A Study on Relationship of Plasmids with Azithromycin and Ciprofloxacin Resistance in Isolates Causing Urinary Tract Infection

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 that the thesis project titled **"A Study on Relationship of Plasmids with Azithromycin and Ciprofloxacin Resistance in Isolates Causing Urinary Tract Infection"** has been written and submitted by me Faizah Zaima and has been carried out under the supervision of Namista Islam, 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 Parents

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Abstract

The urinary tract infection is caused by the members of *Enterobacteriaceae* family. The emergence of multi-drug resistant bacteria is a great challenge and concern worldwide. The aim of this research was to determine the transmission of antibiotic resistance gene. This study included antibiotic susceptibility tests on the isolated organisms using both Kirby Bauer method and agar diffusion method, followed by plasmid extraction by Kado and Liu method and the bands were observed by agarose gel electrophoresis. The selected ten isolates are then treated with different concentrations of curing agents (Ethidium Bromide and SDS). After treatment with the curing agents, the above mentioned procedures were repeated for the cured isolates. Plasmid extraction and gel electrophoresis were repeated after curing treatment to determine the loss of plasmid bands. Following gel electrophoresis (before and after curing treatment), the results indicated that all the 10 isolates have lost plasmid bands. Kirby Bauer disk diffusion method is done to determine if the antibiotic resistance was plasmid mediated or chromosome mediated. The results of antibiogram were compared with the previous antibiogram results to determine whether the sensitivity of the cured isolates has changed or not. The isolates will lose their resistance after curing if that resistance is plasmid mediated, otherwise the resistance is chromosome mediated. After that, the MIC & MBC values were also compared (both before and after curing treatment) to re-confirm the results of antibiogram. All the results found from this research are necessary to design the transformation procedure. Following plasmid curing, transformation was also done to determine whether the plasmid was transferred via transformation.

Contents:

List of Tables

List of Figures

Chapter 1

Introduction

1. Background:

1.1 Urinary Tract Information:

The infection that affects part of the urinary tract of human body is known as urinary tract infection (UTI). It is a condition in parts of the urinary system- the kidneys, ureters, bladder, and urethra become infected. UTIs are the most common of all bacterial infections and can occur at any time in the life of an individual. 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. Much less often, bacteria spread to the kidney from the bloodstream. Although the urinary system is designed to keep out such microscopic invaders, these defences sometimes fail. When that happens, bacteria may take hold and grow into a full-blown infection in the urinary tract. Most infections involve the lower urinary tract— the bladder and the urethra.

E.coli from the gut is the cause of 80–85% of community-acquired urinary tract infections, with *Staphylococcus saprophyticus* being the cause in 5–10%. Healthcare-associated urinary tract infections (mostly related to urinary catheterization) involve a much broader range of pathogens including: *E.coli* (27%), *Klebsiella* (11%), *Pseudomonas* (11%), the fungal pathogen *Candida albicans* (9%), and *Enterococcus* (7%) among others. It is estimated that over 50% of all women will experience at least one UTI during their lifetime, with 20-30% experiencing recurrent UTI. Women are more likely to develop UTIs than men and their infection are tend to recur, due to anatomical differences; the urethra is shorter in women than in men, and it is closer to the anus, making it more likely that bacteria are transferred to the bladder. Infection in bladder can be painful and annoying. However, serious consequences can occur if a UTI spreads to the kidneys. Typically, doctors treat urinary tract infections with antibiotics.

1.1.1 *E.coli*:

Escherichia coli (or *E. coli*) is the most prevalent infecting organism in the family of gram-negative bacteria known as *Enterobacteriaceae*. *E. coli* is a Gram-negative, facultative (anaerobic and aerobic growth), rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms. The ideal growth temperature is 37ºC. It can be classified into several kinds of serotypes. Complex antigenic structure of *E.coli* is based

on O, K and H antigens. It has been classified into six different types 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* cause several intestinal and extra-intestinal infections such as Urinary Tract Infection. *E.coli* is the most common causative agent for UTI.

1.1.2 *Klebsiella spp.:*

Klebsiella spp. is nonmotile, Gram-negative, oxidase-negative, rod-shaped bacteria with a prominent polysaccharide-based capsule. They are found everywhere in nature. They can be found in water, soil, plants, insects and other animals including humans. *Klebsiella* bacteria tend to be rounder and thicker than other members of the *Enterobacteriaceae* family. They have no specific growth requirements and grow well on standard laboratory media, but grow best between 35 and 37 °C and at pH 7.2. The species are facultative anaerobes and most strains can survive with citrate and glucose as their sole carbon sources and ammonia as their sole nitrogen source.

Klebsiella organisms can lead to a wide range of disease states, notably pneumonia, urinary tract infections, septicemia, meningitis, diarrhea, and soft tissue infections. The majority of human *Klebsiella* infections are caused by *K.pneumoniae*, followed by *K.oxytoca*. *K.pneumoniae* is the most common cause of nosocomial respiratory tract and premature intensive care infections, and the second-most frequent cause of urinary tract infections. Some of the species of *Klebsiella* is *K.granulomatis, K.oxytoca, K.michiganensis, K.pneumonia*, *K.quasipneumoniae, K.grimontii, K.variicola.*

1.1.3 *Proteus spp.:*

Proteus spp. is gram-negative, motile, aerobic rod shaped bacilli, urease positive, non-lactose fermenter, oxidase-negative, catalase, nitrate-positive. They belong to *Enterobacteriaceae* family. *Proteus* are widely distributed in nature as saprophytes, being found in decomposing animal matter, sewage, manure soil, the mammalian intestine, and human and animal feces. They are opportunistic pathogens, commonly responsible for urinary and septic infections.

Three species of *Proteus* – *P.vulgaris*, *P.mirabilis*, and *P.penneri* are opportunistic human pathogens. *Proteus* is the third most common bacteria responsible for many human urinary tract infections. About 10–15% of kidney stones disease is caused by alkalinization of the urine by the action of the urease enzyme of *Proteus* bacterial species. Proteus spp can be motile, but have characteristic "swarming" patterns. Some of the species of Proteus is *P.hauseri*, *P.mirabilis*, *P.myxofaciens*, *P penneri*, *P.vulgaris*.

1.1.4 *Enterobacter spp.:*

Enterobacter spp. is gram-negative, facultatively anaerobic, rod-shaped, oxidase-negative, indole-negative non-spore-forming bacteria of the family *Enterobacteriaceae*. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised hosts. The urinary and respiratory tracts are the most common sites of infection. Two clinically important species from this genus are *E.aerogenes* and *E.cloacae*.

The genus *Enterobacter* is a member of the coliform group of bacteria. It does not belong to the fecal coliforms or thermotolerant coliforms group of bacteria, unlike *Escherichia coli*, because it is incapable of growth at 44.5 °C in the presence of bile salts. *Enterobacter* ferments lactose with gas production during a 48-hour incubation at 35-37 °C in the presence of bile salts and detergents. Some species of *Enterobacter* is *E.aerogenes*, *E.agglomerans*, *E.cancerogenous*, *E.cloacae*, *E.cowanii*, *E.gergoviae*.

1.1.5 *Shigella spp.*:

Shigella spp. is gram-negative, facultative anaerobic, nonspore-forming, non-motile, rod-shaped bacteria of the family *Enterobacteriaceae*, genetically closely related to *E. coli*. The causative agent of human shigellosis is *Shigella spp*. During infection, it typically causes dysentery. *Shigella* is one of the leading bacterial causes of diarrhea worldwide, causing an estimated 80–165 million cases. The number of deaths it causes each year is estimated at between 74,000 and 600,000. Though rare but it still is a causative agent of UTI.

Three *Shigella* groups are the major disease-causing species: *S.flexneri* is the most frequently isolated species worldwide, and accounts for 60% of cases in the developing world; *S.sonnei* causes 77% of cases in the developed world, compared to only 15% of cases in

the developing world; and *S.dysenteriae* is usually the cause of epidemics of dysentery, particularly in confined populations such as refugee camps.

Each of the *Shigella* genomes includes a virulence plasmid that encodes conserved primary virulence determinants. The *Shigella* chromosomes share most of their genes with those of *E. coli* K12 strain MG1655. It has four serotypes and they are- *S.boydii*, *S.dysenteriae*, *S.flexneri* and *S.sonnei*.

1.2 Epidemiology:

Urinary tract infections are the most frequent bacterial infection in women. They occur most frequently between the ages of 16 and 35 years, with 10% of women getting an infection yearly and more than 40–60% having an infection at some point in their lives [11]. Rates of asymptomatic bacteria in the urine increase with age from two to seven percent in women of child bearing age to as high as 50% in elderly women. Rates of asymptomatic bacteria in the urine among men over 75 are between 7-10% [10]. Urinary tract infections may affect 10% of people during childhood. Among children urinary tract infections are the most common in uncircumcised males less than three months of age, followed by females less than one year.

In the United States, urinary tract infections account for nearly seven million office visits, a million emergency department visits, and one hundred thousand hospitalizations every year [6].The cost of these infections is significant both in terms of lost time at work and costs of medical care. In the United States the direct cost of treatment is estimated at 1.6 billion USD yearly [6].

The treatment of this infection is done by antibiotics but the causative agents are getting resistant against the conventional antibiotics at an alarming rate. Therefore, this infection is becoming a major complication for the entire public health worldwide. Moreover, these resistance to the antibiotics is not only restricted to the causative agents but also has been transmitted to other distinctly related or un-related species.

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]. Therefore, treatment of these antibiotic resistant bacterial infections is hence becoming more and more strenuous and has been

identified as the greatest epidemic threats to the human by the World Health Organization. (WHO) [Suhani *et al*, 2017].

1.3 Antibiotic resistance:

Antibiotic resistance occurs when bacteria develop the ability to defeat the drugs designed to kill them. Microbes resistant to multiple antibiotics are called multidrug resistant (MDR).

Antibiotic resistance is one of the most urgent threats to the public's health. Antibiotic resistant bacteria can cause illnesses that were once easily treatable with antibiotics to become untreatable, leading to dangerous infections. Antibiotic-resistant bacteria are often more difficult to kill and more expensive to treat. In some cases, the antibiotic-resistant infections can lead to serious disability or even death.

Resistance arises through one of three mechanisms: natural resistance in certain types of bacteria, genetic mutation, or by one species acquiring resistance from another.

Antimicrobial resistance is increasing globally because of greater access to antibiotic drugs in developing countries. Estimates are that 700,000 to several million deaths result per year [11].

1.3.1 Chromosome mediated resistance:

Chromosome-mediated resistance occurs by spontaneous mutation in a locus that controls susceptibility to the drug. The antimicrobial drug serves as a selecting mechanism to suppress susceptible organisms and favor the growth of drug-resistant mutants. Spontaneous mutation is not a frequent cause of the clinical drug resistance in a given patient. Mutation can result in the loss of PBPs, making such mutants resistant to a-Iactam drugs. Examples are - Rifampicin, streptomycin, erythromycin. Resistance of M. tuberculosis to rifampicin is caused by mutation in RNA polymerase and that to isoniazid by mutation in catalase.

1.3.2 Plasmid mediated resistance:

Plasmid mediated resistance occurs by the extrachromosomal genetic elements called plasmid. Plasmid-mediated resistance is the transfer of antibiotic resistance genes which are carried on plasmids. Plasmid mediated drug resistance is more common than that of chromosome. R factors (drug resistance plasmids) are a class of plasmids that carry genes for resistance to antimicrobial drugs. A single plasmid can carry genes that code for resistance to several drugs (multi-drug resistance -MDR) such as streptomycin, chloramphenicol, tetracycline and sulphonamides. Plasmid genes control the formation of enzymes capable of destroying the antimicrobial drugs, e.g. Plactamases destroy P-lactam ring of penicillins and cephalosporins.

Antibiotic resistance mediated by MDR plasmids severely limits the treatment options for the infections caused by gram-negative bacteria, especially *Enterobacteriaceae* family.

1.4 Plasmid:

Plasmids are self-replicating extra chromosomal DNA molecules found in bacteria as well as in some yeast and other fungi. Plasmid is a circular double stranded DNA having several to 100 kbp. Plasmids replicate autonomously and they code for functions which are normally not indispensable. Plasmid ranges from about 1 kb to over 250 kb. The copy number refers to the number of molecules of an individual plasmid that are normally found in a single bacterial cell. In some cases, plasmids may be transferred from one cell to another and thus may carry sets of specialized genetic information through a population, e.g. Drug resistance plasmids (R factors) may render diverse bacteria resistant to antimicrobial drugs. Plasmids carry genes associated with specialized function. Artificial plasmids are widely used as vectors in molecular cloning, serving to drive the replication of recombinant DNA sequences within host organisms.

Plasmids can be classified by function. There are five main classes:

- Fertility F-plasmids, which contain *tra* genes. They are capable of conjugation and result in the expression of sex pili.

- Resistance (R) plasmids, which contain genes that provide resistance against antibiotics or poisons. Historically known as R-factors, before the nature of plasmids was understood.
- Col plasmids, which contain genes that code for bacteriocins, proteins that can kill other bacteria.
- Degradative plasmids, which enable the digestion of unusual substances, e.g. toluene and salicylic acid.
- Virulence plasmids, which turn the bacterium into a pathogen.

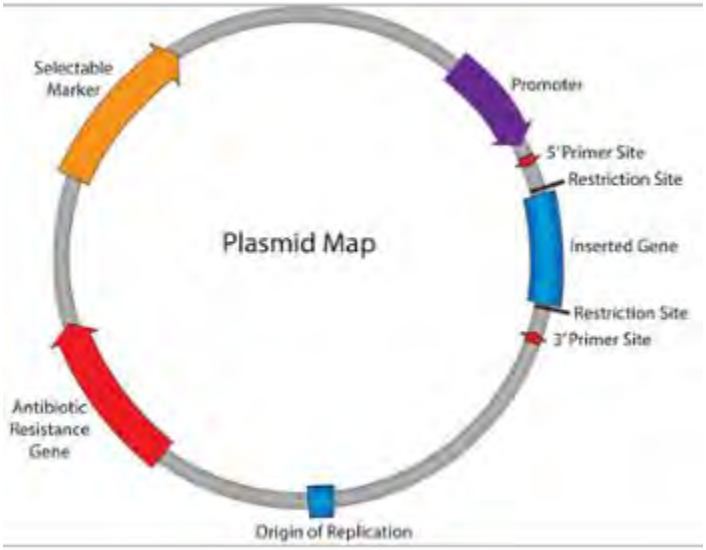


Figure 1.1: Architecture of Plasmid.

1.5 Plasmid Curing:

Bacterial plasmids are known to harbor genes for resistances to antibiotics and metals; catabolic pathways such as lactose utilization and degradation of hydrocarbons; and biosynthesis of certain antibiotics. Curing of plasmids from bacteria strains is a way to eliminate the bacteria plasmid and determine the antibiotic resistance mediation. There are several methods involving chemical and physical agents that have been developed to eliminate plasmids. 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.

The mechanism of plasmid curing starts from the inhibition of plasmid replication resulted from a single nick, outside of the origin of replication of the superhelical structure. The process leads to further relaxation of plasmid DNA, an increase in melting point and circular dichroism. The intercalating agents would then break the superhelical form of plasmid DNA subsequently forming an open circular or linear form plasmid DNA (Spengler *et al.*, 2006).

1.6 Plasmid Curing Agents:

There are several chemical and physical agents that have been developed to eliminate plasmids. Chemical agents are- Sodium Dodecyle Sulfate (SDS), Ethidium bromide (EB), Acridine Orange (AO) etc. And the physical agents are –UV ray, change of optimal growth temperature etc. The effectiveness of a curing agent does vary considerably in ranging of 100- to 1000-fold. This depends on the organisms being treated, curing agent efficiency and efficacy, and the mode of action of respective the curing agent (Carlton and Brown, 1981). Due to these factors, it is essential to use a wide range of curing agent concentration especially when the bacteria are isolated from environmental sources (Trevors, 1986).

Intercalating agents such as AO and EB have been successfully used in curing bacterial plasmids. The modes of action of intercalating agents are through preferential inhibition of plasmid replication. Ethidium bromide with a formula molecule $C_{21}H_{20}N_3Br$ is an intercalating agent which resembles a DNA base pair. Due to its unique structure, EB can easily intercalate into DNA strand. EB was selected as a favorable curing agent in comparative to AO because it is difficult to disposal of AO (Molina-Aja et al., 2002).

Sodium Dodecyl Sulfate is an anionic detergent that is used as a chemical curing agent in *Vibrio* species. Plasmid containing cells are possibly more sensitive to SDS because of plasmid-specified pili on cell surface. The chemical acts in dislodging the indigenous plasmid from its site of attachment.

1.7 Horizontal Gene Transfer:

Horizontal Gene Transfer is the process where the movement of genetic material or information occurs between organisms to related or closely related organisms. It is the primary mechanism for the spread of antibiotic resistance in bacteria, thus very important for evolution. There are three types of horizontal gene transfer and they are-

- Conjugation
- Transformation &
- Transduction

1.8 Transformation:

In molecular biology, transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material from its surroundings through the cell membrane(s). For transformation to take place, the recipient bacteria must be in a state of competence, which might occur in nature as a time-limited response to environmental conditions such as starvation and cell density.

In this process a susceptible or "competent" bacterial cell acquires new genetic material from its environment. Here, exogenous DNA is taken up into the recipient cell through the cell membrane which leads to the alteration of genetic makeup of a cell. It is one of the most popular techniques of molecular genetics among the three basic mechanisms of bacterial DNA exchange method-transformation, transduction and conjugation. Initially, this technique was first discovered in bacteria, but other ways have been designed to transform many types of animal and plant cell as well.

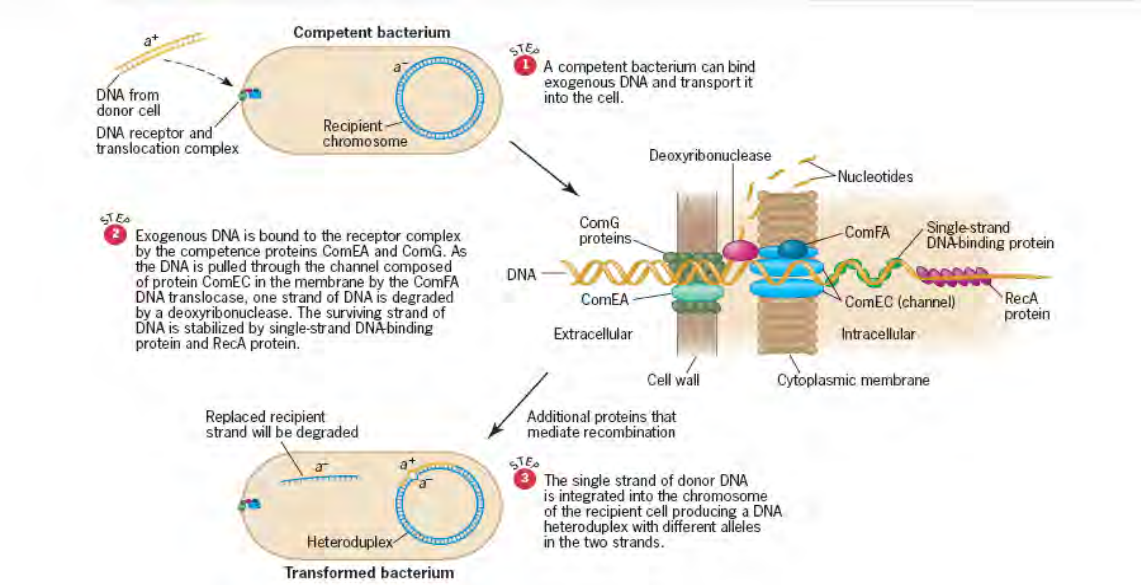


Figure 1.2: Transformation process.

1.9 Literature Review:

Trevors 1985, is a study based on plasmid curing and the roles of curing agents. It also discussed the mode of actions of different curing agents and their efficiency, different procedures of plasmid curing.

Zaman, Pasha & Akhter, (2010) conducted an experiment to investigate the curing effects of Ethidium Bromide, Sodium Dodecyle Sulfate and Acridine Orange against antibiotic resistant *E.coli* isolated from UTI patients. The result shows that Et.Br is the most successful curing agent while AO is better than SDS. Also, the plasmids were cured on the higher concentration of Et.Br.

Tomoeda, Inuzuku, Kubo & Nakamura, (1967) experimented on *Escherichia coli* K-12 strains by treatment with sodium dodecyl sulfate (SDS) to eliminate the drug resistance (R) and sex (F) factors. The results show that the E.coli lost its drug resistance factor and became sensitive to tetracycline by treatment with SDS while the sex (F) factor was intact.

Salisbury, Hedges & Datta, (1971) designed a study where they eliminated the F'lac by acridine orange efficiently from *Escherichia coli* K I 2. But with SDS, only selected cultures were cured. It proved that acridine orange is a true curing agent while SDS acted onlyby selection of spontaneous variants.

Spengler, Molna, Schelz, Amaral & Sharples, (2006) conducted an experiment to eliminate the metabolic functions expressed by plasmid in *E.coli lac* gene by different curing agent. The relation of the metabolic gene with plasmid is complex to achieve a successful curing result.

Lauritsen, Porse, Sommer & Norholm, (2017) designed a study where CRISPR-Cas9 was used to perfore plasmid curing in both *E.coli* and *Pseudomonas putida.* The results shows that the curing efficiencies for those organism was between 40 and 100% for the plasmids most widely used for expression and engineering purposes.

Adeyemo & Onilude, (2015) studied plasmid curing and its effects on the growth and physiological characteristics of *Lactobacillus plantarum* which was isolated from fermented cereals. The curing agent was Acridine Orange and the result was successful.

Letchumanan, Chan & Lee, (2015) conducted an experiment where they used the traditional curing assay in Vibrio spp. they used Ethidium Bromide, Acridine Orange and SDS as chemical agents and increased the optimal growth temperature as physical agent. The result indicated a positive curing assay. Also, it showed that Et.Br is a better curing agent than AO and SDS.

Suhani *et al*., (2017) investigated on *E.coli*, isolated from UTI patient, contains antibiotic resistant plasmid and its ability to transfer resistance gene through horizontal gene transfer. The isolated *E.coli* was resistant against AMX, AMP and CIP. Therefore it was used as donor and *E.coli* DH5α which was sensitive to these antibiotics, was selected as recipient. Both Conjugation and Transformation were conducted. The results showed that the *E.coli* DH5α inherited the resistance gene transferred via Conjugation and Transformation.

1.10 Aims and Objectives:

The aim of this research is to determine the transmission of antibiotic resistance gene, whether it is plasmid mediated or chromosome mediated also, to investigate if the plasmid transmission emergence is caused by Transformation. We conducted this research on a variety of organisms (*E.coli, Klebsiella spp., Enterobacter spp., Shigella spp.* & *Proteus spp.*).

Our objectives for this research was to-

- Understand the plasmid curing assay.
- Investigate the effect of curing agents on the isolates.

- Understand the mechanisms of bacterial survival and pathogenicity
- Understand the antibiotic transfer process via Transformation.

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 November 2017 to June 2018.

2.3 Microbiological Sample:

A total of 30 samples were collected from UTI of a Diagnostic Centre. Then those bacterial samples were isolated and identified using 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.

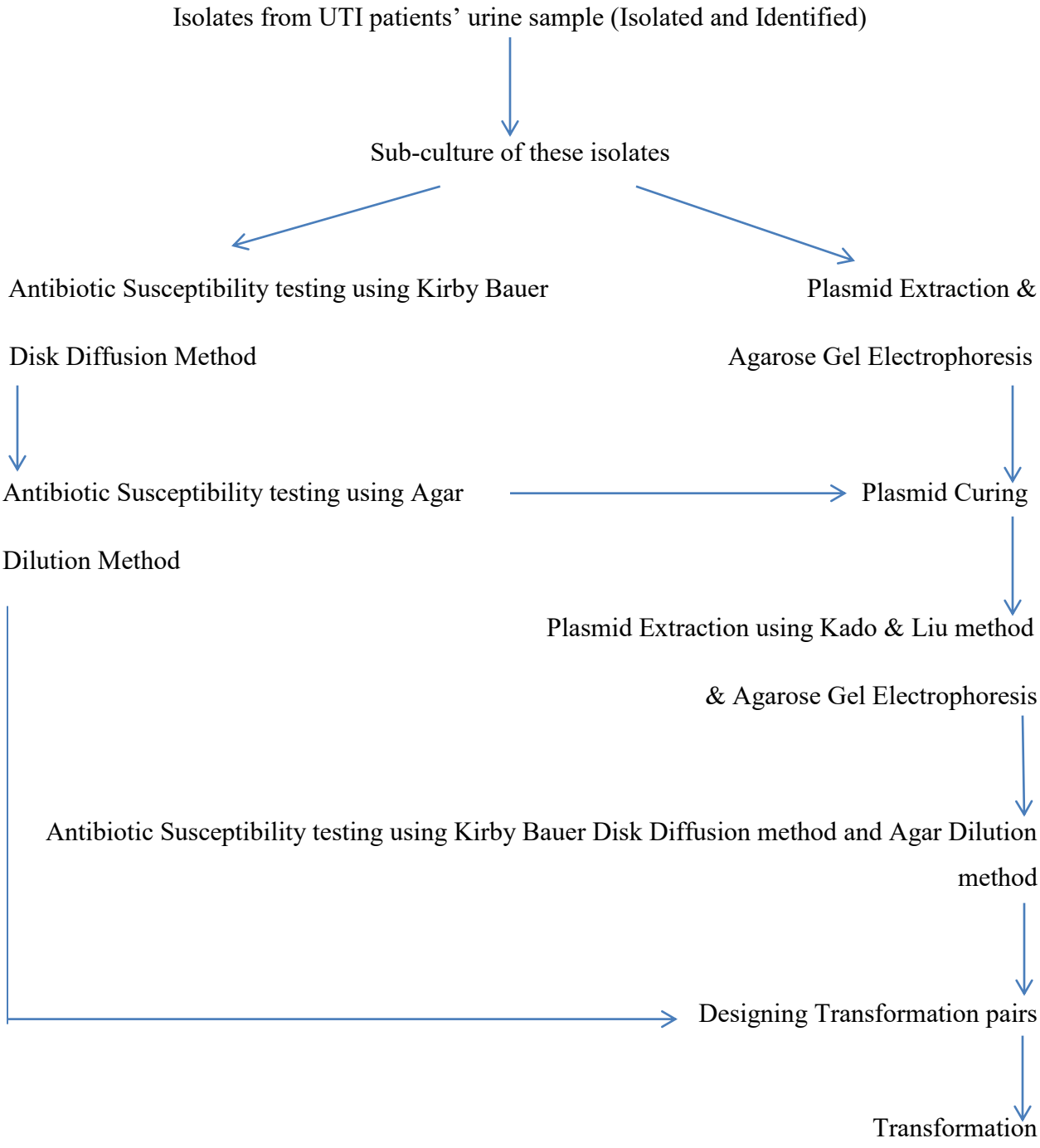
Table 2.1: Name of the Organisms.

SL. No.	Name of the organism	Organism ID
1.	*E.coli*	j
2.	*E.coli*	U8
3.	*Klebsiella spp.*	g
4.	*Klebsiella spp.*	U33
5.	*Klebsiella spp.*	U43
6.	*Enterobacter spp.*	d
7.	*Enterobacter spp.*	U50
8.	*Enterobacter spp.*	i4
9.	*Shigella spp.*	r
10	*Proteus spp.*	p

2.4 Microbiological culture of the sample:

The samples were initially cultured on nutrient agar and incubated at 37ºC for 24 hours. Nutrient Agar media is a general purpose medium for the growth of a wide variety of non-fastidious organisms consisting of appropriate amount of necessary nutrients for growth and replication of the organisms. The sample o be cultured was taken from the stock and then streaked in the four quadrant method on nutrient agar plates. 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 Kirby Bauer Method (Disk Diffusion Method):

By definition, antibiotics are molecules that kill, or stop the growth of, microorganisms, including both bacteria and fungi.They may either kill or inhibit the growth of bacteria. Though antibiotics are specific for bacteria, it may kill one species of bacteria but on the other hand it may be inactive against another species. There are 3 types of antibiotics based on their production, such as- 1) natural antibiotic (produced by microorganisms, e.g; penicillin) 2) semi-synthetic antibiotic (produced by microorganisms then modified in lab, e.g: ampicillin) 3) synthetic antibiotic (produced syntheically in laboratory, e.g; fluroquinolones).

Kirby Bauer Method is a qualitative assay where Mueller Hinton Agar is considered as best for the routine susceptibility testing since it has batch-to-batch reproducibility, low concentration of inhibitors of sulphonamide, trimethoprim and tetracyclines and produce satisfactory results for most of the non-fastidious and fastidious pathogens. Then again, starch of the agar absorbs toxins and therefore antibiotic activity is not inhibited in any way. It provides good diffusion as well which is good for disk diffusion method.

Procedure:

- Using a sterile loop a colony from pure culture plate of the organism to be tested was aseptically emulsified into a test tube containing sterile saline solution and vortexed properly.
- A McFarland solution of 1 was taken and compared with the OD of the saline test tube containing the organism.
- A sterile cotton swab was taken and dipped into the broth culture, streaked thoroughly on the surface of MHA plate to make a lawn culture.
- By using sterilized forceps, antibiotics disks were gently pressed on the surface of the agar plate.
- The plates were incubated at 37 ºC for 24 hours.
- After incubation, the growth zone around each antibiotic disk is observed. If growth zone is observed around the disk, then that organism is sensitive against that particular antibiotic. If not, then the organism is resistant against that antibiotic.

- The zone of inhibition is measured and compared against the CLSI reference chart to indicate antibiotic susceptibility as either Resistant (R), Intermediate (I) or Sensitive(S).

8 different antibiotics were used for this research. Their name is given in the following table.

Table 2.2: List of Antibiotics.

No	Name of the Antibiotic	Code of the Antibiotic	Potency (µg)
1.	Streptomycin	S	10
2.	Ampicillin	AMP	25
3.	Amoxicillin	AMX	30
4.	Azithromycin	AZM	15
5.	Ciprofloxacin	CIP	5
6.	Gentamycin	GEN	10
7.	Vancomycin	VAN	30
8.	Kanamycin	K	10

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

Agar Dilution Method is another technique that is used for antibiotic sensitivity testing. This method is both quantitative and comparative at the same time. Minimum Inhibitory Concentration (MICs) is defined as the lowest concentration of an antibiotic that will inhibit the visible growth of an organism after overnight incubation. Maximum Bacterial Concentration (MBCs) is defined as the lowest concentration of antibiotic that inhibits the growth of an organism by 99.9%. The MBC is complementary to the MIC.

Procedure:

- An inoculum of the organism to be tested is prepared in a test tube containing sterile 10ml of Luria Bertani and kept for overnight incubation at 37ºC.
- Different concentrations of antibiotics are put into different plates along with the MHA medium.
- Both the antibiotic and the agar medium are thoroughly mixed together.

- After overnight incubation, 10µl of each organism were put onto the agar plates containing different concentration of antibiotics.
- A grid was used on the plate to organize the culture drops.
- The plates were kept at 37ºC for 18-24 hours.
- After incubation period, the growth on the plates (if present) and the size of growth zone is observed. The growth zone will determine the sensitivity of the organisms at different concentrations, along with the MIC, MBC value.

The concentrations of the antibiotics used are mentioned in the table below:

Table 2.3: Concentrations of Antibiotics used for Agar Dilution Method.

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

2.7 Plasmid Extraction:

Plasmid extraction of these isolates are done in two methods-

1. Alkaline Lysis Method &

2. Kado and Liu Method.

2.7.1 Alkaline Lysis Method:

Procedure:

- An inoculum of the organism to be extracted is prepared in a conical flask containing sterile 25ml of LB and kept in shaking incubator for overnight incubation at 37ºC.
- Transfer 1.0 ml cells to harvest by centrifugation at 10000 rpm for 2 min (repeat this step 3 times).
- Re-suspend the pellet in 200 µl of Solution 1 (25 mM Tris-HCl pH-8.0, 10 mM EDTA and 50mM glucose) and then add freshly prepared 400 µl of solution 2 (0.2 N NaOH, 1% SDS), mixed by gentle inversion.
- Incubate the cells at room temperature for 5 mins for cell lysis.
- To this, add ice cold 300 µl of solution 3 (5M Sodium Acetate pH-5.2, glacial acetic acid, distilled water) and mix by inversion and incubate on ice for 10 mins.
- Centrifuge the mixture at 12000 rpm for 15 min.
- Collect the supernatant in a fresh tube.
- To this, add equal volume of phenol: chloroform: IAA (25:24:1), mix by vortexing and centrifuge at 12000 rpm for 2 min.
- Collect supernatant carefully and add equal volume of chloroform : IAA (24:1), mix by vortexing and centrifuge at 12000 rpm for 5 min.
- Collect supernatant and then add 0.6 volume of isopropanol to this, mix by inversion and centrifugation at 12000 rpm for 15 minutes.
- Wash the DNA pellet in 70% ethanol, air-dry and re-suspend in 20 µl of TE buffer.
- Store the plasmid at -20 °C.

2.7.2 Kado and Liu Method:

Procedure:

- 1.5 ml of fresh shaking bacterial culture is taken into micro centrifuge tubes.
- The tubes containing bacterial suspension are centrifuged at 14000 rpm for 5 minutes.
- After that, the supernatant is discarded as much as possible and the pellet is taken.
- 60 µl of Kado I (4ml 1M Trsi HCL, 400 µl 0.5 M EDTA, distilled water) is added to the pellet and was mixed properly by pipetting.

- Then, 120 µl of Kado II (0.6g Tris Base, 3g SDS, 6.4 ml 2N NaOH, distilled water) is added and mixed by inverting the tubes (rolling 3 or 4 times).
- The tubes are placed in hot water bath at 55ºC.
- After an hour 400 µl of phenol chloroform mixture (1:1) is added to the tubes and mixed well by upside down the tubes for 30 minutes.
- After mixing, the tubes are centrifuged at 14000rpm for 5 minutes.
- After centrifugation, 3 layers will be observed in the tubes. Top layer contains plasmid DNA, middle layer contains protein debris and the bottom layer contains phenol.
- Plasmid DNA is carefully removed from the top layer by micropipette and is transferred to a new tube.
- The new tubes containing plasmid DNA is then stored at -20ºC for further uses.

2.8 Gel Electrophoresis:

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

Procedure:

- 0.7% gel is prepared separately using 40ml of TBE buffer and 0.28g of Agarose powder.
- 2µl 0f Ethidium Bromide, an intercalating agent is also added to the gel.
- The Electrophoresis Apparatus is set up using the power supply, gel is also set onto the casting tray.
- The solidified gel is placed in the electrophoresis chamber and TBE buffer is added to cover the gel.
- 10 µl of samples are loaded along with 2 µl of dye (Bromophenol Blue).
- The gel run is set at 70 V and it takes nearly 1 hour for the gel run to be completed.
- The bands are then seen under the Ultra Violet (UV) radiation.

2.9 Plasmid curing:

The curing agents which were used for this experiment are Sodium dodecyle (SDS) and Ethidium Bromide (EtBr).

Procedure:

- The organisms to be cured is cultured in 10ml of LB and incubated overnight at 37ºC.
- Then 1ml of overnight culture has been inoculated in test tubes each has 9ml of Nutrient Broth containing different concentrations of curing agents. The concentrations of SDS and ETBr used are listed in the table 2.4.

Table 2.4: Concentrations of SDS and EtBr used for plasmid curing.

No.	Concentration of SDS (%)	Concentration of EtBr (µg/ml)
1.	5	50
2.	8	75
3.	10	100
4.	12	125
5.	15	150

- After overnight incubation, 100µl of culture from each test tube is taken and spread on Nutrient agar and kept at 37ºC for 18-24 hours.
- After incubation, a single colony from each plate is taken and streak on Nutrient agar, kept at 37ºC for 18-24 hours.
- These streak plates are later used for plasmid extraction, antibiotics sensitivity testing to determine if the organisms are cured or not.

2.10 Designing Transformation Pairs:

After the antibiotic sensitivity testing, plasmid isolation and analysis, plasmid curing, extracting the cured plasmid, antibiotic sensitivity testing of the cured isolates, comparing the results would give sufficient information to determine the donor isolate for transformation. The selection of the donor depends on the presence of a certain antibiotic resistance gene. If a isolate is resistant against a particular antibiotic and after curing treatment the same isolate is now sensitive against that particular antibiotic , then that isolate would be the donor strain and that antibiotic would be the transfer antibiotic. The recipient strain is *E. coli* DH5α. The recipient strain should also be

sensitive to that particular antibiotic. The transfer antibiotic is determined from the Kirby Bauer Disk Diffusion Method. The concentration of the transfer antibiotic which will be mixed with NA during plating is determined by MIC & MBC values. The following table shows the pairs for transformation procedure:

Table 2.5: Designing Transformation pairs.

No.	ID of the Donor Isolate	Name of the Donor Isolate	Antibiotic	Concentration of the antibiotic (µg/ml)	Recipient Isolate
1.	J	*E.coli*	Azithromycin	50	*E. coli* DH5α
2.	I4	*Enterobacter spp*	Ampicillin	100	*E. coli* DH5α
3.	G	*Klebsiella spp*	Ciprofloxacin	50	*E. coli* DH5α

2.11 Transformation:

Transformation is a process by which a susceptible or "competent" bacterial cell acquires new genetic material from its environment. Exogenous DNA is taken up into the recipient cell through the cell membrane which leads to the alteration of genetic makeup of a cell. It is one of the most popular techniques of molecular genetics among the three basic mechanisms of bacterial DNA exchange method. Transformation comprises two methods: Competent Cell Preparation and Transformation Process.

2.11.1 Competent Cell Preparation:

Competence is a process by which a cell is able to take up DNA from its surrounding medium. There are two main methods for the preparation of competent cells artificially.

For this experiment cells were made competent by using 0.1 M $CaCl_2$.

Procedure:

Day 1

- A single colony of *DH5α* strain of *E. coli* is taken and inoculated into 20ml of LB medium.
- The culture is kept overnight at 37°C with shaking at 180 rpm.

Day 2

- 1 ml of saturated overnight culture is inoculated in 10 ml LB medium.
- The test tube is kept in shake at 37°C until OD_{600} =0.4 (usually 2-3 hours).
- It is then placed in ice bath for 10 minutes (after this point the cells should never touch anything that is warm).
- The culture is transferred into two pre-chilled 1.5ml micro-centrifuge tube.
- Then the tubes are centrifuged at 5000 rpm for 10 minutes.
- After centrifugation the medium is removed, the cell pellet is re-suspended with 800µL ice cold 100mM $CaCl_2$ by swirling on ice gently.
- The tubes are kept for incubation on ice for 30 minutes.
- Again, the tubes are centrifuged at 5000 rpm for 10 minutes.
- After centrifugation the medium is removed, the cell pellet is re-suspended with 800µL ice cold 100mM $CaCl_2$ by swirling on ice gently.
- The tubes are incubated on ice for 20 minutes.
- The cells are combined into one tube and add 250µL ice cold 80% glycerol and swirl to mix.
- The competent cells are then stored in -20°C (stable for 2 months).

2.11.2 Transformation process [Suhani 2017]:

Procedure:

- 50 µl of plasmid DNA is added to a tube containing 0.2 ml of competent cells.
- The mixture is placed on ice for 20 minutes.
- Then it is exposed to heat shock at 42°C for 1minutes and kept immediately in ice for 10 minutes.
- 800 µl LB is then added to transformation mixture and incubated at 37ºC for 1 hour.
- About 100 µl from transformation mixture is spread on nutrient agar plates containing the appropriate concentration of transfer antibiotic.
- The plates are incubated at 37°C for 48 hours.
- After incubation, the transformant colonies are observed (if present).

Chapter 3

Results

3. Results:

3.1 Antibiotic Sensitivity Testing:

3.1.1 Anti-biogram:

The table below shows the results antibiotic susceptibility testing using Kirby Bauer method.

Table 3.1: Antibiotic Susceptibility Results of the Isolates.

Isolate ID	Name of the organisms	Antibiotics						
		AMP(25) / mm	AMX(30) /mm	AZM(15) /mm	GEN(10) /mm	CIP(5) /mm	VAN(30) /mm	S(10) /mm
j	*E.coli*	- (R)	- (R)	- (R)	23 (S)	32 (S)	24 (S)	27 (S)
U8	*E.coli*	- (R)	- (R)	16 (I)	- (R)	25 (I)	- (R)	23 (S)
g	*Klebsiella spp.*	- (R)	- (R)	19 (I)	- (R)	- (R)	- (R)	20 (S)
U33	*Klebsiella spp.*	- (R)	- (R)	- (R)	- (R)	20 (I)	20 (S)	20 (S)
U43	*Klebsiella spp.*	- (R)	- (R)	- (R)	- (R)	27 (S)	- (R)	29 (S)
d	*Enterobacter spp.*	- (R)	- (R)	25 (S)	- (R)	15 (I)	- (R)	27 (S)
U50	*Enterobacter spp.*	- (R)	- (R)	- (R)	- (R)	- (R)	- (R)	14 (I)
I4	*Enterobacter spp.*	- (R)	- (R)	- (R)	- (R)	19 (I)	21 (S)	32 (S)
r	*Shigella spp.*	- (R)	- (R)	11 (R)	- (R)	37 (S)	- (R)	25 (S)
p	*Proteus spp.*	-	-	-	31	29	-	27

		(R)	(R)	(R)	(S)	(S)	(R)	(S)

From this result it can be said that all the isolates were sensitive to Streptomycin, whereas they showed resistance to Ampicillin, Amoxicillin, Gentamycin, Vancomycin and Azithromycin. Most of these isolates were either sensitive or intermediate to Ciprofloxacin.

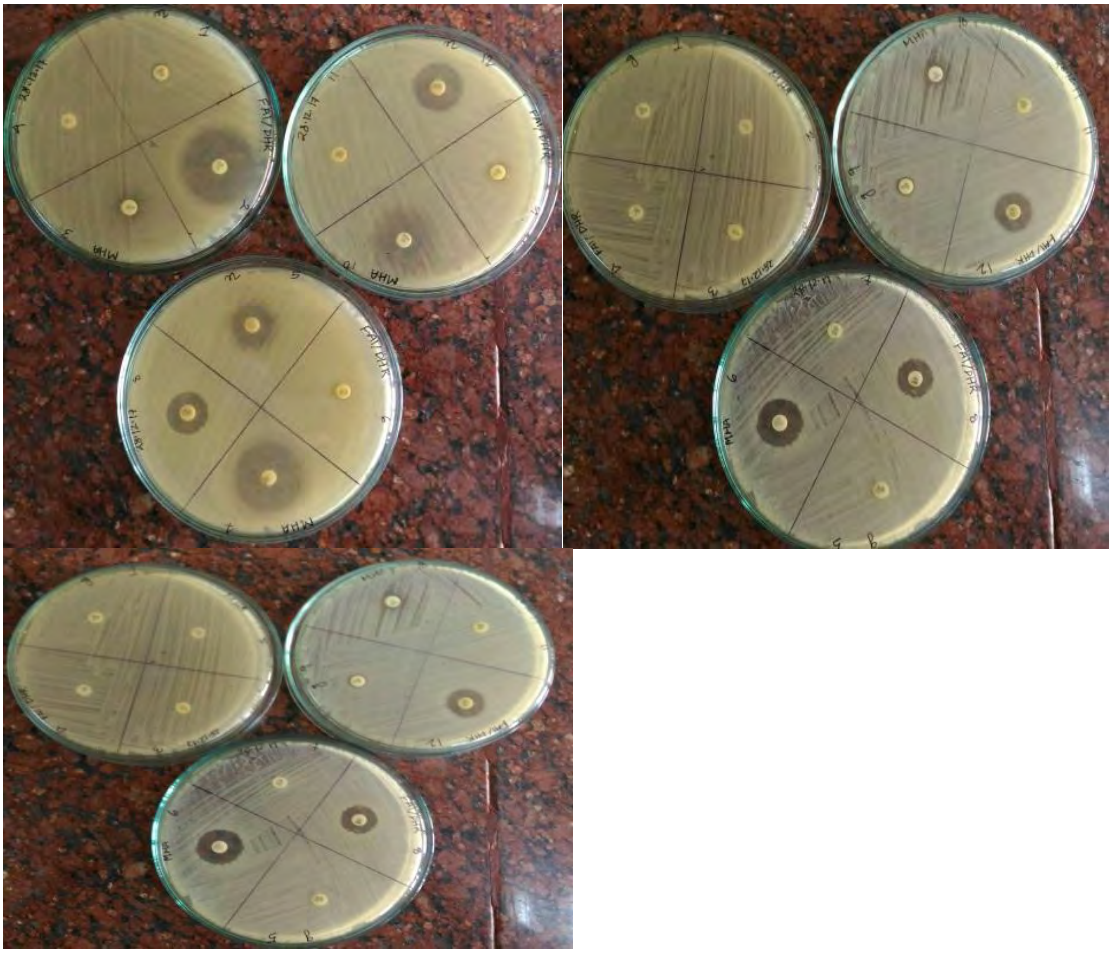


Figure 3.1: These plates show the results of Kirby Bauer method.

3.1.2 MIC and MBC:

For further re-confirmation of antibiotic susceptibility of these isolates, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined following agar dilution technique. All the results are summarized in the table below:

Table 3.2: Antibiotic Susceptibility results for MIC, MBC values by Agar Dilution method.

Isolate ID	Name of the organism	Ampicillin		Azithromycin		Ciprofloxacin	
		MIC Value (µg/ml)	MBC Value (µg/ml)	MIC Value (µg/ml)	MBC Value (µg/ml)	MIC Value (µg/ml)	MBC Value (µg/ml)
j	*E.coli*	Higher	Higher	150	200	10	15
U8	*E.coli*	Higher	Higher	Higher	Higher	Higher	Higher
g	*Klebsiella spp.*	150	200	100 (few colonies)	150	Higher	Higher
U33	*Klebsiella spp.*	Higher	Higher	Higher	Higher	Higher	Higher
U43	*Klebsiella spp.*	Higher	Higher	Higher	Higher	20 (2 colonies)	50
d	*Enterobacter spp.*	Higher	Higher	Higher	Higher	10 (few colonies)	15
U50	*Enterobacter spp.*	Higher	Higher	Higher	Higher	Higher	Higher
I4	*Enterobacter spp.*	150	200	Higher	Higher	1 (2 colonies)	5
r	*Shigella spp.*	150	200	150 (5 colony)	200	0.1 (few colonies)	1
p	*Proteus spp.*	150	200	150	200	10 (8 colony)	15

From this table, it can be said that the results determined from antibiogram and the results determined from agar dilution method were quite similar.

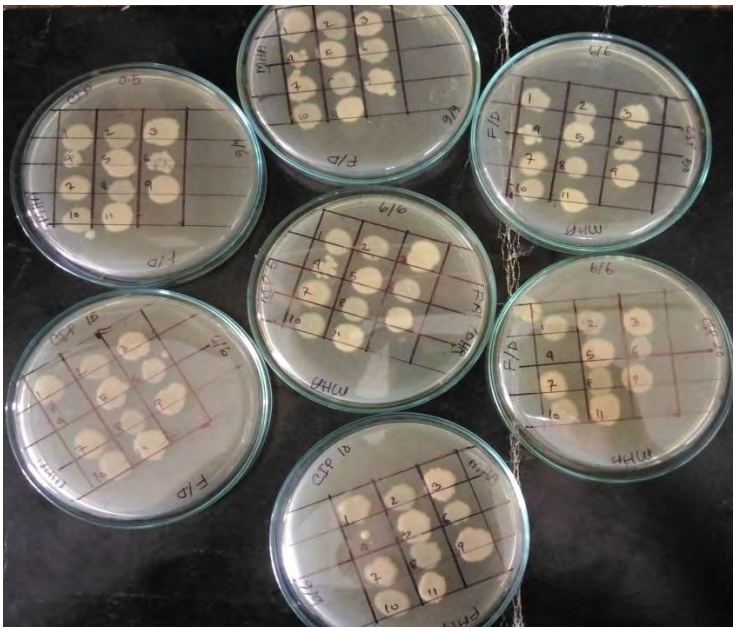


Figure 3.2: These plates show the results of MIC MBC using the Agar dilution method.

3.2 Plasmid Isolation and Gel Electrophoresis:

After the antibiotic susceptibility testing by Kirby Bauer method and Agar Dilution method, plasmid was extracted from these isolates using both Alkaline Lysis method and Kado and Liu method. After successful plasmid extraction, the plasmid bands were separated by gel electrophoresis and observed under UV light. The figure below shows the bands of extracted plasmids of each organism under the UV light. The reference strain is V-157. The extracted plasmid band size varied from 85MDa to 35.6MDa.

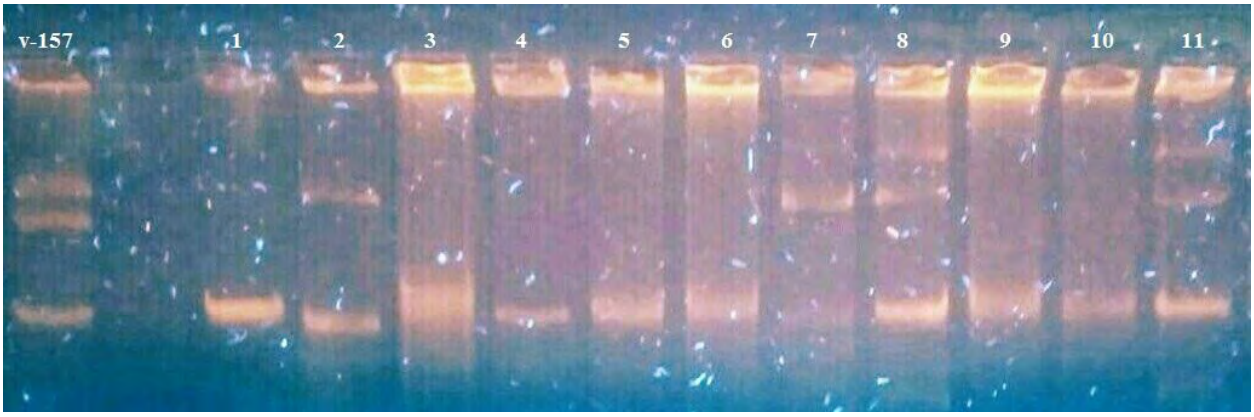


Figure 3.3: The result of Gel Electrophoresis shows the presence or absence of plasmid in the isolates.

3.3 Plasmid Curing:

The isolates (table 2.1) were treated with different concentrations of SDS and Et.Br mentioned in table 2.4. The target of plasmid curing was to see whether the antibiotic resistance gene was removed. After the curing process, to determine if the result of plasmid curing was positive or negative, the bands were checked through plasmid isolation and gel electrophoresis. Also, antibiotic susceptibility testing was done to determine if the antibiotic resistance is either plasmid mediated or chromosome mediated.

3.3.1 Plasmid Isolation and Gel Electrophoresis of Cured Isolates:

I. In the picture below, the extracted plasmid was from isolate j (*E.coli)* which had two bands. After curing treatment, some of the cured isolate lost a single band. The isolates which were treated with 15% SDS, Et.Br 150µg/ml, Et.Br 125µg/ml and Et.Br 100µg/ml had lost a single band. The loss of plasmid bands indicated that plasmid was cured.

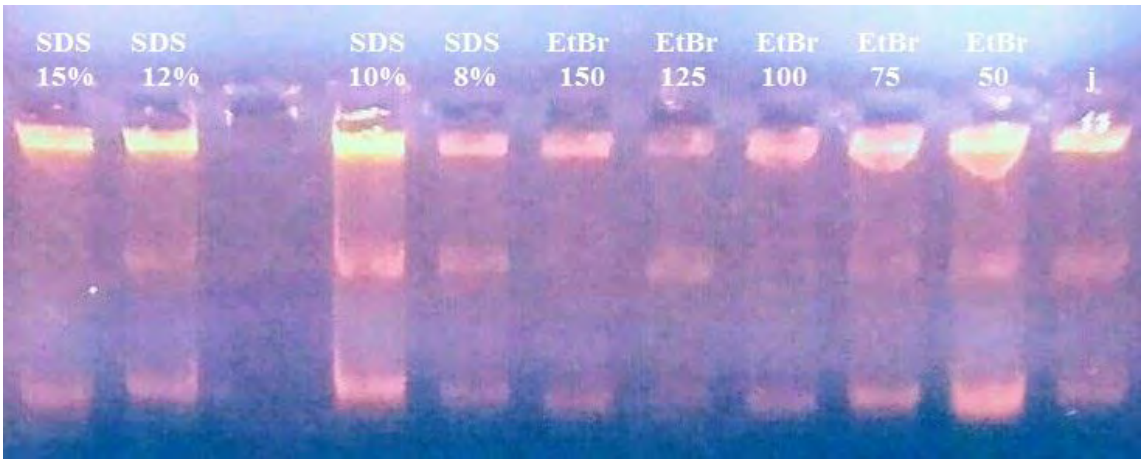


Figure 3.4: The result of gel electrophoresis showing the loss of plasmid bands.

II. The extracted plasmid in the figure below was from isolate U8 (*E.coli)* which had two plasmid bands. After treating with curing agents some of the cured isolates lost a single band. The isolates treated with 15% SDS, 10% SDS, Et.Br 150µg/ml and Et.Br 75µg/ml had lost a single band. So, it can be said that plasmids were successfully cured

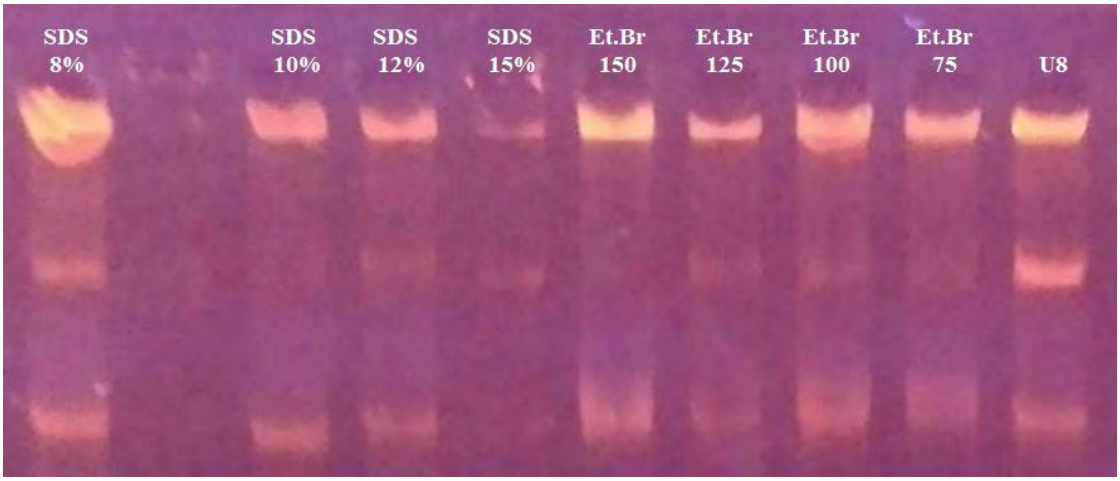


Figure 3.5: The result of gel electrophoresis showing the loss of plasmid bands.

III. Similarly in the following picture, the extracted plasmid was from isolate g (*Klebsiella spp.)*. A single band was lost after curing treatment. The loss of plasmid band was observed with 15% SDS, 12% SDS, 10% SDS, 8% SDS, Et.Br 100µg/ml and Et.Br 150µg/ml curing treatments. The loss of plasmid bands indicated successful curing.

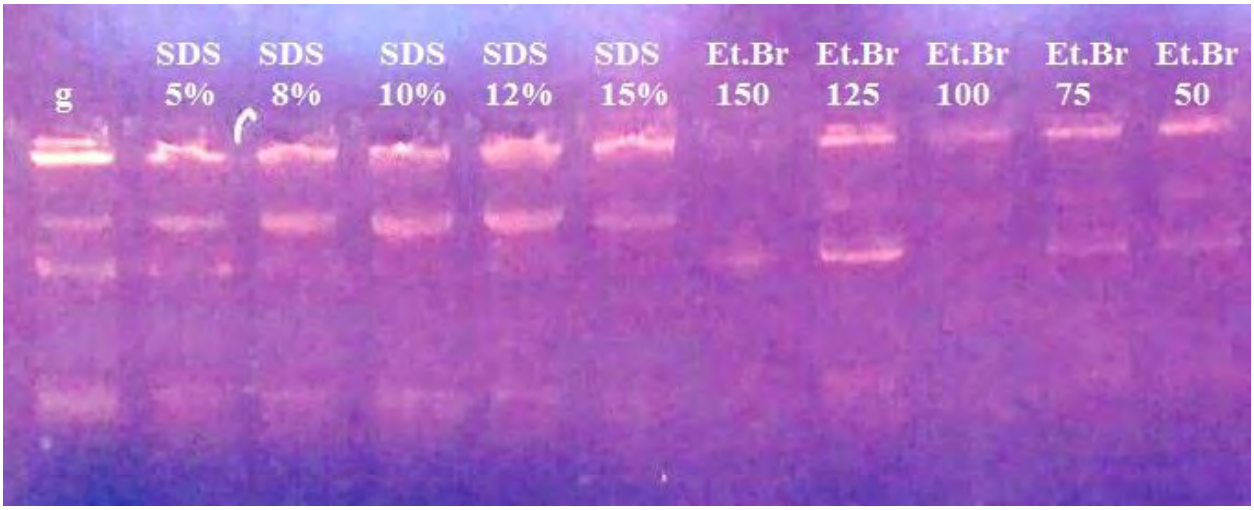


Figure 3.6: The result of gel electrophoresis showing the loss of plasmid bands.

IV. In figure 3.7, the extracted plasmid was from isolate U33 (*Klebsiella spp.)*. The isolates treated with 10% SDS, 12% SDS, Et.Br 75µg/ml, Et.Br 100µg/ml, Et.Br 125µg/ml and Et.Br 150µg/ml had lost a single band. Therefore, a successful curing result is indicated.

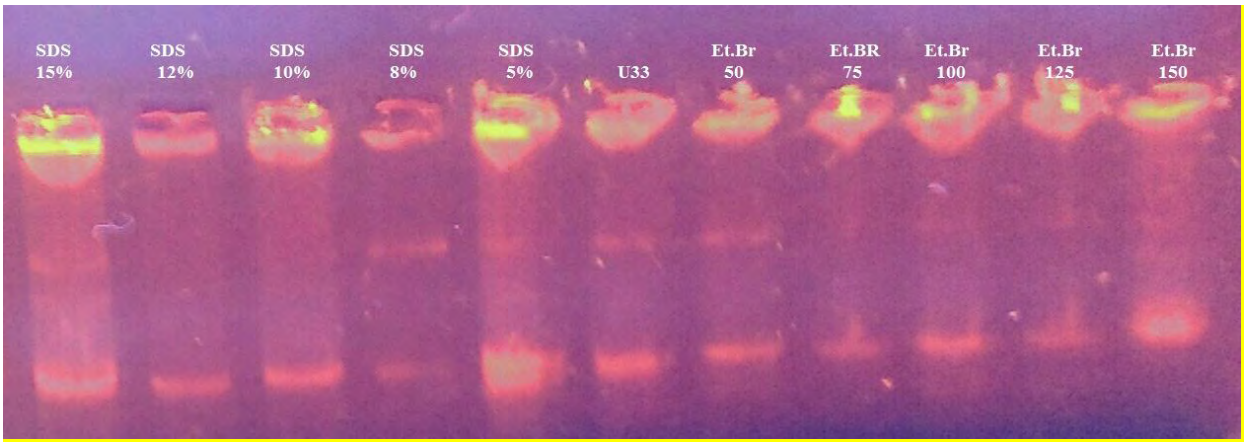


Figure 3.7: The result of gel electrophoresis showing the loss of plasmid bands.

V. The following figure was of isolate U43 (*Klebsiella spp.)* which had two bands. After curing, it can be seen that with 10% SDS, 12% SDS, 15% SDS, Et.Br 50µg/ml, Et.Br 100µg/ml, Et.Br 125µg/ml and Et.Br 150µg/ml, a single band was lost. So, it indicated that curing was successful.

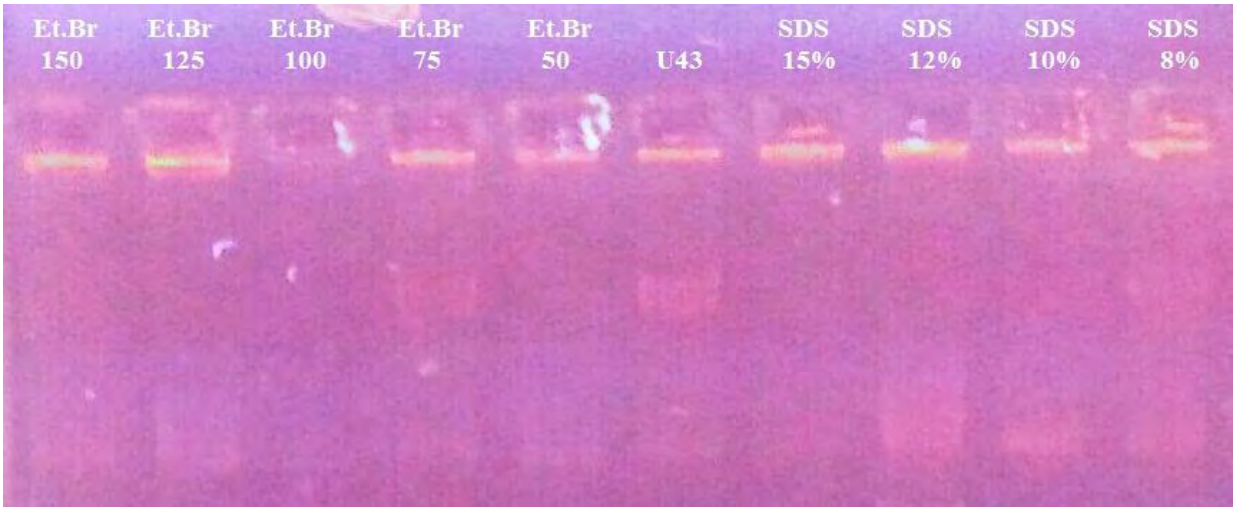


Figure 3.8: The result of gel electrophoresis showing the loss of plasmid bands.

VI. Similarly in figure 3.9, the extracted plasmid was from isolate d (*Enterobacter spp.)*. The isolates treated with 12% SDS, Et.Br 50µg/ml, Et.Br 75µg/ml, Et.Br 100µg/ml, Et.Br 125µg/ml and Et.Br 150µg/ml had lost a single band. Therefore, it can be said that curing was successful.

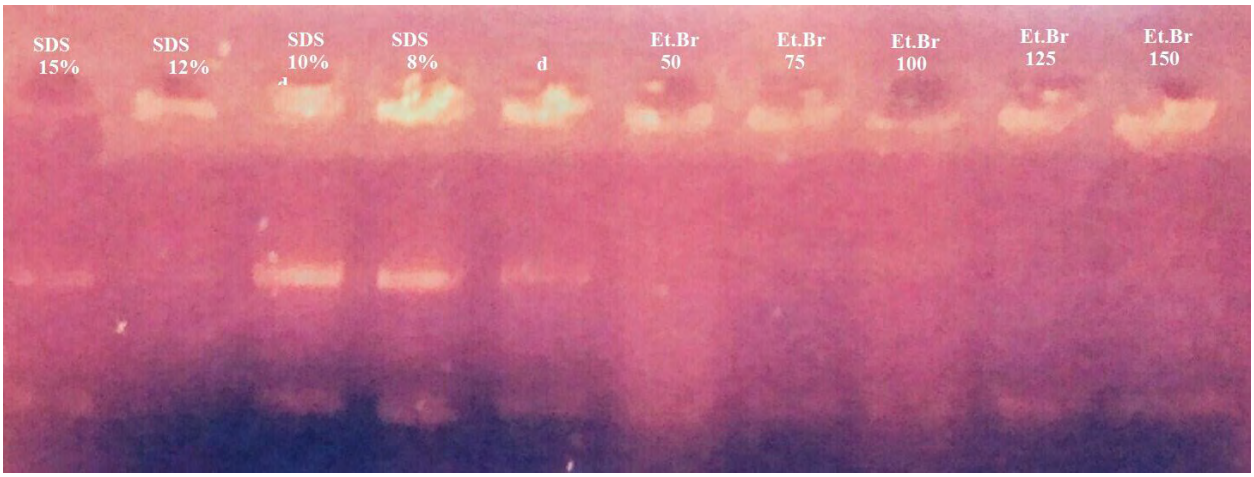


Figure 3.9: The result of gel electrophoresis showing the loss of plasmid bands.

VII. The following figure was of isolate U50 (*Enterobacter spp.)* which had a single band. With the concentrations 10% SDS, 12% SDS, 15% SDS, Et.Br 100µg/ml, Et.Br 125µg/ml and Et.Br 150µg/ml, the band was lost. It indicated successful curing.

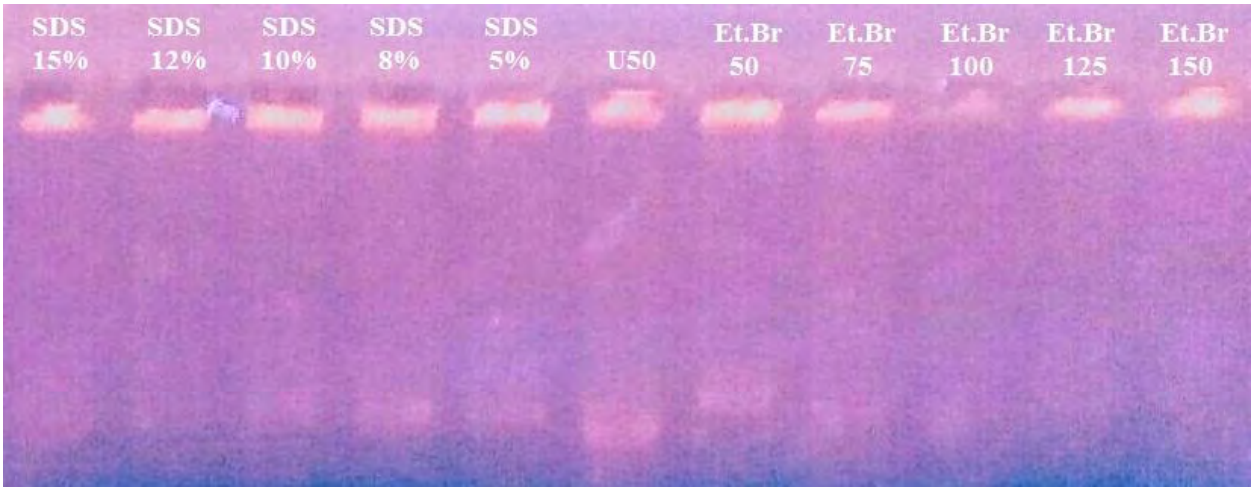


Figure 3.10: The result of gel electrophoresis showing the loss of plasmid bands.

VIII. Similarly, the extracted plasmid was from isolate i4 (*Enterobacter spp.)* in the figure below. The isolates had lost their band when treated with 15% SDS, Et.Br 75µg/ml, Et.Br 100µg/ml and Et.Br 150µg/ml. So, it can be assumed a successful curing.

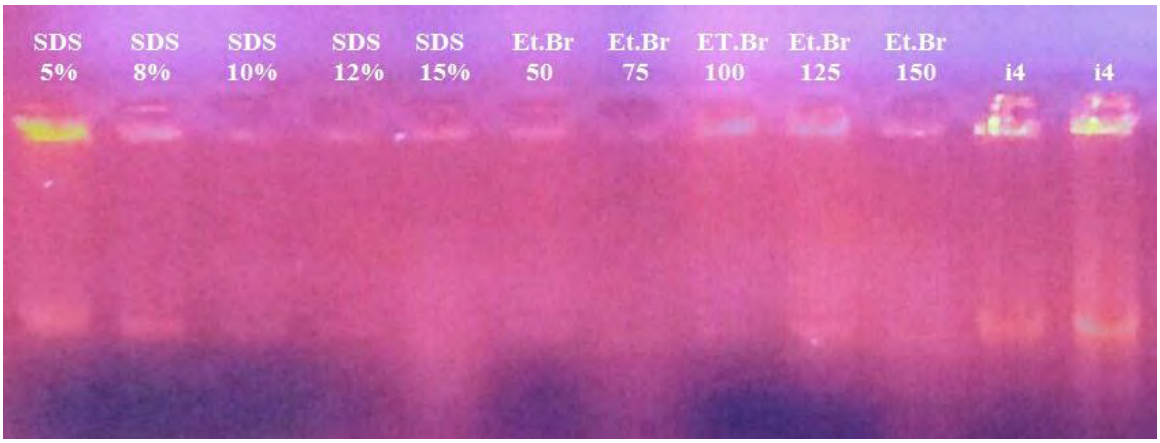


Figure 3.11: The result of gel electrophoresis showing the loss of plasmid bands.

IX. Again the following figure was from isolate r (*Shigella spp.)* which had three bands. When the isolates were treated with 8% SDS, 15% SDS, Et.Br 125µg/ml and Et.Br 150µg/ml, the plasmid band was lost. It indicated that curing was successful.

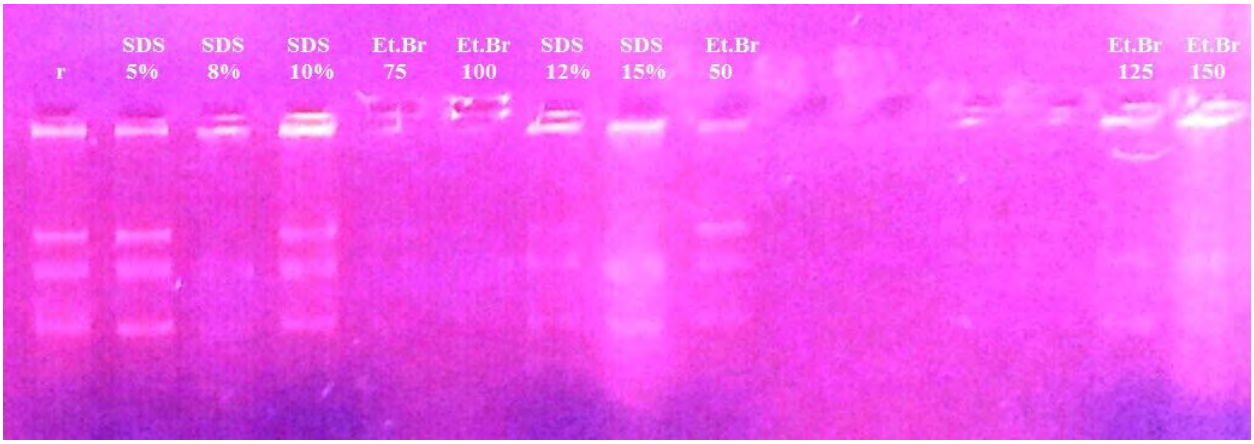


Figure 3.12: The result of gel electrophoresis showing the loss of plasmid bands.

X. In the figure below, the extracted plasmid was from isolate p (*Proteus spp.)* which had a single plasmid band. After treating the isolates with curing agents 15% SDS, Et.Br 75µg/ml and Et.Br 100µg/ml, the loss of band was observed. Therefore, the result showed a successful curing.

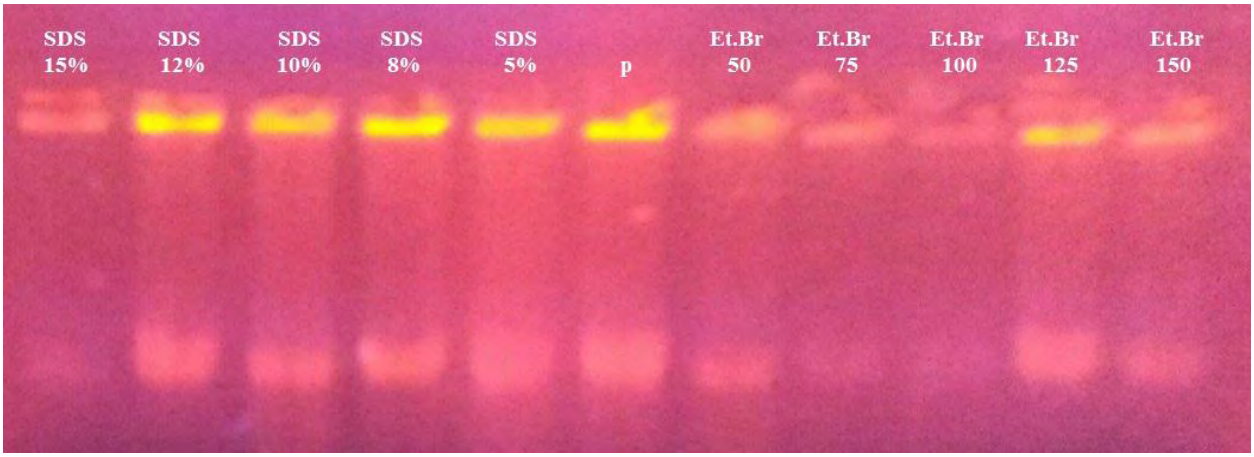


Figure 3.13: The result of gel electrophoresis showing the loss of plasmid bands.

3.3.2 Antibiogram (Kirby Bauer Disk Diffusion method):

The following tables showed the results of antibiotic susceptibility testing by disk diffusion method of these cured isolates for determination whether the transferred antibiotic resistance was plasmid mediated or chromosome mediated.

- As per previous results one *E.coli* isolate was resistant to AMP (25), AZM (15) and AMX (30). After curing treatment it was sensitive to AZM though resistance against AMP and AMX was still persistent. Therefore, it indicated that AZM resistance was plasmid mediated while AMX and AMP resistance was chromosome mediated.

Table 3.3: Antibiotic Susceptibility results of cured j (*E.coli*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AZM(15) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
1.	j5	15% SDS	25	Sensitive	-	Resistant	-	Resistant
2.	j8	Et.Br 100 µg/ml	27	Sensitive	-	Resistant	-	Resistant
3.	j9	Et.Br 125 µg/ml	24	Sensitive	-	Resistant	-	Resistant
4.	j10	Et.Br 150 µg/ml	28	Sensitive	-	Resistant	-	Resistant

- From table 3.4, it can be said that, another *E.coli* isolate was resistant to AMP (25), GEN (10) and AMX (30). After treating with curing agents, this isolate lost its sensitivity to GEN though it was resistant to AMP and AMX. Therefore, the results indicated that GEN resistance was plasmid mediated while both AMX and AMP resistance was chromosome mediated.

Table 3.4: Antibiotic Susceptibility results of cured U8 (*E.coli*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AMP(25) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity
1.	U8-3	10% SDS	-	Resistant	21	Sensitive	-	Resistant
2.	U8-5	15% SDS	-	Resistant	23	Sensitive	-	Resistant
3.	U8-9	Et.Br 125 µg/ml	-	Resistant	25	Sensitive	-	Resistant
4.	U8-10	Et.Br 150 µg/ml	-	Resistant	27	Sensitive	-	Resistant

- After comparing with the previous result of one *Klebsiella spp.* isolate, it can be said after curing treatment, this isolate had become sensitive to CIP, and with GEN they gave intermediate zone. Thus, it was indicated that for this isolate CIP and GEN resistance was plasmid mediated rather than chromosome mediated.

Table 3.5: Antibiotic Susceptibility results of cured g (*Klebsiella spp*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AMP(25) mm	Sensitivity	GEN(10) mm	Sensitivity	CIP(5) mm	Sensitivity
1.	g2	8% SDS	--	Resistant	18	Intermediate	23	Sensitive
2.	g3	10% SDS	-	Resistant	15	Intermediate	21	Sensitive
3.	g4	12% SDS	-	Resistant	19	Intermediate	20	Sensitive
4.	g5	15% SDS	-	Resistant	19	Intermediate	23	Sensitive
5.	g8	Et.Br 100 µg/ml	-	Resistant	15	Intermediate	25	Sensitive
6.	g10	Et.Br 150 µg/ml	-	Resistant	17	Intermediate	25	Sensitive

- From the following table, it can be observed that one *Klesiella spp.* isolate was sensitive to AZM, intermediate sensitive to GEN and resistant to AMP after curing treatment. As per previous result, this isolate was resistant to all the mentioned antibiotics. So, it was indicated that for this isolate AZM and GEN resistance was plasmid mediated while AMP resistance was chromosome mediated.

Table 3.6: Antibiotic Susceptibility results of cured U33 (*Klebsiella spp*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AMP(25) mm	Sensitivity	GEN(10) mm	Sensitivity	AZM(15) mm	Sensitivity
1.	U33-3	10% SDS	-	Resistant	15	Intermediate	21	Sensitive
2.	U33-4	12% SDS	-	Resistant	18	Intermediate	23	Sensitive
3.	U33-7	Et.Br 75µg/ml	-	Resistant	17	Intermediate	25	Sensitive
4.	U33-8	Et.Br 100 µg/ml	-	Resistant	16	Intermediate	24	Sensitive
5.	U33-9	Et.Br 125 µg/ml	-	Resistant	15	Intermediate	27	Sensitive
6.	U33-10	Et.Br 150 µg/ml	-	Resistant	19	Intermediate	25	Sensitive

- From table 3.7, it can be said that, another *Klebsiella spp.* isolate was resistant to AMP (25), GEN (10) and AZM (15). After curing, this isolate had become sensitive to AZM, intermediate sensitive to GEN and resistant to AMP. Therefore, it indicated that for this isolate AZM and GEN resistance was plasmid mediated while AMP resistance was chromosome mediated.

Table 3.7: Antibiotic Susceptibility results of cured U43 (*Klebsiella spp*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AMP(25) mm	Sensitivity	GEN(10) mm	Sensitivity	AZM(15) mm	Sensitivity
1.	U43-3	10% SDS	-	Resistant	15	Intermediate	21	Sensitive
2.	U43-4	12% SDS	-	Resistant	17	Intermediate	20	Sensitive
3.	U43-5	15% SDS	-	Resistant	18	Intermediate	22	Sensitive
4.	U43-6	Et.Br 50µg/ml	-	Resistant	17	Intermediate	-	Resistant
5.	U43-8	Etbr 100µg/ml	-	Resistant	14	Intermediate	23	Sensitive
6.	U43-9	Etbr 125µg/ml	-	Resistant	16	Intermediate	21	Sensitive
7.	U43-10	Etbr 150µg/ml	-	Resistant	18	Intermediate	23	Sensitive

- From the following table, it can be said that one *Enterobacter spp.* isolate gave intermediate zone to GEN though the resistance to AMP and AMX was still persistent after treating with curing agents. So, it can be assumed that for this isolate GEN resistance can be plasmid mediated while AMX and AMP resistance was chromosome mediated.

Table 3.8: Antibiotic Susceptibility results of cured d (*Enterobacter spp*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AMP(25) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity
1.	d4	12% SDS	-	Resistant	19	Intermediate	-	Resistant
2.	d6	Et.Br 50µg/ml	-	Resistant	19	Intermediate	-	Resistant
3.	d7	Et.Br 75µg/ml	-	Resistant	18	Intermediate	-	Resistant
4.	d8	Etbr 100µg/ml	-	Resistant	18	Intermediate	-	Resistant
5.	d9	Etbr 125µg/ml	-	Resistant	19	Intermediate	-	Resistant
6.	d10	Etbr 150µg/ml	-	Resistant	17	Intermediate	-	Resistant

- Similarly from table 3.9, it was observed that another *Enterobacter spp.* isolate lost its resistance to AZM (15), GEN (10) and VAN (30). Therefore, it indicated that for this isolate AZM, GEN and VAN resistance was plasmid mediated.

Table 3.9: Antibiotic Susceptibility results of cured U50 (*Enterobacter spp*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	VAN(30) mm	Sensitivity
1.	U50-3	10% SDS	21	Sensitive	23	Sensitive	-	Resistant
2.	U50-4	12% SDS	23	Sensitive	21	Sensitive	13	Intermediate
3.	U50-5	15% SDS	21	Sensitive	21	Sensitive	13	Intermediate
4.	U50-8	Etbr 100 µg/ml	22	Sensitive	24	Sensitive	14	Intermediate
5.	U50-9	Etbr 125 µg/ml	23	Sensitive	23	Sensitive	15	Intermediate
6.	U5010	Etbr 150 µg/ml	24	Sensitive	23	Sensitive	13	Intermediate

- The following table showed that another *Enterobacter spp.* isolate had become sensitive to AMP and AZM after curing treatment. So, for this isolate AMP and AZM resistance was plasmid mediated.

Table 3.10: Antibiotic Susceptibility results of cured i4 (*Enterobacter spp*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AMP(25) mm	Sensitivity	AMX(30) mm	Sensitivity	AZM(15) mm	Sensitivity
1.	i4-5	15% SDS	-	Resistant	-	Resistant	-	Resistant
2.	i4-7	Etbr 75µg/ml	22	Sensitive	-	Resistant	24	Sensitive
3.	i4-8	Etbr 100µg/ml	23	Sensitive	-	Resistant	25	Sensitive
4.	i4-10	Etbr 150µg/ml	23	Sensitive	-	Resistant	23	Sensitive

- From table 3.11, it can be said that one *Shigella spp.* isolate was resistant to AMP (25), GEN (10) and AZM (15). However, this isolate lost its resistance to AZM and GEN after curing treatment. Thus the result indicated that for this isolate AZM and GEN resistance can be plasmid mediated while AMP resistance was chromosome mediated.

Table 3.11: Antibiotic Susceptibility results of cured r (*Shigella spp*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AMP(25) mm	Sensitivity	GEN(10) mm	Sensitivity	AZM(!0) mm	Sensitivity
1.	r2	8% SDS	-	Resistant	27	Sensitive	21	Sensitive
2.	r5	15% SDS	-	Resistant	26	Sensitive	23	Sensitive
3.	r9	Etbr 125µg/ml	-	Resistant	27	Sensitive	21	Sensitive
4.	r10	Etbr 150µg/ml	-	Resistant	29	Sensitive	22	Sensitive

- As per previous result, one *Proteus spp.* isolate was resistant to AZM (15), AMP (25) and VAN (30). By comparing previous result with the following table, it can be said that the isolate lost its resistance to AZM and VAN after curing treatments. So, for this isolate AZM and VAN resistance was plasmid mediated while AMP resistance was chromosome mediated.

Table 3.12: Antibiotic Susceptibility results of cured p (*Proteus spp*) isolates.

No	Isolate Id	Concentration of curing agents	Antibiotics					
			AZM(15) mm	Sensitivity	AMP(25) mm	Sensitivity	VAN(30) mm	Sensitivity
1.	p5	15% SDS	23	Sensitive	-	Resistant	26	Sensitive
2.	p7	Et.Br 75 µg/ml	24	Sensitive	-	Resistant	25	Sensitive
3.	p8	Et.Br 100 µg/ml	22	Sensitive	-	Resistant	27	Sensitive

3.3.3 MIC and MBC (Agar Dilution method):

Agar dilution method was then done for the cured isolates In the following table the summary of MIC and MBC values of the cured isolates are given:

Table 3.13: Agar Dilution method test results of the cured isolates.

Isolate ID	Name of the organism	Ampicillin		Azithromycin		Ciprofloxacin	
		MIC Value (µg/ml)	MBC Value (µg/ml)	MIC Value (µg/ml)	MBC Value (µg/ml)	MIC Value (µg/ml)	MBC Value (µg/ml)
j	*E.coli*	Higher	Higher	30	60	1	10
U8	*E.coli*	Higher	Higher	100	150	Higher	Higher
g	*Klebsiella spp.*	100	150	100	150	15	20
U33	*Klebsiella spp.*	Higher	Higher	Higher	Higher	Higher	Higher
U43	*Klebsiella spp.*	Higher	Higher	30	60	20 (2 colonies)	50
d	*Enterobacter spp.*	Higher	Higher	100 (6colonies)	150	10	15
U50	*Enterobacter spp.*	Higher	Higher	100	150	Higher	Higher
I4	*Enterobacter spp.*	Total Sensitivity	Total Sensitivity	10	30	0.1	1
r	*Shigella spp.*	150	200	60 (3colonies)	80	0.1	1
p	*Proteus spp.*	100	150	60 (2colonies)	80	10	15

After comparing between table 3.2 and 3.13, it was observed that the MIC values for most of the cured isolates was lower than before. Most of the changes were observed for AZM. One *Enterobacter spp.* isolate has showed total sensitivity against AMP after the isolate was successfully cured. Therefore, it can be said that the results of agar dilution method was correlated with the results of antibiogram.

3.4 Transformation:

To observe if the transmission of antibiotic resistance gene occurs via transformation, three transformation pairs were selected. The selection of pairs are given in table 2.5. As per procedure, antibiotics containing plates were incubated with the transformant mixture. After 48 hours of incubation, there was no growth on the plates, indicating a negative result. The organisms did not acquire the resistance gene to grow on the plates. Therefore, it can be said that transformation did not occur between the donor plasmids and the recipient strain. The figure below shows no visible growth on the plates.

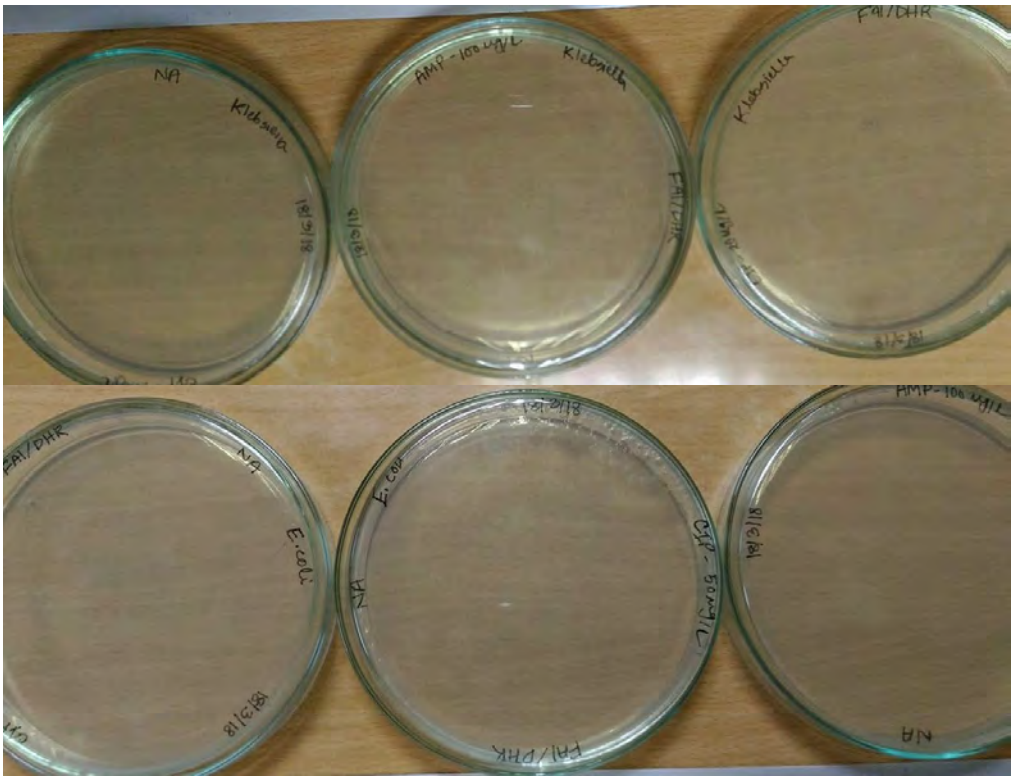


Figure 3.14: The plates show no visible growth after 48 hours of incubation, indicating a negative result.

Chapter 4

Discussion

4. Discussion:

Urinary Tract Infection is a very common bacterial infection nowadays and contributes a frequent cause of morbidity every year. The causative agents of UTI are members of *Enterobacteriaceae* family. *Escherichia coli* is the single most common microorganism, followed by *Klebsiella spp, Proteus* spp., and *Enterobacter spp.*, gram positive bacteria such as *Enterococcus* and *Staphylococcus* are also responsible for UTI. To treat this infection different types of antibiotics are used. Unfortunately, most of these causative bacteria have become resistant to more than one type of antibiotics, so treating this infection with conventional antibiotics is becoming difficult. That is why urinary tract infection has become a major threat and public health concern. The primary goal of this research was to understand the transmission of antibiotic resistance gene present in bacteria which are isolated from urine samples of UTI patients. Apart from determining whether the resistance was plasmid mediated or chromosome mediated, this research also focused on the transfer of plasmid via transformation.

This research included antibiotic susceptibility testing on the isolated organisms via Kirby Bauer Disk Diffusion method to determine if the isolates were multi drug resistant and Agar Dilution method to determine the MIC and MBC values of these isolates. Later, plasmid extraction and gel electrophoresis were done to observe the plasmid bands. It was assumed that the plasmid contains the antibiotic resistance gene. So, the isolates were selected based on the presence of plasmid to conduct further experiments. For this research ten isolates were selected. These are *E.coli* (2), *Klebsiella spp* (3), *Enterobacter spp* (3), *Shigella spp* (1) and *Proteus spp* (1). These ten isolates were then treated with different concentrations of plasmid curing agents: SDS and Et.Br. After curing treatment, plasmid extraction and gel electrophoresis were done to observe the loss of plasmid bands (if any). Antibiotic susceptibility tests were done again on the cured isolates to determine and compare the results of antibiogram and agar dilution method with the previous results of antibiogram and agar dilution method of these isolates. From these results three pairs of donor strain and recipient strain were selected and transformation was conducted.

For Kirby Bauer Disk Diffusion method, 8 different antibiotics disk (names and potency given in table 2.2) were used. The result of this method (table 3.1) showed that all these ten isolates were

resistant to Ampicillin and Amoxicillin. Gentamycin was also resistant to all these isolates except j (*E.coli)* and p (*Proteus spp*). All of these isolates were resistant to Azithromycin expect U8 (*E.coli)*, d (*Enterobacter spp*) and g (*Klebsiella spp*). In case of Ciprofloxacin, most of these isolates were either sensitive or intermediate. After that, MIC and MBC values were also determined to re-confirm the results of antibiogram. The results of Agar Dilution method (table 3.2) showed the MIC value of these isolates against AZM, AMP and CIP. Following antibiotic susceptibility testing, plasmid extraction by both Alkaline Lysis method and Kado and Liu method were conducted. Gel electrophoresis confirmed the presence of plasmid bands.

These isolates were then treated with different concentrations of curing agents (table 2.4). Again, plasmid extraction and gel electrophoresis were done to determine the loss of plasmid bands. From figure 3.5-3.14, it can be seen that all of these ten isolates have lost at least one plasmid band in different concentrations of SDS and Et.Br. So, the result of curing treatment is positive. For example, isolate r (*Shigella spp.*) had three plasmid bands before curing. After the treatment the same isolate now has two plasmid bands.

Again, antibiotic susceptibility tests on the cured isolates were done. Kirby Bauer Disk Diffusion method was done so that we can determine whether the antibiotic resistance is plasmid mediated or chromosome mediated. From table 3.3 – 3.12 it can be determined that after curing treatment all these isolates have lost their resistance to a particular antibiotic/antibiotics. So, it can be said that the isolates had plasmid mediated resistance. For example, before curing isolate g *(Klesiella spp.*) was resistant to AMP, GEN and CIP, after curing now the same isolate has become sensitive to CIP and intermediate to GEN. The MIC and MBC values of these cured isolates have also changed. The change in agar dilution method result was similar to the change in antibiogram.

For further investigation, j (*E.coli)*, g (*Klesiella spp.*) and i4 (*Enterobacter spp.*) were selected as donor strains based on the results of antibiotic susceptibility tests of these isolates. As recipient *E.coli* DH5α strain was selected. The antibiotics for these pairs were consequently AZM, CIP and AMP. The concentrations of the antibiotics used on the agar plates were 50µ/ml for AZM and CIP, 100µg/ml for AMP. Figure 3.15 showed that transformation did not occur between these donor and recipient pairs, indicating negative result.

There are several reasons for a negative transformation result. Transformation between donor and recipient occurs at a very slow rate. The factors that affect transformation efficiency are the strain of bacteria, the bacterial colony's phase of growth, the composition of the transformation mixture, and the size and state of the foreign DNA. Transformation is a very sensitive procedure, hence any of the above mentioned factors can cause a negative impact on the procedure causing a negative result.

From this research it can be said that the multi-drug resistance gene of these isolates is both plasmid and chromosome mediated. For example, isolate j (*E.coli*) contained both plasmid mediated resistance (AZM)) and chromosome mediated resistance (AMP and AMX). Also, from all the results it can be determined that AZM and GEN resistance was plasmid mediated while AMP resistance can be both plasmid and chromosome mediated.

Based on this study results it can be said that, Et.Br was more effective than SDS. The loss of plasmid bands mostly occurred on the different concentrations of Et.Br rather than SDS. For instance, the isolate d (*Enterobacter spp.*) has lost plasmid bands with all of the five concentrations of Et.Br but with only one concentration of SDS. Also, curing was more successful with higher concentrations of SDS and Et.Br.

Even after a successful curing, all the plasmid bands were not completely lost due to the high copy number of plasmids. The higher the copy number of plasmid is, the more difficult it is to cure all the plasmid bands. The effectiveness of a curing agent does vary considerably in rang of 100- to 1000-fold. This depends on the organisms being treated, curing agent efficiency and efficacy, and the mode of action the curing agents (Carlton and Brown, 1981). The curing agents are harmful to human beings and can cause potential mutation in the host chromosome which interferes with the functional analysis of the plasmid. Therefore, alternative methods are being developed to overcome these disadvantages.

The emergence of multi drug resistant bacteria is increasing day by day because the antibiotic resistance gene in plasmid is transferring from one strain to several strains in nature. For this reason the detection of resistance gene transmission is very important. This information is necessary in the global surveillance management of environmental multi-drug resistance (Letchumanan, Chan and Lee, 2015). Successful curing research has been done on the organisms

E.coli, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus etc.

Chapter 5

Conclusion

5. Conclusion:

Urinary Tract Infection (UTI) is a very common and major health issue. Most of the UTI causing bacteria are multi drug resistant, thus it is a major threat and challenge for the future. Antibiotic resistance can be either plasmid mediated or chromosome mediated. Transformation is a horizontal gene transfer method which causes transmission of resistance gene between species, thus very important in the evolution. If the transmission of resistance gene does not occur by horizontal gene transfer method, then the emergence of the resistance gene is chromosome mediated. By successful plasmid curing, this can be determined. Plasmid curing assay is fast, cost effective and gives sufficient knowledge to provide an elaborate result. However, modern methods for plasmid curing is available and they are – next generation sequencing (NGS), diagnostic displacement by specific incompatibility, microarray technique. In this current era of science and technology further researches are being conducted to yield more specific knowledge for the analysis of the antibiotic resistance gene transmission in bacteria.

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Appendix

Appendix- I

Media compositions

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

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

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

Luria Bertani Broth

Component	Amount (g/L)
Yeast Extract	5.000
Casein hydrolysate	10.000
Sodium Chloride	10.000
Agar	15.000
Final pH	7.2± 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 and stored at 4ºC.

0.5M EDTA:

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

2N NaOH:

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

TBE Buffer:

In a Durham bottle, 5.4 g of Tris base, 2.75 g of Boric Acid, 2 ml of 0.5M EDTA were added. Then 500 ml distilled water was added to prepare 500 ml TBE Buffer. After that, pH of the bottle was adjusted to 8. Then it was autoclaved and stored at room temperature.

APPENDIX – III

Instruments:

The important equipment used through this study are listed below

Autoclave	Model: WIS 20R Daihan Scientific Co. ltd, Korea
Sterilizer	Model no: NDS-600D, Japan
Balance machine: Adam	UK
Freezer (-20^{o} 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