Determination of transfer of Azithromycin resistance gene though Conjugation, among Enterobacteriaceae, isolated from Urinary Tract Infected patients

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Declaration

I hereby declare this project titled **"Determination of transfer of Azithromycin resistance gene through Conjugation, among Enterobacteriaceae, isolated from Urinary Tract Infected patients."** has been written and submitted by me, Sharika Ferdous and has been carried out under the supervision of Nazneen Jahan, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

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

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Dedicated to my role models— my parents…

and my best friend

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Abstract

Urinary Tract Infections (UTI) are a predominant bacterial infection caused by pathogens that are resistant to a long list of antibiotics and the greater challenge nowadays is to stop the spread of resistance to them. This study aimed to determine whether resistance to the macrolide—Azithromycin was plasmid mediated and could be transferred via conjugation to the neighboring UTI Enterobacteriaceae. In this study, a total of 28 identified isolates obtained from UTI patients of a Diagnostic Center in Bangladesh were investigated, that contained 22 (78%) organisms from the Enterobacteriaceae family and 6 (21%) organisms from the gram-positive genus. Minimum Inhibitory Concentration (MIC) carried out by the agar dilution method revealed that the Enterobacteriaceae had MIC values of ≥200µg/ml against Azithromycin in 77.3% (17 out of 22) of the organisms and ≥50µg/ml against Ciprofloxacin in 63.3% (14 out of 22) isolates. Plasmid profile showed that 40.9% (9 out of 22) harbored one or more plasmids of sizes ranging from ~2 MDa to ~85 MDa. Based on the antibiotic sensitivity patterns and plasmid profiling, donor and recipient strains were selected for the conjugation experiments carried out in three different methods. In this study, *Enterobater spp* were selected as donor strains that had high resistance to Azithromycin (MIC: ≥200µg/ml) and contained 2 plasmids (~85 MDa, ~35.6 MDa). On the other hand, plasmid-less, Azithromycin sensitive (MIC: 10µg/ml) *Klebsiella spp* was selected as a recipient strain for conjugation. Ciprofloxacin was used as a marker to check for the presence of transconjugants. The results revealed that, no transconjugant was observed from the three conjugation assays. Donor strains were then subjected to a plasmid curing procedure with different concentrations of Ethidium Bromide (EtBr) ranging from 1-100µg/ml and 2-20% Sodium Dodecyl Sulfate (SDS). Among the two *Enterobacter* donor strains used in the study, EtBr successfully cured both plasmids in one strain, whereas the other donor still retained one plasmid after treatment with both reagents. Evaluation of MIC of the cured donors showed the same resistance pattern to Azithromycin (MIC: ≥200µg/ml) proving that the gene responsible for this resistance was not plasmid mediated and hence could not be transferred by conjugation to the recipient *Klebsiella*. The results of this study indicate that resistance to Azithromycin may not be transferred from Enterobacter species, although this study was only conducted with in-vitro techniques.

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List of Abbreviations

UTI	Urinary Tract Infections
AMR	Antimicrobial Resistance
MDR	Multi Drug Resistance
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
LB	Luria Bertani
MHA	Mueller Hinton Agar
EtBr	Ethidium Bromide
SDS	Sodium Dodecyl Sulfate
WHO	World Health Organization
CLSI	Clinical and Laboratory Standards Institute
ESBL	Extended-Spectrum Beta-Lactamases
MDa	Mega Daltons
AZM	Azithromycin
CIP	Ciprofloxacin
IV	Intravenous infusion
spp	Species
mg	Milligram
ml	Milliliter
µl	Microliter
µg	Microgram
cfu	Colony Forming Units

Chapter 1

Introduction

1.1 Introduction

The ever increasing concern of microorganisms acquiring resistance to the long list of antibiotics, is a burden that humanity has borne throughout the ages. To make matters worse, the resistance characteristics have been passed on, not only through generations, but also to neighboring microorganisms in a variety of ways, thus spreading among different populations and making it harder to treat them all. The widespread use of antibiotics has been associated with what we now know to be the predictable emergence of resistance (Rice, 2008). In many developing countries, the use of antibiotics tends to be unrestricted, thus exposing the infectious pathogenic populations to the antibiotics repeatedly. According to Davies, Adams & Newell (2018) weak regulation in pharmaceutical companies, leading to inappropriate consumption of antibiotics has resulted in soaring drug resistance rates and resistant bugs are being spread around the world. The repeated exposure causes them to acquire resistance against the antibiotics, either through genetic mutations in a certain population itself, or by the spread to other populations, distant or closely related, by means of gene transfer.

The use of antibiotics is most commonly the preferred choice of treatment for the highly pressing disease—Urinary Tract Infections. Urinary Tract Infections are caused by bacteria, infecting one or more parts of the urinary system such as the kidney, bladder, ureters or the urethra. Nearly 95% of cases of UTIs are caused by bacteria that typically multiply at the opening of the urethra and travel up to the bladder (Puca, 2014). As a result, the otherwise sterile urine is infected with invading organisms that are foreign to the body, as opposed to the normal flora, leading to turbidity in urine, pain and irritation during urination, abdominal pain and several other UTI related symptoms.

Urinary Tract Infections have been dubbed as the most common bacterial infections to occur in humans, by Nicolle (2002). It is estimated that 150 million cases of UTI occur on a global basis per year resulting in more than 4 billion pounds (6 billion dollars) in direct health care expenditure (Harding & Ronald, 1994). In countries such as Bangladesh, UTI accounts for a high rate of hospital visits every year relating to the fact that the majority lack awareness and lead a poor standard of life. Moreover, the abuse of antibiotics has led to the emergence of numerous resistant strains, and the spread of resistance ever more.

The normal flora of the urinary tract exists to keep the system free from the invasion of pathogens. However, when compromised in the situation of an infection, the resident bacteria fail to protect the parts of the urinary system. Among the most common invaders in UTI, *E. coli* is most often encountered. A study conducted by Noor et al (2013) revealed that among 462 urine samples collected from patients with UTI, *E. coli* was the predominant organism, whereas *Klebsiella* and *Enterococcus* were also prevalent. Other gram-negative microorganisms causing UTI include *Proteus*, *Shigella, Citrobacter*, *Enterobacter*, and *Pseudomonas* spp. Gram-positive pathogens, such as *Staphylococcus saprophyticus*, *Bacillus* and group B streptococci, can also infect these parts (Kattan & Gordon, 2013).

Several studies have demonstrated that, these pathogens can horizontally transfer genes encoded in plasmids, via direct cell-to-cell contact amongst them. This is a major contributor to the current public health crisis arising from the increasing occurrence of antibiotic-resistant pathogenic bacteria (Levy & Marshall, 2004). Some of them have now been termed as multi-drug resistant, due to their ability to evade the destructive mechanisms of several lines of antibiotics. The European Center for Disease Prevention and Control (ECDC) states that there have been accounts of emergence of *Klebsiella pneumonia* that are resistant to carbapenems and polymixins, which happen to be the last line of antibiotics.

Aside from laws being set against un-prescribed consumption of antimicrobials, and other awareness programs, many researchers are trying to figure out the root cause and the mechanisms of how these microorganisms are acquiring resistance. Such knowledge may help bring into light new and better procedures for treatment, ones that may sidestep the threat of the birth of resistant organisms. Since antimicrobial resistance tend to be a gene encoded characteristic, the study into such genetic mechanisms may provide the answers.

1.2 Antibiotic Resistance in *Enterobacteriaceae*

Enterobacteriaceae are the most common list of pathogens to cause Urinary Tract Infections. The emerging resistance among *Enterobacteriaceae* due to extended-spectrum beta-lactamases (ESBL) has been reported worldwide (Qi, Pilla, Yu, & Reed, 2010). This is mainly conferred by the enzyme that inactivates the antibiotic by hydrolysis. Fluoroquinolones (ciprofloxacin, norfloxacin and levofloxacin), beta-lactam (penicillin, cephalosporins and carbapenems) and aminoglycosides had once been potent against pathogenic *Enterobacteriaceae,* but resistance to these have already emerged. *Klebsiella pneumonia* strains that carry ESBLs have become predominant and are highly resistant to these antibiotics, as well as sulfonamides (Fontana, et al. 2010). A study conducted by Hayder *et al* (2012) found 647 *K. pneumoniae* isolates in 2800 patients with UTIs, bacteremia, wound infections, and respiratory diseases. Thirty-one carbapenem-resistant isolates were found to harbor *K. pneumonia* carbapenemase. An additional 287 isolates were ESBL positive. Quinolone resistance ranging from around 11%-50% of *Escherichia*, *Proteus*, *Klebsiella* and *Enterobacter* species isolated from UTI patients were seen in a study conducted by Lyonga, *et al*. (2015).

1.3 Dissemination of Resistance

Acquisition of genes needed to evade the various mechanisms of antibiotic resistance is greatly aided by a variety of promiscuous gene transfer systems (Bennett, 2008). Genetic material conferring resistance to antibiotics can be exchanged between neighboring bacteria by means of horizontal gene transfer. This allows the organisms to acquire resistance to a variety of antibiotics, the genes for resistance to which lies in the vector of transfer. Table 1.1 lists the different mobile genetic elements.

Element	Characteristics	Resistance
Plasmid	Circular molecules, 1-1000 kb, conjugative, mobilizable, often with embedded mobile elements.	Multiple resistant genes.
Insertion sequence(IS)	Small molecules with terminal repeats, contains transposase gene.	No resistance gene.
Transposons Composite Complex Conjugative	Flanked by IS and or long inverted repeats. Large, flanked by short terminal inverted repeats, specifies transposase and recombinase. Able to promote self-transfer.	Examples: Tn5: Kan, Bleo, Str. Tn1 and Tn3: Bla Tn7: Tmp, Str, Spc. Tn1546: glycopeptides. Tn916: Tet, Mino Tn1545: Tet, Mino, Kan, Ery.
Integrons	Can capture and disseminate resistance gene cassettes, carry integrase gene, attachment site and promoter for full expression of multiple genes.	Four classes are recognized based on resistance determinants.

Table 1.1: List of different mobile genetic element (Modified from Alekshun & Levy, 2007)

*Kan-kanamycin, *Bleo-bleomycin, *Str-streptomycin, *Bla-beta lactam, *Tmp-Trimethoprim, *Spc-Spectinomycin, *Tet-tetracycline, *Mino-minomycin, *Ery-erythromycin

1.4 Plasmid Mediated Resistance

Plasmids are the small double stranded molecules of circular DNA, that are extrachromosomal and self-replicating. Genes for antimicrobial resistance (AMR) in bacteria is commonly known to be harbored in plasmids, giving the organism a genetic advantage. There are currently 28 known plasmid types in *Enterobacteriaceae* distinguished by PCR-based replicon typing (PBRT) (Rozwandowicz, *et al*., 2018). Their study concluded that, ESBLs are the most frequently described enzymes, conferring resistance to antimicrobials encoded on plasmids. Enzymes hydrolyzing aminoglycosides and genes encoding for resistance to quinolones and sulfonamides are often co-transferred through transposons located on a plasmid.

Multiple antibiotic resistance genes have also been found to be harbored in plasmids, which have been termed as MDR-plasmids (Multi-Drug Resistance). These plasmids mediate the spread of MDR and limit options for treatment with antibiotics. For example, MDR plasmids may contain multiple β-lactamase genes that encode for different substrate specificities, providing a complementary spectrum of activity that may result in resistance to all β-lactam antimicrobial drugs (Schultsz & Geerlings, 2012). Conjugative plasmids can mediate the lateral transfer of antibiotic resistance or virulence determinants between bacteria, allowing bacteria to adapt to otherwise hostile environments (Woodall C.A., 2003), and their ability to replicate in a wide range of hosts, make them perfect vectors for the spread of AMR.

1.5 Conjugative Plasmids

Horizontal gene transfer between two strains of bacteria are mediated by a certain class of plasmids, termed as conjugative plasmids. They are known to carry the 'fertility' factor, or F factor, on the F episome. These plasmids contain a tra-operon that comprises of a set of transfer genes, that assist in transfer of plasmid from a bacterium containing the F factor plasmid (F+ strain) to a bacterium lacking the conjugative F plasmid (F- strain), by the process of conjugation. This mating between the donor (F+) and recipient (F-) strain results in two F+ strains, both of which can then act as a donor. The typical functional components of a conjugative plasmid include an oriT (origin of transfer) which allows for the recognition and initiation of transfer of the plasmid, an oriC (origin of replication) for replication in the recipient cell, and the tra-region which contains the pilin gene and regulatory genes. The pilin gene is what codes for the formation of the 'sex pilus' on the cell surface of the F+ strain which is the passage through which the plasmids are transferred from the donor to the recipient. The tra-operon also codes for other proteins that are required for assembly, attachment to the surface of the F- strain, and also surface exclusion proteins. This sexual function of bacteria is controlled on the F plasmid with a fertility inhibition (Fin) system, and they can range from 30-100kbp in length. Conjugative plasmids can have a broad or narrow host range. Transfer can be restricted to small number of similar species, or can be between widely different species, such as from gram-negative bacteria to gram-positive bacteria (Bennett, 2009). In addition to transfer properties, MDR-F plasmids may contribute to the spread of resistance to many different antibiotics between species when the genes are horizontally transferred.

1.6 Bacterial Conjugation

Conjugation is believed to be the most important transfer mechanism in the acquisition and spread of antibiotic resistance (Barbosa & Levy, 2000), amongst pathogenic bacteria, related or otherwise. This mode of gene transfer contributes to evolution and the acquisition of new traits (Babic, Berkmen, Lee, & Grossman, 2011). It is initiated with the expression of the transfer gene, to extend the pilus and promote cell-to-cell contact. Once the sex pilus has formed between the two bacteria, the F plasmid is nicked at the oriT, and transferred in the 5' – 3' direction as a single strand, the complementary of which is synthesized in the recipient due to the presence of oriC. This results in a population of the recipient strain termed as transconjugants, which exhibit phenotypic characteristics of the donor (Woodall C.A., 2003), most commonly observed to be resistance to antimicrobials.

For the determination of AMR spread among UTI pathogens, conjugation has been extensively studied. Laboratory techniques involve allowing donor and recipient strains to form direct contact and examining transconjugants based on susceptibility to antibiotics as a selectable marker on the plasmid of interest. The *in vitro* results could indicate, if conjugation is playing a part in causing the UTI pathogens to acquire resistance against the drugs of choice for treatment.

1.7 Literature Review

Current research reveals that many pathogenic bacterial species —*Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* etc., and species of *Enterobacter*, *Salmonella*, and *Shigella* — are now resistant to most antibiotics (WHO, 2014 Livermore, 2003).

A study conducted by Suhani *et al*., (2017), investigated the transfer of resistance among bacteria via horizontal transfer of genetic materials. *E. coli* isolates obtained from patients with UTI were seen to transfer its genes of resistance against the antibiotics Amoxicillin (AMX) and Ciprofloxacin (CIP) to the recipient *E. coli* DH5α by conjugation. Plasmids of three different sizes were shown to have been transferred to the recipient after the experiments, and the *E. coli* DH5α was able to survive in the presence of AMX and CIP.

Jost *et al*., (2016) analyzed the susceptibility and mechanism of transfer of the macrolide—Azithromycin of enterohaemorrhagic *E. coli* isolates from France. From a total of 508 isolates, Azithromycin MICs ranging between 0.25mg/L and 16 mg/L was observed. Two different genes for resistance to the macrolide was found and the 2 transconjugants found from different conjugal assays showed identical resistance patterns to the donor. Only one of the genes for Azithromycin resistance harbored on one plasmid, was transferred to the transconjugant and incomplete plasmid transfer was detected probably due to high molecular weight of the plasmid.

Vaidya, (2011) demonstrated rampant spread of new antibiotic-resistant genes carried by extended spectrum β–lactamase (ESBL)-producing Enterobacteriaceae via horizontal transfer. Susceptibility patterns of a wide range of antibiotics was analyzed for 120 ESBL screen-positive clinical isolates *Escherichia coli* and *Klebsiella pneumonia.* Intra-genus conjugation was observed between the clinical ESBL-producing *E. coli* isolates used as donor with auxotrophic strain of *Escherichia coli* (*E. coli* AB 1157) used as recipient. Resistance was transferred at a frequency of $3–4 \times 10^{-5}$. Inter-genus conjugation was also observed between the mating pairs consisting of ESBL-producing *E. coli* and *K pneumoniae* isolates displaying differing antibiotic-resistance patterns with respect to the antibiotics chosen. The results revealed a two-way transfer of antibiotic resistance markers under laboratory (LB broth) as well as under simulated environmental conditions.

Determination of minimum inhibitory concentration (MIC) by broth and agar dilution methods provided quantitative results (Jorgensen & Ferraro, 2009; Wiegand et al., 2008; CLSI, 2014). These studies involved preparing a two-fold dilution of antibiotics in solid (Mueller Hinton Agar) media and inoculating a standardized bacterial suspension of $1\text{-}5\times10^5$ colony forming units (CFU)/ml. Visible growth evidenced by colonies on the agar surface was observed after a defined period and MIC was interpreted qualitatively using an MIC interpretive criteria/breakpoint that categorizes the infecting organism as susceptible, intermediate, or resistant. Erukunuakpor, (2016) evaluated the agar dilution method for identification of ESBL-producing *Klebsiella pneumoniae* in the environment. MIC against ceftriaxone antibiotic revealed *K.pneumoniae* strains were above 4µg/ml, identifying these strains as resistant to ceftriaxone, an indication of ESBL production.

Zaman, Pasha, & Akhter, (2011) investigated the plasmid eliminating abilities of Acridine Orange, Ethidium Bromide and Sodium Dodecyl Sulfate on multi drug resistant *Escherichia coli* from UTI specimens. The frequencies of cured cells were 5.55% (with 50µg/ml) and 11.76% (with 75µg/ml) for Acridine Orange, 21.05% (with 100µg/ ml) and 17.65% (with 125µg/ml) for EtBr and 7.4% (with 10% w/v) & 6.67% (with 10% w/v) for SDS. However, they found no cured cells from 100µg/ml Acridine Orange, 75µg/ml EtBr and 8 and 12% SDS. Analysis of profiles of wild type and plasmid cured strains by electrophoresis yielded bands of varying sizes for wild type cells, but none were obtained for EtBr cured cells.

1.8 Aims and Objectives

The purpose of this study was to determine the horizontal transfer of mobile genetic elements, by the process of conjugation between bacteria that was collected and isolated from UTI patients of a Diagnostic Center in Bangladesh. The objectives of the study have been given below:

- Determining Minimum Inhibitory Concentration (MIC) of isolates from UTI patients
- Studying in-vitro techniques to demonstrate conjugational transfer of Azithromycin resistant gene among Enterobacteriaceae
- Determining plasmid mediated resistance in the donors of conjugation, by plasmid curing

Chapter 2

Materials & 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 Microbiology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

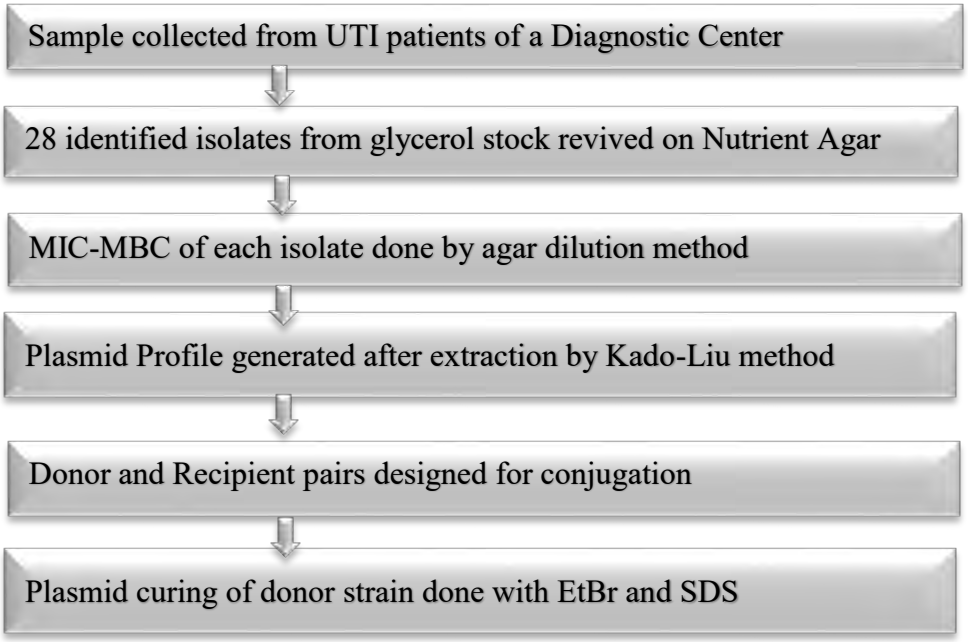
2.2 Study duration

The study was conducted during the period of July, 2017 - April, 2018.

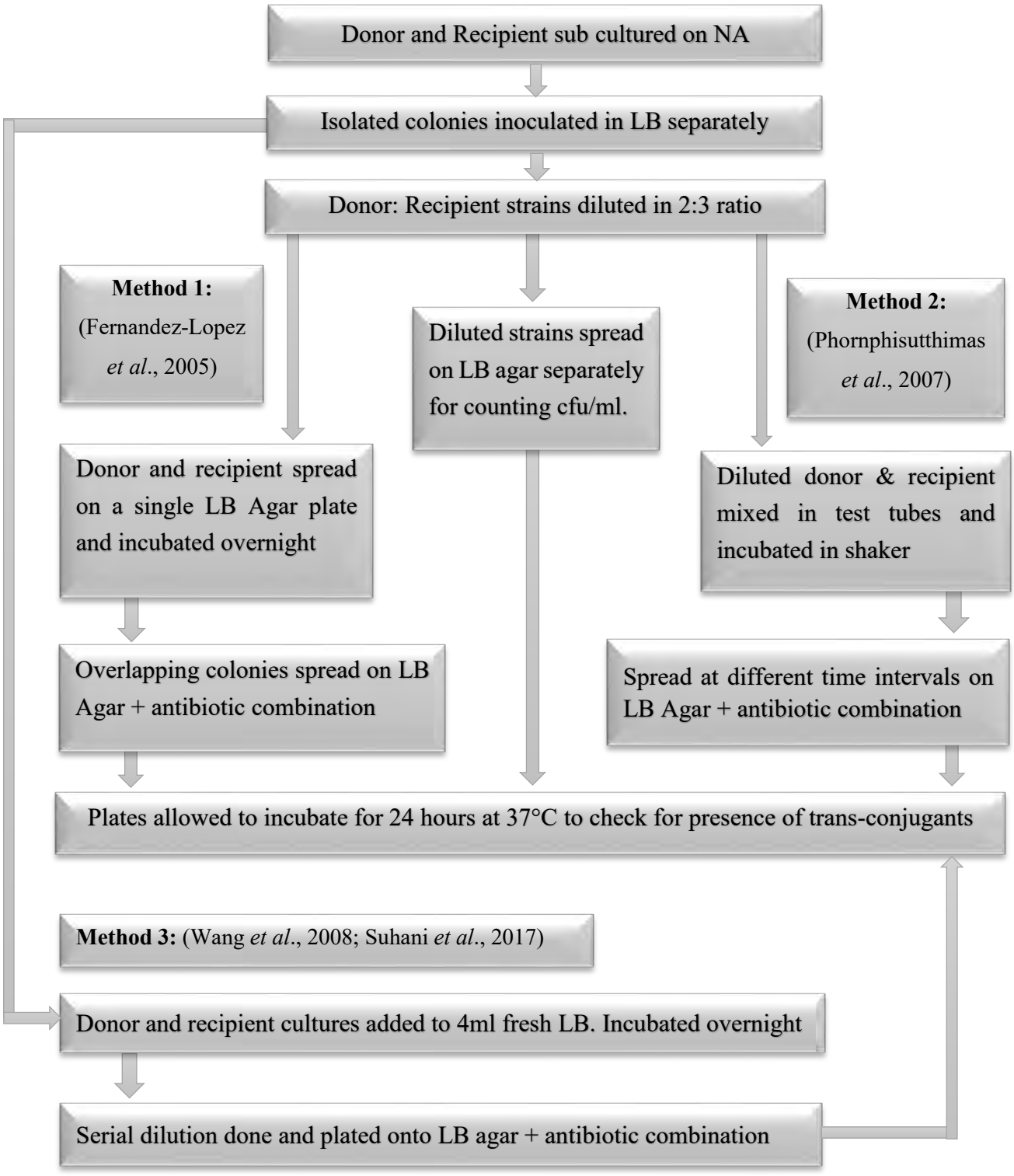
2.3 Sample size

A total of 90 isolates were collected and identified from Urinary Tract Infected patients from a Diagnostic Center in Dhaka. In this study, 28 identified isolates were picked for performing plasmid curing and conjugation experiment.

2.4 Experimental Design



2.5 Conjugation Methods



2.6 Collection & Preparation of Antibiotics

Antibiotics were used in this study for the determination of Minimum Inhibitory Concentration (MIC) and for the conjugation experiments carried out on the organisms isolated from UTI patients. Intravenous injection (IV) of Ampicillin, Azithromycin and Ciprofloxacin were brought from a pharmacy near BRAC University. The list of these antibiotics is given below.

Generic Name	Ampicillin	Azithromycin	Ciprofloxacin
Trade Name	Ampexin 500	Rozith 500	Neofloxacin
Class	Penicillin	Macrolides	Fluoroquinolones
Stock Concentration	5mg/ml	2mg/ml	2mg/ml

Table 2.1: List of antibiotics used for the study

2.7 Methodology

2.7.1 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) is considered the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism. It is widely used for comparative testing and when disk tests are unreliable. For the purpose of this study, the agar dilution method was performed to determine the MIC values. The procedure was derived from the Journal of Antimicrobial Chemotherapy for the Determination of minimum inhibitory concentrations by Andrews, (2001). Mueller Hinton Agar was used as the reference medium since it meets the requirements for the USA National Committee for Clinical Laboratory Standards (NCCLS).

- The $C_1V_1 = C_2V_2$ formula was used to prepare a range of concentrations from the stock of IV solutions of Ampicillin, Azithromycin and Ciprofloxacin.
- The different concentrations of antibiotics were added to separate petri dishes and 20ml of sterile MHA was added and swirled to mix, then allowed to solidify.
- 10µl of pathogenic suspension was placed as a drop onto the plates containing different antibiotic concentrations and allowed to incubate at 37°C, overnight.
- The highest concentration at which lowest growth was seen on the drop was considered to be the MIC value and the lowest concentration at which there was no growth on the drop was considered the Minimum Bactericidal Concentration (MBC) value.

2.7.2 Plasmid Extraction

Plasmid extraction was done for all the isolates, to determine the presence or absence of plasmids by following Kado-Liu method (Kado & Liu, 1981). The following steps were carried out:

- Single colonies were incubated overnight in nutrient broth.
- Cultures were centrifuged in an Eppendorf to obtain the pellet.
- Supernatant was discarded and 60µl of Kado-I and 120µl of Kado-II buffers were added.
- The tubes were then put in 55°C water bath for an hour
- A 1:1 ratio of 400µl Phenol: chloroform was added and mixed for 30mins, to separate the protein layers from the nucleic acids
- The tubes were centrifuged again and the top layer containing the plasmids were carefully pipetted onto separate containers that were stored at -20°C for later use.

2.7.3 Agarose Gel Electrophoresis

The solution containing plasmids, that were extracted by the Kado-Liu method, were subject to Agarose Gel Electrophoresis, and viewed under ultra-violet light to check for the presence of plasmids in the isolates.

- 0.7% agarose gel was prepared with TBE buffer solution
- The gel was stained with 0.5% ethidium bromide.
- 10µl of the extracted sample was mixed with 2µl of the tracking dye—bromophenol blue, using a micropipette.
- These were then loaded onto the wells of the agarose gel and run for approximately 2 hours with the power source at 70V.
- The gel was then removed and placed under UV trans-illuminator for visualization of plasmids.

2.7.4 Conjugation

Information from MIC values and plasmid availability was used to choose a donor and recipient pair for conjugation which was carried out in three different techniques.

Conjugation Method 1: (Fernandez-Lopez *et al*., 2005)

- Single colonies allowed to grow on nutrient broth overnight
- Donor: Recipient pairs serially diluted in a 2:3 ratio
- 100µl of diluted strains spread on LB Agar for counting the colony forming units- cfu/ml
- 200µl mixture of diluted donor and recipient spread on a single LB Agar plate and kept at 37°C for 24 hours
- Overlapping colonies inoculated in saline and 100µl of the solution spread on LB Agar containing a combination of antibiotics that hinder both strains individually
- Plates allowed to incubate for 24 hours at 37°C to check for presence of transconjugants

Conjugation Method 2: (Phornphisutthimas, Thamchaipenet, & Panijpan, 2007)

- Single colonies allowed to grow on nutrient broth overnight
- Donor: Recipient pairs serially diluted in a 2:3 ratio
- 100µl of diluted strains spread on LB Agar for counting the colony forming units- cfu/ml
- 2ml each of diluted donor and recipient were mixed in test tubes and allowed to conjugate in shaking incubator at 37°C
- At time intervals— 20min, 30min and 60mins each, mixture was transferred to another tube and vortexed. 100µl of this was spread on LB Agar containing a combination of antibiotics that hinder both strains individually
- Plates allowed to incubate for 24 hours at 37°C to check for presence of transconjugants

Conjugation Method 3: (Wang *et al*., 2008; Suhani *et al*., 2017).

- Single colonies of donor and recipient strains were inoculated onto 5ml LB broth separately and incubated overnight in shaker at 37°C
- 500µl of donor and recipient cultures was added to 4 ml fresh LB and incubated overnight at 37°C without shaking
- A 10-fold serial dilution was then done with LB and 100µl from each dilution was plated onto LB agar containing selected combination of antibiotics and incubated overnight at 37°C. Colonies observed were assumed to be transconjugants.

2.7.5 Plasmid Curing

Plasmid curing was done to check if the donor strains carried the resistance genes in their plasmids, and to see if they dissipate their plasmids under adverse conditions. The strains were treated with a range of concentrations of Ethidium Bromide and Sodium Dodecyl Sulfate. The procedure that was designed for the experiment was taken from Trevors, (1986).

- 2McFarland solution of donor strain was prepared and 1ml of this was added to 9ml of NB.
- Different concentrations ranging from 1-100µg/ml EtBr were added to one set and 2-20% of SDS to another set.
- The test tubes were incubated overnight at 37°C.
- The highest concentration that showed least growth was plated onto nutrient agar.
- MIC-MBC values and plasmid extraction was done to check if the donor strains had lost their plasmids.

Chapter 3

Results

3.1 Determination of Minimum Inhibitory Concentration (MIC)

Agar dilution method (Andrews, 2001) was employed to get MIC values for a reliable result of antibiotic susceptibility of the organisms against different concentrations of Ampicillin, Azithromycin and Ciprofloxacin. Figure 3.1 show the results obtained against Ciprofloxacin and Table 3.1 depicts the MIC-MBC values against all three tested antibiotics.

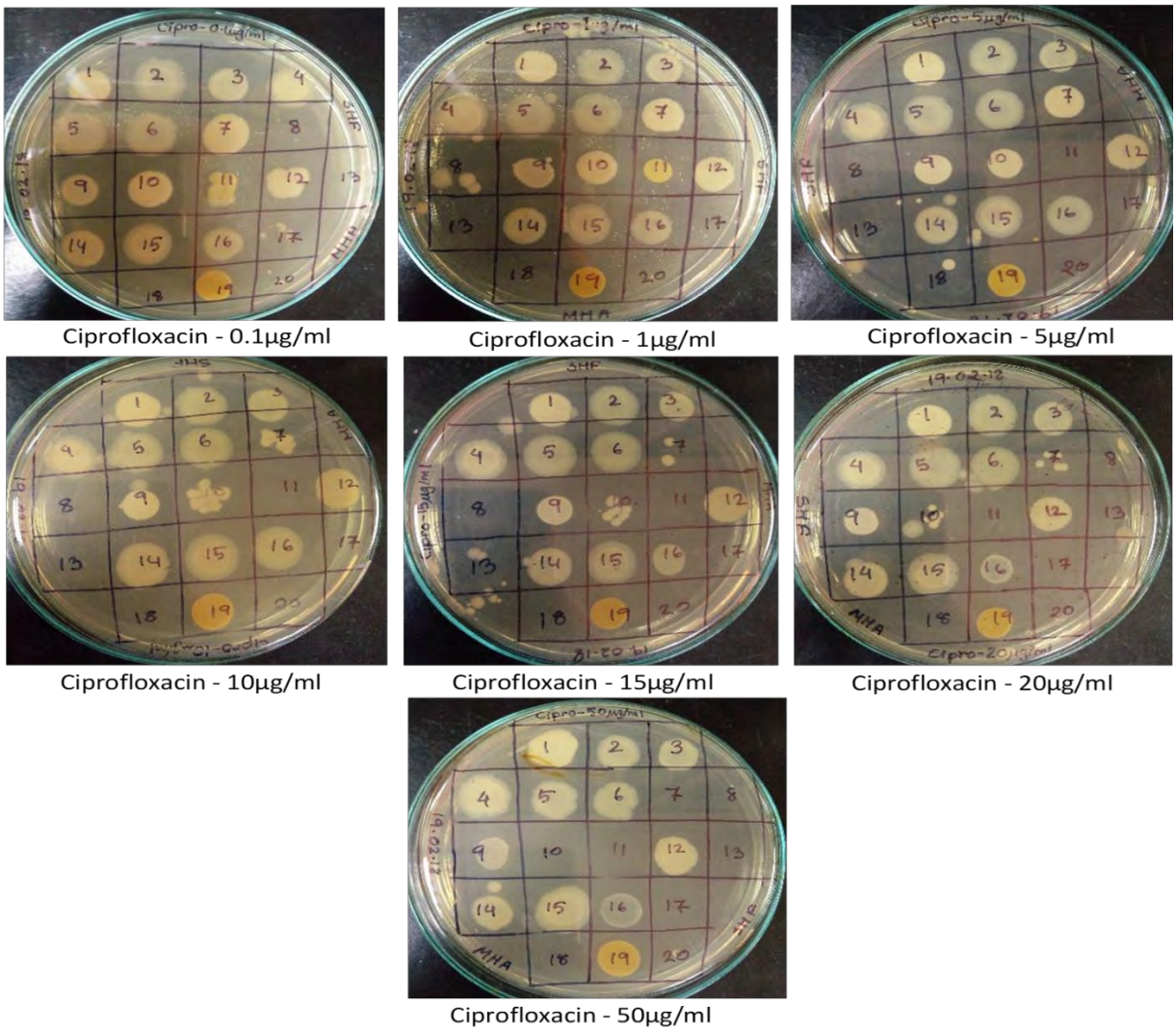


Fig 3.1: Minimum Inhibitory Concentration (MIC) determination of organisms isolated from UTI patients, by using agar dilution method

ID	Suspected Species	MIC-MBC value					
		Ampicillin		Azithromycin		Ciprofloxacin	
		MIC	MBC	MIC	MBC	MIC	MBC
a	*Enterobacter*	150µg/ml	200µg/ml	≥200µg/ml	>200µg/ml	<0.1µg/ml	≤0.1µg/ml
c	*Klebsiella*	≥200µg/ml	>200µg/ml	10µg/ml	30µg/ml	<0.1µg/ml	≤0.1µg/ml
d	*Enterobacter*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
e	*Enterobacter*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	5µg/ml	10µg/ml
g	*Klebsiella*	150µg/ml	200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
h	*Enterobacter*	100µg/ml	150µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
i	*Enterobacter*	150µg/ml	200µg/ml	≥200µg/ml	>200µg/ml	10µg/ml	15µg/ml
q	*Bacillus*	150µg/ml	200µg/ml	≥200µg/ml	>200µg/ml	<0.1µg/ml	≤0.1µg/ml
3a	*Bacillus*	≥200µg/ml	>200µg/ml	1µg/ml	10µg/ml	0.1µg/ml	1µg/ml
3b	*Shigella*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
I4	*Enterobacter*	1µg/ml	10µg/ml	<1µg/ml	≤1µg/ml	1µg/ml	5µg/ml
I5	*Enterobacter*	1µg/ml	10µg/ml	<1µg/ml	≤1µg/ml	<0.1µg/ml	≤0.1µg/ml
I6	*Bacillus*	≥200µg/ml	>200µg/ml	1µg/ml	10µg/ml	0.1µg/ml	1µg/ml
I17	*Klebsiella*	≥200µg/ml	>200µg/ml	10µg/ml	30µg/ml	20µg/ml	50µg/ml
I18	*Klebsiella*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
I36	*Micrococcus*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	1µg/ml	5µg/ml
I40	*Bacillus*	≥200µg/ml	>200µg/ml	1µg/ml	10µg/ml	<0.1µg/ml	≤0.1µg/ml
U5	*E. coli*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U8	*E. coli*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U11	*Staphylococcus*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U14	*Klebsiella*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U33	*Klebsiella*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U34	*Klebsiella*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U35	*Klebsiella*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U42	*Klebsiella*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U43	*Klebsiella*	1µg/ml	10µg/ml	<1µg/ml	≤1µg/ml	20µg/ml	50µg/ml
U47	*Shigella*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml
U50	*Enterobacter*	≥200µg/ml	>200µg/ml	≥200µg/ml	>200µg/ml	≥50µg/ml	>50µg/ml

Table 3.1: The MIC and MBC values for Ampicillin, Azithromycin and Ciprofloxacin for the 28 organisms isolated from UTI patients.

3.2 Plasmid Profile

Plasmid extraction (Kado & Liu, 1981) revealed that among the 28 isolates taken for this study, 9 of them contained one or more plasmids and the rest did not show any plasmids. Each DNA band in the gel represents one plasmid. The size of the bands was determined by comparing with the size of the plasmids of the reference strain, ***E. coli* V-517** that was used for this study. Isolates— 'a', 'e', 'd', 'U8', 'U50' and 'U5' were observed to have plasmids of ~85 MDa in size. Among them, 'a', 'e', 'U8', 'U50' and 'U42' had another plasmid band of approximately ~35.6 MDa in size. These were the two largest plasmids found among all the isolates. Isolates— 'd' and 'g' were seen to have plasmids at around ~2 MDa. Isolates— 'g' and 'U50' also showed a plasmid band at around ~4.8 MDa. Table 3.2 lists the number of plasmids found in the 9 isolates including the sizes and figure 3.2 illustrates the plasmid profile generated after extraction by the Kado-Liu method and agarose gel electrophoresis.

Isolate ID	Number of Plasmids	Approximate sizes of plasmids (MDa)
a	2	85, 35.6
d	2	85, 2
e	2	85, 35.6
g	3	4.8, 3.7, 2
U8	2	85, 35.6
U50	3	85, 35.6, 4.8
U5	2	85, 35.6
U42	1	35.6
U35	1	3.9

Table 3.2: List of the number of plasmids and their approximate sizes found in 9 isolates

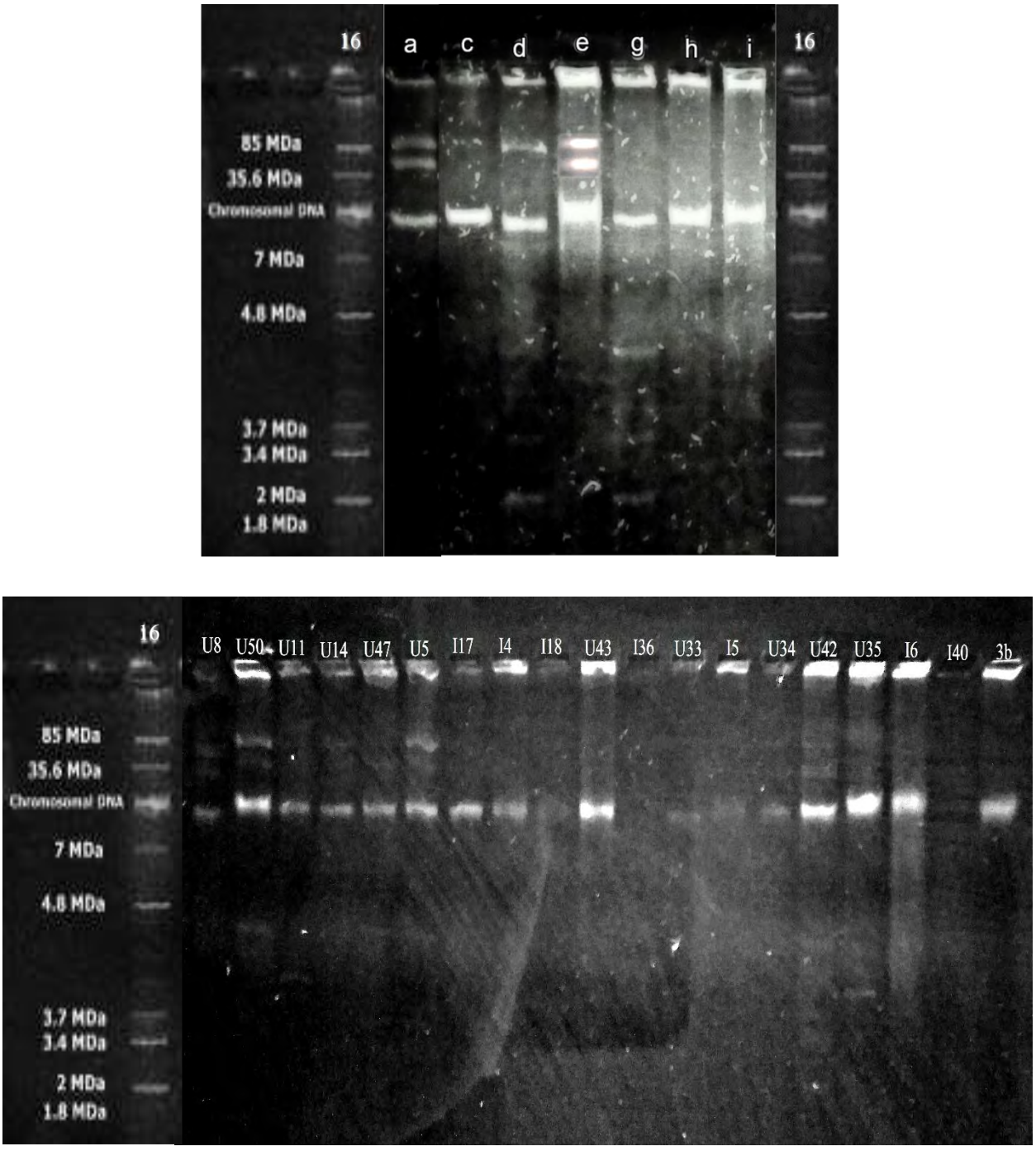


Fig 3.2: Plasmid profile generated after agarose gel electrophoresis of the 28 isolates collected from UTI patients. The lanes labelled "16" represent the plasmid bands of the reference strain—*E. coli* V-517 which was used for comparison of the plasmid band sizes

3.3 Conjugation

From the results obtained above from the MIC values and plasmid profile, two different donor strains and one recipient strain was chosen to use in the conjugation experiments to see if horizontal gene transfer occurs. Table 3.3 shows the details of the donor and recipient pairs chosen according to antibiotic susceptibility and plasmid profiling.

Conjugation methods were carried out to check if horizontal transfer of Azithromycin resistance gene, occurred from *Enterobacter spp* to *Klebsiella spp*. The donor strain was seen to have resistance to high concentrations of Azithromycin (MIC: ≥200µg/ml), whereas the recipient was sensitive at low concentrations (MIC: 10µg/ml). On the other hand, the donor was sensitive at low concentrations of Ciprofloxacin (MIC: <0.1µg/ml, 5µg/ml), whereas the recipient was highly resistant (MIC: 20µg/ml). Hence, Ciprofloxacin was used as a marker, to check for presence of transconjugants. The donor also contained 2 plasmids whereas the recipient was plasmid-less.

	Type	ID	Organism	Transfer Antibiotic	MIC Value	I	Marker Antibiotic	MIC Value	I	No. of plasmid
Pair 1	**Donor**	a	*Enterobacter spp.*	AZM (100µg/ml)	≥200µg/ml	**R**	CIP (10µg/ml)	<0.1µg/ml	**S**	2
	Recipient	I17	*Klebsiella spp.*		10µg/ml	**S**		20µg/ml	**R**	0
Pair 2	**Donor**	e	*Enterobacter spp.*	AZM (100µg/ml)	≥200µg/ml	**R**	CIP (10µg/ml)	5µg/ml	**S**	2
	Recipient	I17	*Klebsiella spp.*		10µg/ml	**S**		20µg/ml	**R**	0

Table 3.3: Donor and recipient pairs chosen for conjugation experiment

*AZM= Azithromycin, CIP= Ciprofloxacin

*I= Interpretation, R= Resistant, S= Sensitive

3.3.1 Method 1 (Fernandez-Lopez *et al.*, 2005)

To check if conjugation occurred, the diluted donor and recipient cells were grown together on LB agar plate. After incubation at 37°C overnight, the colonies of donor and recipient were seen to overlap, which was mixed in saline and spread on another plate containing LB agar with 100µg/ml Azithromycin and 10µg/ml Ciprofloxacin. After incubation overnight at 37°C, no bacterial growth was seen on the plate, indicating that conjugation did not occur, as shown in figure 3.3.

Fig 3.3: Results of each step in conjugation method 1. The plate containing LB agar with Azithromycin & Ciprofloxacin shows no bacterial (transconjugant) growth after incubation.

3.3.2 Method 2 (Phornphisutthimas *et al.*, 2007)

In the 2nd method to simulate conjugation, 2ml each of diluted donor and recipient cultures were mixed in a tube and allowed to conjugate. Tubes were incubated in shaker at 37°C for 60mins. At 20mins, 30mins and 60mins, 100µl of the mixture was vortexed to break any conjugation pilus and spread on plates containing LB agar with 100µg/ml Azithromycin and 10µg/ml Ciprofloxacin. After incubation overnight at 37°C, no bacterial growth was seen on the plate, indicating that conjugation did not occur, as shown in figure 3.4.

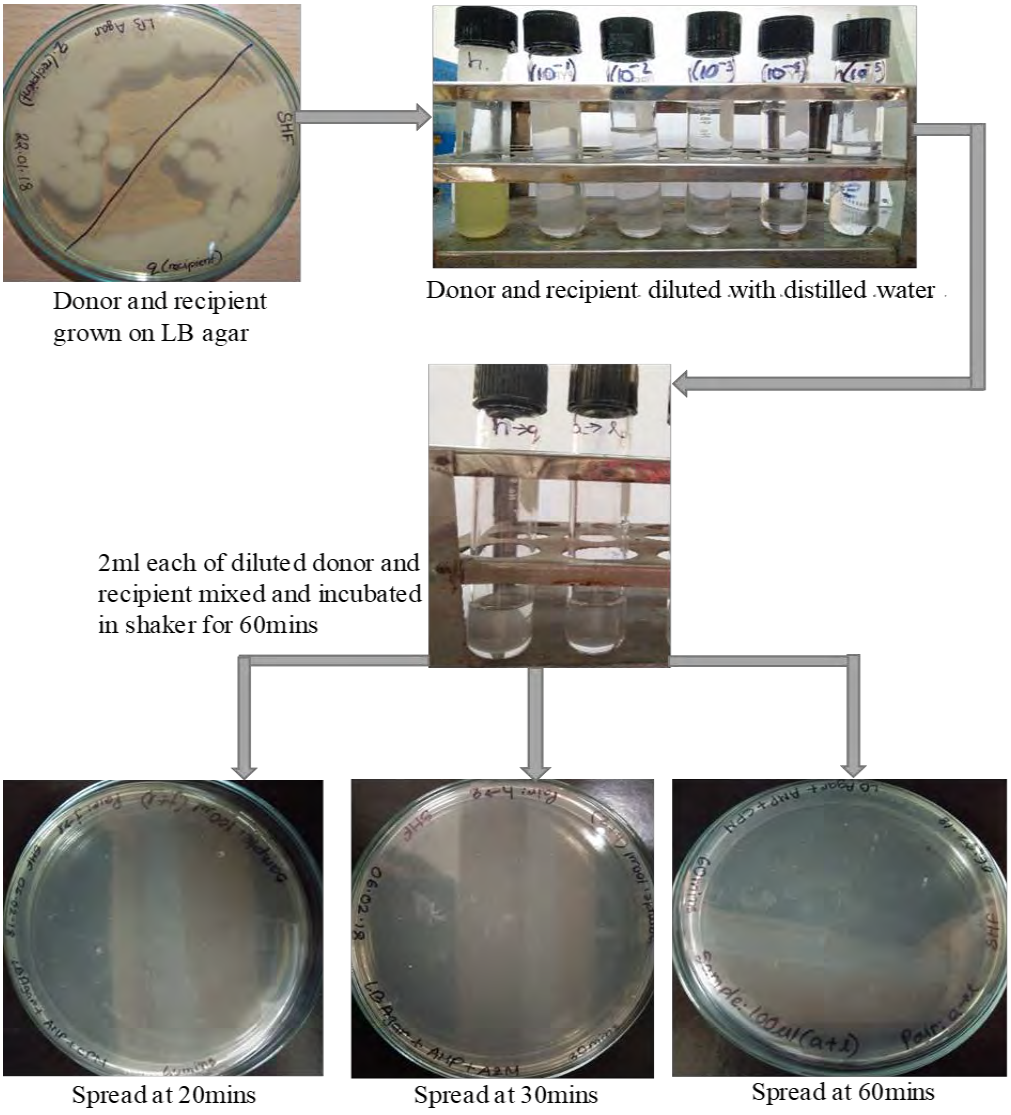


Fig 3.4: Results of each step in conjugation method 2. The 3 plates containing LB agar with Azithromycin & Ciprofloxacin shows no bacterial (transconjugant) growth after incubation.

3.3.3 Method 3 (Wang *et al*., 2008; Suhani *et al*., 2017)

In the 3rd method, the donor and recipient bacteria were inoculated in LB broth and incubated in a shaker at 37°C, overnight. Afterwards, 500µl each, of the donor and recipient was added together, to 4ml of fresh LB broth and allowed to incubated overnight at 37°C, without shaking. The mixture was then diluted 10-fold, using fresh LB broth and each dilution was spread on plates containing LB agar with 100µg/ml Azithromycin and 10µg/ml Ciprofloxacin. After incubation overnight at 37°C, no bacterial growth was seen on the plate, indicating that conjugation did not occur.

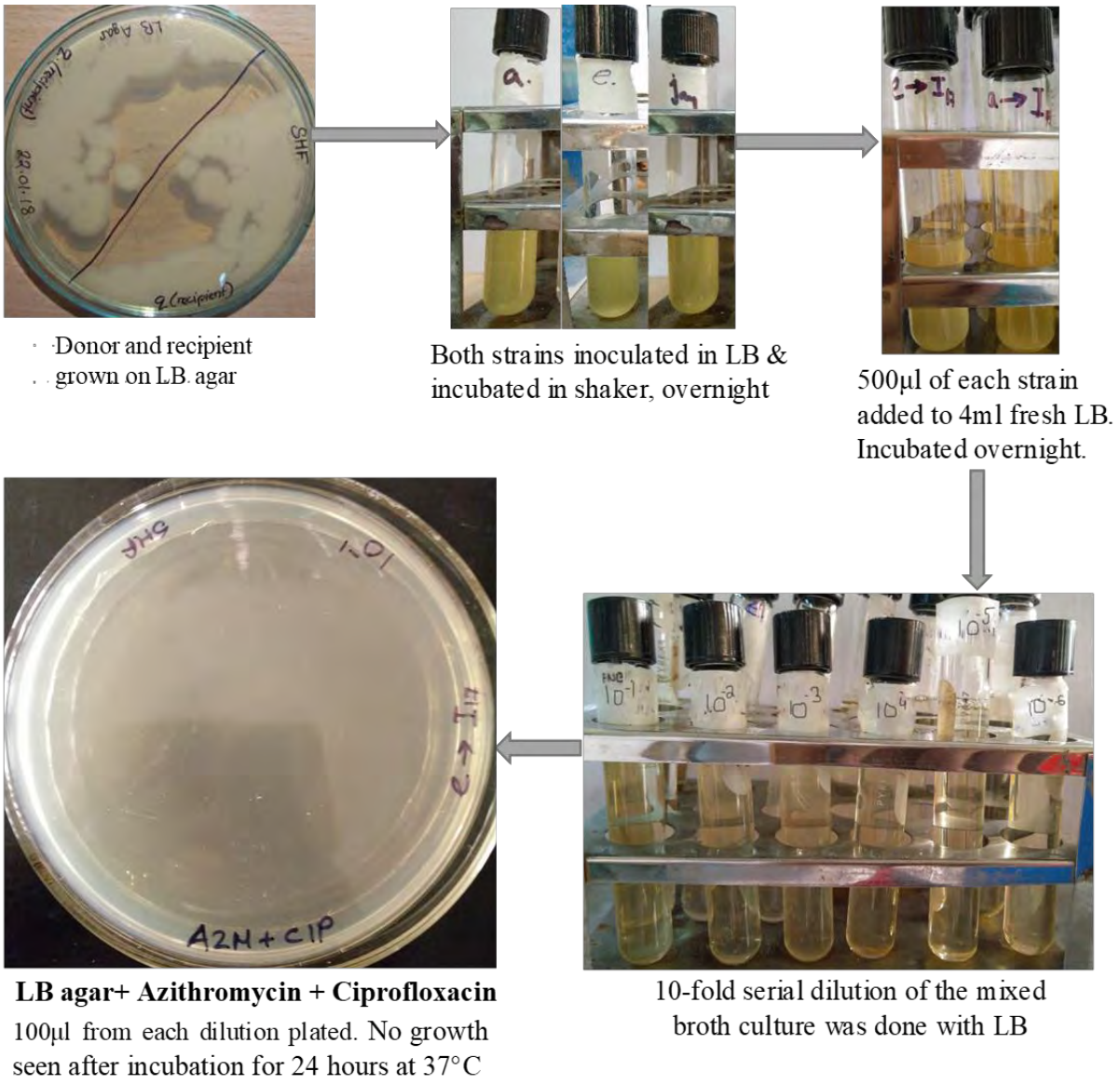


Fig 3.5: Results of each step in conjugation method 3. The plate containing LB agar with Azithromycin & Ciprofloxacin shows no bacterial (transconjugant) growth after incubation.

3.4 Plasmid Curing

The method of plasmid curing (Trevors, 1986) was carried out on the donor strains that were chosen for the conjugation experiments, in order to find out why horizontal gene transfer of the Azithromycin resistance gene had not occurred. The results of the experiment revealed that the donor strains had been cured of one or more of its plasmids, when subject to high concentrations of EtBr and SDS. Figure 3.6 shows the plasmid profile of the cured organisms, beside the original one. Both *Enterobacter* isolates 'a' and 'e' originally had 2 plasmids of length ~85 MDa and ~35.6 MDa. After curing with both EtBr and SDS, isolate 'a' was seen to lose its plasmid of length ~85 MDa, but still retained the other of length ~35.6 MDa. Isolate 'e', on the other hand, lost both plasmids when cured with EtBr, but retained one plasmid of length ~35.6 MDa, when cured with SDS.

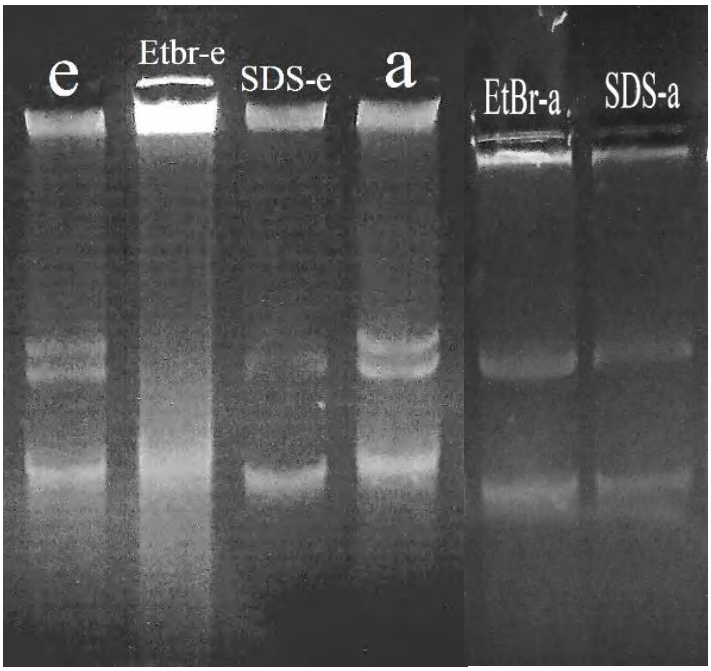


Fig 3.6: Plasmid profile of the donor strains after curing with EtBr and SDS. The lanes labeled 'e' and 'a' represent the plasmid bands of the original uncured organism.

3.4.1 Determination of MIC of Cured Donor

The conjugation experiments were designed to check the transfer of Azithromycin resistance from *Enterobacter spp* to *Klebsiella spp*. In order to determine if Azithromycin resistance was plasmid mediated in the donor strains, they were cured with EtBr and SDS and plasmid profile of the cured organism revealed that one or more of the plasmids had been lost. To check changes in the antibiotic susceptibility of the cured organism, the minimum inhibitory concentration was determined by the agar dilution method (Andrews, 2001).

Both organisms were seen to have the same resistance pattern to Azithromycin, after being cured with EtBr and SDS. esistance to Azithromycin was not plasmid mediated for isolate 'e', since it was still resistant to the antibiotic after being cured of both of its plasmids, with EtBr. Figure 3.7 shows the results of MIC obtained, of both donor strains 'a' and 'e' after being cured and table 3.4 depicts the MIC values of the cured organisms against Azithromycin

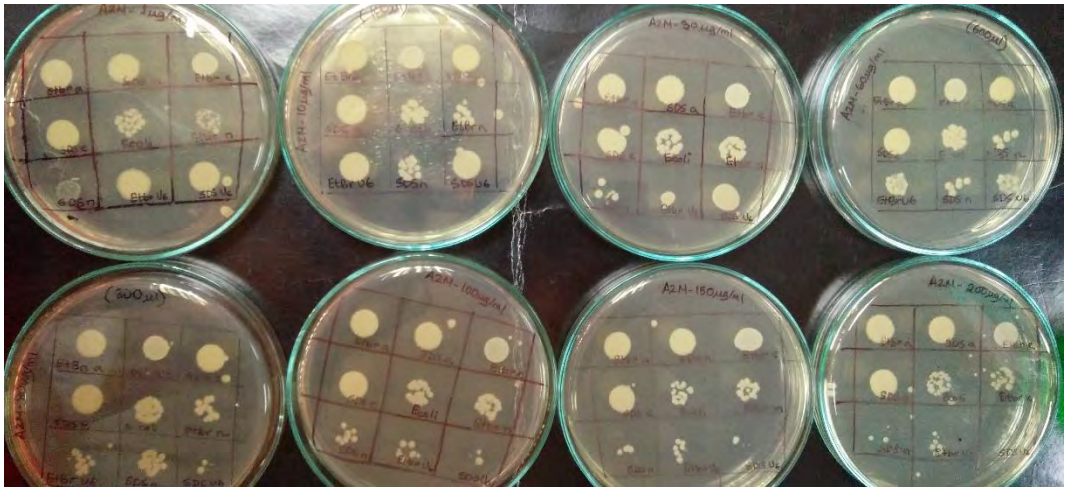


Fig 3.7: Minimum Inhibitory Concentration (MIC) determination against Azithromycin, of the cured *Enterobacter spp*, which were used as donors for conjugation process

Curing Reagent	ID	Suspected Identity	MIC-MBC value	
			Azithromycin	
			MIC	MBC
EtBr	a	*Enterobacter spp.*	≥200µg/ml	>200µg/ml
SDS	a	*Enterobacter spp.*	≥200µg/ml	>200µg/ml
EtBr	e	*Enterobacter spp.*	≥200µg/ml	>200µg/ml
SDS	e	*Enterobacter spp.*	≥200µg/ml	>200µg/ml

Table 3.4: The MIC-MBC values of the cured donor strains, against Azithromycin

Chapter 4

Discussion & Conclusion

4.1 Discussion

Recent decades have seen a drastic and alarming increase in the spread of antimicrobial resistance among microorganisms. A considerable chunk of the resistant bacteria is known to cause Urinary Tract Infections which is a significant burden in the National Health Service. These pathogenic bacteria are becoming increasingly difficult to bring under control since they are acquiring resistance to most antibiotics currently in the market, at a rate faster than ever, and without the introduction of new antibiotics or other approaches for treatment, the human race as a whole, falls under threat.

Elimination of resistance seems unmanageable due to the presence of transposable DNA elements, such as plasmids, which are associated with resistance to antibiotics (Kazemnia, Ahmadi, & Dilmaghani, 2014). The increase in the spread of resistance has been mainly attributed to plasmids which have the capability to carry several resistance genes and have a high ability to be spread from one bacteria to another through conjugative transfer (Nordmann, Naas, & Poirel, 2011).

In this study, 28 identified organisms had been taken, which were isolated from UTI patients of a Diagnostic Center in Bangladesh. Among these, over 78% (22 out of 28) of the isolates were from the Enterobacteriaceae family, with most of them conferring resistance to more than two antibiotics. Determination of Minimum Inhibitory Concentration (MIC) revealed that, among the Enterobacteriaceae family, an MIC value higher than 200µg/ml was observed in 68% (15 out of 22) of the organisms against Ampicillin, and 77% (17 out of 22) against Azithromycin. Furthermore, over 63% (14 out of 22) of these organisms exhibited an MIC value greater than 50µg/ml against Ciprofloxacin. In addition to this, plasmid profile of the organisms portrayed that, among the isolates of the Enterobacteriaceae family, 9 of them (40%) harbored one or more plasmids of different sizes ranging from 2 MDa to 85 MDa, leaving to the assumption that, resistance to the antibiotics may have been present on the plasmids.

It has been reported that Enterobacteriaceae isolates might be a potential carrier of antibiotic resistant genes that could be transferred between bacterial strains of the same or different species of UTI pathogens (Osterblad, *et al*., 2000; Tenover, 2006). This study investigated if resistance to Azithromycin was mediated through plasmids and if they had the ability to be transferred among

pathogens of the Enterobacteriaceae family. In accordance with the antibiotic susceptibility and plasmid profiling, compatible isolates were chosen as donor and recipient, which were used in the conjugation experiments, to determine if horizontal gene transfer occurs among the neighboring UTI pathogens.

The isolates that were chosen as the donor for the conjugation experiments were both *Enterobacter spp*, taken from two different samples. Both were seen to have two plasmids (~85 MDa, ~35.6MDa) and were resistant to Azithromycin with an MIC value ≥200µg/ml. The chosen recipient strain was a plasmid-less *Klebsiella spp*, which was sensitive to Azithromycin with an MIC value of 10µg/ml. Ciprofloxacin was used as marker to check for the presence of transconjugants on the plates, because the donors were sensitive to it with MIC values <0.1µg/ml and 5µg/ml respectively. The recipient *Klebsiella* on the other hand, was resistant to Ciprofloxacin with an MIC value of 20µg/ml. Hence, for the three methods of conjugation that were carried out, LB agar plates were prepared containing Azithromycin and Ciprofloxacin. The Azithromycin concentration would inhibit the recipient, whereas the Ciprofloxacin concentration would inhibit the donor. Thus any bacterial colonies seen on these media can be assumed to be transconjugants, since they will then contain the genes for resistance to both the antibiotics on the plate.

In order to demonstrate the transfer of resistance through plasmids, three different methods for conjugation were followed in this study. In method 1 (Fernandez-Lopez *et al*., 2005), the donor and recipient cells were allowed to conjugate in a plate-mating procedure, where both cells were allowed to grow on a single LB agar plate. Although, when the overlapping colonies were allowed to grow again on LB agar containing the selected antibiotics, no bacterial growth was observed after an overnight incubation. In method 2 (Phornphisutthimas *et al*., 2007) however, the donor and recipient was allowed to conjugate in liquid medium (distilled water) for 1 hour. At different time intervals, the mixture was vortexed to interrupt the mating by breaking the pilus, and was spread on plates containing the selected antibiotics. Similarly, in method 3 (Wang *et al*., 2008; Suhani *et al*., 2017) the donor and recipient was allowed to conjugate in liquid medium (LB broth) for 24 hours and spread on plates containing the selected antibiotics. No bacterial growth was seen after an overnight incubation for either method, indicating that resistance to Azithromycin was not transferred to the recipient.

Several studies have found antibiotic resistances in Enterobacteriaceae to be chromosomally located due to mutations in their genetic structure (Goni-Urriza, *et al*., 2000). Spontaneous mutations in chromosomal genes occur at a frequency of 10^6-10^8/cell division and in relation to antibiotic resistance, such mutations usually involve genes encoding the target site, or cell structures affecting access to the target site (Doss, 1994). In order to determine if the resistance gene to Azithromycin was present in the chromosome or the plasmid, a procedure for plasmid curing was carried out on the donor strains in this study, which would allow a direct comparison to be made between the plasmid-containing and plasmid-cured cell.

Some curing agents work in a non-specific way by damaging and stressing out the cells, while some seem to act much selectively (Hohn & Korn, 1969). Ethidium bromide has been extensively used to cure plasmids in a wide variety of bacterial strains (Trevors, 1986). In 1969, Bouanchaud *et al*., described the use of ethidium bromide to eliminate plasmids in antibiotic-resistant Enterobacteriaceae. Sodium Dodecyl Sulfate (SDS) treatment has also been used to cure plasmids, as has been reported by Tomoeda *et al*., (1968). Hence, these two reagents were used in this study for the plasmid curing procedure.

Ethidium Bromide was seen to be more effective in comparison, since it cured both the plasmids of one of the donor strains. Treatment with SDS on the other hand, had not been able to cure the cells of both plasmids, since the plasmid profile revealed that the larger plasmid (~35.6 MDa) was still present in both the donor strains. The minimum inhibitory concentration against Azithromycin, of the cured organisms was then determined by the agar dilution method (Andrews, 2001), the results of which showed the same pattern as the uncured organisms.

The *Enterobacter* donor strain, which lost both plasmids after treatment with EtBr, was still resistant to Azithromycin, producing the same MIC value of ≥200µg/ml. Hence, it can be concluded that, the resistance gene did not reside in the plasmid, and is assumed to be present chromosomally. However, the other donor strain, retained one of its plasmids after treatment with both reagents, but also produced the same MIC value (≥200µg/ml) against Azithromycin.

Hence, the results of the plasmid curing experiment validates why transfer of Azithromycin gene had not occurred from the conjugation experiments. The donor *Enterobacter spp* did not contain the resistance gene to Azithromycin on its plasmids, hence the recipient *Klebsiella spp* did not confer resistance to it, after being grown in the proximity of the donor, since the gene could not be transferred by conjugation.

Among a few other reasons that result in bacteria not being able to transfer genes by conjugation, is the lack of the conjugative F plasmid, which is the fertility factor that has the ability to be transferred and replicated inside the recipient (Huddleston, 2014). Although, if the recipient is unable to support the replication, it will not be able to confer resistance to the antibiotic present on the transferred plasmid (Bennett, 2008). On the other hand, if the recipient bacteria do not secrete certain chemicals called sex pheromones, that stimulate the donor to extend the pilus, transfer of plasmid cannot take place despite being present in a donor cell (Phornphisutthimas *et al*., 2007). Moreover, the TraN protein at the tip of the pilus which is responsible for binding and interacting with the recipient, may undergo a mutation. A loss of function mutation in the TraN protein has been shown to lead to a tremendous decrease in the efficiency of liquid medium conjugation (Klimke, *et al*., 2005; Klimke & Frost, 1998). Furthermore, recent studies have revealed that, a protein called OmpA found on the outer membrane of the recipient cell, is the target for binding of the F pilus and is hence essential in conjugation, based on the result that no conjugation was observed in OmpA knock-out recipients (Klimke & Frost, 1998). Although these assumptions can be made for the pair of *Enterobater spp* and *Klebsiella spp* that did not conjugate in this study, further research into each cell type is required to come to a conclusion.

This study exhibits its novelty for determining if conjugal transfer of the gene encoding resistance to Azithromycin, occurs among the Enterobacteriaceae pathogens harbored by UTI patients, which has not been documented yet. Although all the procedures were followed with in-vitro techniques, in-vivo validation is required to determine if resistance to Azithromycin was non-plasmid mediated for all strains and if it has the potential to be spread amongst the pathogens in a natural environment.

4.2 Conclusion

Over the course of exposure to antibiotics, bacteria have been seen to respond by evolving and enhancing their genetic armory, to evade their lethal action. Conjugation has long been known as a main player in the transfer of multiple antibiotic resistances among pathogenic bacteria. This transfer from one organism to the other has led to genetic variations, allowing the organisms to survive beyond the treatment with even the last line of antibiotics. Alternative options for the treatment of infections caused by carbapenem resistant strains of Enterobacteriaceae are limited. Current strategies include colistin, fosfomycin, tigecycline and temocillin. Scientists today are not only dealing with the challenge of eliminating resistant pathogens, but also with the seemingly impossible challenge of stopping the spread of these resistance genes in the entire bacterial community. It is therefore crucial to address every event in which a pathogen may acquire resistance to drugs, in order to introduce new techniques of combating the problem down to every individual bacterial cell.

4.3 Future Perspectives

The problem of the birth of superbugs has reached such a point where, discovery of new lines of antibiotics are no longer enough to defeat disease causing pathogens. In order to control the spread of resistance by horizontal transfer among pathogenic bacteria, extensive study into some compounds that may be able to interfere with this process, may prove to be useful in potentially controlling the spread amongst species. Significant research is required to identify conjugation inhibitors that may be able stop the spread, thus allowing the single resistant pathogen to be eliminated without threat of others.

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Appendices

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 hydrolysate	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
Tryptone	10.0
Sodium Chloride	10.0
Final pH: 6.9-7.1	

Luria Bertani Agar (LB Agar)

Component	Amount (g/L)
Casein enzymic hydrolysate	10.0
Yeast Extract	5.0
Sodium Chloride	10.0
Agar	15.0
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