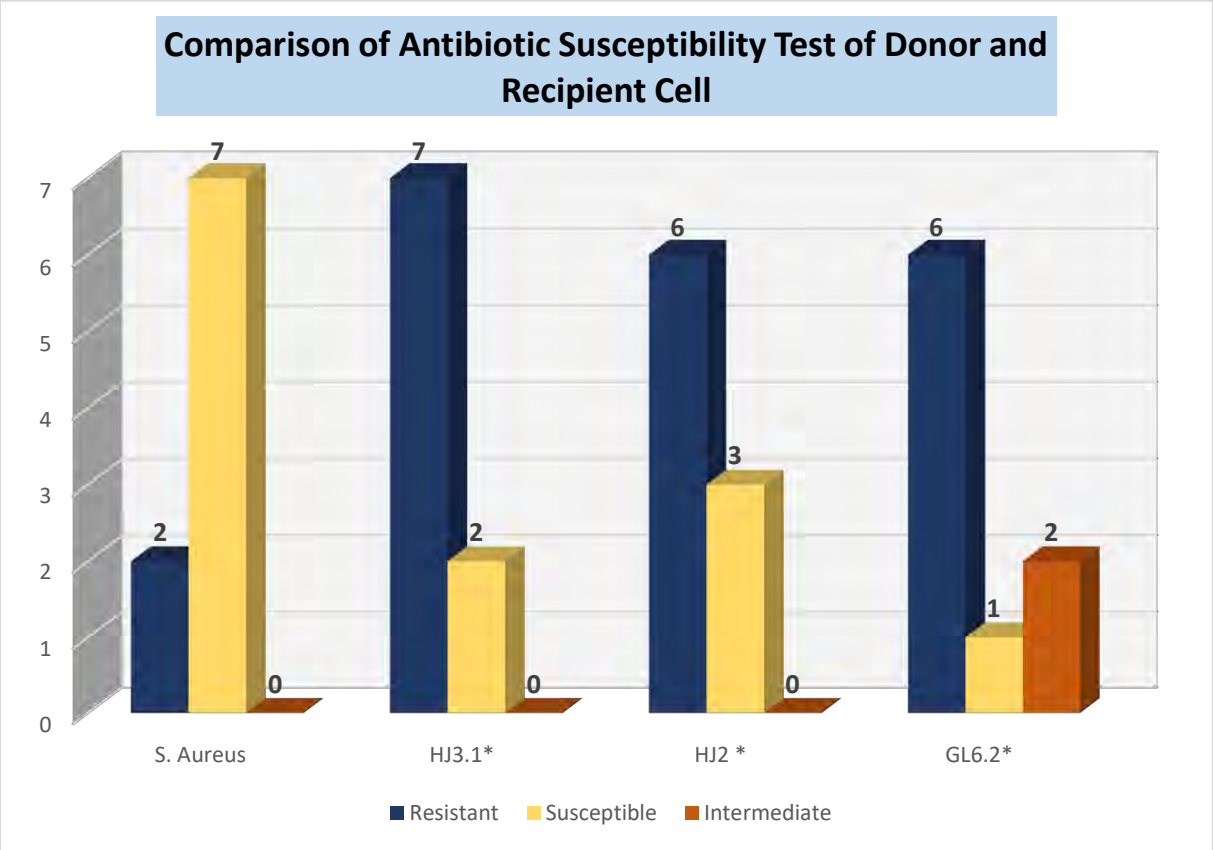


Figure 6: Graphical representation of Antibiotic susceptibility test of recipient and donor strain.

This bar chart of *S. aureus* strain and three *Salmonella* strains (HJ3.1, HJ2 and GL6.2) deals with the resistant pattern of four strains against nine antibiotics. From the above graph, it can be said that three *Salmonella* strains as a donor strains showed maximum resistance against nine antibiotics. In contrast to, *S. aureus* as a recipient strain highlighted maximum sensitivity against nine antibiotics.

Antibiotic sensitivity test of *S. aureus* (Recipient) and Three *Salmonella* Strains (Donor)

This antibiotic sensitivity test was done to ensure authentication of the recipient strain and donor strain selection and to observe their resistant pattern.

Strain Name	Strain ID	AZM 15 (mm)	TE 30 (mm)
S. aureus	**S**	**19(S)**	**25(S)**
Salmonella(GL6.2)	**7**	**nil(R)**	**nil(R)**
Salmonella(HJ2)	**5**	**nil(R)**	**nil(R)**
Salmonella(HJ3.1)	**1**	**nil(R)**	**nil(R)**

This table represents the data obtained from the antibiotic sensitivity test using the Kirby-Bauer method. After analyzing the results of all the isolates, it can be stated that *S. aureus* (S) displays sensitivity against Azithromycin and Tetracycline. This sensitivity of *S. aureus* indicates that it lacks resistance genes on its chromosomal DNA or plasmid DNA against these tetracycline and macrolide antibiotics. In addition to, *Salmonella* strains were repetitively displaying resistance against these two antibiotics. For this reason, we will use this *S. aureus* strain as our recipient so that it will receive resistance genes from our donor *Salmonella* strains via natural transformation.

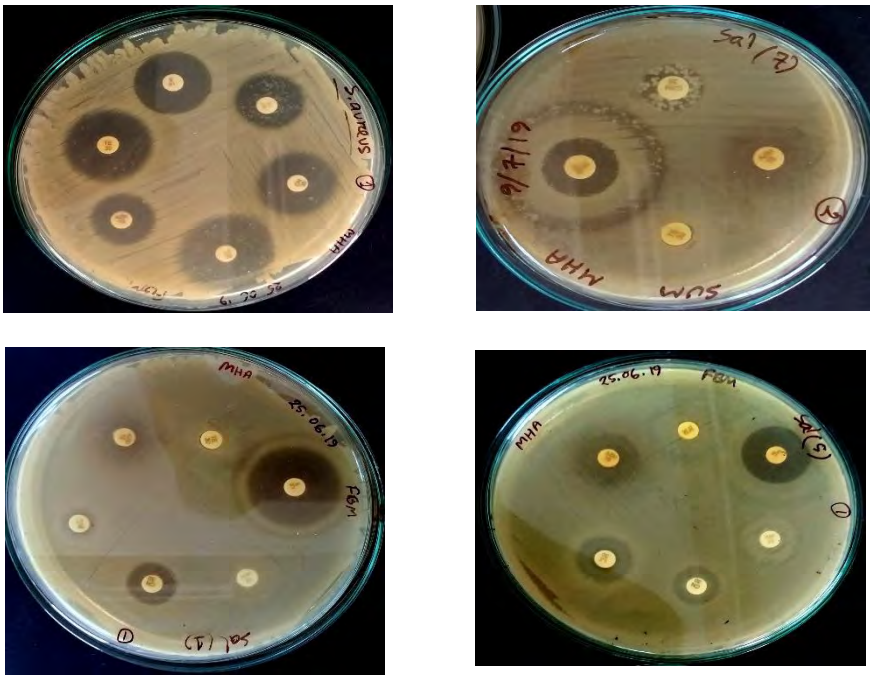


Figure 7: MHA plates with Azithromycin and Tetracycline antibiotic disk and the zone of inhibition. [1st plate – *S. aureus*, 2nd, 3rd & 4th plates – *Salmonella* (7, 5, 1) isolates]

3.3 Chromosomal DNA Extraction and Gel Electrophoresis

After completing the antibiotic sensitivity test of the selected strains, the chromosomal DNA of those strains were isolated using boiling extraction method. After that, gel electrophoresis was done immediately after isolating the chromosomal DNA carefully to observe the separated bands of chromosomal DNA.

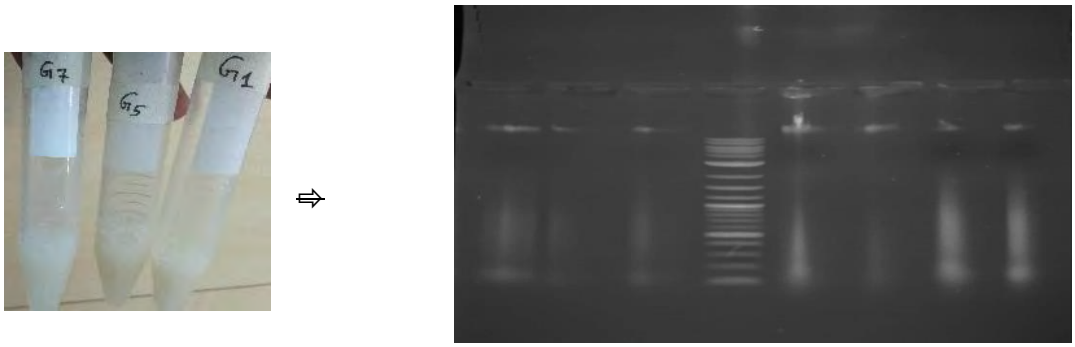


Figure 8: Isolation of Chromosomal DNA and representation of bands on gel electrophoresis

3.4 Plasmid DNA Isolation and Gel Electrophoresis

Using the Kado-Liu method, the plasmids of those isolates were isolated after performing the antibiotic sensitivity test. After isolating the plasmids carefully, gel electrophoresis was performed to observe the distinct bands of plasmids.

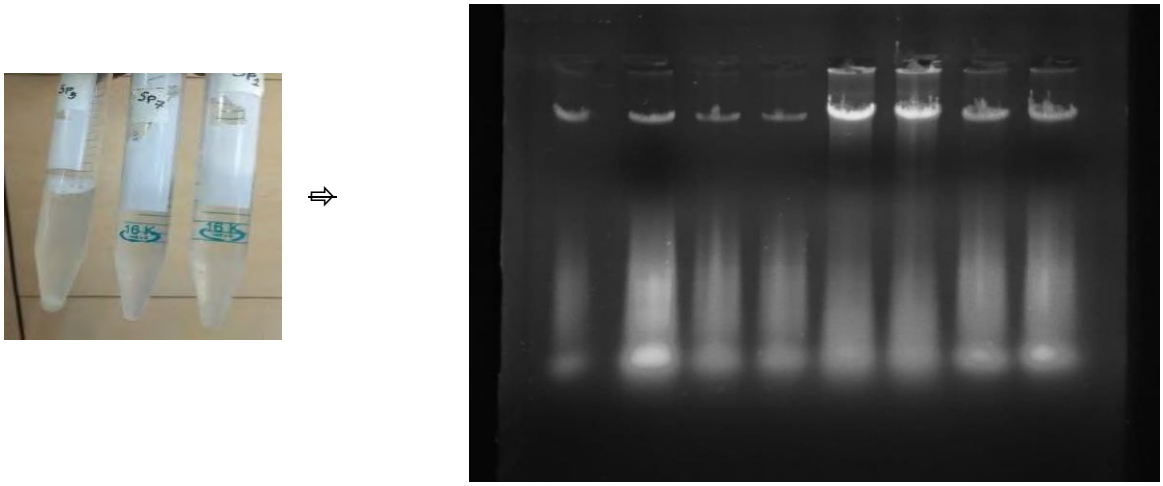


Figure 9: Isolation of Plasmid DNA and representation of distinct bands on gel electrophoresis

3.5 Transformation assay and LB Plate Screening

The bacterial natural transformation assay was done on vial and kept on incubator for overnight after ensuring the correct conformation of chromosomal DNA and plasmid DNA by the gel electrophoresis experiment. After the overnight incubation the transformation mixture on vial was swapped on antibiotic specific LB-Agar plates (azithromycin and tetracycline) to observe the transformed cell growth.

Table 5: List of Recipient sample and Donor Sample for the transformation experiment

Strain Name	Strain ID	Transformation Materials	Experimental Role
S. aureus	S	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

Result interpretation of bacterial natural transformation on Gulshan Lake water:

After analyzing all the antibiotic specific LB-agar plates the confirmation of transformed cell growth was listed down on the table below-

Tetracycline Plates	S + C_1	S + C_5	S+ C_7	S + P_1	S + P_5	S + P_7
30 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
31 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
32 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
33 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
34 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**

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

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

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

Result interpretation of bacterial natural transformation on Hatirjheel Lake water:

After analyzing all the antibiotic specific LB-agar plates the confirmation of transformed cell growth was listed down on the table below-.

Tetracycline Plates	S + C_1	S + C_5	S + C_7	S + P_1	S + P_5	S + P_7
30 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
31 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
32 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
33 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
34 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**

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

Azithromycin Plates	S + C_1	S + C_5	S + C_7	S + P_1	S + P_5	S + P_7
15 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
16 mg/ml	**NG**	**NG**	**NG**	**NG**	**NG**	**NG**
17 mg/ml	**NG**	**NG**	**NG**	**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)

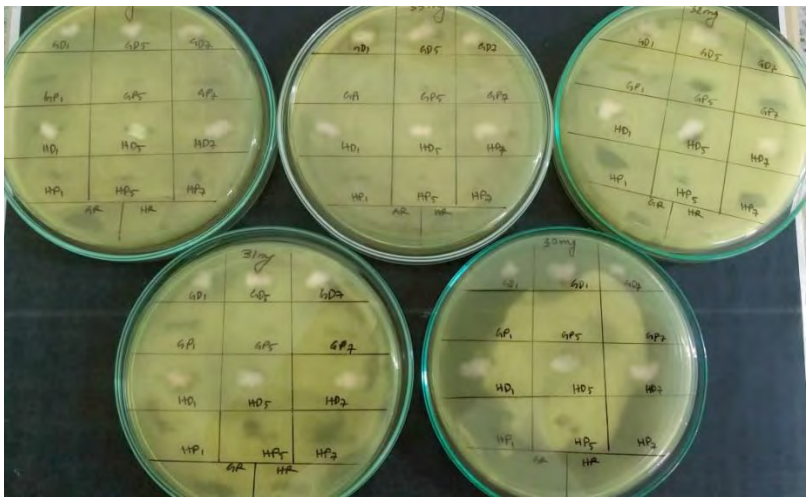


Figure- 10: Screening of Bacterial Transformation results on Tetracycline containing LB Plates in Gulshan and Hatirjheel Lake water

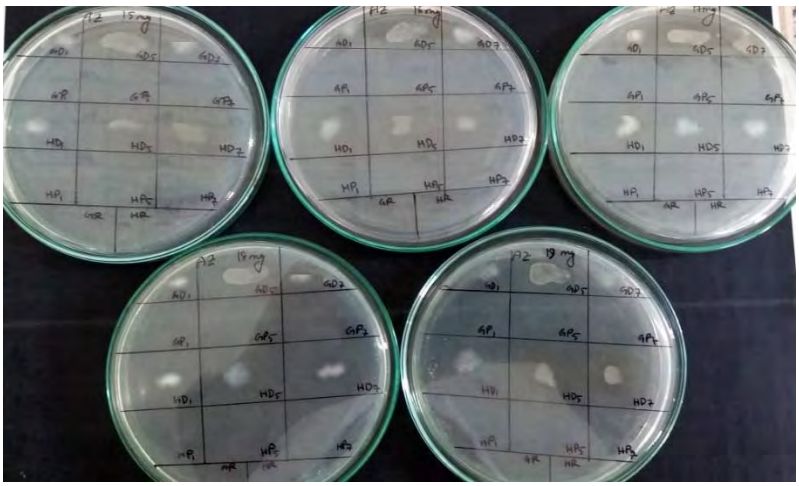


Figure-11: Screening of Bacterial Transformation results on Azithromycin containing LB Plates in Gulshan and Hatirjheel Lake water

3.6 MHA plate screening

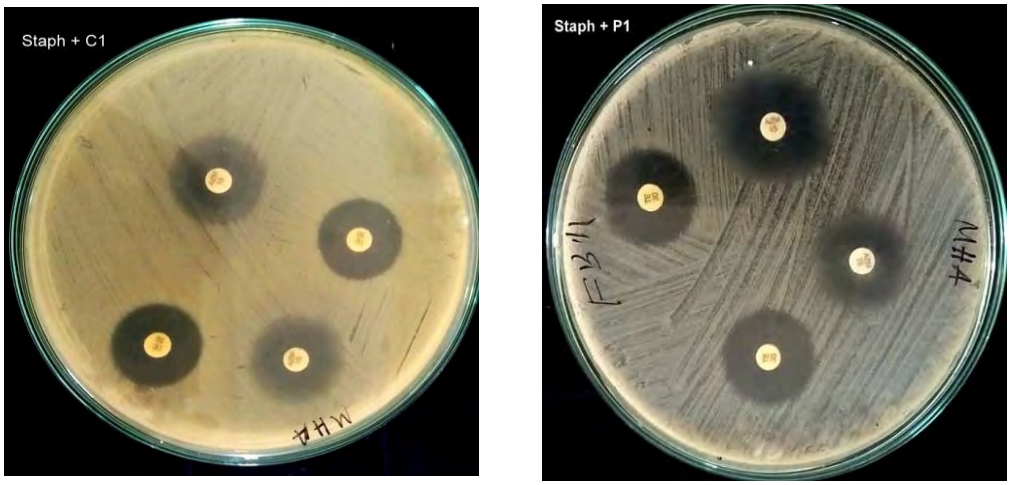
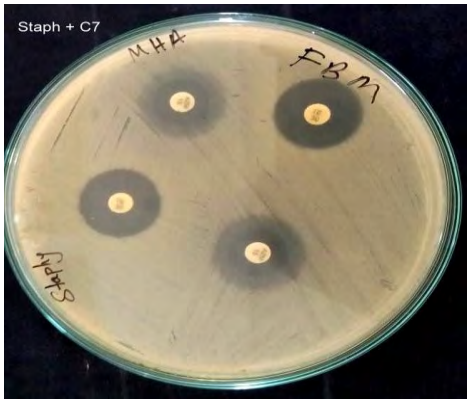


Figure-12: Confirmation test of transformation on MHA for chromosomal and plasmid DNA of donor 1 (*Salmonella HJ3.1)*

Figure-13: Confirmation test of transformation on MHA for chromosomal and plasmid DNA of donor 5 (*Salmonella HJ2)*



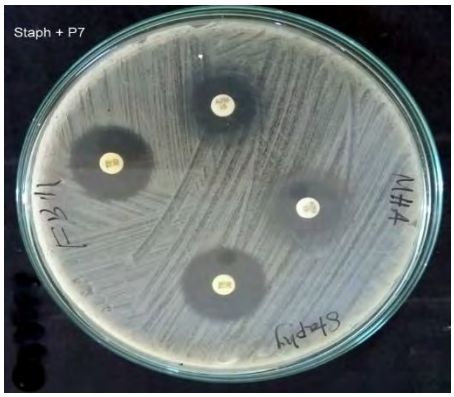


Figure-14: Confirmation test of transformation on MHA for chromosomal and plasmid DNA of donor 7 (*Salmonella GL6.2)*

After analyzing figure 10,11 & figure 12, 13, 14, it can be easily said that the expected transformation has not been occurred between donor DNA and recipient organism. However, in both Tetracycline and Azithromycin plates growth of donor organisms are visible but the transformed sample did not show any growth. Also, the pure recipient *S. aureus* did not grow. Which clearly indicates that the susceptible *S. aureus* did not uptake the resistant donor chromosomal or plasmid DNA. Both in tetracycline and azithromycin plates clear growth of donor samples visible but not the transformed ones.

Chapter 04: Discussion and Conclusion

Discussion

After the evolution of prokaryotes, the horizontal gene transfer has become widespread specially in the organisms that deal with human body (Jeong *et al,* 2019). It has been detected that, up to 60% HGT (Horizontal Gene Transfer) occurs either before colonization in the human body or to the organism of different body parts (Jeong *et al,* 2019). Almost 30% of total human population is the carrier of *S. aureus* as a normal flora (Tong *et al*, 2015). As it's a normal flora organism of human body, becoming pathogenic by acquiring antibiotic resistance gene, it can be really dangerous and even lethal to human. However, methicillin resistant staphylococcus has already been identified and the main concern is that, this methicillin resistant *S. aureus* can be resistant to the other classes of antibiotics through different mechanisms that can lead the limited option of using antibiotics (Stapleton & Taylor, 2007). So as a recipient, *S. aureus* has been selected as a part of this research work in order to determine its foreign antibiotic resistant gene uptake pattern that whether it supports the heterogeneous resistant gene uptake system. As Bangladesh is a land of water and Dhaka city is covered by lakes and other water sources so here Gulshan and Hatirjheel, the two important lake water has been used as sample water for conducting natural transformation. On the other hand, *Salmonella typhi* is one of the most pathogenic human bowel pathogens that preferably are found in the contaminated water, food and in human feces. Approximately 93.8 million foodborne illnesses and 155,000 deaths per year is accounted by this pathogen where 2500 *salmonella* serotypes are identified (Eng, 2015). In addition, from the last few decades, emergence of antibiotic resistant *salmonella* has started to be the major cause of morbidity and mortality in humans (Nair, 2018). As *salmonella* is mainly waterborne pathogen and staphylococcus is commonly found in human skin, so with the experiment the aim was to find out the relationship that when both of these organisms are in close to each other, is there any chance of passing chromosomal or plasmid DNA of pathogenic *salmonella,* which is multidrug resistant to *staphylococcus.*

To begin with, the ATCC strain of *staphylococcus aureus* was tested for the antibiotic susceptibility using the Kirby-Bauer method. Out of 9 antibiotics that were tested, *staphylococcus* showed susceptibility against 7, which are azithromycin, ampicillin, levofloxacin, cefuroxime, ciprofloxacin, vancomycin, tetracycline. *Staphylococcus* was resistant to 2 antibiotics, that were cefixim and colistin. As *S. aureus* showed susceptibility to azithromycin and tetracycline, that means *S. aureus* had no antibiotic resistant gene against macrolid and tetracycline class antibiotics. On the other hand, among 10 isolates of *salmonella* GL6.2, HJ2, and HJ3.1 showed maximum number of resistances which are 6, 6 and 7 respectively. GL6.2 showed resistance against

tetracycline, azithromycin, cefuroxime, vancomycin, colistin and cefixime. HJ2 showed resistance against ampicillin, tetracycline, azithromycin, cefuroxime, vancomycin, colistin and HJ3.1 showed resistance against ampicillin, tetracycline, azithromycin, cefuroxime, vancomycin, colistin, cefixime. As all three isolates of *salmonella* were resistant to azithromycin and tetracycline, these two antibiotics were selected for further testing.

At the same time, in the water collected from Gulshan and hatirjheel lake, recipient *S. aureus* was inoculated with chromosomal and plasmid DNA of three isolates of donor *salmonella* separately for natural transformation. The confirmation test of transformation was performed on LA plate, which were made azithromycin and tetracycline antibiotic specifics in different concentrations. All the plates of azithromycin and tetracycline that were tested for Gulshan lake water gave negative result for the transformed samples and for the recipient, but the resistant donors grew. Also, all the plates of azithromycin and tetracycline, that were tested for Hatirjheel lake water, gave negative result for the transformed samples and for the recipient, but the resistant donors grew. The results both in Gulshan and Hatirjheel lake water gave the clear statement that recipient *S. aureus* did not uptake either chromosomal or plasmid DNA of *S. typhi*. The test was done repeatedly for three times by changing few factors. Attempts were made to make the recipient competent by keeping it in incubation for 5 hours, so that it can reach to its stationary phase and be able to uptake the donor DNA. Each time of incubation, recipient was made starve or the incubation temperature was changed so that it was not kept at its favorable condition. To all these conditions *S. aureus* repeatedly gave negative results of DNA uptake. For further conformation, disk diffusion of transformed samples was done also in MHA plates where the transformed *S. aureus* gave zone against azithromycin and tetracycline, which were the clear confirmation of negative natural transformation.

Methicillin resistant *S. aureus* has already been identified and transformation test over methicillin-sensitive *S. aureus* was performed by providing methicillin-resistant genome of *S. aureus* to be sure whether the sensitive *S. aureus* might uptake resistant gene or not. Also, the test was proved positive for *S. aureus* as a competent cell (Thuy, 2017). Moreover, antibiotic resistant plasmid DNA isolated from *S. aureus* was expressed in another gram-positive organism, named *Bacillus subtilis* (Ehrlich, 1977). So, it can be said that, *S. aureus* can work both as recipient and donor for natural transformation. On the other hand, transfer of ampicillin resistant gene from donor *S. typhi* to recipient *E. coli* was successfully performed (Walsh, 2007). Transformation between gram positive to gram positive and gram negative to gram negative microorganism have successful evidence. Though *S. aureus* has the potential to work as a competent, it has to modify at a large version to

uptake DNA from heterogeneous *S. typhi*. Trans-gram transformation between gram positive and gram-negative bacteria was performed between *Staphylococcus* and *E. coli,* where *Staphylococcus* worked as donor samples and successful transformation was performed through conjugation. Conjugation occurs in *S. aureus*, but it requires a series of 'tra' genes or conjugative plasmids, which are not widespread among *S. aureus* strains and Phage transduction in *S. aureus* is limited to transfer of small DNA fragments (less than 45 kb) due to the size of phage capsid as *S. aureus* phages are particularly host specific (Thuy, 2017). Unlike conjugation and transduction, transformation is entirely directed by the recipient bacterial cell. *S. aureus* possesses the competence gene orthologues that share among transformable Gram-positive bacteria such as *B. subtilis* and *S. pneumoniae*. The expressions of competence genes in *S. aureus* are induced by SigH. SigH is one of the alternative sigma factors in *S. aureus*. It associates with the core RNA polymerase, and renders the resultant holoenzyme the ability to recognize the promoter sequence and initiate the transcription of competence genes (Thuy, 2017). So, it can be said that during being competence the SigH factor sets the information in a way that does not allow *S. aureus* to uptake foreign DNA from heterogeneous bacteria. However, in laboratories artificially cells are made competent where the recipient cells are grown in a complete synthetic medium (CS2 medium), which facilitates the transformation with the frequencies of up to 10-8 ~ 10-9 when purified plasmids were used as donor DNAs and about 10-7 ~ 10-9 in the co-culture with the living donor cells (Thuy, 2017). But the target of this experiment was to observe the whole procedure in a natural system and for this no artificial condition or influence were applied.

This experiment can give few ideas about heterogeneous natural transformation. First of all, antibiotic resistant gene transfer between heterogeneous microorganisms may not be that rapid as homogenous in nature. In case of extreme condition if organisms need to be transformed heterogeneously, they may not prefer the natural transformation among all three horizontal gene transfer system. Phenotypic and genotypic differences between gram positive and gram-negative microorganism is not suitable for trans-gram natural transformation. Most of the spread of antibiotic resistant gene between bacterial species are done by homologous horizontal gene transfer system. For trans-gram transformation recipient bacterium has to be changed its genomic structure a lot that might be too much time consuming and so if it is possible, it will take a huge time to transfer antibiotic resistant gene between heterogeneous bacteria. This experiment was performed in the laboratory by following all the rules of lab and by following the complete protocols of working procedure.

Conclusion

Antimicrobials that kill or inhibit infectious diseases are essential clinical tools, yet resistance continues to emerge, diversify, and spread rapidly (Lerminiaux & Cameron, 2018). Globally, antimicrobial-resistant infections kill at least 700 000 people each year; within 30 years, resistant infections are predicted to kill 10 000 000 per year, greatly exceeding deaths from cancer (O'Neill, 2014). Several studies have already showed that how polluted of the surface water of Dhaka City is. Presence of *Salmonella typhi* is one of the main reasons that yearly causing the death of huge people. On the other hand, *Staphylococcus aureus* has become opportunistic pathogen from normal flora day by day. Antibiotic resistant gene transfer has already proven by conjugation and transduction between homologous organisms. For this reason, the experiment was deigned to observe the trans-gram activities related to antibiotic resistance. The findings of this experiment clearly indicate that, heterogeneous bacteria are not interested in transformation process between them instead of transformation between their homologous pair. So, waterborne diseases that are becoming incurable day by day may be the effect of transformation of antibiotic resistant gene between the homologous organisms.

In future, for more research and finding, comparable experiments can be done between gram negative - gram negative and gram positive - gram positive organisms, that may make the statements of homologous transformation stronger. Apart from that, use of gene marker will make the work more accurate along with PCR and genome sequencing. Following all these steps may lead to finding new ways of transformation between trans-gram organisms and will help to find out a solution of this rapid spreading of antibiotic resistance.

Chapter 5: References

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Chapter 6: APPENDIX

Appendix - I

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