Plasmid profile analysis and curing of plasmids in Enterobacteriaceae isolated from patients of 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 of Authenticity

I hereby humbly declare that this thesis is entitled **"Plasmid profile analysis and curing of plasmids in Enterobacteriaceae isolated from patients of Urinary Tract infection"** submitted by the undersigned has been carried out under the supervision of Ms. Namista Islam (Lecturer, Department of Mathematics and Natural Sciences, BRAC University) at BRAC University, Mohakhali Dhaka.

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

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Dedicated

to

My Beloved Parents

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Abstract

Urinary tract infection (UTI) is one of the most frequent bacterial infections worldwide. It is caused recurrently by *Escherichia coli* and also by *Klebsiella spp., Enterobacter spp*., *Shigella spp*. etc. Theyshow resistance to various commonly prescribed antibiotics. They harbor plasmids that might be responsible for antibiotic resistance. This study was undertaken to find a link between antibiotic resistance and plasmids *of E. coli*, *Klebsiella spp., Enterobacter spp*., *Shigellaspp*. Curing was done to investigate if those antibiotic resistance plasmids can be eliminated by treating the isolates with different concentrations of ethidium bromide and sodium dodecyl sulfate. A total of 10 identified isolates obtained from UTI patients of a Diagnostic Center in Bangladesh were investigated. By using Kirby-Bauer Disk Diffusion method, antibiotic susceptibility to various antibiotics were tested. The isolates showed resistance to Azithromycin, Amoxicillin, Gentamicin and Ampicillin. MIC and MBC values for the antibiotics Azithromycin and Ampicillin were also determined by using agar dilution method. After antibiotic susceptibility test was done, the plasmids of the isolates were extracted by Kado-Liu method and separated by using Agarose Gel Electrophoresis. Then the isolates were subjected to plasmid curing procedure with different concentrations of Ethidium Bromide (EtBr) and Sodium Dodecyl Sulfate (SDS). After curing treatment the isolate‘s different strains underwent another antibiotic susceptibility testing. In this study ethidium bromide was more successful in terms of plasmid curing. The isolates which showed plasmid losses after the curing procedure, showed changes in resistance patterns. These results reveal that in terms of the studied isolates, Azithromycin and Gentamicin resistance might be carried in harboring plasmids whereas Amoxicillin resistance is not carried in the plasmid. In the plasmid curing procedure the most effective concentration was proved to be 150µg/ml for Ethidium Bromide and 15% for Sodium Dodecyl sulfate.

Table of Contents

SL no.	Title	Page Number
1	Introduction	10
2	Materials & Methods	19
3	Results	28
4	Discussion	47
5	Conclusion	54
6	References	56
7	Appendices	63

List of Tables

Table No.	Title	Page
2.3.1	Name of the organisms	20
2.6.1	Concentration of Antibiotics used during Antibiotic Susceptibility testing	21
2.11.1	Concentrations of EtBr abd SDS used in plasmid curing	26
3.1	Antibiotic Susceptibility Results of the initial isolates	29
3.2	Antibiotic Susceptibility testing for MIC MBC of primary isolates	30
3.3	Curing Treatment result summary	36
3.4-3.12	Antibiotic Susceptibility Results of Different Isolates	36-40
3.13-3.14	Primary MIC/MBC	41
3.15	Antibiotic Susceptibility testing for MIC MBC	42
3.16	Final result summary for Plasmid Curing	43-44

List of Figures

Fig No.	Name of the figure	Page no.
3.1	Colony morphology of the isolates	28
3.2.1	Antibiogram Results	30
3.2.2	MIC/MBC determination of primary isolates	31
3.3	The results of Gel Electrophoresis shows the presence of plasmids in the isolates	32
3.4-3.7	Result of gel electrophoresis showing the loss of plasmid bands	33-35
3.8	MIC/MBC determination of curing agent treated isolates	43
3.9	Transformation Plates	45

List of Abbreviations

UTI	Urinary Tract Infections
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
AZM	Azithromycin
AMP	Ampicillin
SPP	Species
mg	Miligram
ml	Mililiter
µl	Microliter
µg	Microgram

Chapter 1
Introduction

1. Background

The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives. Many decades after the first patients were treated with antibiotics; bacterial infections have again become a threat (Golkar, 2014). The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications. A number of bacteria have been classified as urgent, serious, and concerning threats, many of which are already responsible for placing a substantial clinical and financial burden on the health care system, patients, and their families (Sengupta, 2015). 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. Coordinated efforts to implement new policies, renew research efforts, and pursue steps to manage the crisis are greatly needed. In a third world country like Bangladesh where medicines and antibiotics are being sold without prescription the threat of antibiotic resistance is increasing day by day. The occurrence of multidrug-resistant (MDR) bacteria to clinically used antibiotics is a major health issue and a great challenge to the worldwide drug discovery programs (Alanis, 2005).

1.1 Urinary tract infection

Urinary tract infection (UTI) is a serious health problem affecting millions of people each year. This is the second most common type of infection in human. Urinary tract infection is a common community-acquired bacterial disease, which frequently affects female than male (Dromigny, 2006). Increasing rates of resistance among bacterial uropathogens has caused growing concern in both developed and developing countries (Gupta, 2002). *Escherichia coli*, the most common member of the family Enterobacteriaceae, accounts for 75-90% of all urinary tract infections in both in-patients and out-patients. *Klebsiella spp.* is the second most frequently found organism in UTI patients. Antibiotics are the typical treatment for UTIs. Therefore, multidrug-resistant organisms are frequently found in urinary tract infection (UTI) (Calbo, 2006). 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.

1.2 Causative agent:

Urine is generally considered to be sterile and is believed to be germ free. Any source of possible infection occurs through urethra which initiates the incidence of the infection. The predominant pathogen responsible for UTI is *E. coli* which constitutes up to 80-85% and is followed by *Staphylococcus saprophyticus* which accounts to 5-10% (John, 2017). The occurrence of the infection due to viral or fungal agents is a rare phenomenon. In addition to the above mentioned bacterial species, *Klebsiella*, *Proteus*, *Pseudomonas* and *Enterobacter* are associated with UTI. The bacteria enter the bladder through urethra and the infection can also occur through blood and lymph. The microbial etiology of UTIs is deemed to be well established and frequent (Farajnia, 2009). Pathogens like *E. coli* and *S. saprophyticus* are associated with population acquired acute uncomplicated infection whereas *Klebsiella*, *Enterococcus*, *Proteus Species*, *Enterobacter, Bacillus, Shigella* are known to confer uncomplicated cystitis and phylonephritis that are sporadic (Vasudevan, 2014).

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 revealed that among 462 urine samples collected from patients with UTI, *E. coli* was the predominant organism, whereas *Klebsiella* and *Enterococcus* were also prevalent (Noor, 2013). 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 *streptococc*i, can also infect these parts (Kattan & Gordon, 2013).

E. coli causing UTI belongs to several subgroups that are selected by some factors, which enhance extra intestinal survival. Hese factors include structural features such as fimbrae or pili for adherence, flajella for motility and chemical adhesion (Kerenyi, 2003) . He type-1 pili of uropathogenic E. coli are known to be associated with increased severity of UTI by binding to

mannose containing glycoprotein receptors on facet cells lining the bladder or vaginal epithelial cells (Venegas, 1995). Other virulence factors which confer the ability to fecal E. coli to colonize the vaginal mucosa and cause symptomatic UTI have also been identified.

1.3 Antibiotic Resistance in causative agents of UTI

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). However, the ability of E. coli to cause UTIs is increasing, while the ease of treating these infections due to resistance to first generation antibiotics such as cotrimoxazole, ampicillin and nitrofuranton is becoming progressively more elusive. The greater concern is the recent increase in resistance to second or even third generation antibiotics such as ciprofloxacin, levofloxacin and ceftriaxone(Hooton, 2000). These resistances are harbored in the resistance plasmids and in chromosomes.

1.4 Plasmid and Plasmid Curing

Plasmids are extrachromosomal pieces of double-stranded circular DNA which have the capability to replicate independently of the host chromosome, yet coexist with it . To date, many species of bacteria isolated from diverse habitats are known to contain plasmid DNA. Some

plasmids are stable and can be maintained through successive generations by being partioned to each daughter cell during cell division. This allows each cell to receive at least one plasmid copy.

In recent years, plasmids have been observed in a wide variety of bacteria. In part, this is due to the development of new procedures that allow the detection, isolation, and molecular characterization of plasmid DNA. When working with some plasmid-containing bacteria, it is often desirable to obtain a plasmid-cured derivative. This allows a direct comparison to be made between the plasmid-containing and plasmid-cured cells. Some plasmids undergo spontaneous segregation and deletion. However, the majority are extremely stable, and require the use of curing agents or other procedures to increase the frequency of spontaneous segregation. The usefulness of curing agents is unpredictable in many bacterial strains, as there are no standard protocols applicable to all plasmids. However, there are some procedures that have provided good results with certain species.

Plasmid is one of the several environmental and genetic factors that carry the resistance property against a specific drug or a number of drugs in bacteria (Clowers, 1972). R-plasmids from resistant strains of an organism may transfer to a sensitive counterpart that would in due course show the same drug resistance as the donor strain (Datta, 1972). Plasmids can also be eliminated by curing agents which can be used to display the role of R-plasmid in drug resistance. The techniques used to promote curing include exposing the host strain to elevated temperatures, use of chemical agents such as intercalating dyes (acridine orange, ethidium bromide), treatment with crystal violet, sodium dodecyl sulfate (SDS), thymidine starvation and exposure to UV radiation. Different plasmids vary considerably in their property to be cured, and not necessarily depending upon properties of specific plasmid.

As no universally effective curing agent has yet been identified, curing experiments are generally conducted on trial and error basis, both with respect to the choice of the curing agent and the culturing conditions used. Some curing agents work in a non-specific way by damaging and stressing out the cells, while some seem to act much selectively (Hohn, 1969). Curing of plasmids from bacteria strains is a way to eliminate the bacteria plasmid and determine the antibiotic resistance mediation.

The mechanism of plasmid curing starts from the inhibition of plasmid replication resulted from a single nick, outside of the replication origo 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). Resistance is usually classified as “chromosomal” when unaffected by plasmid curing and as “plasmidial” when affected.

1.4.1 Intercalating Agents

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. Basically, overnight bacteria cultures are inoculated into enrichment broths, Tryptic Soy Broth (TSB), or Luria Bertani Broth (LB). Curing agents at a concentration ranging from 0.1 to 0.5 mg/ml is added to the culture broth. The concentration depends on the organism and curing agent used. The cultures are then incubated overnight at 35 or 37°C under constant agitation. After the treatment, antibiograms assay were again performed to find antibiotic resistance phenotypes.

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 & 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).

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.

1.4.2 Sodium Dodecyl Sulfate

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

1.5 Bacterial Transformation

In the 1928 the experimental work by Frederick Griffith using the pneumococcal bacteria, identified two forms or strains. One of them is smooth (S) cells which are virulent, and

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

Enterobacter spp and *E.coli* is not assumed to be naturally transformable; it develops high genetic competence only under artificial conditions, e.g. exposure to high Ca2+ concentrations (Méjean, 1993). However, several reports have shown that E. coli can express modest genetic competence in certain conditions that can arise in its environment and make it subjected to horizontal transformation (Lorenz, 1994). We herein report that inheritance of antibiotic resistance occurs due to transconjugation or transformation of plasmids. It has been further discussed that a particular plasmid is responsible for resistance against a specific antibiotic.

1.6 Literature review

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.

Yano et al. (2014) demonstrated the use of EB to eliminate plasmids in antibiotic resistant *Vibrio* species isolated from shrimp cultured in inland ponds in Thailand. Typically the resistant isolates were grown in TSB supplemented with 0.2 mg/ml EB. The oxytetracycline resistance phenotype was eliminated through plasmid curing. The authors suggested that the resistance to oxytetracycline was related to R-plasmids (Yano et al., 2014).

Manjusha and Sarita (2011) performed plasmid curing using 0.05 to 0.5 mg/ml of EB. It was evident from the curing experiment that all of the *Vibrio* strains loss their plasmids when treated with concentration of 0.3 mg/ml EB and demonstrated a change in their resistance pattern. In their study, 79% of the *Vibrio* strains was devoid of plasmid but showed an antibiotic resistance pattern, which indicate chromosomal resistance. The isolates exhibited a chromosomal borne resistance toward amoxicillin, ampicillin, furazolidone, and tetracycline after curing assay. The authors concluded that some of these resistances may be encoded on plasmids in some strains, while the other isolates may be chromosomal mediated.

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.

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

molecular aspects. The physiological aspects and the treatments were also covered extensively as a part of this study.

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).

1.7 Aims and Objectives

The purpose of this study was to determine the mediation way of antibiotic resistance carrying plasmids of bacteria that was collected and isolated from UTI patients of a Diagnostic Center in Bangladesh through plasmid curing method. The objectives of the study are given below.

- Understanding the mediation method of multiple drug resistance plasmid.
- Understanding the effects of plasmid curing agent on UTI causing organisms.
- Understanding the dissemination way of plasmid transfer.

Chapter 2
Materials and Methods

2. Methodology

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 Period of study

The research work was carried out from November 2017 till June 2018.

2.3 Microbiological Sample

A total of 90 isolates were collected and identified from Urinary Tract Infected patients from a Diagnostic Center in Dhaka. The isolates were a diverse range of species including gram-negative and gram-positive bacteria. In this study, 10 identified isolates were picked for performing plasmid curing and transformation experiment.

Table 2.3.1: Name of the organisms

Sl. no	Name of the organism	Organism ID
1.	*E.coli*	Y
2.	*E.coli*	K
3.	*Klebsiella*	O
4.	*Klebsiella*	S
5.	*Klebsiella*	T
6.	*Shigella*	B
7.	*Shigella*	L
8.	*Enterobacter*	E
9.	*Enterobacter*	I5
10.	*Enterobacter*	H

2.4 Microbiological culture of the sample

The samples were initially cultured in nutrient agar, put away for at least 18 – 24 hours incubation at 37°C. Nutrient Agar media is the general purpose medium for the growth of a wide variety of non-fastidious microorganisms consisting of appropriate amount of necessary nutrients for growth and replication of the organisms. The sample to be cultured was taken from the stock and streaked in the four quadrant method, on the nutrient agar medium. After incubation, the plates had growth of the desired organism and individual single colonies were taken for further procedures, ensuring a pure culture of the desired organism.

2.5 Experimental Design

1. Samples collected from UTI patients of a diagnostic center.
2. Identified isolates from glycerol stock revived on Nutrient Agar.
3. Antibiotic susceptibility testing using the Kirby Bauer Disk Diffusion.
4. MIC- MBC of each isolate done by agar dilution method.
5. Plasmid extraction.
6. Gel electrophoresis.
7. Plasmid curing of the isolates with EtBr and SDS.
8. MIC- MBC of cured strains done by agar dilution method.

2.6 Antibiotic Sensitivity Testing

2.6.1 Disk Diffusion Method (Kirby Bauer Method)

An antibiotic is an anti-microbial chemical that inhibits the growth or destroys the microorganism. These antibiotics are used to treat infectious diseases caused by microorganisms. These antibiotics are both natural and synthetic (man-made) and several generations of these synthetic antibiotics have been invented to meet the requirements of the human population.

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

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

Consequently, the MHA plates were kept at 37 °C for incubation for 24 – 48 hours. After incubation, the zone of inhibition were measured and compared against the CLSI reference chart for the antibiotic susceptibility, as either resistant (R), intermediate (I) or sensitive (S).

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

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

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

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

Table 2.6.1: Concentration of Antibiotics used during Antibiotic Susceptibility testing		
Sl. No.	**Concentration of Azithromycin (μg/ml)**	**Concentration of Ampicilin (μg/ml)**
1	1	1
2	10	10
3	30	30
4	60	60
5	80	80
6	100	100
7	150	150
8	200	200

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

2.7 Plasmid Extraction

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

2.7.1 Kado-Liu Method

This particular method of plasmid extraction is considered as one of the fastest techniques to isolate plasmids. For plasmid isolation, the cells are treated with Tris Buffer, a typical buffering substance for DNA with buffering capacity in the slightly alkaline range (pH 7.5 – 8.2) and EDTA (Ethylene-diamine tetra-acetic acid) which is responsible for the inhibition of the nuclease activity.

This procedure requires fresh culture of organism which is inoculated in nutrient broth, which allows the cells to proliferate. After overnight incubation, 1.5 ml of the culture is taken in a micro-centrifuge tube and is spun at 14000 rpm for 5 minutes. The supernatant was discarded and first solution Kado-I was added and mixed gently via pipetting. Kado-I is a solution that comprises of Tris HCl and EDTA. Consequently, the second solution was added, Kado-II, which essentially contains Tris Buffer, SDS and 2N Sodium Hydroxide and was homogenized by rolling the tube. Then, the mixture was put away in a water bath at 55°C for an hour. After the hour long incubation, 400µl Phenol-Chloroform was added and the mixture was hand-mixed by lateral inversion for 30 minutes. Finally, the mixture was centrifuged again and the top layer consisting of plasmid is separated out in a fresh micro-centrifuged tube for storage at 4°C freezer.

2.7.2 Alkaline Lysis Method

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

2.8 Agarose Gel Electrophoresis

After the successful extraction of plasmid, the next procedure that is carried out is, gel electrophoresis, to test the presence of plasmids in the desired organisms. Agarose Gel Electrophoresis is a technique that separates DNA fragments on the basis of molecular weight and the charge applied by the electric fields in the apparatus. Therefore, in order to analyze the plasmids that have been extracted earlier, at first, 0.7% gel is prepared separately using 80ml TBE buffer solution and 0.56g of Agarose powder. Ethidium Bromide, an intercalating agent used as a fluorescent tag, is also added to the gel. Then, the electrophoresis apparatus is set up using the power supply, gel is set onto the apparatus and the samples are loaded along with the dye (Bromophenol Blue). At the voltage of 70 and time of about 2 hours, the gel run is complete and the bands are seen under the Ultra-violet (UV) radiation.

2.9 Plasmid Curing

Plasmids are known to be extra-chromosomally replicating molecules of DNA that harbors the resistance property against a specific antibiotic or a certain class of antibiotics. Given that plasmids tend to play one of the crucial roles in the gene transfer in transformation and without which transformation may not even take place. There is a type of plasmid, known as the R-plasmids, which is the resistance plasmids, carrying the resistance genes against the antibiotics.

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

The protocol of curing plasmids goes as such, first the organisms to be cured is cultured and incubated overnight at 37°C. The 1ml fresh culture has been inoculated in 9ml nutrient broth containing different concentrations of the curing agents, like ethidium bromide (EtBr) and sodium dodecyl sulfate (SDS). The concentrations of SDS and EtBr used are listed in the table 2.9.1.

Table 2.11.1: Concentrations of EtBr abd SDS used in plasmid curing

Concentration of EtBr / (μg/ml)	**Amount of EtBr / (μl)**	**Concentration of SDS (%)**	**Amount of SDS / (g)**
50	45	5	0.5
75	67.5	8	0.8
100	90	10	1
125	112.5	12	1.2
150	135	15	1.5

After overnight incubation of the cultures in EtBr and SDS were plated on LB agar containing plates with different concentrations of antibiotics. Same as mentioned in table above. Presence or absence of growth in the antibiotic containing plates enables us to determine whether the resistance gene is present in the chromosomal DNA or in the plasmid [Trevors J.T., 1986].

2.10 Transformation

E. coli DH5α, was collected from department of microbiology, Brac University, Bangladesh. The pure culture of studied isolates and *E. coli* DH5α was routinely sub-cultured on nutrient agar.

Competent cells of E. coli DH5α were prepared as described by Sambrook et al. For transformation, 50 μl of plasmid DNA was added to a tube containing 0.2 ml of competent cells. The mixture was placed on ice for 20 min, and then exposed to heat shock at 42°C for 1 min and kept immediately in ice for 10 min. A 800 μl LB broth was then added to transformation mixture and incubated at 37ºC for 60 min. About 100 μl from transformation mixture was spread on nutrient agar plates containing the appropriate antibiotic. An aliquot (100 μl of 5-10 fold diluted)

of competent cells spread on nutrient agar containing the same antibiotics was used as a control. All plates were incubated at 37°C for 48 h.

CHAPTER 3
Results

3. Results

3.1 Microbiological culture of the organisms

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

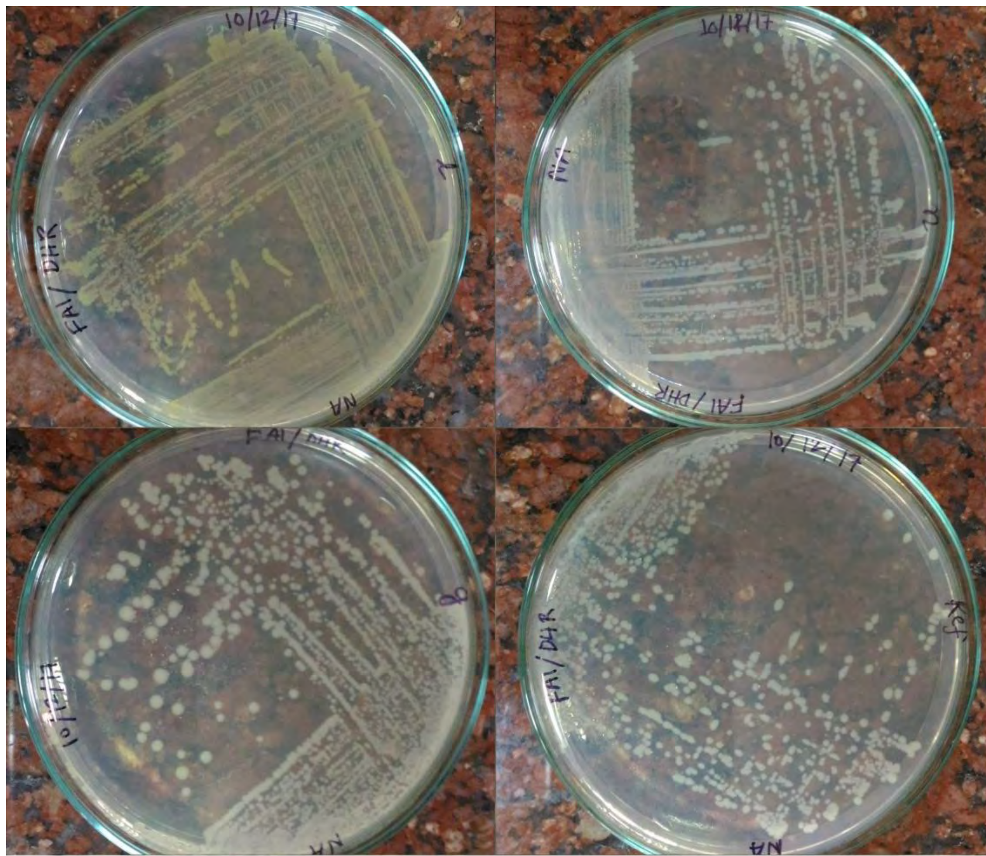

Figure 3.1 Colony morphology of the isolates

3.2 Antibiotic Susceptibility Testing

3.2.1 Antibiogram- 1

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

Table 3.1: Antibiotic Susceptibility Results of the initial isolates									
Isolate Id	Name of the Organism	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
Y	*E.coli*	Nil	R	13	I	Nil	R	Nil	R
K	*E.coli*	15	I	Nil	R	Nil	R	8	R
O	*Klebsiella*	14	I	15	I	Nil	R	Nil	R
S	*Klebsiella*	15	I	13	I	Nil	R	Nil	R
T	*Klebsiella*	17	I	19	S	Nil	R	Nil	R
B	*Shigella*	12	I	14	I	Nil	R	Nil	R
L	*Shigella*	13	I	Nil	R	Nil	R	Nil	R
E	*Enterobacter*	14	I	13	I	Nil	R	Nil	R
I5	*Enterobacter*	10	R	10	R	Nil	R	Nil	R
H	*Enterobacter*	11	R	15	I	Nil	R	Nil	R

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

The table above describes the results obtained from the antibiotic susceptibility testing using the Kirby Bauer method. Given the results and analysis of all the isolates it can be said that two isolates of *E. coli* are resistant to Azithromycin, Amoxicillin and Ampicillin. While one isolate shows intermediate resistance against Gentamicin. Other organisms are resistant to Amoxicillin and Ampicillin.

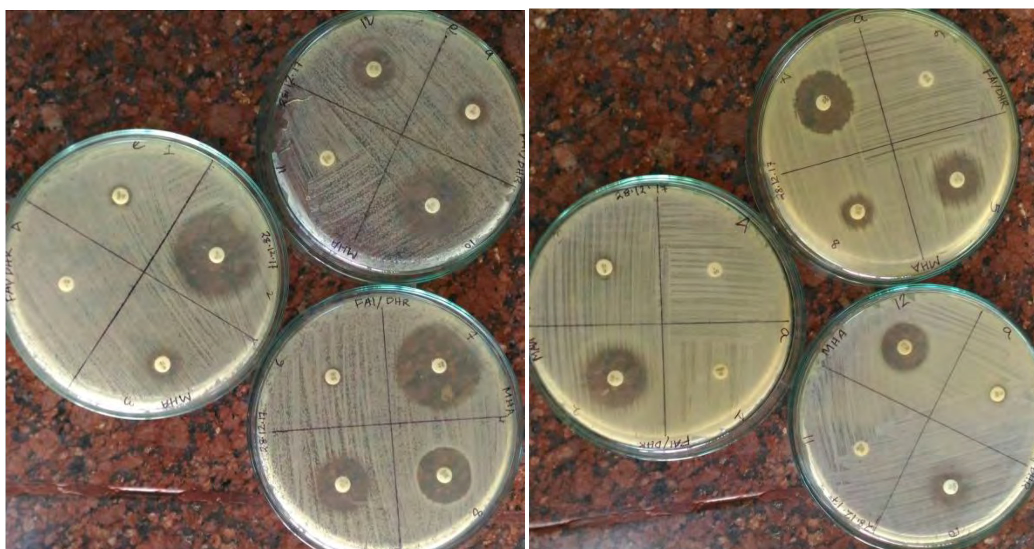

Figure 3.2 the plates shows the zone of inhibitions after Kirby Bauer Disk Diffusion

3.2.2 MIC & MBC Determination -1

Minimum inhibitory concentration and Minimum Bactericidal concentration were also determined by using agar dilution method. The antibiotics that were used are Azithromycin and Ampicillin. The details are given in the following table.

Table 3.2 Antibiotic Susceptibility testing for MIC MBC of primary isolates

		MIC Value (µg/ml)		**MBC Value (µg/ml)**	
Name of the Organism	Organism ID	Azithromycin	Ampicillin	Azithromycin	Ampicillin
E.coli	Y	>200µg/ml	>200µg/ml	>200µg/ml	>200µg/ml
E.coli	K	>200µg/ml	150 µg/ml	>200µg/ml	200 µg/ml
Klebsiella	O	>200µg/ml	>200µg/ml	>200µg/ml	>200µg/ml
Klebsiella	T	>200µg/ml	>200µg/ml	>200µg/ml	>200µg/ml
Klebsiella	S	150µg/ml	>200µg/ml	200µg/ml	>200µg/ml
Shigella	B	>200µg/ml	>200 µg/ml	>200µg/ml	>200 µg/ml
Shigella	L	100 µg/ml	100 µg/ml	150 µg/ml	150 µg/ml
Enterobacter	E	80 µg/ml	>200 µg/ml	100 µg/ml	>200 µg/ml
Enterobacter	I5	>200 µg/ml	>200 µg/ml	>200 µg/ml	>200 µg/ml
Enterobacter	H	>200 µg/ml	>200 µg/ml	150 µg/ml	200 µg/ml

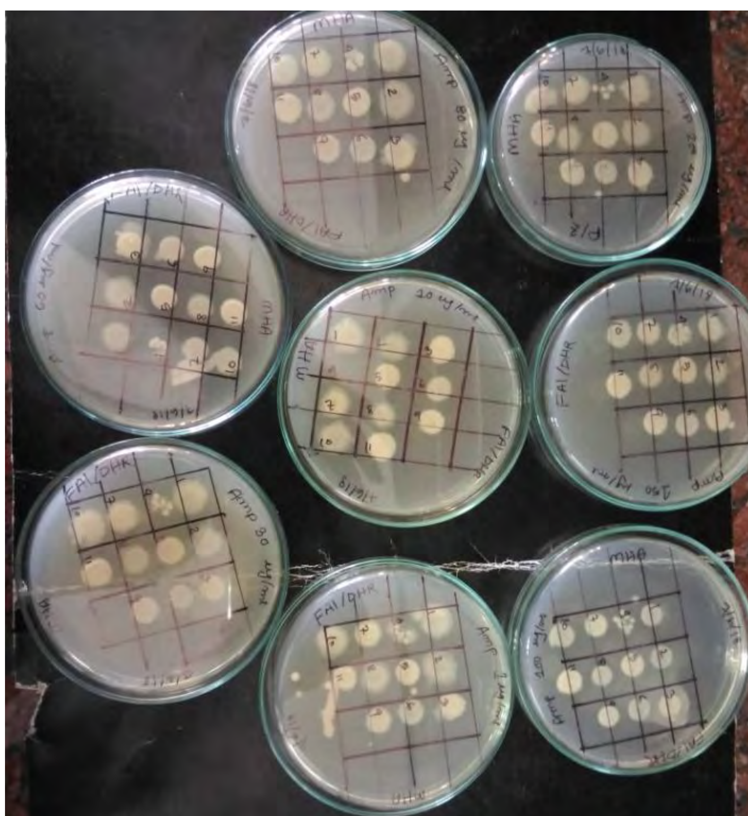

Figure 3.2.2- MIC/MBC determination of primary isolates

3.3 Plasmid Isolation and Gel Electrophoresis

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

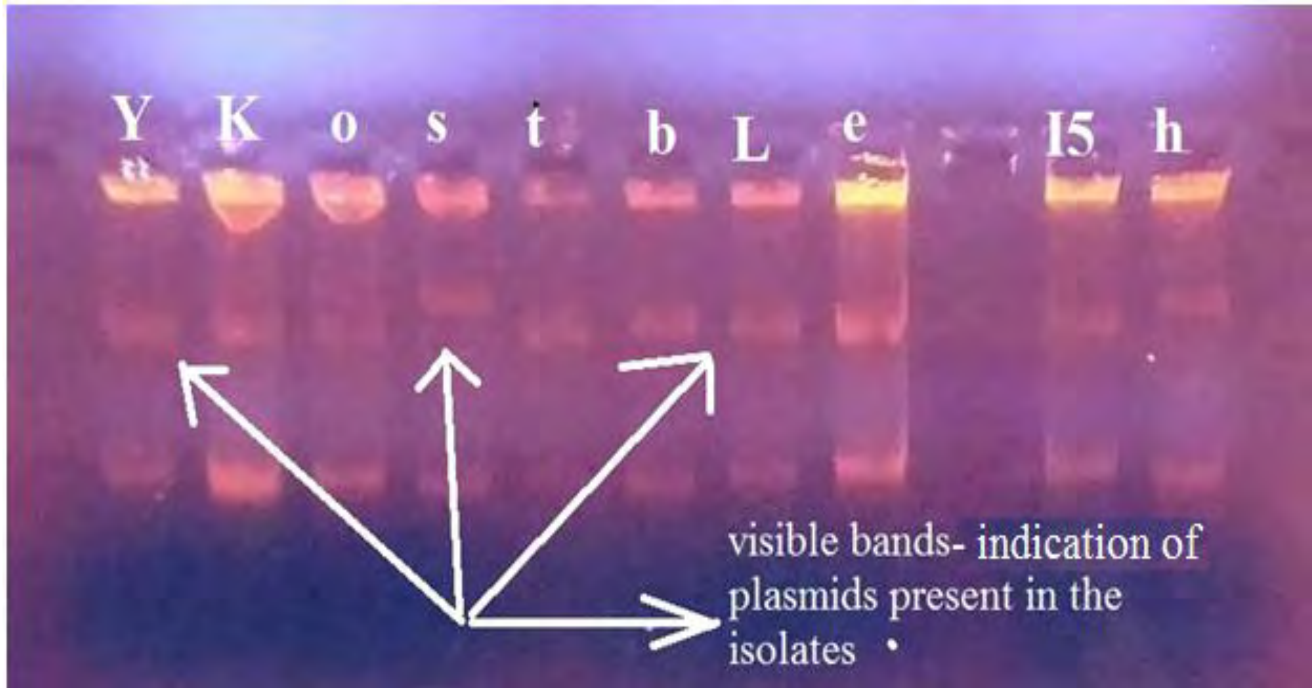

Figure 3.3 The results of Gel Electrophoresis shows the presence of plasmids in the isolates.

3.4 Plasmid Isolation and Gel Electrophoresis of Cured Isolates (Plasmid Curing):

As mentioned in the methods section plasmid curing was conducted using EtBr and SDS. After performing the curing procedures, plasmids were extracted again and gel electrophoresis was performed to observe whether there were any loss of bands which would suggest that particular strains is cured.

I. Organism- *E. coli*

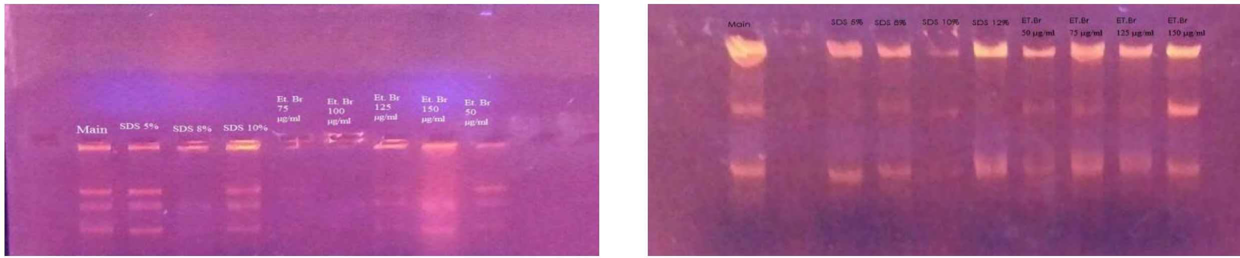

Fig3.4 : The result of gel electrophoresis showing the loss of plasmid bands

The extracted plasmids of isolate Y shows several bands under UV light. It was found that the well which contains 8% SDS treated plasmids, the first band is missing. Same goes for Et. Br- 75 µg/ml, Et.Br- 100 µg/ml, Et. Br- 125 µg/ml treated strains. It can be concluded that these 4 strains were possibly cured. For the isolate K several separated bands are seen when the gel is illuminated by UV light. Here the first well contains the main isolate‘s plasmid. From the picture it can be deduced that the well which contains SDS 15% treated plasmids of isolate K, the first band is not present. Same goes for Et. Br- 75 µg/ml and Et.Br- 100 µg/ml treated strains. It can be concluded that these 3 strains were possibly cured.

II. Organism – *Enterobacter*

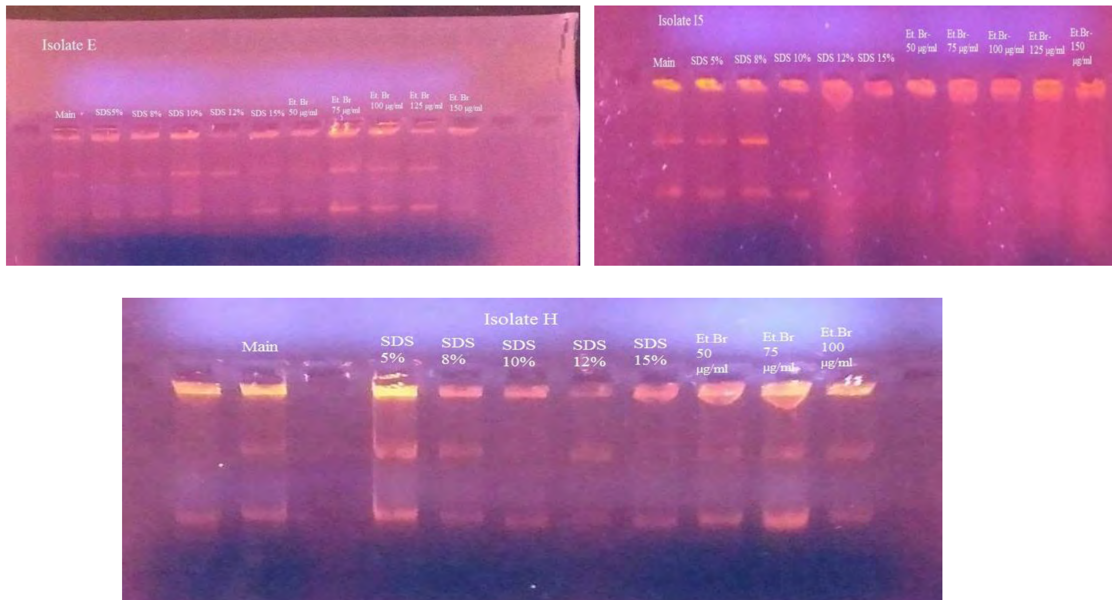

Figure 3.5: The result of gel electrophoresis showing the loss of plasmid bands

When illuminated by the UV light, the extracted plasmid from isolate E shows two bands. After curing, it was observed that some of the cured isolate has lost a band. The isolates which were treated with the concentrations of 5% SDS, 8% SDS, 15% SDS, Et.Br 150µg/ml and Et. Br- 50 µg/ml are among those isolates. Therefore, the result of plasmid curing is positive. The extracted plasmids of isolate I5 shows two several bands under UV light as seen on the picture above. Here the first well contains the main isolate‘s plasmid. The well which contains 10% SDS, 12% SDS and 15% SDS treated plasmids of isolate I5, the first band is missing. Similar result can be seen in Et.Br- 50 µg/ml, Et.Br- 75 µg/ml, Et.Br- 100 µg/m, Et.Br- 125 µg/ml l and Et.Br- 150 µg/ml treated strains. So it can be concluded that these 8 strains were possibly cured. Isolate H shows several bands under UV light. Here the first well contains the main isolate‘s plasmid. It was observed that the well which contains Et.Br- 150 µg/ml treated strain of isolate H the first band is missing. Possibly it is the only cured strain of isolate H.

III. Organism – *Klebsiella*

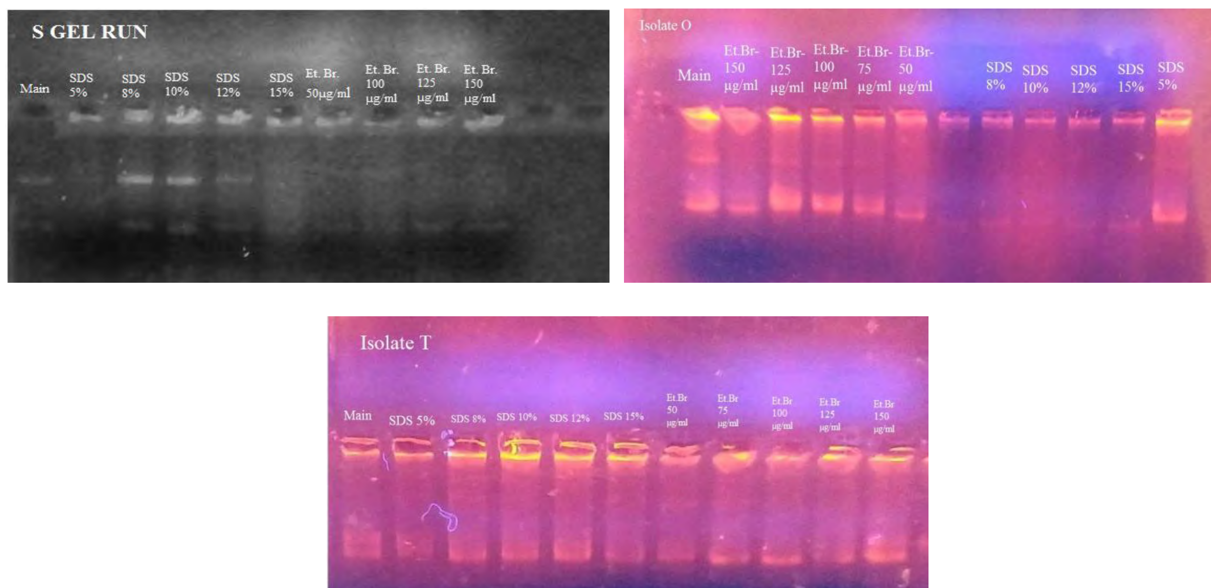

Figure 3.6 - The result of gel electrophoresis showing the loss of plasmid bands

The isolate O of *Klebsiella spp.* shows 2 bands under UV light. It can be fathomed that the well which contains 8% SDS, 10% SDS, 12% SDS and 15% SDS treated plasmids, the first band is missing. Same goes for Et.Br-50 µg/ml, Et.Br- 75 µg/ml and Et.Br- 150 µg/ml treated strains. These 7 are the possible cured strains. It was observed that the well which contains 15%

SDS treated strain‘s plasmids of isolate S the first band is missing. Same goes for Et. Br- 50 µg/ml, Et.Br- 125 µg/ml and Et.Br- 150 µg/ml treated strains. Assumedly, these 4 strains were possibly cured. Isolate T show a single band under UV light. Here the first well contains the main isolate‘s plasmid. It was observed that none of the strains lost the band, so none of the strains were cured.

IV. Organism – *Shigella*

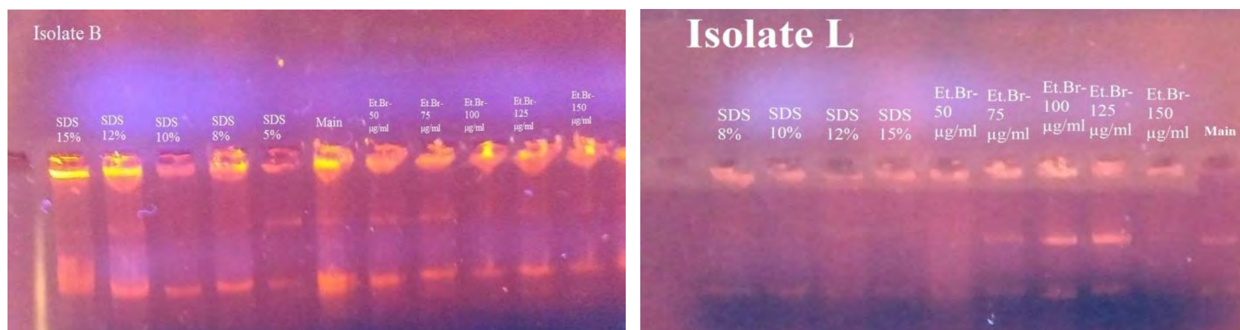

Figure 3.7- The result of gel electrophoresis showing the loss of plasmid bands

The extracted plasmids of isolate B show two bands under UV light. Here the sixth well contains the main isolate‘s plasmid. It was seen that the well which contains 8% SDS and 10% SDS treated strain‘s plasmids of isolate B the first band is missing. Same goes for Et.Br- 125 µg/ml and Et.Br- 150 µg/ml treated strains of isolate B. It can be concluded that these 4 strains were possibly cured. Isolate L showed two bands under UV light. Here the last well contained the main isolate‘s plasmid. The wells which contain 8% SDS, 10% SDS, 12% SDS and 15% SDS treated strain‘s plasmids the first band is missing. Same goes for Et.Br- 150 µg/mlt reated strains of isolate L. These 5 strains were possibly cured.

3.4.1. Summary

Table 3.3: Curing Treatment result summary			
Organism ID	Name of the Organism	Possible Cured strain's concentration	
		EtBr	SDS
Y	*E.coli*	75 µg/ml,100 µg/ml,125 µg/ml	8%,
K	*E.coli*	75 µg/ml,100 µg/ml	15%
O	*Klebsiella*	50 µg/ml ,75 µg/ml, 150 µg/ml	8%, 10%, 12%, 15%
S	*Klebsiella*	50 µg/ml,125 µg/ml, 150µg/ml	15%
T	*Klebsiella*	Nil	Nil
B	*Shigella*	125 µg/ml, 150µg/ml	8%, 10%
L	*Shigella*	150 µg/ml	8%, 10%, 12%, 15%
E	*Enterobacter*	50 µg/ml,150 µg/ml	5%, 8%,15%
I5	*Enterobacter*	50 µg/ml ,75 µg/ml,100 µg/ml,125 µg/ml, 150 µg/ml	10%, 12%, 15%
H	*Enterobacter*	150 µg/ml	Nil

1.5 Antibiogram- 2

After all the isolates underwent curing procedures with different concentrations of EtBr and SDS, antibiotic susceptibility test were performed with the assumed cured strains (which showed fewer bands when seen under UV light). The result is shown below.

Table 3.4: Antibiotic Susceptibility Results of Isolate Y (*E. coli*)									
Isolate Id	Curing Agent Concentration	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
Y	No treatment	Nil	R	13	I	Nil	R	Nil	R
Y2	Sds - 8%	18	S	29	S	Nil	R	Nil	R
Y7	Et. Br- 75	12	I	22	S	Nil	R	Nil	R

	μg/ml								
Y8	Et.Br-100 μg/ml	21	S	28	S	Nil	R	Nil	R
Y9	Et. Br-125μg/ml	25	S	28	S	Nil	R	Nil	R

According to this table the isolate Y was previously resistant to the antibiotic Azithromycin. But after curing treatment it showed sensitivity against it. Isolate Y also changed sensitivity against Gentamicin altering from Intermediate resistance to sensitive.

Table 3.5: Antibiotic Susceptibility Results of Isolate K (*E. coli*)

Isolate Id	Curing agent Concentration	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
K	None	15	I	Nil	R	Nil	R	8	R
K5	Sds- 15%	21	S	Nil	R	Nil	R	9	R
K7	Et. Br- 75 μg/ml	23	S	Nil	R	Nil	R	13	I
K8	Et.Br- 100 μg/ml	26	S	Nil	R	Nil	R	15	I

In table 3.5 similar changes can be observed in terms of Azithromycin sensitivity for isolate K. However no changes are observed for Gentamicin and Amoxicillin. Only K7 and K8 showed intermediate resistance against Ampicillin after curing was done.

Table 3.6: Antibiotic Susceptibility Results of Isolate E (*Enterobacter spp.)*

Isolate Id	Curing agent concentration	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
E	None	14	I	13	I	Nil	R	Nil	R
E1	Sds- 5%	21	S	17	S	Nil	R	Nil	R
E2	Sds-8 %	19	S	16	S	Nil	R	Nil	R
E5	Sds- 15%	20	S	14	I	Nil	R	13	I
E6	Et. Br- 50 μg/ml	18	S	16	S	Nil	R	11	R
E10	Et. Br- 150 μg/ml	19	S	18	S	Nil	R	12	I

There is change in the susceptibility pattern for the isolate E before and after treating with curing agent. It showed intermediate resistance against Azithromycin while curing agent treated strains are showing complete sensitivity. Similar changes are observed for the antibiotic Gentamicin where E1, E2, E6 and E10 turned sensitive from intermediate resistance.

Table 3.7: Antibiotic Susceptibility Results of Isolate I5 (*Enterobacter spp.)*

Isolate Id	Curing agent concentration t	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
I5	None	10	R	10	R	Nil	R	Nil	R
I5-3	Sds- 10%	14	I	17	S	Nil	R	17	S
I5-4	Sds- 12%	19	S	18	S	Nil	R	Nil	R
I5-5	Sds- 15%	19	S	19	S	Nil	R	11	R
I5-6	**Et. Br- 50 µg/ml**	15	I	17	S	Nil	R	Nil	R
I5-7	Et. Br- 75 µg/ml	21	S	22	S	Nil	R	15	I
I5-8	Et. Br- 100 µg/ml	18	S	18	S	Nil	R	18	S
I5-9	Et. Br- 125 µg/ml	17	S	16	S	Nil	R	21	S
I5-10	Et. Br- 150 µg/ml	20	S	18	S	Nil	R	16	S

Isolate I5 showed changes in susceptibility pattern against Azithromycin, Gentamicin and Ampicillin. Most of the strains became sensitive against AZM and GEN from resistant, whereas only I5-8, I5-9 and I5-10 showed changes for AMP, becoming sensitive from resistant.

Table 3.8: Antibiotic Susceptibility Results of Isolate H (*Enterobacter spp.)*

Isolate Id	Curing agent concentration	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
H	None	11	R	15	I	Nil	R	Nil	R
H10	Et. Br- 150 µg/ml	18	S	21	S	Nil	R	Nil	R

The single strain for isolate H which showed plasmid band loss also showed changes in susceptibility pattern against AZM and GEN.

Table 3.9: Antibiotic Susceptibility Results of Isolate O (*Klebsiella*)									
Isolate Id	Treatment	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
O	None	14	I	15	I	Nil	R	Nil	R
O2	Sds- 8%	12	I	26	S	Nil	R	Nil	R
O3	Sds- 10%	15	I	23	S	Nil	R	Nil	R
O4	Sds- 12%	12	R	21	S	Nil	R	Nil	R
O5	Sds- 15%	11	R	25	S	Nil	R	Nil	R
O6	**Et. Br- 50 µg/ml**	17	S	26	S	Nil	R	Nil	R
O7	Et. Br- 75 µg/ml	16	S	19	S	Nil	R	Nil	R
O10	Et. Br- 150 µg/ml	13	I	20	S	Nil	R	Nil	R

According to table 3.9 isolate O showed intermediate resistance against GEN. After curing all of the strains showed sensitivity against it. For AZM O4 and O5 became resistant after curing treatment which is considered anomaly. However, O6 and O7 became sensitive. No changes in susceptibility were found for AMX and AMP.

Table 3.10: Antibiotic Susceptibility Results of Isolate S (*Klebsiella*)									
Isolate Id	Curing agent concentration	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
S	None	15	I	13	I	Nil	R	Nil	R
S5	Sds- 15%	15	I	21	S	Nil	R	Nil	R
S6	Et. Br- 50µg/mı	16	S	16	S	Nil	R	Nil	R
S9	Et. Br- 125 µg/ml	15	I	19	S	Nil	R	Nil	R
S10	Et. Br- 150 µg/ml	19	S	21	S	Nil	R	Nil	R

Changes can be observed in the susceptibility pattern for isolate S against AZM and GEN (from intermediate resistance to complete sensitive). No susceptibility changes against other two antibiotics.

Table 3.11: Antibiotic Susceptibility Results of Isolate B (*Shigella*)

Isolate Id	Curing agent concentration	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
B	None	12	I	14	I	Nil	R	Nil	R
B2	Sds- 8%	15	I	26cm	S	Nil	R	Nil	R
B3	Sds- 10%	13	I	25cm	S	Nil	R	Nil	R
B9	Et. Br- 125 µg/ml	17	S	26cm	S	Nil	R	Nil	R
B10	Et. Br- 150 µg/ml	21	S	23cm	S	Nil	R	Nil	R

There is change in the susceptibility pattern for the isolate B before and after treating with curing agent. It showed intermediate resistance against Azithromycin while two curing agent treated strains B9 and B10 are showing complete sensitivity. Also, all of the treated strains showed sensitivity against Gentamicin, whereas the main isolate showed intermediate resistance.

Table 3.12: Antibiotic Susceptibility Results of Isolate L (*Shigella*)

Isolate Id	Curing agent concentration	Name of the antibiotics and sensitivity							
		AZM(15) mm	Sensitivity	GEN(10) mm	Sensitivity	AMX(30) mm	Sensitivity	AMP(25) mm	Sensitivity
L	None	13	I	Nil	R	Nil	R	12	R
L2	Sds- 8%	15	I	20	S	Nil	R	14	I
L3	Sds- 10%	14	I	21	S	Nil	R	11	R
L4	Sds- 12%	16	S	25	S	Nil	R	16	S
L5	Sds- 15%	19	S	27	S	Nil	R	15	I
L10	Et. Br- 150 µg/ml	16	S	21	S	Nil	R	18	S

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

From table 3.12 it can be fathomed that the isolate L was resistant against Gentamicin. But 5 strains became sensitive after the curing treatment. It showed intermediate resistance

against Azithromycin, but 3 strains showed sensitivity afterwards. Changes in susceptibility for the antibiotic Ampicillin can also be observed.

Taking the whole disk diffusion antibiogram result in consideration, it can be deduced that in terms of antibiotics Azithromycin and Gentamicin several cured strains showed complete sensitivity and intermediate sensitivity after treating with curing agents. One *Enterobacter* strain showed changes in sensitivity in terms of the antibiotic Ampicillin. However, none of the strains showed any kind of change in susceptibility in terms of the antibiotic Amoxicillin. So it can be safely assumed that **Amoxicillin** resistance is **not plasmid mediated,** whereas there is a high possibility that **Azithromycin** and **Gentamicin** resistance is plasmid mediated for the studied isolates.

1.6 MIC & MBC

In order to reconfirm the antibiotic susceptibility result, Minimum inhibitory concentration (MIC) and Minimum Bactericidal concentration were determined by using agar dilution method. The antibiotics that were used are Azithromycin and Ampicillin. The details of the result are given below.

Table 3.13

		Azithromycin Concentration (μg/ml)									
Name of the Organism	Organism ID	1	10	30	60	80	100	150	200	MIC value	MBC value
E.coli	Y	√	√	√	√	√	√	-	-	100	150
E.coli	K	√	√	-	-	-	-	-	-	10	30
Klebsiella	O	√	√	√	√	√	-	-	-	80	100
Klebsiella	S	√	√	√	√	√	-	-	-	80	100
Shigella	B	√	√	√	-	-	√	-	-	30	60
Shigella	L	√	√	√	√	√	√	√	√	Higher	Higher
Enterobacter	E	√	√	√	-	-	-	-	-	30	60
Enterobacter	I5	√	√	√	√	-	-	-	-	60	80
Enterobacter	H	√	√	√	√	√	√	-	-	100	150

Table 3.14											
		Ampicillin Concentration (µg/ml)									
Name of the Organism	Organism ID	1	10	30	60	80	100	150	200	MIC value	MBC value
E.coli	Y	√	√	√	√	√	√	√	√	Higher	Higher
E.coli	K	√	√	√	√	√	√	√	-	150	200
Klebsiella	O	√	√	√	√	√	√	√	-	150	200
Klebsiella	S	√	√	√	√	√	√	√	√	Higher	Higher
Shigella	B	√	√	√	√	√	√	√	-	150	200
Shigella	L	√	√	√	√	-	-	-	-	60	80
Enterobacter	E	√	√	√	√	√	√	-	-	100	150
Enterobacter	I5	√	-	-	-	-	-	-	-	1	10
Enterobacter	H	√	√	√	√	√	√	√	-	150	200

Table 3.15 Antibiotic Susceptibility testing for MIC MBC					
		MIC Value (µg/ml)		**MBC Value (µg/ml)**	
Name of the Organism	Organism ID	Azithromycin	Ampicillin	Azithromycin	Ampicillin
E.coli	Y	100	Higher	150	Higher
E.coli	K	10	150	30	200
Klebsiella	O	80	150	100	200
Klebsiella	S	80	Higher	100	Higher
Shigella	B	30	150	60	200
Shigella	L	Higher	60	Higher	80
Enterobacter	E	30	100	60	150
Enterobacter	I5	60	1	80	10
Enterobacter	H	100	150	150	200

By observing the MIC and MBC chart it can be deduced that in terms of isolate K of organism *E. coli* there is change in the MIC/MBC value for the antibiotic Azithromycin. Before curing both the MIC and MBC value were more than 150µg/ml. Now it has been reduced to 10µg/ml and 30 µg/ml. The MIC/MBC value has also been decreased for isolates E and I5 of *Enterobacter spp.* Moreover for isolate I5 of *Enterobacter spp*the MIC/MBC value has reduced to 1 µg/ml and 10 µg/ml for the antibiotic Ampicillin after treating with curing agents. This further confirms that the antibiotic resistance genes for these specific antibiotics are plasmid mediated.

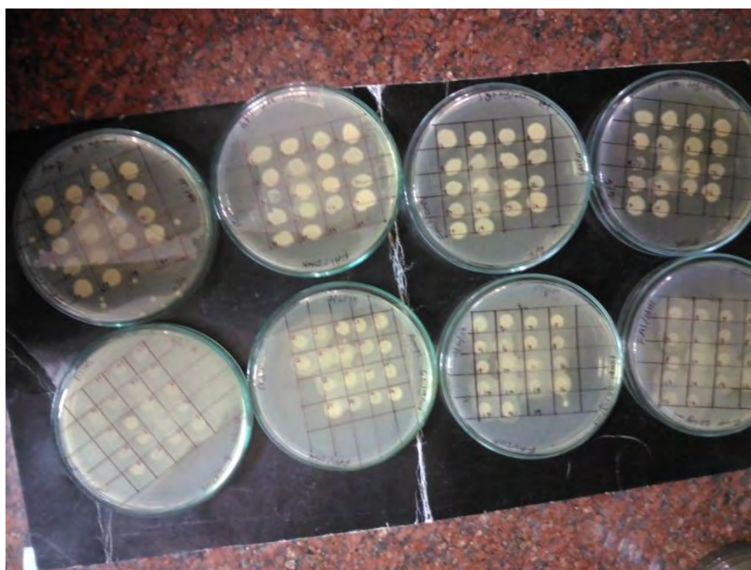

Figure 3.8- MIC/MBC determination of curing agent treated isolates

Table 3.16: Final result summary for Plasmid Curing

		Cured strain's concentration			
Organism ID	Name of the Organism	Azithromycin		Gentamicin	
		EtBr (µg/ml)	SDS	EtBr (µg/ml)	SDS
Y	*E.coli*	100,125	8%,	75,100,125	8%,
K	*E.coli*	75,100	15%	Nil	Nil
O	*Klebsiella*	50,75	Nil	50, 75,150	8%, 10%, 12%, 15%
S	*Klebsiella*	50,150	Nil	50,125, 150	15%

T	*Klebsiella*	Nil	Nil	Nil	Nil
B	*Shigella*	125, 150	Nil	125, 150	8%, 10%
L	*Shigella*	150	12%, 15%	150	8%, 10%, 12%, 15%
E	*Enterobacter*	50,150	5%, 8%, 15%	50,150	5%, 8%,
I5	*Enterobacter*	75,100,125, 150	12%, 15%	50,75,100,125, 150	10%, 12%, 15%
H	*Enterobacter*	150	Nil	150	Nil

By considering the data of this table it can be deduced that in terms of plasmid curing most effective concentration for EtBr is 150 µg/ml and most effective concentration for SDS is 15% SDS.

1.7 Transformation

Pair 1	Type	Organism ID	Organism	Antibiotic	Result
	Donor	K	*E. coli*	Azithromycin	No Transformation
	Recipient	DH5α	*E. coli*		

Pair 2	Type	Organism ID	Organism	Antibiotic	Result
	Donor	I5	*Enterobacter spp.*	Ampicillin	No Transformation
	Recipient	*DH5α*	*E. coli*		

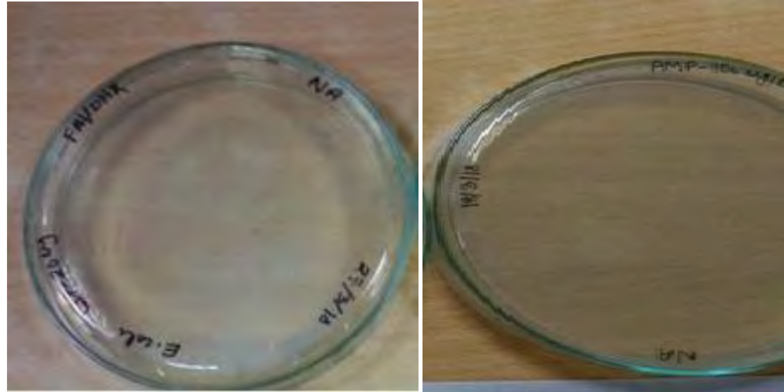

Figure 3.9- Transformation Plates

Two different donor strains and one recipient strain were chosen to use in the transformation experiment to see if the resistance plasmid is transferred. The antibiotic concentration were chosen from the MIC/MBC table. According to the procedure the plates containing antibiotics were plated with selected organisms and were kept for incubation for 24 hrs. No growth was observed. This indicates that transformation did not take place between the donor and recipient strains. As a result the recipient strain did not acquire the resistance required to grow on the plates.

Chapter 4
Discussion

5.1 Discussion

Urinary tract infections are one of the most prevalent bacterial infections found in humans. Possible causative agents include *Escherichia coli* followed by *Klebsiella spp., Shigella spp., Proteus spp., Enterobacter spp.* and *Staphylococcus*. Women and children are more affected by UTI *(Sule ,2016)*. The prevalence of UTI varies with age, race, sex and temperature *(Guernion, 2001).*According to Foxman,(2010) about 40% of women and 12% of men experience at least one symptomatic UTI duringtheir lifetime, and approximately 25% of affected women show recurrent UTI (RUTI). The rapid emergence of antibiotic resistance of bacteria is occurring globally, jeopardizing the efficacy of antibiotics, which have transformed medicine and saved millions of lives (*Golkar,2014)*.A project commissioned by the British government, Review on Antimicrobial Resistance (AMR) estimates, antibiotic resistance creates 700,000 deaths per year worldwide. Patients who suffer from UTI are generally prescribed antibiotics which work against the causative agents. However, ability of *Escherichia coli,Klebsiella spp., Shigella spp.* and *Enterobacter spp.* are increasing, while the ease of treating these infections due to resistance to first generation antibiotics such as Ampicillin, Gentamicin and Nitrofuranton is becoming more difficult. Taking preventive measure in order to decrease the resistance rate is being proven difficult due to the presence of transposable DNA elements, such as plasmids, which are associated with resistance to antibiotics (*Ahmadi, & Dilmaghani, 2014*).This study focuses on finding if the resistance of UTI causing pathogens is plasmid mediated and whether those plasmids can be eliminated by plasmid curing procedure which includes treating the isolates with various concentrations of Ethidium Bromide and Sodium Dodecyl Sulfate. It had been also observed if the resistance gene carrying plasmid can be transferred between the organisms through transformation procedure.

In this study, 10 identified organisms had been taken, which were isolated from UTI patients of a Diagnostic Center in Bangladesh. They were of *E. coli*, *Klebsiella spp., Shigella spp.* and *Enterobacter spp.* At first antibiotic susceptibility test was done via Kirby Bauer Disk Diffusion method and Agar Dilution method to determine the MIC/MBC value of the isolates. After the antibiotic susceptibility testing, the chosen isolates went through plasmid extraction and gel electrophoresis to determine and identify the presence of plasmid. The presence of plasmids was needed to be ensured. Then according to the data collected from antibiotic susceptibility testing

and gel electrophoresis the studied isolates underwent plasmid curing procedures. After treating the isolates with different concentrations of EtBr and SDS, electrophoresis was done again to observe whether the different strains of the isolates had lost plasmid bands which were assumed to be carrying the antibiotic resistance gene. For further confirmation antibiotic susceptibility test was done again via Kirby Bauer Disk Diffusion method and Agar Dilution method to determine the MIC & MBC of the assumed cured isolates.

For antibiotic susceptibility testing via Kirby Bauer disk diffusion method, four antibiotics were chosen. They are Azithromycin, Ampicillin, Amoxicillin and Gentamicin. Three isolates showed complete resistance to Azithromycin and 7 isolates showed intermediate resistance. Again, 3 isolates showed complete resistance, 6 showed intermediate resistances and only 1 isolate was sensitive to Gentamicin. For the antibiotics Amoxicillin and Ampicillin, each of the 10 isolates showed complete resistance.

After acquiring these results, MIC and MBC value were determined for the same isolates for the antibiotics Azithromycin and Ampicillin. These results showed that isolates of *E. coli* and *Klebsiella spp.* grew on plate containing Azithromycin (concentration 200µg/ml), which is very high dosage. One isolate of *Klebsiella spp.* grew on plate containing Azithromycin concentration 150µg/ml which is also very high dosage. One isolate of *Shigella spp.* and 2 isolates of *Enterobacter spp.* also grew on plate containing high concentration of Azithromycin. One isolate of *Shigella spp.* showed MIC value of 100 µg/ml and MBC value of 150 µg/ml to Azithromycin and 1 isolate of *Enterobacter spp.* showed MIC value of 80 µg/ml and MBC value of 100 µg/ml.

One isolate of *E. coli,* all 3 isolates of *Klebsiella spp.*and *Enterobacter spp.*grew on plate containing high concentration of Ampicillin. One isolate of *E. coli*showed MIC value of 150 µg/ml and MBC value of 200 µg/ml. Nevertheless, these are all high concentrations which is not unlikely as most of the isolates showed resistance to Azithromycin and Ampicillin in disk diffusion method.

After the antibiotic susceptibility testing the isolates underwent plasmid extraction procedure via Kado-Liu method and Alkaline Lysis method. Then gel electrophoresis was done. When illuminated by the UV light the gel showed presence of separate bands of plasmids for each isolates. These confirmed the presence of plasmids. After that plasmid curing was done using different concentrations of the curing agents. The agents are Ethidium Bromide and Sodium Dodecyl Sulfate. Ethidium bromide has been extensively used to cure plasmids in a wide

variety of bacterial strains *(Trevors, 1986).* According to Korn *et al*, Some curing agents work in a non-specific way by damaging and stressing out the cells, while some seem to act much selectively Plasmid eliminating ability of Ethidium Bromide was tested before (Bouanchaud, 1969). In 1968 Tomoeda used Sodium Dodecyl Sulfate (SDS) treatment to cure plasmids.

Table 5.1: Curing Treatment result summary

Organism ID	Name of the Organism	Possible Cured strain's concentration	
		EtBr	SDS
Y	*E.coli*	75 µg/ml,100 µg/ml,125 µg/ml	8%,
K	*E.coli*	75 µg/ml,100 µg/ml	15%
O	*Klebsiella*	50 µg/ml ,75 µg/ml, 150 µg/ml	8%, 10%, 12%, 15%
S	*Klebsiella*	50 µg/ml,125 µg/ml, 150µg/ml	15%
T	*Klebsiella*	Nil	Nil
B	*Shigella*	125 µg/ml, 150µg/ml	8%, 10%
L	*Shigella*	150 µg/ml	8%, 10%, 12%, 15%
E	*Enterobacter*	50 µg/ml,150 µg/ml	5%, 8%,15%
I5	*Enterobacter*	50 µg/ml ,75 µg/ml,100 µg/ml,125 µg/ml, 150 µg/ml	10%, 12%, 15%
H	*Enterobacter*	150 µg/ml	Nil

After the treatment was done, the treated isolates plasmids were extracted and they underwent gel electrophoresis procedure again. The gel run picture of these isolates showed whether there was any loss of plasmid bands which were present previously. For Ethidium Bromide there were 5 different concentrations which were. From the table it can be deduced that among this 50 strains only 22 strains lost plasmid bands. For Sodium Dodecyl sulfate again there were 5 different concentrations. From the table it can be deduced that among this 50 strains only 19 strains showed loss in plasmid bands. After this step, it was needed to check whether these plasmid losses had any effect on antibiotic susceptibility. Antibiotic susceptibility testing was done again via Kirby Bauer disk diffusion method. For the antibiotic Amoxicillin there was no

change in the result. So it can be deduced that Amoxicillin resistance gene is not carried on the plasmid for the studied organisms.

However, for the antibiotic Azithromycin, changes were observed in the antibiotic susceptibility in 9 different isolates. For instance, one isolate of *E. coli* showed complete resistance to Azithromycin. But 3 of the curing agent treated strains (SDS 8%, Et. Br- 100 µg/ml, Et. Br- 125 µg/ml) were tested sensitive against Azithromycin. One strain showed intermediate resistance after treating with curing agent (Et.Br- 75 µg/ml). Again for another isolate of *E. coli,* changes were observed in the antibiotic susceptibility. Before curing treatment, the organism showed intermediate resistance to Azithromycin, but after the treatment the strains which showed plasmid loss, also showed sensitivity to Azithromycin (15% Sds, Et.Br- 75 µg/ml and Et. Br- 100 µg/ml). Onw isolate of *Enterobacter spp.* showed same kind of changes in Azithromycin susceptibility. The strains which were sensitive against Azithromycin from isolate 5% Sds, 8% Sds, 15% Sds, Et. Br- 50 µg/ml and Et. Br- 150 µg/ml. For the organism O of *Klebsiella spp.*the strains which showed plasmid losses are O2, O3, O4, O5,O6,O7 and O10 (8% Sds, 10% Sds, 12% Sds, 15% Sds, Et. Br- 50 µg/ml, Et. Br- 75 µg/ml and Et. Br- 150 µg/ml). Only 2 from these strains showed sensitivity to Azithromycin. Rest of the strains showed complete resistance and intermediate resistance. For the organism L of *Shigella spp.*5 strains showed plasmid loss. Thy are 8% Sds, 10% Sds, 12% Sds,15% Sds and Et. Br- 150 µg/ml. Among them 12% Sds, 15% Sds and Et. Br- 150 µg/ml treated strains showed sensitivity to Azithromycin. But the main isolate showed intermediate resistance to Azithromycin. From these results it can be assumed that Azithromycin resistance is plasmid mediated as many of the strains which showed plasmid losses after curing treatment and showed changes in their susceptibility nature. Plasmid curing ability of Ethidium Bromide proved to be more effective. Among 5 concentrations of EtBr- 150 µg/ml seemed to be the most efficient concentration for plasmid curing. For this concentration 6 strains showed losses in plasmid bands and changes in susceptibility.

For Gentamicin too, changes were observed in the antibiotic susceptibility result in 8 different strains. For instance, one isolate of *E. coli* showed intermediate resistance to Gentamicin in primary antibiotic susceptibility test. But 4 of the curing agent treated strains were tested sensitive to Gentamicin. These four strains also showed plasmid loss after curing procedure. For one isolate of *Enterobacter spp.* changes were observed in antibiotic susceptibility. It showed complete resistance to Gentamicin. But after curing treatment the 8

strains which showed plasmid loss, also showed sensitivity against Gentamicin. For one isolate of *Klebsiella spp* .changes were observed in antibiotic susceptibility too. It showed intermediate resistance to Gentamicin on primary susceptibility test. But after curing treatment the 7 strains which showed plasmid loss, also showed sensitivity to Gentamicin. All 5 of the them showed sensitivity against Gentamicin. Isolate L showed complete resistance against Gentamicin. From these results we can deduce that the isolates showed changes in Gentamicin resistance due to plasmid loss after plasmid curing procedure. So it can be assumed that Gentamicin resistance is plasmid mediated. EtBr seemed more effective between EtBr and SDS for Gentamicin. For SDS the most effective concentration is 15%.

For the antibiotic Ampicillin however, very few of the curing agent treated strains showed changes in susceptibility. More specifically only two isolates of *Enterobacter spp.* and *Shigella spp.* showed these changes. The *Enterobacter spp.* showed complete resistance against Ampicillin on primary susceptibility test. After curing treatment among 4 strains showed sensitivity against Gentamicin. One isolate of *Shigella spp.* showed complete resistance to Ampicillin susceptibility test. But after curing treatment among the 5 strains which showed plasmid loss only 2 strains showed sensitivity against Ampicillin. The strains are 12% Sds and Et. Br- 150 µg/ml treated strains. From these, we can deduce that plasmid curing by EtBr and SDS for the antibiotic Gentamicin is not that much effective. As only 2 isolates among 10 showed changes in antibiotic susceptibility after curing procedure we can not assume accurately whether the Ampicillin resistance is plasmid mediated or not. There is a high probability that resistance against Ampicillin is chromosome mediated.

The comparison of MIC and MBC value for the antibiotic Azithromycin before and after curing treatment also confirms the deduced possibility as the MIC and MBC values reduced after curing treatment. However, the comparison of MIC and MBC value for the antibiotic Ampicillin does not show enough changes to deduce any result.

Lastly a transformation experiment was performed to see whether the Azithromycin resistance carrying plasmid can be transferred in vitro via transformation procedure. A similar experiment was also designed for the antibiotic Ampicillin. These would‘ve helped us to presume whether the resistance plasmid can be mediated via transformation process. But the results were negative on both circumstances. As transformation is a very intricate procedure and some parameters might not have been achieved which resulted in the negative result.

Ultimately, this study exhibits its novelty on investigating the possibility whether the antibiotic resistance is carried in plasmids for the antibiotics – Azithromycin, Ampicillin, Gentamicin and Amoxicillin in the causative agents of Urinary Tract infections- *E.coli, Enterobacter spp, Klebsiella spp and Shigella spp*and whether plasmid curing procedure have any possibility to eliminate those resistance carrying plasmid. All procedures were performed with in-vitro conditions, so in-vivo validation is also required to determine whether resistance to Gentamicin and Azithromycin is plasmid mediated and if it can spread amongst the organisms in natural environment.

Chapter 5
Conclusion

5.2 Conclusion

Urinary tract infection is a significant public health concern. Though antibiotic usage has proven to be beneficial in counteracting the UTI but consuming more antibiotics are harmful for our body. The alarming fact is that the causative agents of UTI are showing resistance against most of the major antibiotics and these resistances are proving hard to contain with time. Plasmid mediated resistance that can be transferred between cells enable rapid spread of the disease. The present study investigated whether UTI causing bacteria carry drug resistance on plasmids. Plasmid curing can determine this. The results obtained were a preliminary indication of association of Azithromycin and Gentamicin resistance of the clinical isolates of *E.coli, Enterobacter spp, Klebsiella spp and Shigella spp* with plasmids. Between the two curing agents Ethidium Bromide showed more effectiveness than Sodium Dodecyl Sulfate. Future study may involve additional optimization to strengthen this result that might go deeper within the molecular biology aspects. Researchers nowadays are trying to find ways that would eliminate the resistant pathogens. So, it is necessary to know the process by which bacteria gains resistance to antibiotics and invent new ways to face the problem at primary level.

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Chapter 6
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Chapter 7
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
Sodium Chloride	10.0
Tryptone	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	15.0
Agar	10.0
Final pH: 7.5±0.2 at 25°C	

Appendix- II

Reagents and Buffers:

1M Tris HCl:

In a McCartney bottle, 1.576g Tris HCl was added. Then 10 ml distilled water was added to prepare 10 ml 1M Tris HCl. After that pH was adjusted to 8. Then it was stored at 4ºC.

0.5M EDTA:

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

2N NaOH:

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

Kado-I Buffer:

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

Kado-II Buffer:

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

1X TBE Buffer:

In a Durham bottle, 5.4 g of Tris base, 2.75 g of Boric Acid, 2ml of 0.5M EDTA were added. Then 500 ml distilled water was added to prepare 500 ml 1X TBE Buffer. After that pH of the buffer was adjusted to 8. Then it was autoclaved at 15psi 121ºC. After autoclave, it was stored at room temperature.

Appendix- III

Equipment:

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