Study of Antibiotic Resistance Gene transfer between Isolates from patients with Urinary Tract Infection via Conjugation

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Declaration of Authenticity

I hereby humbly declare that this thesis is entitled "***Study of Antibiotic Resistance Gene transfer between Isolates from patients with Urinary Tract Infection via Conjugation"*** submitted by the undersigned has been carried out under the joint supervision of Ms. Namista Islam (Lecturer, Department of Mathematics and Natural Sciences, BRAC University) as supervisor and Ms. Nazneen Jahan (Lecturer, Department of Mathematics and Natural Sciences, BRAC University) as co-supervisor at BRAC University, Mohakhali Dhaka.

The presented dissertation is based on original research work carried out by myself and has not been submitted to any other institution for any degree or diploma. Any reference to work done by any other person or institution or any material obtained form other sources have been accordingly cited and referred to.

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Dedicated

to

My Beloved Parents

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Abstract

The Urinary Tract Infection is caused recurrently by *Escherichia coli* and also by *Proteus spp., Klebsiella spp., Enterococcus spp*., *Staphylococcus spp*. etc. [3, 11] Recently, UTIs are considered a severe public health concern due to the increasing antibiotic resistance in between the strains and species of the Enterobacteriaceae family. These multi-drug resistant forms (MDRs), due to subsequent use of antibiotics, transfer their antibiotic resistance genes by horizontal gene transfer methods. Conjugation allows gene transfer not only within species but also in-between closely related species, given that the cells have direct cell-to-cell contact. These resistance genes could be a part of either the resistance plasmids (R-plasmids) or Fertility plasmid (F-plasmid) and often times chromosomal DNA as well. Thus, this investigation aims to determine whether horizontal gene transfer takes place between the naturally occurring causative agents leading to an increase in MDRs. Therefore, the study tests the antibiotic susceptibility of the isolates from UTI patients using both the Kirby-Bauer Disk Diffusion and Agar Dilution, to determine the MIC and MBC values of the isolates. After the interpretation of antibiotic sensitivity using the Rapid Plasmid Extraction Technique by Kado-Liu, the plasmids of the isolates were extracted and separated using the Agarose Gel Electrophoresis. Using the plasmid extraction results, the presence or absence of plasmids were determined which would lead to the determination of donor and recipient strains for conjugation. After conjugation, the donor organisms were cured to further strengthen the results and outcome of the investigation. The results shows a suspected isolate *Escherichia coli*, azithromycin resistant (MIC>100µg/ml), as the donor, and another suspected isolate *Shigella spp*. azithromycin sensitive (MIC>60 µg/ml), as the recipient did not conjugate together and the likely reason would be due to absence of resistance genes in the plasmid which is the only form of DNA that is transferred via pillus adjoining the donor and recipient cell. This conclusion was drawn from plasmid curing where the results shows that even at higher concentrations of EtBr (100µg/ml) and SDS (20%), the donor isolate grew and showed resistance against azithromycin, despite their plasmids being removed (as seen from plasmid extraction and gel electrophoresis). Hence it could be deduced that conjugation does not transfer the chromosome mediated antibiotic resistance gene. However, further confirmation on the plasmid being a Fertility plasmid or not, would have further backed up the results of this investigation and shed light on some interesting future aspects of this investigation that could lead to a positive result for conjugation and even further enquire about the resistance gene.

Contents

List of Tables

Table of figures

CHAPTER 01

Introduction

1. Background

1.1 Escherichia coli (E.coli)

Escherichia coli (E.coli) is a common member of the intestinal tract flora of humans and animals and falls under the family Enterobacteriaceae.It was first discovered in 1885 by Theodor Escherich, a German bacteriologist. It is a facultative (aerobic and anaerobic growth), gram-negative, rod shaped bacteria that can be commonly found in animal feces, lower intestines of mammals, and even on the edge of hot springs. They grow best at 37°C and cannot sporulate. It can be classified into several kinds of serotypes. Complex antigenic structure of *E.coli* is based on O, K, and H antigens [1]. *E.coli* has been classified into six different categories based on its virulence properties such as enterotoxigenic *E.coli* (ETEC), enteropathogenic *E.coli* (EPEC), enteroinvasive *E.coli* (EIEC), enterohemorrageic *E.coli* (EHEC), enteroadherentaggregative *E.coli* (EAggEC), and verotoxigenic *E.coli* (VTEC). These enteric *E.coli* can cause several intestinal and extra-intestinal infections such as Urinary Tract Infection and mastitis.

E.coli, an enteric pathogen mostly in the developing countries, is known as the primary causative agent responsible for the Urinary Tract Infections (UTIs). [2] *E.coli* strains that cause UTIs are termed as uropathogenic *E.coli* (UPEC). In general, *E.coli* strains that are responsible for causing the UTIs, are a subset of strains that tend to colonize the colon. However, UPECs differ from common bacterial strains, as they possess extragenic material [3]. Moreover, there are a couple of factors that enables them to survive in the intestines. These factors include structural features such as fimbriae or pilli for adherence and flagella for motility and chemical adhesion [4]. UPEC strains can attach specifically to receptors of the renal pelvis mucosa with the pyelonephritis-associated pilli (PAP, P fimbriae) or non-fimbrial adhesions (NFA). They produce the hemolysin HlyA. [1]

In addition to the fact that *E.coli* has many different strains, the natural biological process of mutation in genomes is the major cause to produce so many different strains. Moreover, similar to most bacteria, *E.coli* can transfer its DNA materials through bacterial conjugation with other related species of bacteria to produce more strains and spread resistance gene into the existing population.

1.2 Shigella spp.

Shigella also belongs to the family of Enterobacteriaceae and is a gram-negative rod, aerobe or facultative anaerobes, non-motile, which aids in the facilitation of intracellular pathogens. It is able to survive the proteases and acids in the intestinal tract, which allows the bacteria to infect in very small amounts. There are four different groups which *Shigella* has been divided into, *Shigella dysenteriae, Shigella flexneri, Shigella boydii,* and *Shigella sonnei* [5]. *Shigella dysenteriae* is known to be mostly responsible for the bacillary dysentery, also commonly known as shigellosis. However, in terms of its genomic structure, *Shigella* shares many genes with *Escherichia coli*, hence, could be said to be related. Moreover, *Shigella* is capable of both receiving and sharing its resistance gene via conjugation.

Shigella is primarily known to be the causative agent for Gastrointestinal infections, and urinary tract infections caused by *Shigella spp* and *Shigella sonnei* are very unusual. However, despite being rare, it still remains a cause of the Urinary Tract Infection (Karakas, 2015).

1.3 Urinary Tract Infection

Urinary Tract Infection, UTI, is a common bacterial disease, often contributes to a frequent cause of morbidity in out-patients as well as hospitalized patients [9]. An estimated 50% of women report having had a UTI at some point in their lives. 8.3 million office visits and more than 1 million people getting admitted into the hospital, for an overall annual cost is greater than $ 1 billion [10]. Urinary Tract Infection can occur due to two main reasons, either because of infection by the pathogenicity of the causative agent/organism or the susceptibility of the host or even a combination of both. UTIs that typically affect individuals who are healthy and have no structural or neurological urinary tract abnormalities, these infections are differentiated into lower UTIs (cystitis) and the upper UTIs (pyelonephritis). Several risk factors are involved with cystitis, including female gender, a prior UTI, sexual activity, vaginal infections, diabetes, obesity and genetic susceptibility. UTIs can also be complicated if it is associated with factors that compromise the host defense or urinary tract, which could further lead to complications like, neurological disease, immunosuppression or renal failure [11].

Causative agents of UTIs could be both gram-negative and gram-positive, given that the most common causative agent all types of UTIs would be Uropathogenic *Escherichia coli*, (UPEC) followed by *Klebsiella spp*. [21]. Other prevalent species responsible for the UTIs includes, *Staphylococcus saprophyticus, Enterococcus faecalis, Streptococcus spp., Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus and Candida spp.* [11].

Patients suffering from UTIs, are commonly treated with antibiotics; as a result these treatments leads to long-term alteration of the normal microbiota of the vagina and the gastro-intestinal tract and also leads to the development of multi-drug resistant microorganisms [11].

1.4 Global emergence and epidemiology

An estimated 150 million urinary tract infections results per annum worldwide, which clearly shows that it is one those pressing bacterial infections that occurs in all the populations and ages. Although, this infection happens to be more pre-dominant in women compared to males. The reason is due to certain aspects like anatomic differences between males and female, hormonal effects and behavioral pattern. The major pathogens contributing to the UTIs are *E.coli*, *Pseudomonas spp., Proteus spp., Klebsiella spp.,* etc.

One of the major concerns of the infection is how the infection is treated with antibiotics and how the causative agents are becoming resistant to these antibiotics at an alarming rate. The wide and extensive use of antibiotics against this infection has been altering the microbiological and antibiotic susceptibility patterns of these pathogens [12]. This matter at present could become a major complication for the entirety of public health worldwide. Moreover, these resistance to the antibiotics is not only restricted to the causative agents, but has also been transmitted to other distinctly related or un-related species. The ability of E.coli to cause UTIs and the resistances against the first generation antibiotics is becoming progressively more elusive [13].

In the light of recent events, where multiple organisms turn up resistant against multiple antibiotics, it is also known as multi-drug resistance (MDR), is in accordance with the chromosomal genetic material as well as the existing plasmids [13]. Plasmids are extrachromosomal DNA mainly containing the resistant genes against multiple antibiotics.

Antibiotic resistance can be both chromosomal and plasmid-mediated. Therefore, treatment of these antibiotic resistant bacterial infections are hence becoming more and more strenuous and has been identified as the greatest epidemic threats to the human health by the World Health Organization, (WHO) [Suhani et al., 2017].

1.5 Horizontal gene transfer methods

The discovery of horizontal gene transfer has been a major advancement in molecular biology, and can even be seen as its founding experiment. By the demonstration in 1928, almost 70 years ago, the non-virulent pneumococcus bacteria can become pathogenic simply by contact with virulent bacteria [16]. Therefore, soon after its discovery, one of the issues that arose was of increasing antibiotic resistance of pathogenic bacteria. This problem stimulated the research in bacterial genetics which revealed that the horizontal gene transfer is somewhat involved in the genetic variations caused due to antibiotics [17].

Horizontal Gene transfer is the movement of the genetic material or information between organisms, related or unrelated, a process that includes the spread of antibiotic resistant genes among bacteria, thus fueling evolution [14]. These antibiotic resistant genes that flows between different species and genera, gives the bacteria ability to exploit new environments and respond to selective pressures in an enhanced manner [15]. Identifiably, there different methods of horizontal gene transfer; transduction, conjugation and transformation. Transduction is the type of transfer process that occurs via a bacteriophage that transmits the DNA from one cell to another. Conjugation is the type of process that is more of one-way transmission mechanism of DNA from one cell to another via a “sexual pilus” by which DNA is transported. Lastly, Transformation, is an active mechanism by which free DNA present in the medium, typically derived from dead organisms and is taken up into the cytoplasm of the cell [16].

1.5.1 The discovery of bacterial transformation

In the 1928 the experimental work by Frederick Griffith using the pneumococcal bacteria, identified two forms or strains. One of them is smooth (S) cells which are virulent, and the other is rough (R) cells which are not virulent. These have been observed upon an infection of mice in laboratory test, where the mice were infected with both forms of the bacteria. Given that the R variants were living and the S variants were heat-killed. After some time of the infection, many of the mice had developed pneumonia and living S variants were found in the blood of the mice. These results indicated that some sort of recombination had occurred between the living R form of the bacteria and the substance of the heat-killed S form. In 1944, Biologists Oswald T. Avery, Colin M. MacLeod and MaclynMcCarty identified a highly purified DNA fraction to bring about the recombinants, whereas no other fraction of the heat-killed S bacteria caused recombination. The results clearly stipulated that DNA is the 'transforming principle'. Both these results were milestones in the elucidation of the molecular nature of the genes [18]. After the revelation that DNA was the agent that determines the virulence, later it was further demonstrated that exogenous DNA, is incorporated into the bacterial chromosome by a breakage-and-insertion process. Thus leading to the fact that DNA is transferred from one cell to another as isolated pieces of external DNA.

1.5.2 The discovery of bacterial transduction

In 1951, Joshua Lederberg and Norton Zinder were testing for recombination between the bacterium *Salmonella typhimurium* by using similar techniques, as they did with *E.coli.* Which lead to the conclusion similar to that of E.coli. Moreover, researchers also confirmed via a U-tube experiment eliminating the possibility of conjugation which further investigations down the hill, lead to the advancement that the recombination had occurred via an agent and, the size of it was about the size of a virus particle, specifically P22, a known temperate phage of *Salmonella.* Thus, Lederberg and Zinder, instead of confirming conjugation in *Salmonella,* had discovered a new type transfer method mediated by a virus. This process is called Transduction. Transduction has subsequently been shown to be quite common among both temperate and virulent phages.

There are two types of Transduction; generalized and specialized. Generalized transducing phages can carry any part of the chromosome, whereas specialized transducing phages carry only restricted parts of the bacterial chromosome. Therefore, transduction occurs when newly forming phages acquire host genes and transfer them to other bacterial cells. Generalized transduction can transfer to any host and it occurs when phage packaging accidentally incorporates bacterial DNA instead of phage DNA. Specialized transduction is due to faulty separation of the prophage from the bacterial chromosome, so the new phage includes both phage and bacterial genes.

1.5.3 The discovery of bacterial conjugation

Conjugation was the first extensively studied method of gene transfer. The discovery of conjugation came about when biologists questioned, "Do bacteria possess any processes similar to sexual reproduction and recombination?" This question was answered in 1946 by the elegant work of Joshua Lederberg and Edward Tatum. The experimental work involved studying two strains of *Escherichia coli* with different nutritional requirements, where one would grow on a minimal medium only if the medium were supplemented with methionine and biotin and the other would grow on a minimal medium only if it were supplemented with threonine, leucine and thiamine. Thus, Strain A would be met^{-}, bio^{-}, thr^{+}, leu+, thi+ and strain B would be met –, bio +, thr-, leu-, thi-. Hence, the strains were mixed together and the results were, some of them wild type and have gained the ability to grow without the added nutrients. Therefore, the plates (un-supplemented minimal media) containing the both the strains A and B, had shown growth and lead to the conclusion of some sort of recombination.

Moreover, the U-tube experiment conducted by Bernard Davis, consist of a filter that would not allow bacteria to pass through. After a couple of hours of incubation and after the testing of the contents of minimal media, it was found that there were no cells found. Eventually, which lead to the conclusion that physical contact between the two strains were required for the recombination to take place. This process which required a tight contact between strains for recombination lead to the invention of the process Conjugation.

1.6 Conjugation

Conjugation is the process described as a one-way transmission mechanism of DNA from one cell-to another and the only process that involves a cell-to-cell contact. The bacterium is required to carry a plasmid. Plasmid is a small, replicative and circular piece of extrachromosomal DNA. It normally carries genes associated with drug resistance or antibiotic resistance along with genes that encode to mediate transfer from one organism to another. Plasmids can either be non-transmissible (cannot transmit independently) or self-transmissible plasmids. When plasmids contain genes that assist to mediate transfer between cells, it is called a Fertility plasmid (F plasmid). The bacterium containing the F plasmid is referred to as F+ or male and confers donor characteristics. Those without the F plasmid, are referred to as F- or female or recipients of the bacterial gene transfer. Therefore, through conjugation, the recipient receives the genetic information from the donor, which changes certain characteristics that helps the recipient to adapt in their current environment.

During conjugation, all the proteins associated with the pilus (morphological characteristic of bacteria that enables a direct contact with the recipient cell) are transcribed and translated from the genes of the F plasmid (F factor). The pilus tends to pull the two cells together that allows a conjugative junction to form, containing a pore. The pore allows the DNA to be transferred from one cell to the other. However, the F plasmid is not transferred to the F- cell rather, the F plasmid unwinds and one of those strands are transferred. The single-stranded DNA in both the donor and the recipient cell then undergoes a rolling replication model to become double stranded. Therefore, the recipient cell then becomes an F plasmid containing F factor.

One of the major breakthroughs of the conjugation was when Luca Cavalli-Sforza discovered a derivative of an F+ strain. This derivative is when the F plasmid becomes integrated into the host cell genome and is capable of inducing more than thousand times the number of genetic recombination than seen in F+ and F- cells. Such a donor strain is called a high frequency of recombination (HFr) strain. The HFr chromosome (originally circular) unwinds and, is transferred to the F- cell in a linear manner. The unwinding and transfer of the HFr chromosome begin form a specific point at one end of the integrated F, called the origin or O. The farther a gene is from O, the later it is transferred to the F-; the transfer process most likely will stop before the farthermost genes are transferred. The HFr chromosome replicates while it is transferring a single strand to the

F- cell; this replication ensures a complete chromosome for the donor cell after mating. The transferred strand is replicated in the recipient cell, and donor genes may become incorporated in the recipient's chromosome through cross overs, creating a recombinant cell. Otherwise, the transferred fragments of DNA in the recipient might get lost in the course of cell division.

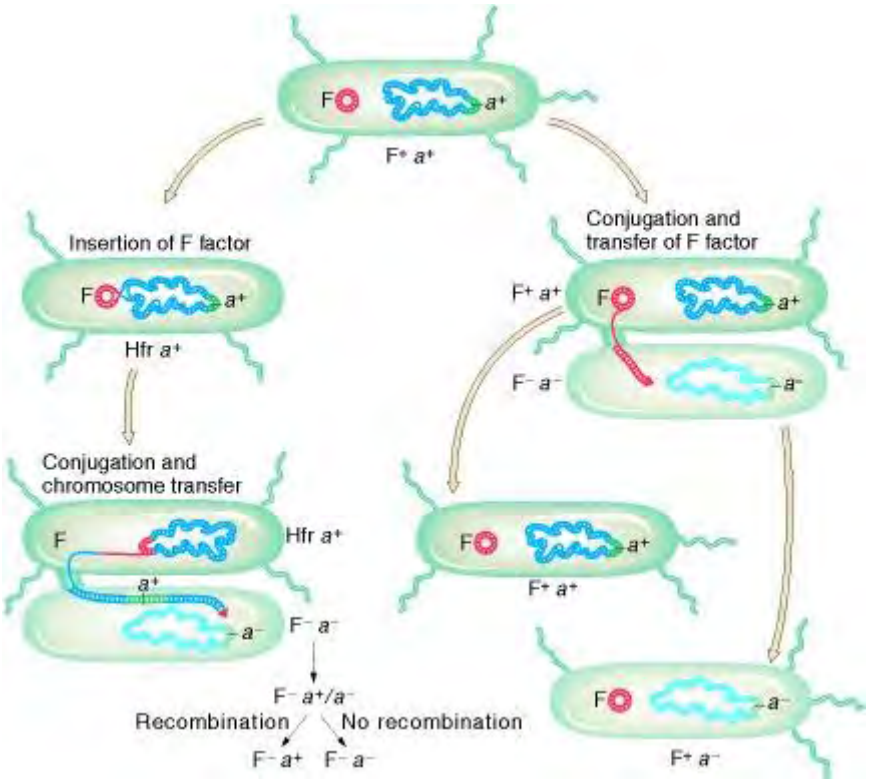


Figure 1.6 Mechanism of Conjugation

1.7 The role of horizontal gene transfer in Biological Evolution

Given that micro-organisms are single-celled organisms and that their genome sizes vary by little more than an order of magnitude in length, bacterial species show extraordinary variation in their metabolic properties, cellular structures and lifestyles. Even within relatively narrow taxonomic groups, such as enteric bacteria, the phenotypic diversity among species is remarkable. Several mechanisms could be responsible for the differences evident among bacterial species. Point mutations leading to modification, inactivation and differential regulation of existing genes have significantly contributed to the diversification of microorganisms on an evolutionary timescale. However, it is difficult to account for the ability of bacteria to exploit new environments by the

accumulation of point mutations alone. In fact, none of the phenotypic traits that are typically used to distinguish the enteric bacteria *Escherichia coli* from its pathogenic sister species *Salmonella enterica,* can be attributed to the point mutational evolution of genes common to both. Instead there is a growing evidence that, lateral gene transfer has played an integral role in the evolution of bacterial genomes and, in the diversification and speciation of the enterics and other bacteria [20].

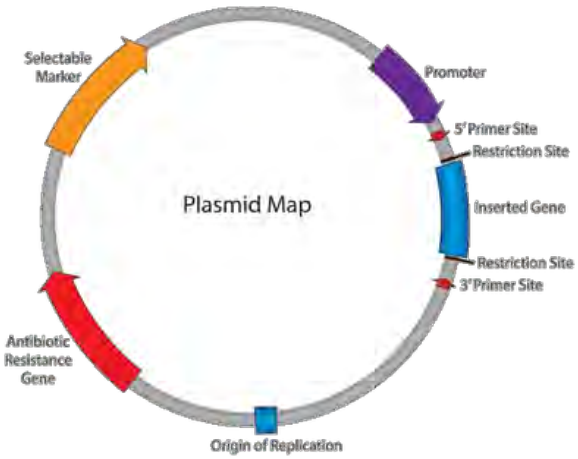
The significance of lateral (horizontal) gene transfer for bacterial evolution was not recognized until the 1950s, when multi-drug resistance patterns emerged on a worldwide scale. The facility with which certain bacteria developed resistance to the same spectrum of antibiotics indicated that these traits were being transferred among taxa [20]. Even though widespread impact of lateral or horizontal gene transfer on bacterial evolution was not been appreciated until much later, it is important to realize its implications on different aspects. Urinary Tract Infection has been one of the most commonly contracted infection and the causative agents are on the rise of spreading antibiotic resistance at an alarming rate. This research work is a project on the transfer of antibiotic resistance genes with *Escherichia coli (E.coli)* and *Shigella spp.* via conjugation from a sample of UTI patients.

1.8 Plasmid and Plasmid Curing

Plasmids are extra-chromosomal, double stranded circular pieces of DNA. They replicate independent of the host chromosome and yet co-exist with it [Trevors, 1985]. In 1952, Joshua Lederberg coined the term plasmid, which can be passed from one bacterium to another by a type of horizontal gene transfer (conjugation). Plasmids are essential in the field of molecular biology due to a number of reasons, which includes, convenience to work with, since their size is in between 1-20 kilo-base pairs; their nature to self-replicate and be stable; lastly they are functional in many different species which allows for a diverse range of applications.

Plasmids are identified as mobile genetic elements that contribute significantly to their diversity and adaptability. These MGEs do not carry anything essential for the survival under non-stressful conditions but is important for specialized conditions. Bacterial plasmids are known to harbor

genes for resistances to antibiotics and metals, catabolic pathways such as lactose utilization and degradation of hydrocarbons, biosynthesis of certain products.



The diagram beside shows a plasmid which contains the following elements.

Figure 1.8 Genetic Map of Plasmid

Plasmid curing is a technique adapted to substantiate a relationship between a genetic trait and carriage of that specific trait in the plasmid. The process involves the removal of plasmid from the bacteria with the help of curing agents. These curing agents could be either chemical or physical, some of which tends to mutate DNA, interfere specifically with its replication or affect particular structural components or enzymes of the bacterial cells. Protocols for plasmids consist of frequent exposure of a culture to sub-inhibitory concentrations of some chemical agents such as Acridine orange, acriflavine, and sodium dodecyl sulfate (SDS) or to a super-optimal temperature followed by selection of cured derivatives. The DNA intercalating agents such Acridine Orange and Ethidium Bromide are most commonly used because they are found to be effective against plasmids in a wide variety of genera.

1.9 Literature Review

Suhani et al., (2017) investigated *Escherichia coli (E.coli)*, a causative agent for Urinary Tract Infection, containing antibiotic resistant plasmids and its ability to transfer resistance through horizontal gene transfer. The investigation goes on to show that the isolate *E.coli* was resistant to amoxicillin (AMX), ceftriaxone (CTR) and ciprofloxacin (CIP) and E.coli DH5α were sensitive to all these antibiotics. The *E.coli* isolate was taken as the donor and *E.coli DH5α* was taken to be

the recipient. Both these strains were conjugated and after successful conjugation, antibiotic susceptibility testing was conducted to confirm the transfer of the resistance. Moreover, plasmids were also extracted to show the *E.coli DH5α* which initially had no plasmids acquired multiple. *E.coli DH5α* was also made competent and then underwent transformation. The results were positive for transformation. Hence, the investigation revealed that *E.coli DH5α* inherited resistance via conjugation and transformation.

Karakas et al., (2015) conducted a research on the prevalence of *Shigella spp*. as causative agent for Urinary Tract Infections. Given that urine is the second most abundant source for positive isolates, after stool, *Shigella* isolates were mostly recovered from the stool however, *Shigella* species were also isolated from the urine samples. The isolates from the urine samples were identified using biochemical tests particularly, triple sugar iron agar, urease, citrate and indole. Moreover, susceptibility of these isolates to antibiotics were tested by the Kirby-Bauer Disk Diffusion method using E.coli ATCC strain as control strain. It was found that *Shigella* isolates were resistant to first and second generation cephalosporins, ciprofloxacin and aminoglycosides. The research also stated that aged people with underlying diseases could be more susceptible to UTI caused by *Shigella*.

Arber, (2014) studied extensively the horizontal gene transfer among bacteria and its role in biological evolution. The study describes in detail about the discovery of bacterial transformation and highlights DNA as a genetic carrier and its role in carrying virulence. Moreover, the study discusses conjugation as another method of the horizontal gene transfer that requires a direct cell-cell connection for the transfer of plasmid. Moreover, the donor must possess a fertility plasmid containing the resistance genes. In addition, transduction was also extensively discussed along with bacterial modification or restriction system that could limit the horizontal gene transfer. The study sheds light upon the natural barriers like the lack of bacterial surface compatibility for conjugational pair or for the uptake of DNA from the medium, requirement for functional harmony between the recipient's genome and CRISPR-Cas. Further into the study, the implications of horizontal gene transfer and its role in evolutionary biology was stressed upon, as it contributes to genetic variation and how this variation could also lead to an eradication of forms of life.

Zaman, Pasha & Akhter, (2010) conducts an experiment to investigate the ability of Ethidium Bromide, Sodium Dodecyl Sulfate and Acridine Orange as curing agents against Escherichia coli

isolated from urinary tract infection specimens. Plasmids are referred to as one of the several environmental and genetic factors that harbors the resistance genes against certain antibiotics. Plasmids are eliminated from these multi-drug resistant strains to outline the role of R-plasmid in drug resistance. Primary methods of curing involves, exposure to elevated temperatures, use of chemical agents such as intercalating agents (EtBr, acridine orange), treatment with crystal violet, Sodium Dodecyl sulfate and thymidine starvation. The investigation involves antibiotic sensitivity assay of the isolates and plasmid extraction of these isolates and curing of plasmids afterwards. The result shows that Ethidium Bromide was the most successful curing agent amongst Sodium Dodecyl Sulfate and Acridine Orange, and the results of Acridine orange, on the other hand were better than SDS. The plasmids were cured at a concentration of 100µg/ml and 125µg/ml. Plasmid profiling was carried out for these isolates to further strengthen the results. Therefore, the investigation concluded by stating uro-pathogenic E.coli as the major culprit for UTI and they are capable of transferring their plasmid mediated resistance gene and Ethidium Bromide as the more successful curing agent.

Eco Sal Plus 2013, is a study on uro-pathogenic Escherichia coli as the causative agents of the Urinary Tract Infection. The study talks about the urinary tract infection being the most common bacterial infections, stating that women are at a higher risk than men. The study talks about the clinical syndromes and routes of infection. Moreover, the study elaborates onto the consequences of the recurring UTIs and other major illnesses that associates with it. The study also discusses the different strains and their genomic maps which focuses on the different pilli, related adhesins and fimbriae type 1 and their roles in virulence. Overall the study looks into the details of the Urinary Tract Infections which includes the microbiological aspects as well as the molecular aspects. The physiological aspects and the treatments were also covered extensively as a part of this study.

Trevors 1985, is based on the curing of plasmids and the curing agents and its roles in curing. The different curing agents and their mode of actions were discussed very elaborately. The paper also discusses the procedure to follow for the curing of plasmids.

1.10 Aims and Objectives

The work presented in this thesis aims to illustrate horizontal (lateral) gene transfer via conjugation and how they acquire their resistance through this method. In this research we aim to conjugate different species of bacteria on the basis of their antibiotic susceptibility, from the urine samples of the UTI patients. Although there have been similar researches this paper looks into aspects differently than the ones already present. Given that biologists have been conducting different kinds of research on conjugation itself, the following is what is believed to be achieved through this research:

1. Understand the natural events that occur during conjugation
2. Transfer antibiotic resistant gene from one species to another
3. Understand the mechanisms of bacterial survival and pathogenicity in other organisms.

CHAPTER 2

Methodology

2. Methodology

2.1 Place of study

The research was conducted at the Microbiological Laboratory of the Department of Mathematics and Natural Sciences Department of BRAC University.

2.2 Period of Study

The research work was carried out from July 2017 till April 2018.

2.3 Microbiological Sample

A total of 30 samples collected from UTI patients of a Diagnostic Centre, these bacterial samples were isolated then identified using the biochemical tests and put into the selective media for re-confirmation of the organism. The isolates were a diverse range of species including gram-negative and gram-positive bacteria. The table below shows only 10 isolates out of the total sample.

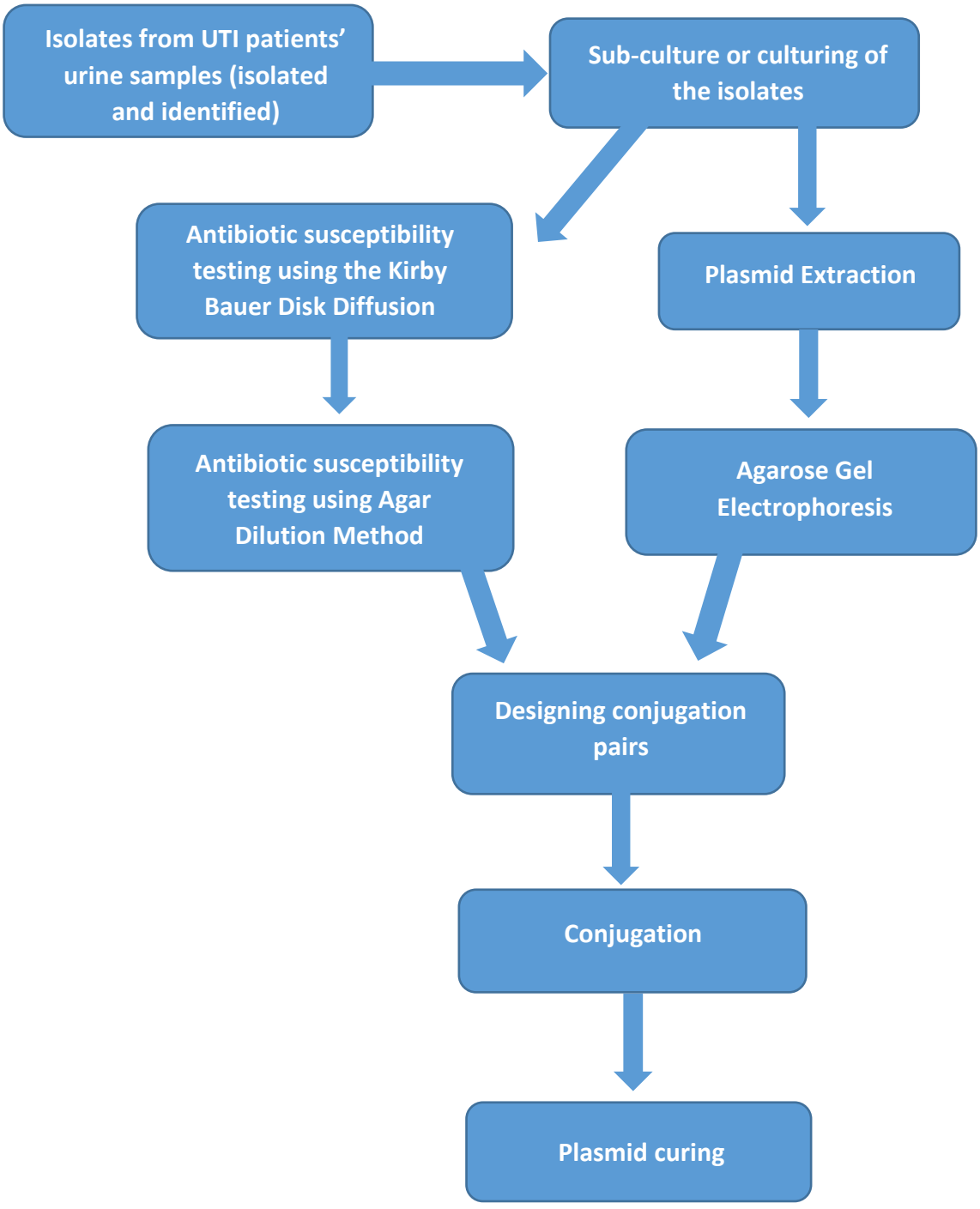
Table 2.3.1: Name of the organisms

Sl. No.	Name of the organism	Organism ID
1.	*Klebsiella*	**C**
2.	*Klebsiella*	**G**
3.	*E.coli*	**J**
4.	*E.coli*	**K**
5.	*Shigella*	**L**
6.	*Shigella*	**M**
7.	*E.coli*	**N**
8.	*Shigella*	**R**
9.	*E.coli*	**U6**
10.	*Shigella*	**I8**

2.4 Microbiological culture of the sample

The samples were initially cultured in nutrient agar, put away for at least 18 – 24 hours incubation 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 in the four quadrant method, 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.

2.5 Experimental Work Flow



2.6 Antibiotic Sensitivity Testing

2.6.1 Disk Diffusion Method (Kirby Bauer 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. These antibiotics are both natural and synthetic (man-made) and several generations of these synthetic antibiotics have been invented to meet the requirements of the human population.

Kirby Bauer Disk Diffusion technique is normally the standard procedure to check for the antibiotic sensitivity of these organisms. It was first developed in the 1950s and refined by Kirby and A. Bauer and then finally standardized by the World Health Organization (WHO). This technique allows in determination of whether the organism in question is resistant or sensitive to the antibiotic (anti-microbial) in question.

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 nutrient broth. Nutrient Broth is a non-selective liquid medium used for the general cultivation and enumeration of less fastidious micro-organisms. The organisms are incubated for three hours. Afterwards a sterilized cotton swab is dipped into culture broth and that swab is used to inoculate the surface of the fresh plate containing Mueller Hinton Agar. Mueller Hinton Agar (MHA) is the commonly used medium for the susceptibility testing since it's a non-selective, non-differential medium allowing most organisms to grow. Moreover, it contains starch, which is responsible for the absorption of toxins, released from bacteria, so that they cannot interfere with the antibiotics and it mediate the rate of diffusion of the antibiotics through the agar. Adding to it, it is a loose agar which allows for better diffusion of antibiotics giving a truer zone of inhibition.

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).

2.6.2 Minimum Inhibitory Concentration (MIC) and Maximum Bactericidal Concentration (MBC) using the Agar Dilution Method

Minimum Inhibitory Concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Minimum Bactericidal Concentrations (MBCs) are defined as the lowest concentration of antimicrobial agent that inhibits the growth of an organism by 99.9%. The MBC is complementary to the MIC.

Among the common techniques, Agar dilution is the one that have been used. This method is both quantitative and comparative at the same time. In the agar dilution method, different known concentrations of anti-microbial agent are put into different plates along with the agar medium. Both the antibiotic and the agar medium are thoroughly mixed together and 10µl of each organism or sample were put onto the plate. A grid was used on the plate to organize the culture drops on the plate.

The agar medium used was Mueller Hinton agar and the antibiotic used were azithromycin and ciprofloxacin. The concentrations of the antibiotics used are mentioned in the table below:

Table 2.6.1: Concentration of Antibiotics used during Antibiotic Susceptibility testing

Sl. No.	Concentration of Azithromycin (µg/ml)	Concentration of Ciprofloxacin (µg/ml)
1.	1	0.1
2.	10	1
3.	30	5
4.	60	10
5.	80	15
6.	100	20
7.	150	50
8.	200	-

After the organisms were put on the grid of the plates as drops, they were put away for incubation at 37°C for 18 to 24 hrs. Following incubation, the growth on the plate would determine the sensitivity of the organisms at different concentrations and along with that the MIC, MBC values.

2.7 Plasmid Extraction

Plasmid extraction procedures are based on the fact that plasmids usually occur in the covalently closed (supercoiled) CCC configuration within the host cells. After gentle cell lysis all intracellular macromolecules have to be eliminated whereas plasmid DNA is enriched and purified. The smaller a plasmid the easier is the isolation of intact CCC molecules. Therefore the following includes the methods used during the duration of the research to isolate plasmid from the desired organisms.

2.7.1 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 for storage at 4°C freezer.

2.7.2 Alkaline Lysis Method

Alkaline lysis is another commonly used technique in molecular biology to isolate plasmid DNA or other cellular components via cell lysis. The cell was lysed with the help of an alkaline lysis buffer which contains, Sodium Dodecyl Sulfate (SDS) and Sodium Hydroxide (a strong base). SDS acts as the detergent and cleaves the phospholipid bilayer of membrane, whereas, the alkali denatures the proteins which are involved in maintaining the structure of the cell membrane. After a series of steps that involves agitation, precipitation, centrifugation, and the removal of supernatant containing cell debris, the plasmid isolated and collected.

2.8 Agarose Gel Electrophoresis

After the successful extraction of plasmid, the next procedure that is carried out is, gel electrophoresis, to test the presence of plasmids in the desired organisms. 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. Therefore, in order to analyze the plasmids that have been extracted earlier, at first, 0.7% gel is prepared separately using 80ml TBE buffer solution and 0.56g of Agarose powder. 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.

2.9 Designing of Conjugation pairs

After the antibiotic susceptibility testing, plasmid isolation and analysis of the extracted plasmids using gel electrophoresis, the results would give sufficient information to determine the donors and recipients for conjugation. The donor organism must be resistant against certain antibiotic to which the recipient organism is sensitive/susceptible. This particular antibiotic would be the transfer antibiotic. The transfer antibiotic is determined from the Kirby Bauer Disk Diffusion and

the antibiotic susceptibility testing provided information regarding concentration of antibiotic to be used when designing the plates for conjugation. Moreover, a marker antibiotic was also required to ensure the growth of recipient after conjugation, which was further backed up by the MIC and MBC values. The selection of both the donor and recipient was also dependent on the presence of plasmids. In order for conjugation to take place, it is a basic requirement for the donor to have a plasmid. The bands visible from the gel electrophoresis, allow us to determine that.

For instance, the table 2.9.1 shows the designing of the conjugates from the collected sample.

Table 2.9.1: Designing conjugation pairs

Name of the organism	**Organism ID**	**Transfer Antibiotic**	**Marker Antibiotic**	**Number of plasmids**	**Comments**
Escherichia coli	n	Azithromycin Resistant	Ciprofloxacin Sensitive	2	Donor
Shigella spp.	I8	Azithromycin Sensitive	Ciprofloxacin Resistant	None	Recipient

2.10 Conjugation

After determining the conjugants (i.e. donor and recipient) for conjugation, we culture both the donor and recipients on Nutrient agar and incubated at 37°C. Individual colonies were chosen and inoculated in Luria Bertani (LB) broth, separately. The inoculated broth was incubated at 37°C overnight in a shaking incubator. The following day, five hundred micro-litre of donor and recipient cultures were added to 4ml fresh LB. and incubated overnight without shaking [Suhani S. et.al., 2017] afterwards, a 10-fold serial dilution was done till 10^{-5} and then, 100µl from each dilution were plated on Luria Bertani, (LB) agar plates containing the antibiotic of the concentration determined from the MIC and MBC values. [Suhani S. et.al. 2017].

The growth on the plates after conjugation would indicate a positive result since, the plates were designed to allow the growth of only trans-conjugants (recipient after receiving the resistant gene from the donor).

2.11 Plasmid Curing

Plasmids are known to be extra-chromosomally replicating molecules of DNA that harbors the resistance property against a specific antibiotic or a certain class of antibiotics. Given that plasmids tend to play one of the crucial roles in the gene transfer in conjugation and without which conjugation may not even take place. The plasmids responsible for the conjugation are commonly known as the Fertility, F-plasmids. The F-plasmids contains the *tra* gene that is responsible for conjugation to take place and also for the expression of sex-pilli. The other type of plasmid, known as the R-plasmids is the resistance plasmids, carrying the resistance genes against the antibiotics.

Often times, the resistant properties carried by the bacterial strains happens to be chromosome mediated rather than plasmid-mediated. Due to which, despite having a plasmid, it is a quite likely reason for the bacteria to be not be able to transfer antibiotic genes. Plasmid curing happens to be one of those techniques that includes chemicals and physical agents, some of which can mutate DNA, interfere specifically with its replication, or affect particular structural components or enzymes of the bacterial cell. The DNA intercalating agents for example, Acridine orange and Ethidium Bromide are the most commonly used agents since, they have been found to be successful among all the different genera of the bacteria.

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 2.11.1.

Table 2.11.1: Concentrations of EtBr used in plasmid curing			
Concentration of EtBr / (µg/ml)	**Amount of EtBr / (µl)**	**Concentration of SDS (%)**	**Amount of SDS / (g)**
1	1	2	0.2
10	10	5	0.5
20	20	8	0.8
40	40	10	1.0
60	60	12	1.2
80	80	15	1.5
100	100	20	2.0

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 enable us to determine whether the resistance gene is present in the chromosomal DNA or in the plasmid [Trevors J.T., 1986].

CHAPTER 3

Results

3. Results

3.1 Microbiological culture of the organisms

The isolates that were collected from the UTI patients were a diverse group of organisms and all the isolates were cultured, the following isolates in the figure below are suspected to be either *Escherichia coli* (*E.coli*) or *Shigella spp.* and both were cultured from the stock in nutrient agar and incubated for 24 hours at 37°C.

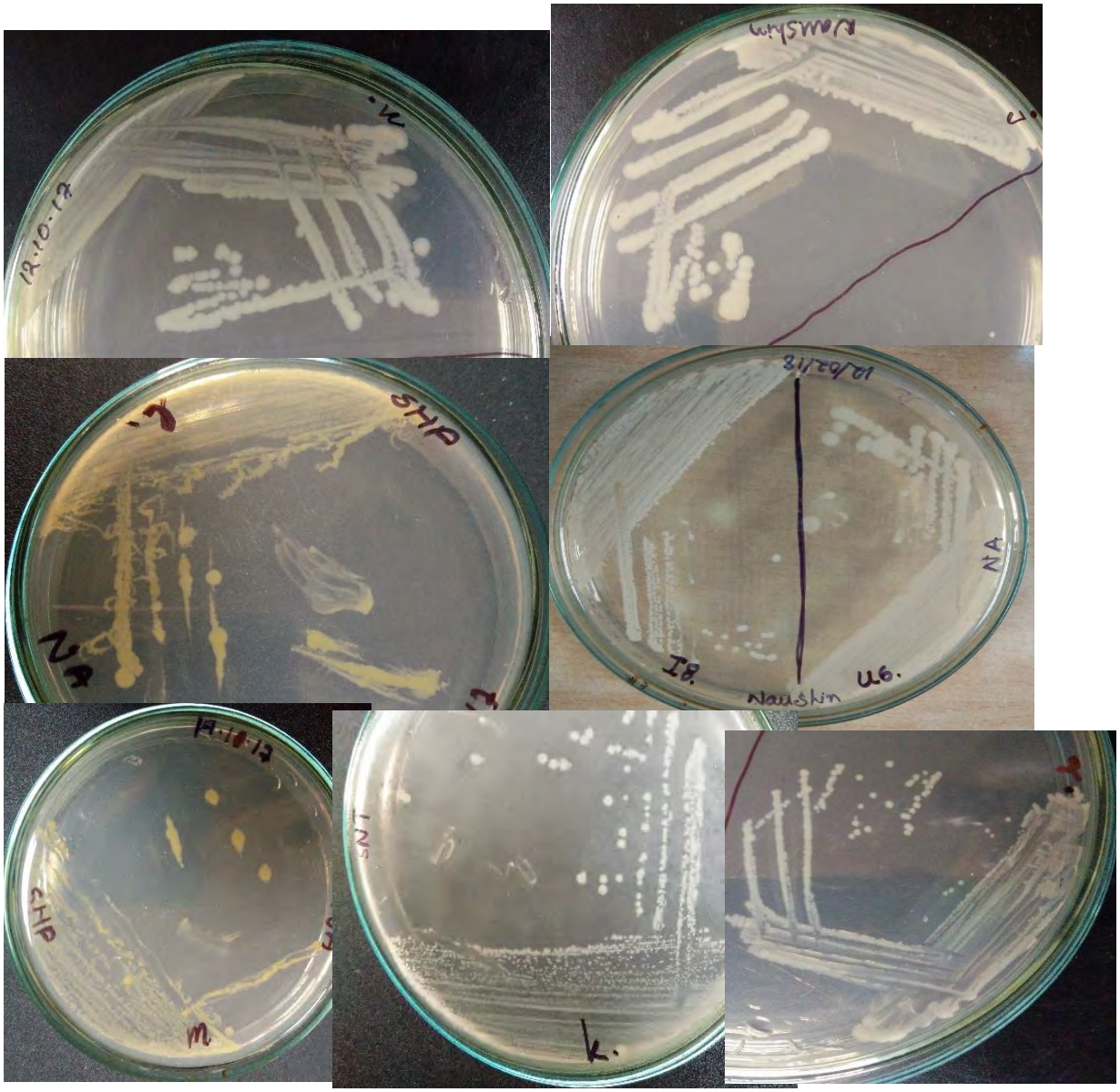


Figure 3.1 Colony morphology of the isolates

Both the organisms, *Escherichia coli* and *Shigella* were cultured on nutrient agar for fresh culture before any procedure to obtain best results. *E.coli* has mostly shown to have medium sized colonies, with off-white color, circular form and raised elevation. *Shigella* on the other hand, had shown to possess two different types of colon morphology. Either medium sized off white colonies with a circular form or medium sized orange colonies with a rhizoid form and filamentous margin. For the re-confirmation of the identity of the isolates, suspected *E.coli* isolates were grown on EMB agar and suspected growth of the *Shigella* isolates were grown on Salmonella Shigella (SS) agar.

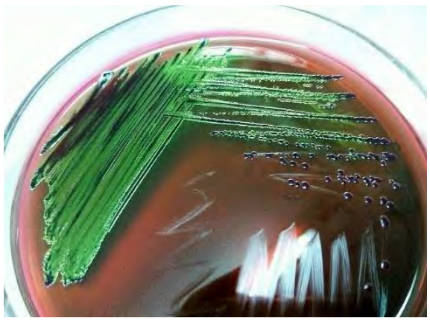


Figure 3.2 growth of E.coli on EMB agar

Figure 3.3 Growth of Shigella spp. on SS agar

3.2 Antibiotic Susceptibility Testing

3.2.1 Anti-biogram

Table 3.1: Antibiotic Susceptibility Results of the isolates

Isolate ID	Name of the organism	Antibiotics					
		CIP(5) /mm	Sensitivity	AZM(30) /mm	Sensitivity	AMP(25) /mm	Sensitivity
j	*E.coli*	32.0	S	16.0	I	Nil	R
k	*E.coli*	36.5	S	19.0	I	17.0	R
l	*Shigella spp.*	28.0	I	12.5	R	30.0	S
m	*Shigella spp.*	25.0	I	0.0	R	29.5	S
n	*E.coli*	32.0	S	11	R	19.0	S
r	*Shigella spp.*	37.0	S	25.0	S	0.0	R
U6	*E.coli*	Nil	R	11	R	Nil	R
I8	*Shigella spp.*	16.0	I	13	I	Nil	R

Key: S – Sensitive (or Susceptible); I – Intermediate; R - Resistant

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 was deduced that isolate n, suspected *E.coli* was sensitive against ciprofloxacin with a large zone diameter in millimeters and resistant against azithromycin with certain zone diameter in the range that can be considered resistant.

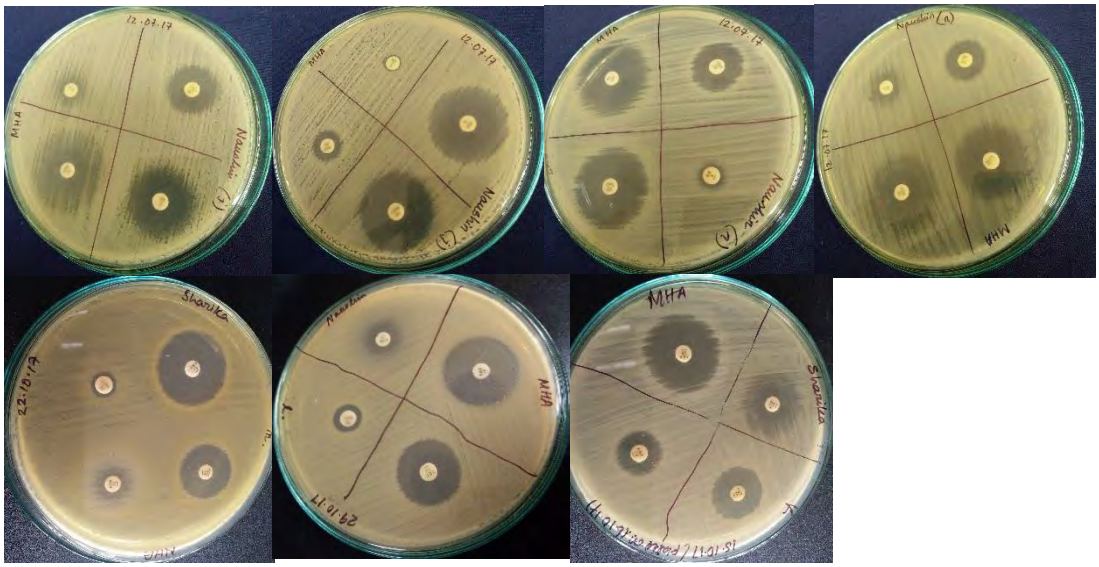


Figure 3.4 the plates shows the zone of inhibitions after Kirby Bauer Disk Diffusion

3.2.2 MIC & MBC

For further re-confirmation of antibiotic susceptibility of the organisms, minimum inhibitory concentration (MIC) and maximum bactericidal concentration (MBC) were determined following an agar dilution technique. The figure above shows the variation in the growth of the organisms, due to the presence of the antibiotics. The antibiotics present were Ampicillin, Azithromycin, and Ciprofloxacin. The details of the result are provided in table 3.2, 3.3 and 3.4 respectively. All the data are summarized in table 3.5.

Table 3.2

		Ampicillin Concentration / microgram per milliliter (µg/ml)									
Name of the Organism	**Isolate ID**	**1**	**10**	**30**	**60**	**80**	**100**	**150**	**200**	**MIC Value**	**MBC Value**
E.coli	j	✓	✓	✓	✓	✓	✓	✓	-	150	200
E.coli	k	✓	✓	✓	✓	✓	✓	✓	-	150	200
Shigella	l	✓	✓	✓	✓	✓	✓	-	-	100	150
Shigella	m	✓	-	✓	✓	✓	✓	✓	-	150	200
E.coli	n	✓	-	✓	✓	✓	✓	-	-	100	150
Shigella	r	✓	-	✓	✓	✓	✓	-	-	100	150
E.coli	U6	✓	✓	✓	✓	✓	✓	✓	✓	Higher	Higher
Shigella	I8	✓	✓	✓	✓	✓	✓	✓	✓	Higher	Higher

Table 3.3

		Azithromycin Concentration / microgram per milliliter (µg/ml)									
Name of the Organism	**Organism ID**	**1**	**10**	**30**	**60**	**80**	**100**	**150**	**200**	**MIC Value**	**MBC Value**
E.coli	j	✓	-	-	✓	-	-	-	-	1	10
E.coli	k	✓	✓	✓	-	-	-	-	-	30	60
Shigella	l	✓	✓	✓	✓	-	✓	-	✓	80	100
Shigella	m	✓	✓	✓	✓	-	✓	-	-	60	80
E.coli	n	✓	✓	✓	✓	-	✓	✓	✓	Higher	Higher
Shigella	r	✓	✓	✓	✓	-	✓	-	-	60	80
E.coli	U6	✓	✓	✓	✓	✓	-	-	-	80	100
Shigella	I8	✓	✓	✓	-	-	✓	-	-	30	60

Table 3.4

		Ciprofloxacin Concentration/ microgram per milliliter (µg/ml)								
Name of the organism	**Organism ID**	**0.1**	**1**	**5**	**10**	**15**	**20**	**50**	**MIC Value**	**MBC Value**
E.coli	j	-	✓	✓	✓	-	-	✓	10	15
E.coli	k	✓	✓	-	✓	-	-	-	10	15
Shigella	l	✓	✓	✓	✓	-	-	-	10	15
Shigella	m	✓	✓	✓	-	-	-	-	5	10
E.coli	n	-	-	✓	-	-	-	-	Totally sensitive	Totally sensitive
Shigella	r	✓	-	-	-	-	-	-	0.1	1
E.coli	U6	✓	✓	✓	✓	✓	✓	✓	Higher	Higher
Shigella	I8	✓	✓	✓	✓	✓	✓	✓	Higher	Higher

Table 3.5 Antibiotic Susceptibility testing for MIC MBC							
		MIC Value (µg/ml)			MBC Value (µg/ml)		
Name of the Organism	Isolate ID	Ampicillin	Azithromycin	Ciprofloxacin	Ampicillin	Azithromycin	Ciprofloxacin
E.coli	j	150	1	10	200	10	15
E.coli	k	150	30	10	200	60	15
Shigella spp.	l	100	80	10	150	100	15
Shigella spp.	m	150	60	5	200	80	10
E.coli	n	100	Higher	Totally sensitive	150	Higher	Totally sensitive
Shigella spp.	r	100	60	0.1	150	80	1
E.coli	U6	Higher	80	Higher	Higher	100	Higher
Shigella spp.	I8	Higher	30	Higher	Higher	60	Higher

Following the previous results of antibiogram and the results of MIC and MBC, it can be deduced that the isolate n, *E.coli,* being resistant to azithromycin could be a possible donor with the azithromycin resistant gene and the possible recipient in this case would be *Shigella spp*. since it is sensitive to azithromycin at a concentration of 60µg/ml and higher.

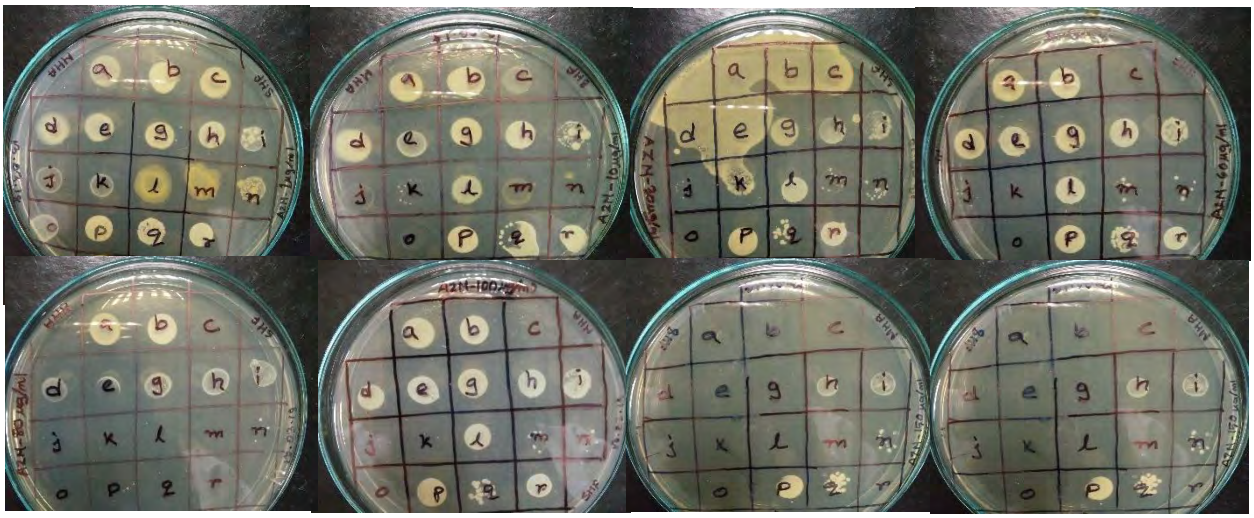


Figure 3.5 the plates showing the results of MIC MBC using the Agar dilution method

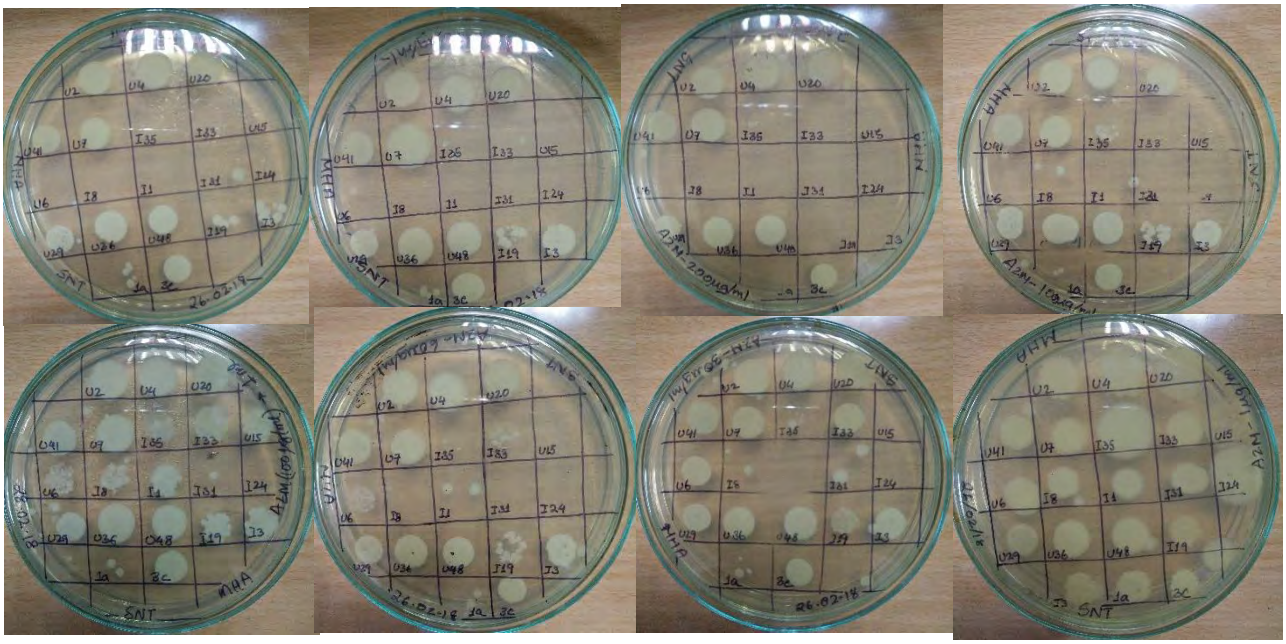


Figure 3.6 the figure above shows the plates of MIC MBC of azithromycin of all the samples in total

3.3 Plasmid Isolation and Gel Electrophoresis

After the antibiotic susceptibility testing via Kirby Bauer method and the Agar dilution method, all the samples underwent plasmid extraction using both the Alkaline Lysis method and the KADO LIU method. After successful extraction of plasmids, extracted plasmids underwent gel electrophoresis as well and the separated bands of plasmid DNA were then observed under UV light.

The following figure shows the plasmids each organism contained as illuminated by the UV light. As per the results, we can clearly see that the isolate n contains two bands above the chromosomal DNA band indicating two plasmids. Whereas, isolate I8 does not have any other bands other than the chromosomal indicating no plasmids. Thus, it can be deduced that isolate n is the potential donor containing two plasmids and isolate I8 is the potential recipient containing zero plasmids.

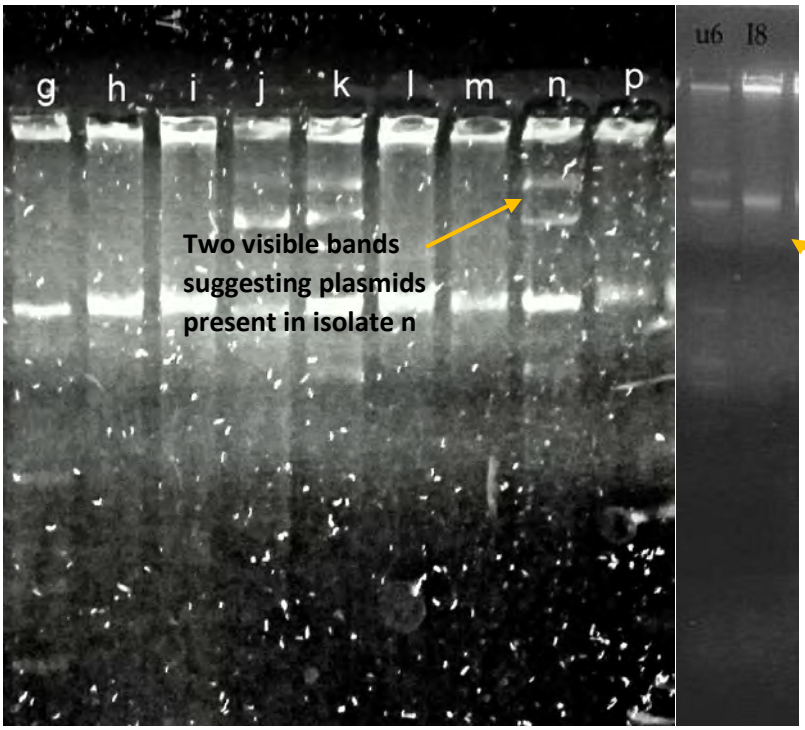


Figure 3.7 The results of Gel Electrophoresis shows the presence or absence of plasmid in the isolates.

3.4 Conjugation

As per the procedure, the plates containing antibiotics were plated with the mixture of the organisms kept for 24 hours incubation, the image below shows no growth on the plate. This indicates, conjugation did not take place between the organisms, as a result, the organisms did not acquire the resistance required to grow on the plates. There were five different 10-fold dilutions of the mixtures, hence, the five different plates.

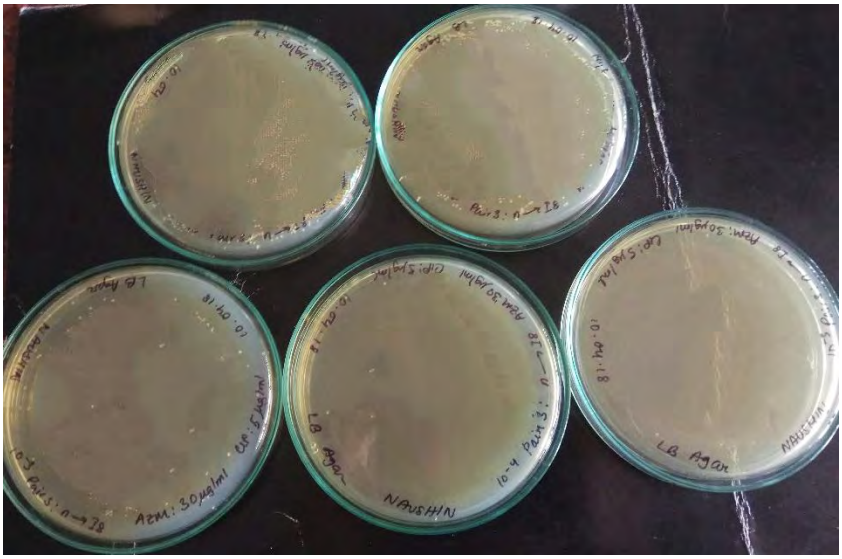


Figure 3.8 the plates shows no visible growth after 24 hours incubation period of conjugation. Starting from the top left, the dilutions increase till the bottom right.

3.5 Plasmid curing

Plasmid curing was conducted, using EtBr on the donor isolates and then antibiotic susceptibility testing was conducted on those cured isolates. The figure beside shows the isolate n which initially consisted of plasmids, shown above the chromosomal bands, were not present in the cured organism. Which indicates a positive result for plasmid curing.

The table below shows the results of the antibiotic susceptibility testing of these cured organisms, to help us determine whether the resistance possessed were plasmid-mediated or chromosome-mediated.

Table 3.6 Antibiotic Susceptibility of Cured isolates			
Organisms (ID)	**Antibiotics**		
	Ampicillin	**Azithromycin**	**ciprofloxacin**
Esherichia coli (E.coli) treated with EtBr	Resistant	Resistant	Sensitive

As per previous results, *Escherichia coli* was resistant to azithromycin with the plasmids, however, after plasmid curing, the antibiotic resistance was persistent, showing that despite the plasmids not being present, the organism still carried its resistance. Therefore, we can say that azithromycin resistance was chromosome mediated rather than plasmid mediated.

Figure 3.9 Plasmid Extraction observed after gel electrophoresis after plasmid curing.

CHAPTER 4

Discussion

4. Discussion

Urinary Tract infections is a bacterial infection in the human anatomy and accounts for significant morbidity and high medical costs. The prevalent causative agents would include *Escherichia coli* followed by *Klebsiella spp., Proteus spp., Enterobacter spp.*, and *Staphylococcus*, given that women and children are more likely to be affected by this [21]. Patients suffering from Urinary Tract Infections are commonly treated with antibiotics that these causative agents are sensitive to. Considering these current issues and antibiotic resistance on the rise, among the diverse variety of species causing UTIs or the group of *Enterobacteriaceae*, it is one of the major public health concerns. Therefore, one of the primary goals of this research was to identify whether species present in the urine samples of the UTI patients were able to transfer their resistance gene to other species or within species via conjugation. Moreover, an extension to this research was to understand the underlying meaning behind this mechanism of transfer. The transfer mechanism was conjugation and to further investigate, plasmid curing was also conducted.

The research involves antibiotic susceptibility testing of the isolated and suspected species via Kirby Bauer Disk Diffusion method and Agar Dilution method to determine the MIC & MBC of the isolates. Of all the isolates, this study focuses on *E.coli* and *Shigella spp*. identified, since the resistance transfer is not very common between *E.coli* and *Shigella* spp. Following the antibiotic susceptibility testing, the isolates went through plasmid extraction and gel electrophoresis to determine and identify the presence of plasmid. Presence of plasmid is an essential requirement if conjugation is to take place. Given that conjugation is amongst the three horizontal/lateral gene transfer mechanisms that requires direct cell to cell contact. Then, the data acquired from antibiotic susceptibility testing and plasmid extraction were used to determine different combinations of isolates as donors and recipients for conjugation. Thereafter designing, conjugation was conducted and afterwards plasmid curing using EtBr.

The results of antibiotic susceptibility shows that one particular isolate, suspected to be a strain of *E.coli* was sensitive to ciprofloxacin, with a significant zone diameter, and resistant to azithromycin and ampicillin. Similarly, one particular species, suspected to be a strain of *Shigella spp.* bore results intermediate (I) against ciprofloxacin and resistant (R) towards ampicillin and azithromycin. Following these results, MIC and MBC were determined for the same isolates,

which shows that the suspected isolate of *E.coli,* grew on plates containing 150µg/ml and 200µg/ml, which is considered to be a relatively high concentration of antibiotic. Due to the presence of similar growth on all concentrations of azithromycin and ampicillin plates, the MIC and MBC values could not have been pin-pointed out. Although, we could clearly deduce the isolate was clearly resistant to both of these antibiotics. As per the results presented in the tables 3.2, 3.3, 3.4 and 3.5, we observe that the isolate *E.coli* had no growth on plates containing ciprofloxacin. The isolate did not grow even on the least antibiotic containing plate that is, 0.1µg/ml. Hence, it was safe to deduce that the isolate, suspected *E.coli*, was completely sensitive to ciprofloxacin. Moreover, when the data for the other isolate, suspected strain of *Shigella spp*., was analyzed, the results showed, complete resistance to ampicillin and ciprofloxacin, as the plates had growth even on the highest concentrations, and susceptible to azithromycin at concentrations 60µg/ml and higher.

Furthermore, as plasmid extraction was carried out, there were multiple strains containing plasmid and amongst them four isolates stood out distinctly whereas, the other isolates did not show any bands. With the data that was obtained from the procedures conducted, isolate *E.coli* stood out clearly as a potential donor and isolate *Shigella spp.* as a recipient, for conjugation. After following the procedure of the conjugation [Suhani, 2017], the plates containing the mixture of organisms, showed no growth. Neither the donor nor the recipient showed growth of the organisms. Thus, it could be concluded that the conjugation did not take place, since the plate containing both the donor and recipient strain were unable to conjugate, the recipient did not receive any resistance gene, and hence were unable to grow. The concentration of antibiotics used on the plates were determined using the MIC and MBC values, since the donor was sensitive to ciprofloxacin, and it didn't grow at all on any concentrations of it, an intermediate significant concentration, 5µg/ml, was taken. Adding to it, ciprofloxacin was taken to be the marker antibiotic in this particular pair as the recipient was completely resistant to it. The transfer antibiotic was azithromycin and since, the recipient was sensitive to azithromycin at 60µg/ml, the concentration appropriate for the plate was 60 µg/ml. The purpose of these concentrations was to allow the growth of trans-conjugants only. The marker antibiotic would inhibit the donor and the transfer antibiotic would inhibit the recipient. Therefore, conjugation was unsuccessful.

All things considered, conjugation depends on multiple factors, however, two of them are, the fertility plasmid, F-plasmid and second the organism needs to possess the resistance gene in the plasmid, meaning it should be plasmid-mediated and not chromosome mediated. Therefore, the procedure that was carried out following conjugation was plasmid curing. Plasmid curing is a technique to substantiate the relationship between a genetic trait and carriage of that specific trait in the plasmid. Various methods involving chemicals and physical agents have been developed however, ethidium bromide has been considered to be a successful curing agent. Using Ethidium Bromide, EtBr, plasmid curing was conducted and donor of the conjugation pair, isolate E.coli, was successfully cured. Afterwards, the cured isolate was tested for its antibiotic susceptibility and the results shows that the isolate was still fully sensitive to ciprofloxacin and fully resistant to both ampicillin and azithromycin. As per the results of the initial antibiotic susceptibility tests, the isolate pertained the same results as it did earlier. Hence, it clearly shows that, even after the loss of plasmid, the organism did not lose its resistance.

In the light of the following information and taking into consideration the process of conjugation, a possible reason for unsuccessful conjugation was due to the antibiotic resistance genes being present in the chromosomal DNA rather than in the plasmid. According to process of conjugation, the F plasmids encodes gene for the synthesis of pilli required for the contact between the donor and the recipient cell, which is expressed allowing conjugation to take place and transfer of the F plasmid to the recipient cell. Due to the absence of the resistance gene in the plasmid, there was no transfer between the donor and recipient hence, the conjugation was unsuccessful.

Even though plasmid curing suggests a possible reason for conjugation being unsuccessful, further confirmation of the presence of the Fertility plasmid specifically would have strengthened the results and research deeper in terms of molecular aspect and assisted in the determination of a more significant and concrete reason for the unsuccessful conjugation. Moreover, further optimization of other factors involved during conjugation would have shed on the circumstances that influences the organisms to share their resistances. These factors could include the bacterial surface compatibility and functional harmony between the individual bacterial strains.

There has been a handful number of research conducted on conjugation and plasmid curing to fulfill the gap in knowledge regarding the wide range of antibiotics and the resistances that these enteric organisms have been acquiring. Successful conjugation has been the conclusion to many

literature and there has been many where E.coli has donated multiple antibiotic resistances to multiple species including *Proteus, Klebsiella, Salmonella* and *Enterobacter* etc.

CHAPTER 05

Conclusion

5. Conclusion

Urinary Tract Infection is a significant bacterial infections and concern. It is caused by *Escherichia coli*, which have been showing resistance towards most of the major antibiotics, which possesses threat to different strains of its own kind and also, other species as well. One of the ways through which resistance gene is transferred is via conjugation, a lateral or horizontal gene transfer method. Conjugation occurs between *E.coli* and many other species, however in this case, unsuccessful conjugation has led to the conclusion that many antibiotic resistances are chromosome mediated and conjugation does not transfer chromosome mediated resistances. This has been substantiated by successful plasmid curing. Moreover, future study could involve further optimization to strengthen this result that would go deeper in the molecular biology aspects.

References

6. Literature Cited

1. Kayser, F. H. (2005). *Medical microbiology*. Stuttgart: Georg Thieme Verlag.
2. Baron, S. (1986). *Medical microbiology*. Menlo Park, CA: Addison-Wesley.
3. Mobley, H. L., Donnenberg, M. S., & Hagan, E. C. (2009). Uropathogenic Escherichia coli. *EcoSal*. doi:10.1128/ecosal.8.6.1.3
4. Suhani, S., Purkaystha, A., Begum, M. K., Islam, M. J., & Azad, A. K. (2017). Plasmids for Amoxicillin and Ciprofloxacin Resistance in Escherichia coli Isolate Causing Urinary Tract Infection. *Clinical Microbiology: Open Access,06*(04). doi:10.4172/2327-5073.1000284
5. Choudhury, M. R. (1999). *Modern medical microbiology*. Dhaka: Bishwa Parichaya.
6. Karakaş, A., Coşkun, Ö, Kiliç, A., Bedir, O., & Beşirbellioğlu, B. A. (2016). Urinary tract infections caused by Shigella species. *Travel Medicine and Infectious Disease,14*(2), 167-169. doi:10.1016/j.tmaid.2015.12.002
7. Gupta, A., Polyak, C. S., Bishop, R. D., Sobel, J., & Mintz, E. D. (2004). Laboratory-Confirmed Shigellosis in the United States, 1989-2002: Epidemiologic Trends and Patterns. *Clinical Infectious Diseases,38*(10), 1372-1377. doi:10.1086/386326
8. Anatoliotaki, M., Galanakis, E., Tsekoura, T., Schinaki, A., Stefanaki, S., & Tsilimigaki, A. (2003). Urinary Tract Infection Caused by Shigella sonnei. *Scandinavian Journal of Infectious Diseases,35*(6-7), 431-433. doi:10.1080/00365540310009077
9. Baral, P., Neupane, S., Marasini, B., Ghimire, K., Lekhak, B., & Shrestha, B. (2012). High prevalence of multidrug resistance in bacterial uropathogens from Kathmandu, Nepal. *BMC Research Notes,5*(1), 38. doi:10.1186/1756-0500-5-38
10. I don't know how to cite it
11. Flores-Mireles, A. L., Walker, J. N., Caparon, M., & Hultgren, S. J. (2015). Urinary tract infections: Epidemiology, mechanisms of infection and treatment options. *Nature Reviews Microbiology,13*(5), 269-284. doi:10.1038/nrmicro3432
12. Trends in Antimicrobial Resistance among Urinary Tract ... (n.d.). Retrieved from http://www.bing.com/cr?IG=DF9DA4686B7B4C75AF73E5584D07C10B&CID=1FA6F65164EC6BE02B8CFDB565436A45&rd=1&h=CrD-

0JbmDryfEn835OWAwMx0LyRZ_zu4bQ_wSdu4YV8&v=1&r=http://aac.asm.org/content/46/8/2540.full.pdf&p=DevEx.LB.1,5502.1

13. Suhani, S., Purkaystha, A., Begum, M. K., Islam, M. J., & Azad, A. K. (2017). Plasmids for Amoxicillin and Ciprofloxacin Resistance in Escherichia coli Isolate Causing Urinary Tract Infection. *Clinical Microbiology: Open Access,06*(04). doi:10.4172/2327-5073.1000284

14. Burmeister, A. R. (2015). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4536854/?log$=activity

15. Davison, J. (1999). Genetic Exchange between Bacteria in the Environment. *Plasmid,42*(2), 73-91. doi:10.1006/plas.1999.1421

16. Daubin, V., & Szöllősi, G. J. (2016, April). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4817804/?log$=activity

17. Arber, W. (2014). Horizontal Gene Transfer among Bacteria and Its Role in Biological Evolution. *Life,4*(2), 217-224. doi:10.3390/life4020217

18. Griffiths AJF, Miller JH, Suzuki DT, et al. An Introduction to Genetic Analysis. 7th edition. New York: W. H. Freeman; 2000. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21766/?log$=activity

19. Daubin, V., & Szöllősi, G. J. (2016). Horizontal Gene Transfer and the History of Life. *Cold Spring Harbor Perspectives in Biology,8*(4). doi:10.1101/cshperspect.a018036

20. Ochman, H. (2006). Lateral Gene Transfer And The History Of Bacterial Genomes. doi:10.2172/876510

21. Bacterial plasmids: Their extraordinary contribution to molecular genetics. (2003, January 16). Retrieved from https://www.sciencedirect.com/science/article/pii/037811199390050D

22. Sule, H. (2016). The Prevalence of Klebsiella Species Causing Urinary Tract Infections in Murtala Muhammad Specialist Hospital, Kano, Nigeria. *American Journal of Biomedical and Life Sciences,4*(2), 11. doi:10.11648/j.ajbls.20160402.11

23. Bacterial Plasmids. (n.d.). Retrieved from https://www.csun.edu/~hcbio027/biotechnology/lec2/PL/pl.htm

24. What is a Plasmid? (n.d.). Retrieved from https://www.addgene.org/mol-bio-reference/plasmid-background/

25. Objectives. (n.d.). Retrieved from http://vlab.amrita.edu/?sub=3&brch=186&sim=1097&cnt=1

26. Chen, H. (2011). Urinary Tract Infection in Children. *Clinical Management of Complicated Urinary Tract Infection*. doi:10.5772/24595

Appendix

7. Appendix

Appendix –I

Media and compositions

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

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

Mueller Hinton Agar

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

Luria Bertani Agar Miller

Component	Amount (gms/litre)
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
Agar	15.000
Final pH (at 25°C)	7.5±0.2

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.