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The effect of sub-lethal concentration of ciprofloxacin on the transfer of multidrug resistance plasmids, fitness costs on the host and the stability of the newly acquired plasmids

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"Everyone can rise above their circumstances and achieve success if they are dedicated to and passionate about what they do." — Nelson Mandela

Dedication

I dedicate this work:

- To my mother and father for their unlimited love, support and for always believing in me.
- To my son James Besa Junior for the joy you have brought in my life.
- And to my relatives, friends, and colleagues.

Acknowledgements

I would like to take this opportunity to thank my brother Kabanda Mwansa for the advice he gave me before I started this master program. I was initially reluctant to come to Tromsø because of the misconceptions I had about the weather in this city. After the talk he gave me I knew that coming here was the best thing to do. The decision to come here is probably the best decision I have ever made in my life. I have had an enjoyable time here.

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To my son James Besa Junior, I know I owe you a "father-son bonding time," I will surely make up for the lost time now that I have come to the end of this project. I am looking forward to holding you in my arms and maybe teach you one or two important life lessons.

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Summary

The increase in reported cases of antimicrobial resistance has raised a lot of concerns in the public health community. This has prompted efforts aimed at finding out factors that have been contributing to this situation.

In Gram-negative bacteria plasmids have been singled out as the most important vehicles behind the spread of antimicrobial resistance. Some plasmids can harbour several resistance genes resulting into host bacteria with multiple resistance profiles.

These plasmids are spread horizontally from one bacterium to another. Of major concern is the increased dissemination of the carbapenemases among Gram-negative bacteria particularly New Delhi metallo-β-lactamase (NDM-1) and oxacillinase-48 (OXA-48).

In this study the aim was to study the effects sub-lethal concentrations of ciprofloxacin (0.02μg/ml) have on the transfer frequency of plasmids carrying bla_{NDM-1} and bla_{OXA-48} genes. Further studies were carried out to analyse the stability of the newly acquired plasmids in the host in the absence of antimicrobial selection. In addition, studies were carried out to analyse the fitness costs the plasmids imposes on the host.

In vitro conjugations experiments using two clinical strains of *Escherichia coli* containing plasmid borne bla_{NDM-1} and bla_{OXA-48} genes respectively were used as donors. Clinical plasmid-free *E. coli* strains with different genetic backgrounds were used as recipients.

Our study showed a marked increase in plasmids transfer frequency in the presence of 0.02 μg/ml ciprofloxacin.

In order to determine the fitness cost the plasmids imposes on the host, competition experiments that were done between the transconjugants, G2-06 (NDM-1) and G2-07 (OXA-48) respectively and the recipient (K56-75-1).

G2-06 was found to have a fitness cost of 17 %, while G2-07 was found to have a fitness cost of 8 %.

The plasmid stability tests that were done over a period of 10 days found that the plasmids in G2-06 and G2-07 were 100% stable.

1. Introduction

1.1 Gram-negative bacteria

Bacteria can be classified either as Gram-negative or Gram-positive depending on its ability to retain a specific stain colour. A chemical stain called the gram stain is used to make this possible. The gram stain was developed by Hans Christian Gram in 1884. In this test the bacterial cell wall is viewed through the microscope and the classification depends on the colour of the cell wall. Gram-negative bacteria stain red with carbol fuchsin (or safranin) which is a counterstain, while Gram-positive bacteria stain purple (or blue) after retaining crystal violet (primary stain) as Figure 1 shows [1, 2].

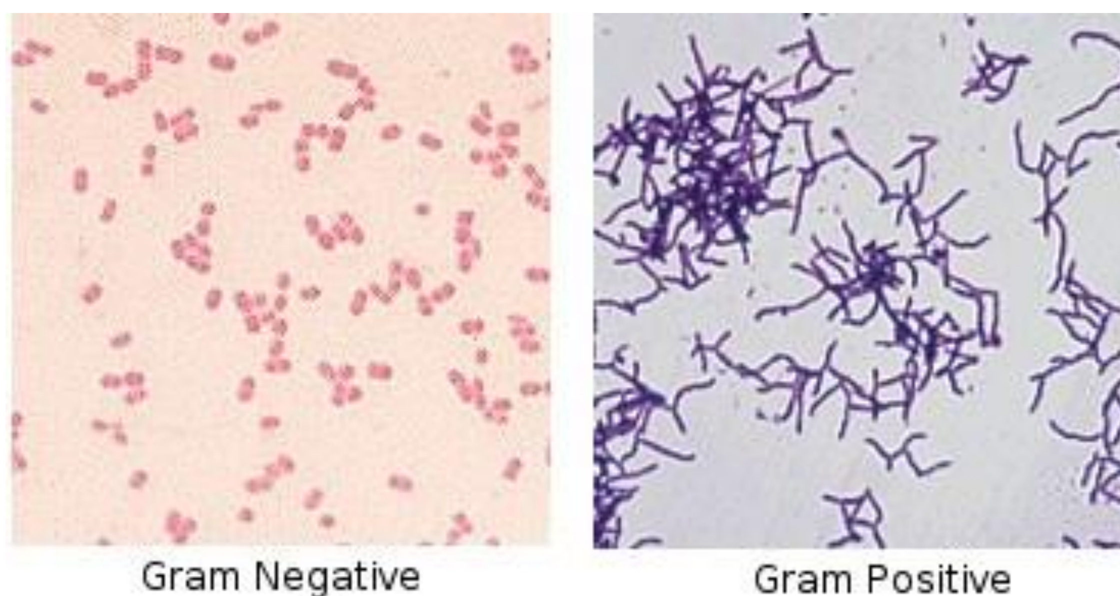

Figure 1. Showing the appearance of Gram-positive and Gram-negative bacteria after staining. (http://water.me.vccs.edu/courses/env108/lab2.htm)

The structure of the bacteria cell envelope determines the stain that the bacteria can retain. The cell membrane structure in Gram-negative bacteria differs from that of Gram-positive bacteria. Gram-negative bacteria contain an additional outer membrane which is mainly composed of phospholipids and lipopolysaccharides. Additionally they contain a cytoplasmic membrane and a thin peptidoglycan layer. The space between cytoplasmic membrane and the outer membrane forms what is called the periplasmic space. Gram-positive bacteria are able to retain the primary stain due to their thicker peptidoglycan layers, while Gram-negative

bacteria are unable to do so due to their thinner peptidoglycan layers [1, 3]. Figure 2 below gives a comparison of Gram-positive and Gram-negative bacteria.

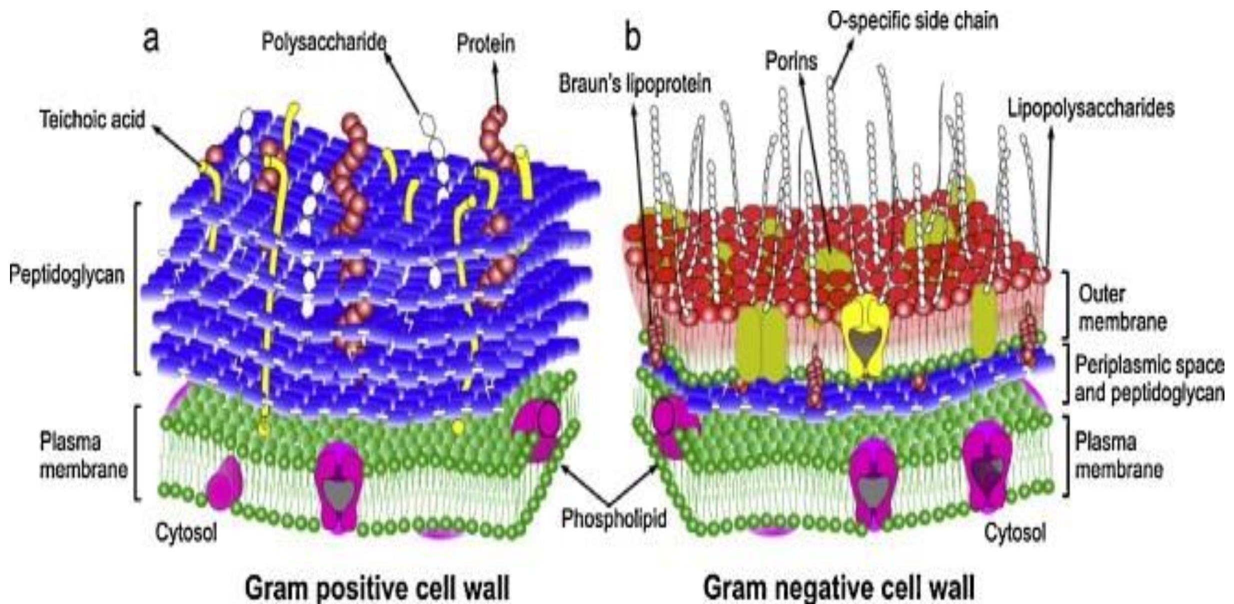

Figure 2. Showing the general comparison of Gram-positive and Gram-negative bacterial cell wall structures.
(https://microbewiki.kenyon.edu/index.php/File:Cell_wall.jpeg)

Gram-negative bacteria are commonly associated with nosocomial infections especially among patients admitted in the intensive care unit (ICU) [4].

For example the Enterobacteriaceae which are a family of bacteria that are a major cause of opportunistic infections (including pneumonia, septicaemia, meningitis and urinary tract infections) are Gram-negative. Prominent among Enterobacteriaceae are *Salmonella*, *Escherichia coli*, *Klebsiella pneumoniae* and *Shigella* [1, 2].

1.1.1 *Escherichia coli*

E. coli was first described in 1885 by a German paediatrician Theodor Escherichia. *E. coli* is a rod shaped bacilli that can exist singly or in pairs. It is approximately 0.5 µm in width by 2 µm in length and has 1-2 peptidoglycan layers. Furthermore it can exist either as a facultative anaerobic or as an aerobic bacilli. Some strains are motile by flagella while others are non-motile [2, 5]. The structure of *E. coli* is illustrated in Figure 3.

E. coli is transmitted by contaminated food or water. Despite living as a commensal in the intestines of both humans and animals certain strains have acquired virulence factors and as a result have become one of the leading causes of diarrhoea, septicaemia, neonatal meningitis and urinary tract infections [5, 6].

The emergence and spread of multidrug resistant *E. coli* strains has now become a global public health concern. This has been attributed to increased travel as well as the widespread use of antibiotics in clinical and veterinary medicine which as a consequence has resulted into resistant strains having a selective advantage. The increased transfer of resistance genes from pathogenic strains to commensal strains has further contributed to this problem [1, 7-8].

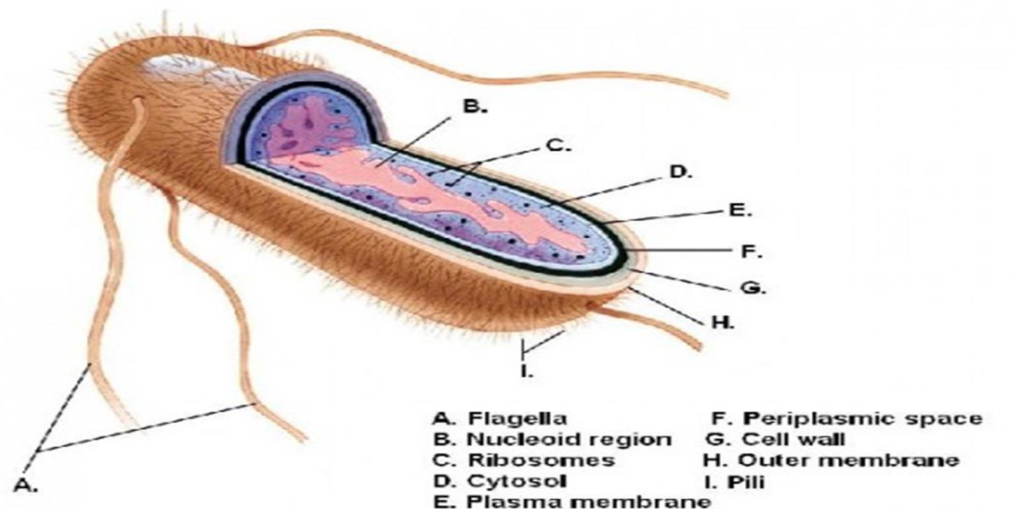

Figure 3. The structure of *E. coli*. The different parts are represented by the letters. (http://melpor.hubpages.com/hub/Understanding-The-E-Coli-Bacteria#slide5143394)

1.2 Antimicrobial agents

An antimicrobial agent is a chemical compound that kills microorganisms or inhibits their growth. Antimicrobial agents is a general term that encompasses bactericidal (kills microbes) and bacteriostatic (inhibit microbial growth) products derived from both natural and synthetic sources. The term antibiotic is specifically used for antimicrobial agents derived from natural sources like microorganisms. Penicillin which is produced by fungi is a good example of an antibiotic. On the other hand antimicrobial agents like fluoroquinolones are by the strict definition not antibiotics because they are derived from synthetic sources, but the two terms are often used interchangeably. An antimicrobial agent can be categorized as broad-spectrum if it is effective against many types of bacteria, while narrow-spectrum is applied for antimicrobial agents which has activity against a more limited range of bacteria [1, 2].

1.2.1 Brief historical background on the use of antimicrobial agents

For many centuries infectious diseases have had a major contribution to the number of recorded deaths. It was only during the latter half of the 19th century that micro-organisms were singled out as the main cause of infections. This discovery gave way to the discovery and development of antimicrobial agents. Salvarsan was the first antimicrobial agent that was developed by Paul Ehrlich in 1910 as an effective treatment for syphilis [9, 1].

1928 was an important year in medical history. It was a year in which penicillin was discovered. Alexander Fleming observed that growth of the bacterium *Staphylococcus aureus* was inhibited in an area surrounding a mould on his petri dish. The mould was later identified as *Penicillium notatum*. A while later the active compound in the mould was isolated and it was named penicillin. The discovery of penicillin later proved to be a major turning point in the treatment and management of infectious disease as it led to the saving of many soldiers lives during the second world war. 1935 saw the development of sulphonamides by Domagk and other researchers. These were synthetic compounds.

The following two decades after this saw the introduction of newer classes of antimicrobial agents, for example streptomycin, an aminoglycoside discovered from soil bacterium called *Streptomyces griseus* in 1944. Later years resulted in the

discovery of other antimicrobial agents like macrolides, chloramphenicol, tetracycline and nalidixic acid [9, 1]. A timeline chronicling the discovery of the major antimicrobial agents that have had proved vital in the treatment of infections is illustrated in Figure 4. From the timeline it shows that there have not been any further discoveries of novel agents since 1987. This perhaps points toward the need for more investment in new antimicrobial agents research.

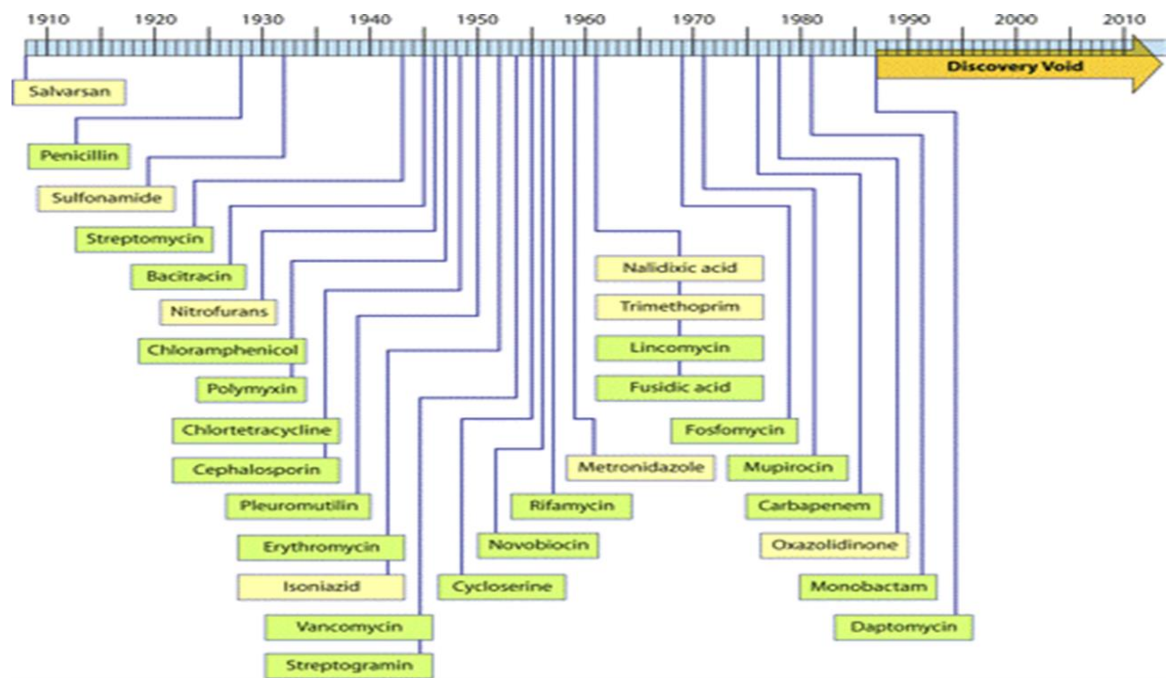

Figure 4. Timeline showing the order in which antimicrobial agents were developed between 1910 and 1990 [10].

1.2.2 Mechanism of action of most antimicrobial agents

Mechanism of action refers to a biochemical reaction that describes how an antimicrobial agent interacts with its molecular target. It usually includes an identification of the agent`s molecular target and the biochemical changes that takes place in the target once the agent binds [1, 2]. Table 1 below shows some of the antimicrobial agents used in clinical care and their mechanisms of action.

Table 1. The mechanism of action of most antimicrobial agents (adapted from Tenover 2006 [11].

Mechanisms of action	Antimicrobial agents
Interference with cell wall synthesis	β-Lactams: Penicillins, cephalosporins, carbapenems, monobactams. Glycopeptides: Vancomycin, teicoplanin
Protein synthesis inhibition -Bind to 50S ribosomal subunit -Bind to 30S ribosomal subunit	Macrolides, chloramphenicol, clindamycin, quinupristin-dalfopristin, linezolid Aminoglycosides, tetracyclines
Interference with nucleic acid synthesis -Inhibit DNA synthesis -Inhibit RNA synthesis	Fluoroquinolones Rifampicin
Inhibition of metabolic pathway	Sulfonamides, folic acid analogues
Disruption of bacterial membrane structure	Polymyxins, daptomycin

1.2.3 β-lactams agents

β-lactams are a large class of antimicrobial agents. This group of agents contain a four membered β-lactam ring in their molecular structure. The different side chains that attaches to the β-lactam ring differentiates the individual agents from each other (Figure 5). They work by inhibiting bacterial cell wall synthesis resulting in bacterial cell death, hence cannot be used against microorganisms that do not contain a cell wall. β-lactams consists of penicillins, cephalosporins, monobactams, and carbapenems. They are the most widely used antimicrobial agents [2, 12].

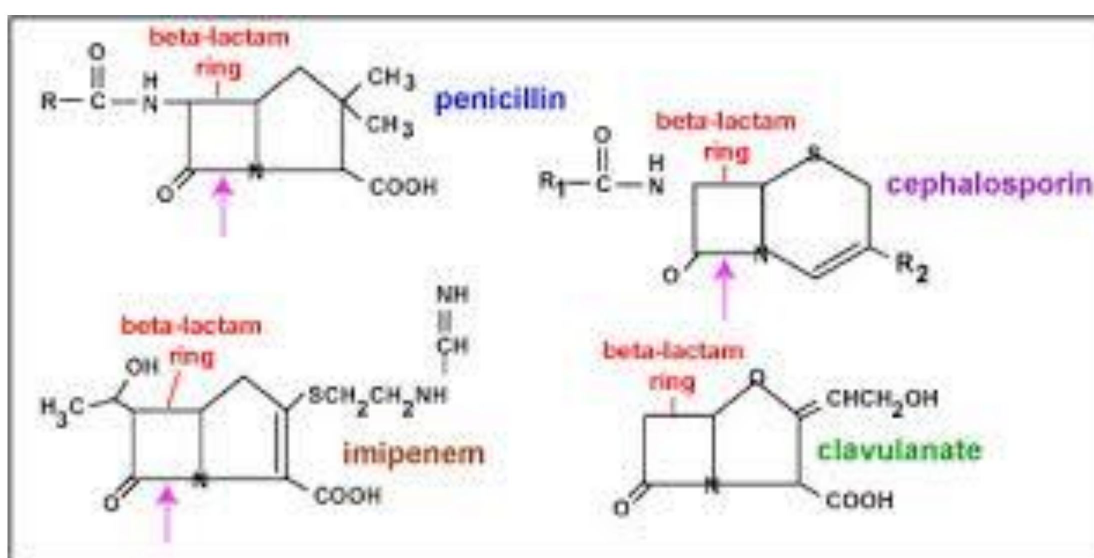

Figure 5. Chemical structures of some β-lactam agents [13].

1.2.3.1 Penicillins

Penicillins can be classified according to their antimicrobial activity. They can be produced both naturally and semi-synthetically [1, 2].

Examples of naturally produced penicillins include penicillin G and penicillin V. These penicillins are used to treat staphylococci (Gram-positive bacteria) infections. However they have a narrow spectrum of activity. To overcome disadvantages that natural penicillins had, scientists developed semi-synthetic penicillins by chemically manipulating the naturally produced ones. A typical example of a semi-synthetic penicillin is methicillin [1, 2].

After methicillin was introduced it was eventually discovered that many strains of staphylococci became resistant. This led to the development of a more broader

spectrum of semi-synthetic penicillins (extended-spectrum penicillins) which are more effective against many Gram-negative and Gram-positive bacteria. A typical example of these antimicrobial agents is ampicillin [1, 2].

1.2.3.2 Cephalosporins

Cephalosporins are similar to penicillins in the manner in which they inhibit bacterial cell wall synthesis. However they have a more broad spectrum of activity compared to natural penicillins and are less susceptible to penicillinases (enzymes produced by bacteria which hydrolyse and inactivate penicillin). There have been introductions of second, third and fourth generation cephalosporins in recent years. Each of the newer generation is more effective and has a broader spectrum of activity than the previous generation. Good examples of cephalosporins are cefotaxime and ceftazidime [1, 14].

1.2.3.4 Monobactams

Monobactams are synthetic antimicrobial agents that have been developed in order to circumvent the effects of penicillinases. Unlike most β-lactam agents monobactam has a single ring (instead of double) hence the name. A good example of a monobactam is aztreonam [1].

1.2.3.5 Carbapenems

Carbapenems are considered agents of last resort due to the increase in resistance to other β-lactams. They have a broad spectrum of activity. They were developed as a result of an increase in the spread of extended spectrum β-lactamases (enzymes that inactive cephalosporins and monobactams). There has been a considerable increase in the use of carbapenems in recent years as a result of a rising resistance to cephalosporins in Enterobacteriaceae. This class of β-lactam agents substitutes a carbon atom with a sulphur atom and add a double bond to the penicillin nucleus. Good examples of carbapenems are imipenem, meropenem and ertapenem [1, 2, 15].

1.3. Antimicrobial resistance

Antimicrobial resistance can defined as the ability of microorganisms to grow in the presence of an antimicrobial agent to which they were initially sensitive. The widespread use of antimicrobial agents in agriculture and medicine has been cited as the most contributing factors in the increased cases of antimicrobial resistance. The presence of these agents in the environment has led to selection of the resistant strains [1].

1.3.1 A brief history of antimicrobial resistance

The discovery of antimicrobial agents has been considered as one of the greatest discoveries of the 20th century. The discovery drastically reduced the mortality rate. However it did not take long before resistance to penicillin was discovered in some strains of staphylococci. Similar incidences of bacterial resistance toward streptomycin, chloramphenicol and tetracycline were observed soon after the introduction of these agents in the late 1940`s. In the early 1950`s, during an outbreak of Shigella in Japan, multi-drug resistant strains of *Shigella dysenteriae* were discovered. During the course of years, starting from the time resistance to penicillin was first observed to the present time, it has been noted that almost all the known bacterial pathogens have developed resistance to one or more antimicrobial agents in medical use [16, 2].

1.3.2 Mechanism of antimicrobial resistance

There are four major mechanisms by which bacteria can become resistant to antimicrobial agents [1].

1. By destroying or inactivating the agent (one example is penicillinases which are enzymes that can hydrolyse the β-lactam ring of penicillins, rendering them ineffective).
2. The microbes develops an ability that enables them to reduce the amount of agents that can penetrate through their cell envelope leading to reduced intracellular levels of the accumulated agents (a mechanism frequently seen in bacteria which are resistant to tetracycline).
3. Alteration of the antimicrobial target site. For example a single amino acid change in the ribosome can make a microbe resistant to macrolides.

4. Efflux mechanisms whereby the agent is pumped out of the cell before it can interact with the antimicrobial target. This mechanism is common in tetracycline resistant *E. coli*.

   Since the focus in this thesis is on β-lactam resistance we will now therefore focus on β-lactamases.

1.4 β-lactamases

β-lactamases are enzymes that are produced by some microbes. They have been singled out as the most common cause of antimicrobial resistance to β-lactam agents. These enzymes acts on the β-lactam ring of these agents destroying it in the process. This deactivates the agent's antimicrobial properties. These enzymes are usually produced by bacteria when there are β-lactam agents in the environment [2, 17].

In this study our focus is on β-lactamase enzymes called carbapenemases.

1.4.1 Carbapenemases

Carbapenemases are a group of enzymes that can efficiently hydrolyze carbapenems. They belong to the molecular classes A, B and D β-lactamases based on their molecular structure and amino acid homology. Class A and D possess a serine residue at their active site important for the hydrolysis of substrates, while class B are metallo-β-lactamases and uses zinc at their active site for hydrolysis [2, 18].

1.4.1.1 Serine carbapenemases (class A and D)

Members among the Class A carbapenemases include variants of GES (Guiana extended spectrum), KPC (*Klebsiella pneumoniae* carbapenemase), SME (Serratia marcescens enzyme), IMI (imipenem-hydrolysing β-lactamase) and NMC-A (not metalloenzyme carbapenemase) [19].

Class D comprises of the oxacillinases (OXA) which are a group of enzymes that are capable of hydrolysing oxacillin and cloxacillin. More than 250 different types of oxacillinases have been reported and only in a few cases have these enzymes been found to have low levels of carbapenem-hydrolysing activity. It has also been found that the majority of these enzymes can be inhibited *in vitro* using sodium chloride (NaCl), while commercially available β-lactamase inhibitors have been found to have no effect on the enzymes. Many different types of oxacillinases have been reported in *Acinetobacter baumannii* and Enterobacteriaceae. Examples are OXA-23, OXA-24, OXA-25, OXA-26, OXA-27, OXA-48 variants, OXA-51, OXA-66, OXA-69, OXA-58, and OXA-143. At least six OXA-48-like variants (OXA-48, OXA-162, OXA-163, OXA-181, OXA-204 and OXA-232 for example) have now been identified. These variants differ from each other by a few amino acids substitutions or deletions. OXA-48 is the most widespread class D carbapenemase in *Enterobacteriaceae* and has emerged as a very serious threat to global health and it is the enzyme of interest in this study, so we will concentrate on it here [20, 21].

OXA-48 was first identified in carbapenem-resistant *K. pneumoniae* isolates in 2001 from Istanbul, Turkey. Since their first identification reports of OXA-48 are now widespread. They have now been a number of reported hospital outbreaks of OXA-48 in western European countries like France and Spain (Figure 6). Some of the outbreaks have been attributed to the movement of people from countries like Turkey, India and Morocco where OXA-48 is endemic [20, 21].

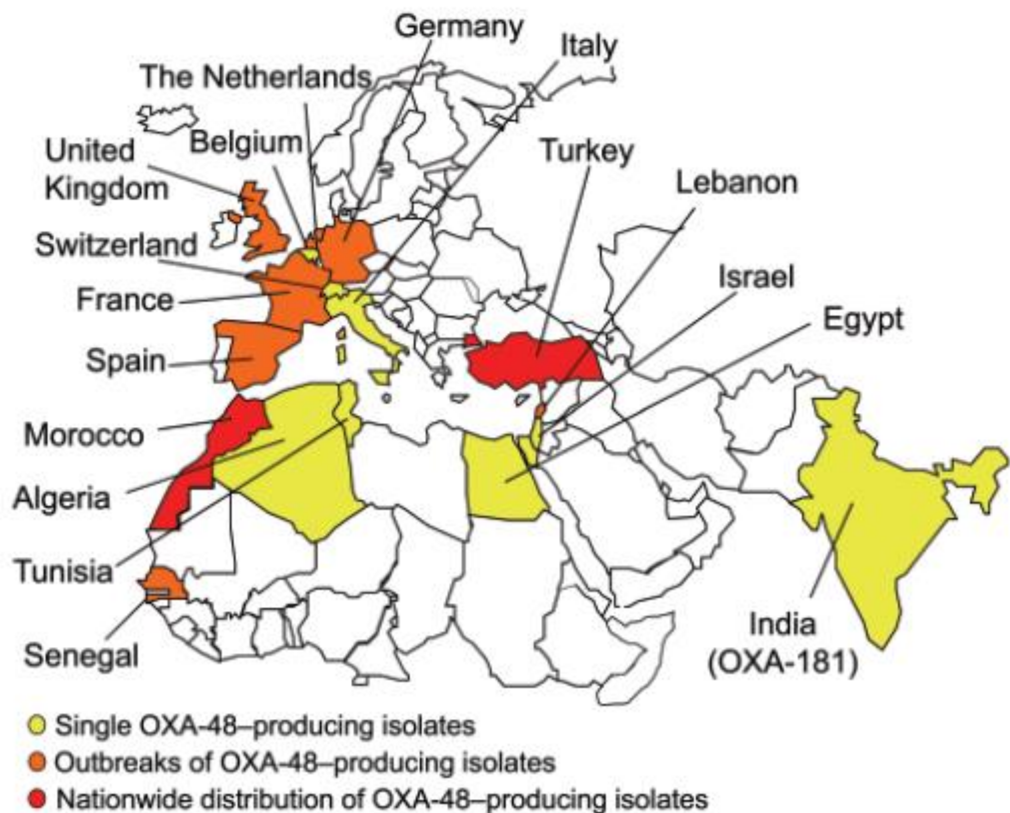

Figure 6. The global distribution of OXA-48 as of 2011[22].

The first isolates were found to have a high of level resistance to cephalosporins, cephamycins, monobactams and carbapenems. In the further investigations that followed it was found that these OXA-48-producing *K. pneumoniae* also co-expressed other β-lactamases like OXA-47 and ESBLs (Extended-spectrum β-lactamases), especially SHV-2 and TEM-1. They were also found to have a lot of defects in some of their outer membrane proteins. All these factors contributed to their high-level antimicrobial resistance pattern. The identification of OXA-48 in *E. coli* is now a major concern because *E. coli* happens to be a commensal in humans. Despite having a high level of hydrolysis to penicillins OXA-48 only weakly hydrolyse carbapenems and extended-spectrum cephalosporins. It has now been established that the $bla_{\text{OXA-48}}$-type genes are plasmid-borne. These genes have insertion sequences that helps them during their acquisition and eventual expression. In the majority of cases the spread of the $bla_{\text{OXA-48}}$ gene has been attributed to a single 62 kb self-conjugative IncL/M-type plasmid. This plasmid does not carry additional resistance genes [20, 21-22].

1.4.1.2 Metallo-β-lactamases (MBLs)

This group of β-lactamases includes for example IMP (active on imipenem), VIM (Verona integron encoded metallo-β-lactamase) and NDM-1 (New Delhi metallo-β-lactamase). Due to the zinc in their active sites these enzymes can be inhibited by metal-chelating agents like EDTA (ethylenediaminetetraacetic acid). The expression of MBLs in Gram-negative bacteria makes them resistant to cephalosporins, penicillins and carbapenems. They are however susceptible to monobactams (e.g. aztreonam). We will concentrate on NDM-1 here since it is one of the major areas of interest in this study.

The first reported case of NDM-1 was in 2009. It was from an isolate of *K. pneumoniae* obtained from a Swedish patient of Indian descent who had earlier travelled to India for medical treatment. It was later discovered that that the resistance genes were located on a 180 kb plasmid and conferred a high level resistance to all penicillins, aztreonam, cephalosporins and carbapenems. The isolate was only susceptible to colistin. From the time NDM-1 was first detected to the present Gram-negative bacilli containing $bla_{\text{NDM-1}}$ has been reported in many parts of the world (Figure 7). India has been singled out as being the epicentre of the spread these NDM-1 producers. The $bla_{\text{NDM-1}}$ gene has been detected on several types of plasmids which includes IncA/C, IncF and IncL/M replicon types. This shows that the $bla_{\text{NDM-1}}$ gene is not associated with a single plasmid backbone. In some cases plasmids carrying the $bla_{\text{NDM-1}}$ gene can carry additional resistance genes like cephalosporinase genes, macrolide resistance genes, aminoglycoside resistance genes and ESBL genes. This can result into isolates with a multi-drug resistance profile. Many NDM-1 producers however remain susceptible to tigecycline and colistin. The identification of NDM-1 in *E. coli* sequence type 131 (ST-131) is now a major concern because this *E. coli* sequence type is known for efficient global mobilization of CTX-M-15 (an ESBL variant). This raises serious concerns of co-mobilization of resistance genes. The convenience of international travel and medical tourism has had a major impact on the global dissemination of NDM-1 [20, 22-23].

Figure 7. Countries in which NDM positive bacteria has been reported as of 2013[23].

1.5 Horizontal gene transfer

Horizontal gene transfer is the transfer of genes from one bacterium to another without the recipient bacteria being the offspring of the donor. The transfer can take place between bacteria of the same or different species. The recipient bacterium that receives the donor DNA and incorporates it into its own genome becomes a recombinant bacterium. There are three mechanisms in which horizontal gene transfer happens which are transformation, transduction and conjugation (Figure 8) [1].

1.5.1 Transduction

In this process DNA is transferred from a donor bacterium to the recipient bacterium through a virus. Viruses called bacteriophages have an ability to infect bacteria. These bacteriophages are able to use the bacteria they infect to reproduce. When these viruses infect a new bacterium they incorporate DNA from their previous host into the new bacterium. During the assembling of the bacteriophages small pieces of the host DNA are sometimes packaged together with that of the bacteriophage. In other cases some of the phage DNA is left behind in the host DNA.

This knowledge has helped scientists to take advantage of this process to introduce some genes of interests into target bacteria [1].

1.5.2 Transformation

In this process genes are transferred from one bacterium to the other as naked DNA (DNA without associated cells or proteins). This process works well between related donor and recipient cells. This DNA must pass through the recipient's cell wall. This process can be enhanced if the recipient cell is in a physiological state that can make it easier for it to receive the DNA from the donor. Bacteria which have an ability to take up naked DNA are called competent. Scientists have discovered ways in which to manipulate bacteria to make them competent. *E. coli* is one example of a bacterium which is not naturally competent but can easily be made competent by scientists in the lab [1].

1.5.3 Conjugation

Conjugation is another mechanism in which DNA can be transferred from one bacterium to another. This process usually occurs between related species. This form of genetic transfer was first reported by Lederberg and Tatum in 1946. It was while they were working with *E. coli* when they observed a sex-like exchange between two *E. coli* strains. This process requires direct cell to cell contact to take place. Through this process plasmids are transferred from the donor to the recipient cell. In Gram-negative bacteria, the plasmids containing genes codes for a sex-pilli which is a bridge like projection which a donor bacterium can use to transfer the genetic material to the recipient cell. In *E. coli* which is the bacterium of interest in our study this process is driven by a DNA plasmid called the F factor (fertility factor). Donor cells containing the F factors (F^+ cells) transfers plasmids to recipient cells (F^-cells) making them F^+ cells in the process. This form of horizontal gene transfer plays a very big role in the acquisition and spread of resistance genes. To initiate conjugation an origin of transfer (*ori*-T) is needed. A plasmid encoded protein makes a single stranded cut (cleavage) at this site. This cleavage leads to rolling circle replication. This further leads to the displacement of a single stranded DNA molecule to the recipient cell. Once in the recipient cell this single DNA strand is circularized and a complementary strand is made [1, 2].

Figure 8 below illustrates the three horizontal gene transfer mechanisms and how the process takes place.

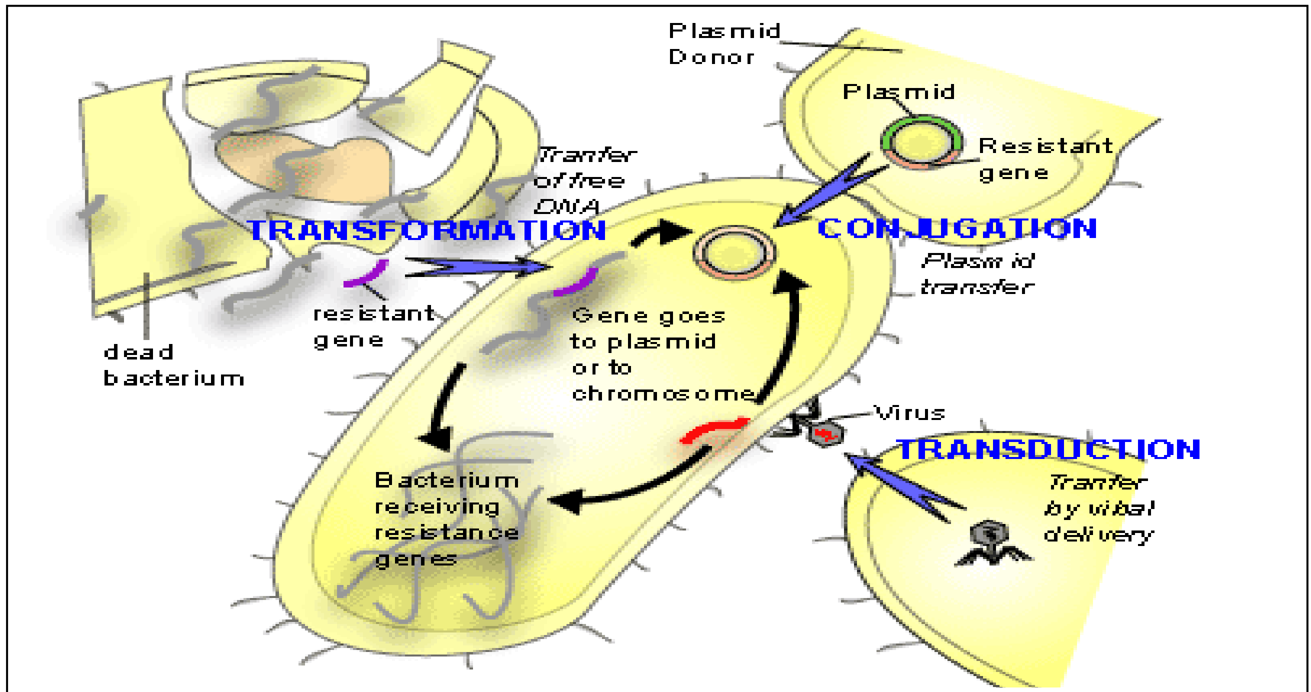

Figure 8. Horizontal gene transfer mechanisms.
(http://textbookofbacteriology.net/resantimicrobial_3.html)

1.6 The role of plasmids in the spread of carbapenemases

Plasmids are extrachromosomal pieces of DNA. They range between 1 and 1000Kbp in size. They are circular in shape and are self-replicating. Plasmids have in recent decades generated a lot of interest among scientists because of the role they are playing in the global dissemination of antimicrobial resistance. Plasmids conferring resistance to host bacteria were first discovered in Japan in the 1950`s after a dysentery outbreak. It was during this outbreak when a pathogen which was very resistant to a number of antimicrobial agents was isolated. Soon after this, more resistant pathogens (*E. coli* for example) were discovered. The researchers soon discovered that the resistance was as a result of gene transfer between bacteria of the same or different species. They further discovered that plasmids were the vehicles that were mediating these gene transfers. Plasmids gives the host bacterial cell a selective advantage [1, 24].

Apart from resistance genes, plasmids can also carry genes coding for virulence and resistance to toxic heavy metals such as cadmium and silver [1, 25].

Plasmids are classified depending on their mode of replication and partitioning incompatibility (Inc) grouping. In this system two related plasmids cannot occupy the same host. Related plasmids can thus be said to belong to the same Inc-group [26].

Plasmids can provide a platform on which genes can be assembled or rearranged. Plasmids have now been singled out as being responsible for the increasing reports of carbapenemase producers in Enterobacteriaceae. They are a major contributor in the global spread of NDM-1 and OXA-48. The acquisition of useful genes through plasmids can help the host bacteria to have an ability to thrive in environments in which they were initially unable to survive, facilitating their spread and adaption in the process. Most of the reported cases of antimicrobial resistance in bacteria have been attributed to plasmids [22, 27].

1.7 Plasmid fitness and stability

While the acquisition of plasmids carrying resistance genes might give the host bacterium a selective advantage of surviving in an environment with antimicrobial agents, they impose a biological cost on the host. In environments free of antimicrobial agents resistant bacteria may have a lower fitness compared to their susceptible counterparts [28].

The concept of fitness has a foundation in Darwin's theory of natural selection. In this theory Darwin argued that individuals with favourable traits suited for a certain environment can outlive those with less favourable traits (e.g. height, eyesight and high reproduction), and in the process are more likely to leave more offspring. Fitness is now widely defined as "an ability for an organism to survive and reproduce in a particular environment" [1, 2, and 29].

Reduction in fitness usually manifests itself as reduced growth and spread. The resistance determinants imposes energy demands on the host. This energy is required for replication of the acquired DNA and expression of the encoded novel proteins. The novel proteins produced may interfere with the physiology of the host bacterium. These processes can

as a result lead to reduction in bacterial fitness. In a nutshell the biological costs the host incurs determines how long the plasmids can be stably maintained in subsequent generations [28, 30].

1.8 The role of clones (genetic backgrounds) in the spread of resistance.

Scientists are able to group bacterial isolates into different sequence types (ST). The technique that they use to achieve this is called multilocus sequence typing (MLST). In this process a number of housekeeping genes are sequenced. Sequencing of the housekeeping genes at seven loci allows them to distinguish isolates of bacteria based on their genetic variation. Alleles with similar nucleotides profiles are assigned into the same ST designation, while all unique sequences are given a new allele number. Hence this technique makes it possible to infer relationships among isolates by comparing their allelic profiles. The numbers are assigned depending on the order of discovery. Databases are now available online where all the sequence information for isolates is stored. This development has brought flexibility and allows scientists working in different part of the world to compare their strains [31]

In an aim to find out whether or not the spread of resistance is only confined to certain bacterial clones, MLST has been the technique of choice. We will only concentrate on NDM-1 and OXA-48 in this study.

Studies that have been done on *E. coli* isolates have so far shown that the current dissemination of NDM-1 is not related to the spread of specific clones. In a recent study which conducted in Taiwan and China in 2012 *bla*$_{\text{NDM-1}}$ genes were discovered in a ST345 *E. coli* isolate [32].

In 2010, a NDM-1 positive *E. coli* isolate was reported in Norway. After MLST analysis was done it was discovered that it belonged to ST410 [33].

A surveillance program that was conducted in Indian hospitals from 2006-2007 listed ten *E. coli* ST types in which the *bla*$_{\text{NDM-1}}$ genes were found, these were; ST90,

ST101, ST405, ST410, ST648, ST156, ST131, ST167, ST224, and ST38. These findings proves that *bla*$_{NDM-1}$ genes can be harboured in several clones [34].

In an intercontinental survey that was conducted from 2001 to 2011 with an aim of understanding the spread of OXA-48 genes in Enterobacteriaceae it was found that the genes were predominantly spread in ST38 *E. coli* isolates. Other *E. coli* isolates where the genes were found includes ST10, ST617, ST648, ST46, ST69, ST95, ST101, ST362, ST410, ST746, ST963, ST1092 and ST2969 [35]. Just like in the case of NDM-1, we can conclude that the spread of OXA-48 is not restricted to a single clone.

2. Aims of the study

Plasmids have been singled out as the main vehicles behind the global spread of antimicrobial resistance in Gram-negative bacteria. It is not rare to find plasmids harbouring two or more resistance genes, leading to host bacteria with multi-resistance profiles [1, 36].

The involvement of plasmids in the spread of resistance genes in *E. coli* particularly $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes is a matter of public health concern. The knowledge that *E. coli* is a normal commensal in humans and has a tendency to spread easily between humans through contact and the oral-faecal route has heightened these concerns [8, 22].

A lot of studies have highlighted the role plasmids plays in the global dissemination of resistance genes, but few have focused on the probable influence external factors like temperature and antimicrobial agent concentrations might have on the rate of transfer. For example studies that were done on *Staphylococcus aureus* showed that sub-lethal concentrations of β-lactam agents increases the transfer frequency of plasmids carrying tetracycline resistance genes between the different derivatives of the bacteria [37].

Another study that was done on zebra fish infected with antimicrobial resistant *Aeromonas hydrophilic* showed that treating the fish with sub-lethal concentrations of flumequine (a fluoroquinolone) induced expression of plasmid transfer genes in pRAS1 plasmids [38].

In this study the overall aim was to study the effect sub-lethal concentration of ciprofloxacin have on the transfer frequency of plasmids carrying $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes. Further, the aims included investigating the stability and fitness costs of the acquired plasmids in different genetic backgrounds.

3. Materials and methods

3.1 Strain collection

3.1.1 Recipient strains

The recipient strains in this study were from taken from the ECO-SENS collection. This collection consists of *E. coli* strains from four different countries namely Greece, Portugal, United Kingdom and Sweden. The strains were G1-38 (ST73), G1-40 (ST95), K56-50-1 (ST100) and K56-75-1 (ST69). These strains have been verified as plasmid-free. Their susceptibility to 24 antimicrobial agents has also been verified [39].

3.1.2 Donor strains

The donors which were used in this study were clinical *E. coli* isolates that were submitted to the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance by other laboratories. The laboratory reference numbers are K71-77 (ST410) and 50579417 (ST405) respectively. K71-77 has bla_{NDM-1} bearing plasmids, while 50579417 has plasmids that harbours bla_{OXA-48} genes [33, 40].

3.2 Generation of rifampicin resistant mutants

Mutation in bacteria can arise spontaneously as a consequence of mistakes during DNA replication, due to chemical or radiation exposure [1, 41]. In *E. coli* rifampicin resistance arises as a consequence of mutations in the *rpoB* gene that encodes the β-subunit of RNA polymerase [42, 43]. The presence of antimicrobial agents in the environment can also select for antimicrobial resistance [28].

In this study rifampicin resistant mutants of isolates from the ECO-SENS collection were created using 100 µg/ml rifampicin.

Procedure

1. Plate the clinical *E. coli* isolates on green agar plates (Appendix A: Table 1) and incubate overnight at 37°C.

2. Pick one colony from each plate and inoculate into respective 5ml LB broth (Appendix A: Table 1). Incubate overnight at 37°C with shaking at 180 rpm.

3. Plate 100µl of the overnight culture onto LB agar plates with 100 µg/ml rifampicin (Appendix A. Table 1). Incubate overnight at 37°C.

4. Re-plate colonies onto LB agar plates with 100 µg/ml rifampicin and incubate overnight at 37°C.

5. Prepare stock cultures (see procedure 3.3).

6. Determine the rifampicin minimum inhibitory concentration (MIC) using gradients strips (see procedure 3.5.2).

3.3 Preparation of stock cultures

In this study the stock cultures were made using glycerol which acts as an osmotic protector (Appendix A. Table 1).

Procedure

1. The isolates were cultured on green agar plates (Appendix A. Table 1)

2. After overnight incubation at 37°C, 7-10 colonies were picked from the overnight culture and inoculated into 1ml freeze broth (Appendix A. Table 1). An inoculation loop was then used to homogenously mix the colonies in the broth.

3. The stock cultures were stored at -80◦C.

3.4 Vitek identification

The Vitek 2 is an automated instrument (bioMérieux,) that performs bacterial identification. It operates on fluorescence-based technology. It uses colorimetric identification cards (reagent card). The cards consists of substrates that undergoes biochemical reactions, it is the pattern of these reactions that determines the species [44].

Procedure

1. Colonies from an overnight culture grown at 37°C on Green agar or on green agar with 100 mg/ml ampicillin were inoculated into 3 ml 0.45% NaCl (Appendix A. Table 1). The suspension was adjusted to 0.5 McFarland.

2. The tube with a 0.5 McFarland suspension was put into the rack and then a colorimetric identification card was inserted into the neighbouring slot while making sure that the transfer tube of the card was in the suspension. The card was then scanned for registration.

3. The rack was placed into the VITEK2 machine. In the machine each test card was filled automatically with the bacterial suspension.

4. Data was analysed by the software and the results were displayed on the computer screen ready to be printed out.

3.5 Susceptibility testing

Susceptibility testing is a test performed to find out if bacteria are susceptible or resistant to one or several antimicrobial agents. Susceptibility testing can help us to know if a certain antimicrobial agent is likely to cure an infection or not [45]. Having this knowledge can help refrain clinicians from prescribing broad-spectrum agents which have been singled out as one of the causes behind the development and increase in antimicrobial resistance witnessed in recent decades. Making sure that no unnecessary drugs are prescribed to patients can further help reduce health care costs [46].

In this study two methods were used to determine antimicrobial susceptibility, EUCAST disc diffusion and determination of the minimum inhibitory concentration (MIC) using gradient strips.

3.5.1 EUCAST Disk Diffusion

Disk diffusion is one of the most common tests employed in determining antimicrobial susceptibility in most of the routine laboratories in the world. European Committee on Antimicrobial Susceptibility Testing (EUCAST) met during the 2009-2010 period to discuss ways in which laboratories can have a standardized way of testing for susceptibility in Europe after it was noted that different countries had their own breakpoints [47, 48].

This method uses paper discs impregnated with antimicrobial agents that diffuses out into the agar, inhibiting bacterial growth (Figure 9). The diameter of the zone of inhibition is measured and the values are compared with the breakpoints set up by EUCAST to determine if the bacteria can be categorized as susceptible (S), intermediate (I) or resistant (R) to an antimicrobial agent [48].

In this study disk diffusion was done to determine the susceptibility to ciprofloxacin and rifampicin.

Procedure

1. A 0.5 McFarland bacterial suspension was made from a fresh overnight culture grown on LB media with 100 µg/ml rifampicin for the mutants or green agar with 100 mg/ml ampicillin (Appendix A. Table 1) for the transconjugants.
2. The MH agar plates (Appendix A. Table 1) were inoculated using a cotton swab and rotator.
3. The discs were put on the plates within 15 minutes.
4. The plates were incubated for 18+/-2 hours at 37°C.
5. The inhibition zones were measured using a slide calliper.

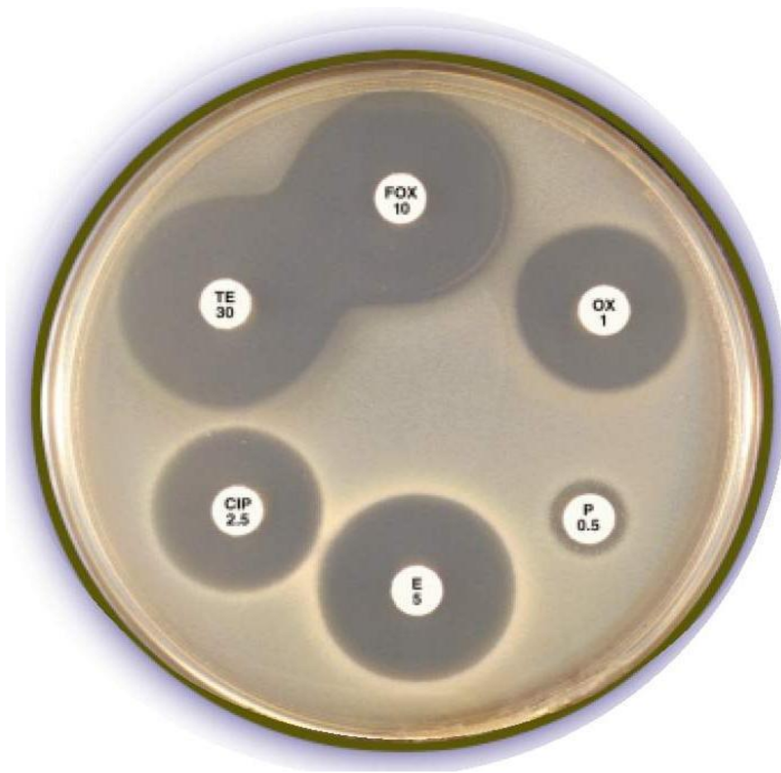

Figure 9. An example of a disc diffusion test, showing a MH-plate with six different discs and inhibition zones. (http://web.med.unsw.edu.au/cdstest/)

3.5.2 Minimum inhibitory concentration (MIC) determination

MIC determination using gradient strips is one of the most commonly used quantitative methods to determine the susceptibility of antimicrobial agents in many microbiology labs in the world. This test works on a similar principle as agar diffusion. The test comprises of rectangular strips that have been impregnated with a gradient of antimicrobial agents of interest. In this test a 0.5 McFarland suspension of bacteria is spread on MH agar plate and then a gradient strip is placed on top. When the strip is placed on the agar the antimicrobial agent diffuses into the agar, producing an exponential gradient of the agent. After incubation an elliptical zone of inhibition is formed (Figure 10). The position at where the zone of inhibition meets the strip gives us the MIC of the agent [49, 50]

Procedure:

1. A 0.5 McFarland bacterial suspension was made from an overnight culture grown on green agar with 100 mg/ml ampicillin.
2. The MH agar plates were inoculated using a cotton swab and rotator.

3. The gradient strips (Liofilchem) were applied on the inoculated plates using the strip applicator Simplex C76 machine (bioMérieux).
4. The plates were incubated at 37°C for 16-18 hours.
5. After incubation the plates were read.

Figure 10. An example MIC determination using the gradient strips. On this MH-plate are six different discs and their characteristic elliptical inhibition zones. (http://www.biomerieux-diagnostics.com/etest)

3.6 DNA isolation

In this study the DNA was isolated using the QIAGEN GenoM-48 BioRobot (QIAGEN, Hilden, Germany) and the procedure was followed according to the manufacturer's recommendation. The robot performs all the necessary steps needed to isolate the DNA. The Qiagen BioRobot M48 sample preparation is magnetic particle based. In the machine the bacterial cells are first lysed, releasing the DNA in the process. Then the released DNA binds to the magnetic particles. The DNA is eventually eluted in water after undergoing several cycles of washing [51].

Procedure

1. A 0.5 McFarland suspension were made in 0.85% NaCl (Appendix A. Table 1) from an overnight culture grown on appropriate agar plates.
2. 200µl of the suspension was transferred into sample tubes.
3. The samples were placed into the robot and the program started.
4. The isolated samples were kept refrigerated at 2-8°C.

3.7 Polymerase Chain Reaction (PCR)

PCR is a rapid method that can be used to amplify DNA segments from a few into billions of copies. PCR is now widely used to diagnose diseases, testing for paternity, identification of bacteria and in forensic laboratories. To synthesise the new DNA strands PCR uses a heat stable enzyme (e.g. Taq polymerase) as well as oligonucleotide primers that matches the beginning and the end of a target sequence. Deoxynucleotide triphosphates (dNTPs) provide the building blocks from which the enzyme can synthesise the new DNA strands. The whole PCR cycling process is automated and can be completed in just a few hours [52].

PCR is generally divided into three steps

1. Denaturation: During this stage the reaction is heated to a high temperature (approximately 95°C). This breaks the hydrogen bonds that hold the double helix together resulting into single strands.
2. Annealing: This stage happens between 50-60°C at which temperature allows the primers to attach or anneal to complimentary strands on single stranded DNA.
3. Extension/elongation: This takes place at approximately 72°C, a temperature at which the DNA polymerase (Taq polymerase in our case) works at its optimum capacity. At this stage the DNA polymerase binds to the annealed primers. The DNA polymerase adds nucleotides (dNTPs) to the 3´end of the primers. This results into synthesis of new DNA strands which are complementary to the template strand (parent strands).

These cycling conditions of denaturing, annealing and synthesizing of DNA is often repeated as many as 30-40 times (Figure 11). To monitor the efficiency and performance of PCR positive and negative controls are included in the run (Table 2). The former confirms if the PCR is working properly and while the later confirms if there is any contamination.

In this study the PCR was done using JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma Aldrich). We followed the manufactures instructions in all our tests [53].

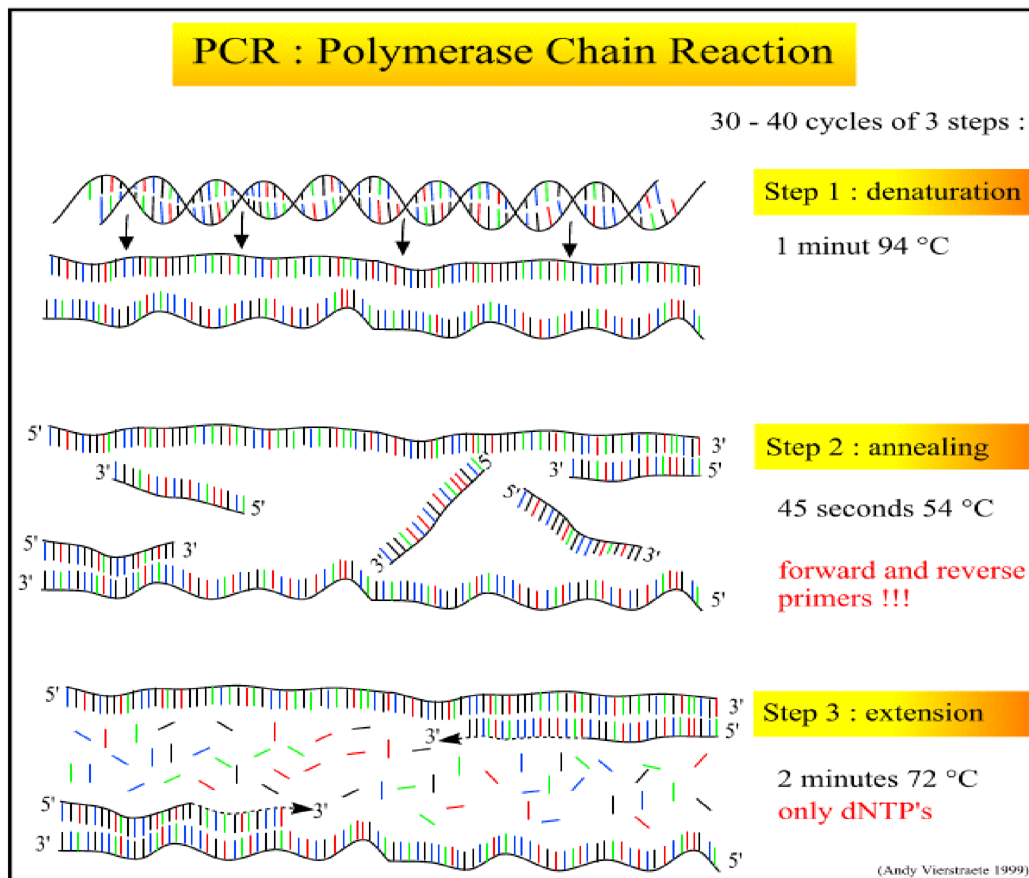

Figure 11. Showing the different steps in PCR.
(http://users.ugent.be/~avierstr/principles/pcr.html)

General procedure for the PCR

1. For each of the reactions were added 2.5 µl DNA to 22.5 µl PCR master mix (Appendix B. Table 1)
2. Both the positive and the negative control were added (Table 2). Additionally ddH_2O was used as a master mix control.
3. The PCR was run on a thermocycler (Applied Biosystems, Foster City, Ca, USA), annealing temperature was dependent on the melting temperature of the primers used (Appendix B. Table 2).

Table 2. Showing the primers, primer sequences, products size, and the negative and positive controls used in this study.

PCR type	Primer name	Sequence(5'-3')	Positive control	Negative control	Product size	Reference
NDM-1	NDM-1-F	CAA TAT TAT GCA CCC GGT CG	A5-28 *E. coli*	A-81 *E. coli*	621bp	[33,53]
	NDM-1-R	ATC ATG CTG GCC TTG GGG AA				
OXA-48	OXA-48-F	GCG TGG TTA AGG ATG AAC AC	A5-13 *E. coli*	A-81 *E. coli*	438bp	[53]
	OXA-48-R	CAT CAA GTT CAA CCC AAC CG				

3.8 Agarose gel electrophoresis

Agarose gel electrophoresis is a highly sensitive method that is used to separate, identify and purify DNA and RNA fragments using an electric field. Since DNA and RNA are negatively charged their movement in the electric field is toward the positive electrode. In this method the DNA/RNA fragments are separated by size. This is made possible by taking advantage of the fragment`s charge-to-mass ratio as the migration of the fragments in the electric field is inversely proportional to their molecular weight. Smaller fragments moves furthest in the gel while the larger fragments does the opposite. The migration of these fragments is compared with the migration of fragments with known size and weight called molecular ladders [52, 54].

Location of the DNA fragment after the electrophoresis has been run is made possible using GelRed . GelRed fluoresces after binding to DNA. It is this property that enables scientists locate the DNA under ultra-violet light [52, 55].

Procedure:

1. 1% agarose (Seakem LE agarose, Lonza, Rochland, ME, USA) was added to 0.75X Tris/Borate/EDTA-buffer (TBE) and boiled in the microwave oven to dissolve it completely.
2. 0.5 µl of GelRed (Appendix A: Table 2) was added for staining of the DNA fragments.
3. The agarose-GelRed solution was poured into an assembled gel tray with a comb to form wells (Bio-Rad laboratories, Hemel Hemstead, UK) and was allowed to solidify at room temperature for about 30 minutes.
4. After removing the comb the gel was placed in a running chamber containing 0.75xTBE buffer (appendix A. Table 1). 5 µl of each PCR reaction was loaded into the gel wells.
5. 5µl of 1 Kb Plus DNA molecular marker was loaded into the flanking wells.
6. Electrophoresis was then run at 120 V for about 90 minutes.
7. The DNA bands were finally visualized using the GelDoc system (BioRad)

3.9 Pulsed-field gel electrophoresis (PFGE)

Pulse-field gel electrophoresis (PFGE) is a technique that is used to separate large fragments of chromosomal DNA. Using this technique DNA fragments from 100 bp to 10 Mbp can be separated. This method involves agarose gel electrophoresis. The electrophoresis in this technique involves two electrical fields are that are applied alternatively at different time points. When the first electric field is activated it causes the DNA to move through the pores of the agarose gel in a horizontal plane. Interruption of this first electric field and application of a second field forces the DNA to move in a new direction. The mass to charge ratio determines the movement of DNA with the smaller molecules (restriction digests) able to easily re-align themselves with the new field while the opposite is occurs for the larger DNA molecules. Consequently the smaller molecules moves faster than the larger ones [56].

To protect the chromosomes from fragmentation as a result of mechanical damage during sample processing the bacterial cells are moulded into agarose plugs. The plugs are then treated with lysosome. This is done in order to lyse the cells contained in the plugs, which results into release of DNA from the cells. The plugs are then treated with proteinase K (Sigma-Aldrich). The main function of proteinase K is to remove DNA degrading enzymes (nucleases) which are naturally present in the cells. The DNA in the plugs is then subjected to digestion with restriction enzymes [52, 56-57].

In this study the restriction enzyme used was XbaI (New England BioLabs), which recognizes a few restriction sites in the genome. This results into generation of restriction fragments which are separated in the agarose gel during electrophoresis (PFGE). (http://www.neb.com/nebecomm/products/productr0145.asp).

Procedure:

A. Preparation of PFGE plugs:

1. A single bacterial colony from a fresh green agar plate or green agar with 100 mg/ml ampicillin plate was inoculated into 15ml falcon tubes containing 5ml BHI-media (Appendix A. Table 1). This was then incubated overnight at 37^0C with shaking at 180 rpm.

2. 50µl of the overnight culture was transferred into 15 ml falcon tubes with 5ml BHI-media and then incubated at 37^0C for 4 hours with shaking at 180 rpm.

3. The cultures were transferred to a 15 ml falcon tubes and then centrifuged at 3500 rpm for 10 min.

4. The supernatant was removed and the pellets were re-suspended in 1ml cold PIV- buffer (Appendix A. Table 1).

5. 495µl of the suspension was transferred into eppendorf tubes and 5µl of lysozyme (100mg/ml) (Sigma-Aldrich) was added into the individual tubes. The suspension was mixed by vortexing and then equilibrated at 50^0C in a water bath.

6. To each of the individual bacterial suspensions was added 500µl of 2% agarose (BioRad) dissolved in PIV-buffer and equilibrated at 50^0C. The suspensions were mixed by vortexing and then carefully transferred to the plug mould. The plugs were left to solidify at 4^0C for 15 minutes.

7. The plugs were transferred to new tubes containing 2ml lysisbuffer (Appendix A. Table 1). They were then incubated at 37^0C for 2 hours at with careful slow shaking.

8. After removing the lysisbuffer the plugs were washed in 1 ml ddH_2O for 15 minutes. The ddH_2O was removed and 1 ml of ESP buffer (Appendix A. Table 1) containing 50µl/ml proteinase K solution (VWR International, West Chester, PA, USA) was added. This was then incubated at 50 ^{0}C overnight.

9. The proteinase K-solution was removed and the plugs were washed in 1ml TE-buffer (Appendix A. Table 1) for 30 minutes. This was step was repeated twice.

10. The plugs were stored in TE-buffer at 4°C.

B. Restriction enzyme digests of DNA and gel electrophoresis.

1. A thin slice of the plug was cut and transferred to an eppendorf tube.

2. The plug was washed 2x30 min in TE buffer.

3. The plug was washed in 125µl 10 x buffer 2 (New England BioLabs, Ipswich, USA) for 30 min.

4. 125 µl of restrictionenzyme mix (20 U XbaI) (New England BioLabs, Ipswich, USA) was prepared. The plug was then transferred to this mix. The plug was then incubated overnight at 37^{0}C.

5. The plug was then washed 2x30 min in TE buffer.

6. 0.5x TBE (100ml 10xTBE + 1900 ml dH2O) was prepared. 1% agarose was then dissolved in 0.5X TBE (i.e. 1 g agarose/100ml 0.5X TBE) by boiling the solution in a microwave oven and then equilibrated in a water bath at 50°C.

7. The gel equipment was prepared. The plugs were placed straight onto the gel comb and left for a few minutes to attach. The comb was then carefully inserted before the agarose was poured into the chamber. The gel was then left to solidify for approximately for 30 minutes at room temperature.

8. The electrophoresis chamber was filled with 1.8-2 l of 0.5X TBE and the circulation was turned on in order to cool the buffer down to 12°C.

9. After the gel had solidified the comb was removed.

10. A low range PFG marker (New England BioLabs, Ipswich, MA, USA) (Appendix A. figure 2) was cut into thin slices and then inserted directly into the appropriate empty wells in the gel. The gel was carefully placed into the electrophoresis chamber, the program parameters set and then the electrophoresis was run.

Programparameters:

Pulsetime	1-20 sec
Total runtime	21 hours
Voltage	6.0 V/cm = 200V
Angle	120^{0}C
Temperature	12 ^{0}C

11. The program was switched off and the gel was stained in a 3X GelRed solution (Appendix A. Table 2) for 60 min.

12. The DNA banding patterns were analysed using GelDoc system (BioRad, Hercules, CA, USA).

3.10 Plasmid transfer studies

Plasmids have played a very big role in the horizontal dissemination of genes. There are a large number of genes whose existence across many species has been attributed to plasmids. It is perhaps not surprising that the recent increase in the dissemination of antimicrobial resistance genes has largely been attributed to plasmids. Some bacterial populations have adapted to strong selection pressures due their plasmid acquisition. Perhaps a better understanding of the way plasmids spreads might help us to control the spread of resistance genes and to also reduce mortality [58].

Bacterial conjugation can be regarded as the bacterial equivalent of sexual reproduction since an exchange of genetic material takes place between two mating pairs of bacteria. This process involves direct cell to cell contact through the sex-pili which is a bridge like connection. During this process a donor cell transfers a mobile genetic element (which is most often a plasmid) through the pili to a recipient cell [1, 2].

Conjugative plasmids codes for genes that they need for their own transfer. Some of the proteins encoded by these genes includes relaxases, origin of transfer (oriT), type IV coupling proteins (T4CP) and type IV secretion system (T4SS) proteins. The relaxase recognizes the origin of transfer (oriT) which is a short plasmid DNA sequence necessary for plasmid transfer. It is the nicking of the oriT by the relaxases that starts the plasmid transfer process. A membrane-associated mating pair formation (MPF) provides the mating channel through which genetic material eventually passes. The MPF is part of the T4SS. Some plasmids called mobilizable plasmids lacks MPF to enable them transfer to other cells by conjugation, but can do so if the co-resident plasmids has a MPF. The interaction of the relaxase with both the T4CP and the components of the T4SS plays an important role in DNA transfer. The pumping activity of the T4CP (an ATPase) ensures transfer of DNA into the recipient cell (Figure 12) [59].

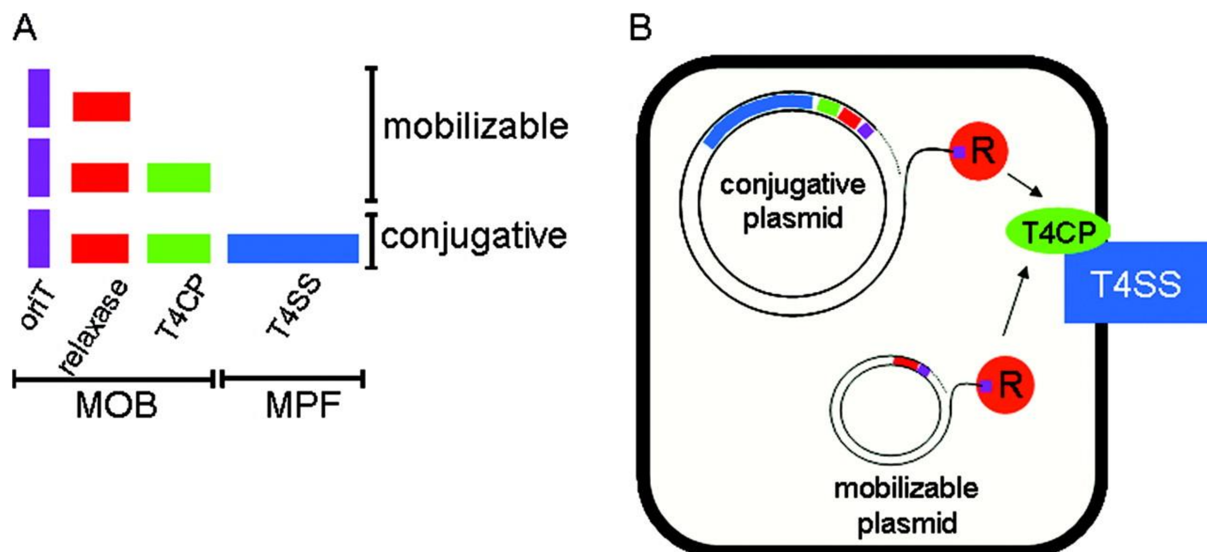

Figure 12. A) Showing the genetic constitution of conjugative and mobilizable plasmids including the components they code. (B) Showing some important steps that take place during conjugation (http://mmbr.asm.org/content/74/3/434/F1.expansion.html)

In this study two comparative plasmids transfer studies were done. In the first part the transfer of plasmids carrying OXA-48 and NDM genes from clinical *E. coli* isolates to laboratory generated rifampicin mutants (*E. coli* strains) were done in the absence of sub-lethal concentrations of ciprofloxacin. In the second part the transfer studies were done in the presence of 0.02µg/ml ciprofloxacin.

Procedure

1. The donor and recipients strains were inoculated into LB-medium (Appendix A. Table 1) and incubated overnight at 37^{0}C 180 rpm.

2. The overnight cultures were diluted 1:100 in LB medium and incubated at 37°C with shaking at 180 RPM until the cultures were in exponential growth (OD600 = 0.3-0.5) measured on a spectrophotometer.

3. The donor and recipient cultures were mixed in a 1:9 ratio with or without 0.02µg/ml ciprofloxacin. The mixed cultures were then incubated for 2hrs with slow shaking at 20 rpm.

4. The cultures were diluted and spread on LB media as shown in the table 3 below

Table 3. Showing the way the dilutions and plating were done.

LB medium with	Dilution						
	10^0	10^1	10^2	10^3	10^4	10^5	10^6
RD + ETP (T)	100 µl	100 µl	100 µl	100 µl	100 µl		
RD + AMP (T)	100 µl	100 µl	100 µl	100 µl	100 µl		
RD + CAZ (T)	100 µl	100 µl	100 µl	100 µl	100 µl		
ETP (D)					100 µl	100 µl	100 µl
AMP (D)					100 µl	100 µl	100 µl
CAZ (D)					100 µl	100 µl	100 µl
RD (R)					100 µl	100 µl	100 µl

RD + ETP = 100 µg/ml rifampicin + 0.25 mg/L ertapenem, RD + AMP = 100 µg/ml rifampicin + 100mg/L ampicillin, RD + CAZ = 100 µg/ml rifampicin + 50 µg/ml ceftazidime. ETP= 0.25 mg/L, AMP= 100mg/L ampicillin, CAZ= 50 µg/ml ceftazidime and RD=100 µg/ml rifampicin. T=Transconjugants, D=Donors and R= recipients.

5. After overnight incubation at 37^0C possible transconjugants, donors and recipients were counted and transfer frequencies counted.

6. The possible transconjugants were further plated on green agar with100 mg/L ampicillin and incubated at 37°C overnight.

7. Possible transconjugants were frozen for subsequent analysis.

3.11 MALDI-TOF

MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) is one of the technologies used in the routine identification of bacteria in some microbiology laboratories. In this method mass spectrometry is used to identify the bacterial species. It has been found that bacterial extracts from different species and genera give unique mass spectrums. These unique mass spectrums for the different bacterial species have been recorded and put into a database. The mass spectrums can be considered as the molecular fingerprint of the individual organisms. In this study we used the MALDI Biotyper which measures the proteins found in bacteria. It compares or matches the characteristic patterns of these proteins with proteins from already identified organism in the database. The sample (bacteria) for analysis in this method is first mixed with a compound called matrix on a metal plate slide. The mixture is then vaporized with a laser. The purpose of the matrix is adsorption of laser energy and then transferring the energy to the sample. This leads to desorption of the sample under analysis into a plume. The desorbed analyte gets ionized in the process. The matrix also prevents the aggregation of the analyte. These ions (electrically charged) are then separated according to their mass to charge ratio. The quantity of each ion is measured, and using this information the MALDI Biotyper is able to identify the different bacterial species [60, 61].

Procedure

1. One colony from a fresh agar plate was picked and spread on a metal plate slide.
2. The colony was mixed with 1 µl of matrix.
3. The mixture was left to air dry at room temperature.
4. The sample was inserted into the machine (Bruker) for processing
5. A print out of the results was produced at the end of the machine run.

3.12 Bacterial fitness competitions

The horizontal acquisition of plasmids by bacteria may come at a cost. The extent of the costs depends on the evolutionary history between the plasmid and the host. It affects their fitness and it is the biological cost of the acquired traits that determines if the trait will be maintained in subsequent generations or not. The fitness costs that the newly acquired resistance genes impose on the host can be measured in terms of relative fitness. Relative fitness compares the rates at which susceptible and resistant bacteria grow or die under the same conditions. The theory is that when two strains of bacteria are allowed to grow in the same environment the fittest strains outcompetes the less fit strains [30, 62, and 63].

An equation called a Malthusian parameter ($m = \ln (N_1/N_0)/d$) is used to measure the growth of the competing isolates. N_1 and N_0 denote the final and initial densities of the competing strains respectively.

Another equation $w = m_1/m_2$ was used to derive the relative fitness of the competitors. Finally an equation $s=w-1 = (m_1-m_2)/m_2$ was used to measure the selection coefficient of the isolates (fitness effect) [30, 62].

In this study 24-hour fitness assays were done to find out if there were any fitness costs incurred by *E .coli* (K56-75-1) after its horizontal acquisition of NDM-1 and OXA-48 genes. In this assay the competitors (susceptible and resistant bacteria) were mixed together in growth medium and allowed to compete.

Procedure

1. The competitors from freeze stocks were inoculated into LB broth and incubated overnight at 37^0C with shaking at 180 rpm.
2. 0.1 of each culture was transferred into 9.9 ml of pre-warmed LB broth and incubated overnight at 37^0C with shaking at 180 rpm.
3. The competitors were adjusted to the same optical density (OD_{600nm}) using the photometer. After the competitors were adjusted to the same optical density, 0.05 ml of each competitor was added to 9.9 ml pre-warmed LB medium. Immediately after mixing the competitors 0.1 of the culture was taken out for initial density (N_0) determination of the competitors. The competition cultures were then incubated overnight at 37^0C with shaking at 180 rpm.

4. Dilute the sample taken from the initial culture with 0.85 NaCl to 10^{-4}, 10^{-5}, and 10^{-6} respectively. Aliquots of the cultures were plated on appropriate selective and non-selective medium.
5. Dilute the overnight competition culture to 10^{-4}, 10^{-5}, and 10^{-6} respectively using 0.85 NaCl.
6. 0.1 ml of the mixed cultures were plated on appropriate selective and non-selective media and incubated overnight at 37^0C.
7. The colony counts were taken and the relative fitness and selection coefficient (fitness effect) were calculated.

3.13 Plasmid stability

Plasmids puts physiological burdens on their host cells. How long plasmids can be maintained in subsequent generations depends on the effect they have on their host [30].

In this study the stability of plasmids were assessed in a duration of 10 days through successive sub-culturing of the transconjugants.

Procedure

1. A single colony was emulsified in 10 ml pre-warmed LB broth and incubated overnight at 37°C with shaking at 180 rpm.
2. 100 µl from the overnight culture was taken out and inoculated into 9.9 ml LB broth. This was incubated overnight at 37°C with shaking at 180 rpm.
3. 100 µl from the overnight culture was serially diluted to 10^{-6} using 900 µl 0.85 % NaCl and then plated on green agar and incubated overnight at 37^0C. A further 100 µl of the overnight culture was inoculated into 9.9 ml LB broth as shown in step 2.
4. All single colonies from green agar were picked one at a time with an inoculation stick and transferred to selective plates (green agar with ampicillin).
5. Steps 2-4 were repeated in a 10 days duration.

4. Results

4.1 Generation of rifampicin mutants

To make rifampicin resistant mutants for counter selection in the plasmid transfer studies 100 µg/ml rifampicin was used (see procedure 3.2). Four plasmid free *E. coli* isolates susceptible to rifampicin were chosen from the ECO-SENS collection. These isolates have been verified as having no phenotypic antibiotic resistance to at least 24 antimicrobial agents in including rifampicin. These isolates belongs to different genetic backgrounds [39]. The isolates were from sequence types; ST100, ST215, ST69 and ST1235. After the isolates were grown in the presence of 100 µg/ml rifampicin it resulted into selection for rifampicin resistant isolates as shown in Table 4.

Table 4. Showing the initial colony counts obtained on LB media with 100 µg/ml rifampicin and the resulting MIC values.

Sequence type	ST100	ST215	ST69	ST1235
Ref. No	K56-50-1	K56-65 -1	K56-75-1	K56-78-1
Colonies	1	5	2	2
MIC to RD	>256	>256	>256	256

RD= Rifampicin, Ref. No=Reference number

4.2 Bacterial identification

Two automated methods were used in this study to identify the bacteria species, these were Vitek 2 and MALDI-TOF.

The Vitek 2 identifies bacteria using cards that have been infused with chemicals and it is the pattern of the biochemical reactions in these cards that helps determine the species (see procedure 3.4).

The second method, MALDI-TOF uses mass spectrometry to identify the bacterial species. This is done by mixing the sample with the matrix. The mixture is then vaporized using a

laser light. It is the mass spectrum created from the ionization of the mixture that is used to identify the bacterial species as shown in procedure 3.11.

Using these methods the bacterial species were identified as *E. coli*.

4.3 Plasmid transfer studies

In order to find out if sub-lethal concentrations of antimicrobial agents increases plasmid transfer frequency we did comparative conjugation studies. The first part was done in the absence of antimicrobial agents, while second part was done in the presence of 0.02µg/ml ciprofloxacin. The transfer studies were done by conjugation a process where two strains are grown in the same media. The physical contact between the strains leads to gene transfer resulting into formation of recombinants. The process was done as shown in procedure 3.10.

4.3.1 Plasmid transfer studies without ciprofloxacin

Four rifampicin resistant *E. coli* mutants were selected to act as recipients. These were G1-38 (ST73), G1-40 (ST95), K56-50-1 (ST100) and K56-75-1 (ST-69). G1-38 (ST73) and G1-40 (ST95) were generated in a previous study. The selection of these isolates gave us a possibility of assessing the transfer frequencies in recipients belonging to different genetic backgrounds.

The donors were *E. coli* strains with laboratory reference numbers 50579417 (ST405) and K71-77 (ST410) respectively. 50579417 harbour the $bla_{\text{OXA-48}}$ genes and K71-77 harbour the $bla_{\text{NDM-1}}$ genes [40, 33].

In the first part of the experiment K71-77 (NDM-1) was selected as the donor, while G1-38, G1-40, K56-50-1 and K56-75-1 were the recipients.

G1-38 and G1-40 did not give us transconjugants. However K56-50-1 and K56-75-1 gave us transconjugants on all the selective media used in this study as shown by the transfer frequencies in Table 5.

In the second part of this study 50579417 (OXA-48) was selected as the donor while G1-38, G1-40, K56-50-1 and K56-75-1 were the recipients.

G1-38 did not give any transconjugants, while G1-40, K56-50-1 and K56-75-1 gave transconjugants. The frequency for G1-40 was however not as high as that of K56-50-1 and

K56-75-1. This shows that K56-50-1 and K56-75-1 easily take up plasmids compared to G1-40. Table 5 shows the results obtained in the study.

Table 5. Plasmid transfer studies without ciprofloxacin

Donor	K71-77 (NDM-1)				50579417 (OXA-48)			
Recipient ref. no	G1-38	G1-40	K56-50-1	K56-75-1	G1-38	G1-40	K56-50-1	K56-75-1
Sequence type	ST73	ST95	ST100	ST69	ST73	ST95	ST100	ST69
Transconjugation frequency-Amp			$1.6x10^{-5}$	$1.1x10^{-4}$		$1.7x10^{-6}$	$1.5x10^{-6}$	$7.1x10^{-5}$
Transconjugation frequency-CAZ			$3.0x10^{-6}$	$2.3x10^{-4}$				
Transconjugation frequency-ERT			$1.3x10^{-5}$	$1.5x10^{-4}$				

AMP= ampicillin, CAZ= ceftazidime, ERT=Ertapenem

4.3.2 Plasmid transfer studies in the presence of 0.02µg/ml ciprofloxacin

In this part of the study the transfers were done in the presence of 0.02µg/ml ciprofloxacin. K71-77 (NDM-1) was used as a donor and G1-38 and K56-75-1 were used as recipients. G1-38 transconjugants grew on selective media containing 50 µg/ml ceftazidime. It is perhaps surprising that it grew on this media without growing on an ampicillin containing media. K56-75-1 transconjugants grew on all the selective media used.

In the second part 50579417 (OXA-48) was used as a donor and K56-75-1 as a recipient, the transconjugants grew on an ampicillin plate. Table 6 shows the results.

Table 6. Plasmid transfer studies in the presence of 0.02µg/ml ciprofloxacin

Donor	K71-77(NDM-1)		50579417 (OXA-48)
Recipient ref. no	G1-38	K56-75-1	K56-75-1
Recipient-ST	ST73	ST69	ST69
Transconjugation frequency-Amp		3.9×10^{-4}	5.4×10^{-7}
Transconjugation frequency-CAZ	1.6×10^{-7}	4.4×10^{-4}	
Transconjugation frequency-ERT		4.4×10^{-4}	

AMP= ampicillin, CAZ= ceftazidime, ERT=Ertapenem, Ref.no =reference number.

4.3.3 Comparing plasmids transfer results

Comparing the two studies, G1-38 only took up plasmids in the presence of 0.02µg/ml ciprofloxacin, while K56-75-1 showed increased frequency during transfer of *bla*$_{\text{NDM-1}}$ genes carrying plasmids under similar conditions, indicating increased transfer frequency under sub-lethal concentration of ciprofloxacin. However unlike in the two previously mentioned studies K56-75-1 showed reduced frequency during transfer of *bla*$_{\text{OXA-48}}$ genes from 50579417 in the presence of the antimicrobial.

4. 4 Ciprofloxacin disc diffusion test

Ciprofloxacin disc diffusion tests were done in order to verify if the isolates obtained from the transfer studies were true transconjugants. Donor isolates are highly resistant to ciprofloxacin due to chromosomal mutations, while the recipients are fully susceptible to ciprofloxacin, as a result this test can help identify true transconjugants. This is the first confirmatory test that the transfer of plasmids was successful (before PCR verification). Discs impregnated with antimicrobial agents are used. The discs are placed on agar plates that has been spread with bacteria and incubated for 18+/-2 hours at 37°C. The diameters of the zone of inhibition formed after the diffusion of the antimicrobial agents into the agar are measured and used to verify the sensitivity of the bacteria. The bigger the zone the more effective the agent is or the more sensitive the bacteria is.

The results obtained showed that the isolates were true conjugants due to their susceptibility to ciprofloxacin (Table 7 and 8).

There were no noticeable difference in the disc diffusion test for transconjugants made in the absence of 0.02µg/ml ciprofloxacin and those made in the presence of 0.02µg/ml ciprofloxacin. The EUCAST recommended clinical breakpoints for ciprofloxacin are listed in Appendix B. Table 3.

Table 7. The ciprofloxacin disc diffusion test results for the possible transconjugants transferred in the absence of ciprofloxacin.

Donor	K71-77 (NDM-1)		50579417 (OXA-48)		
Transconjugant	K56-50-1 ST100	K56-75-1 ST69	G1-40 ST95	K56-50-1 ST100	K56-75-1 ST69
Zone diameter (mm)	38	38	34	38	40

Table 8. The ciprofloxacin disc diffusion test results for the possible transconjugants transferred in the presence of 0.02µg/ml ciprofloxacin.

Donor	K71-77(NDM-1)		50579417 (OXA-48)
Transconjugant	G1-38	K56-75-1	K56-75-1
Zone diameter (mm)	38	40	42

4.5 PCR verification of plasmid transfer

In order to verify if the isolates obtained on the culture plates after the plasmid transfer experiments were done were true transconjugants DNA from the isolates was extracted using the QIAGEN GenoM-48 BioRobot that uses a magnetic based technology to isolate and purify DNA, the steps are shown in procedure 3.6. The DNA that was isolated using the robot was amplified into many copies using PCR as shown in procedure 3.7.

The PCR products were later analysed using agarose gel electrophoresis. The agarose gel electrophoresis separates DNA by size.

By running the DNA alongside the DNA ladder the size of the fragments can be accurately measured.

Formation of 621 bp bands confirms successful transfer of *bla*$_{NDM-1}$ genes, while formation of 438bp bands confirms successful transfer of *bla*$_{OXA-48}$ genes. Procedure 3.8 shows how this test was run in this study.

4.5.1 PCR verification of plasmid transfer-results for NDM-1

The results obtained after agarose gel electrophoresis was done showed that all the isolates from this part of the transfer study were positive for *bla*$_{NDM-1}$ genes. The transconjugants were identified as positive for *bla*$_{NDM-1}$ after the formation of 621 bp bands. Table 13 shows the results.

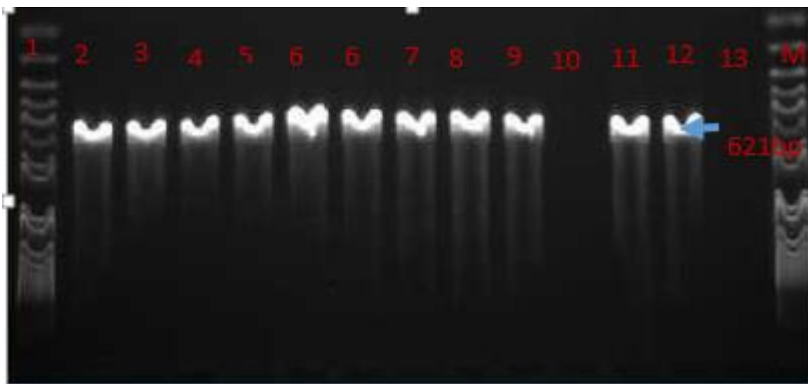

Figure 13. Agarose gel electrophoresis of PCR products. Lane M is molecular size marker, lane 1-5 shows NDM positive K56-50-1 transconjugants. Lane 6-9 further shows NDM-1 positive K56-75-1 transconjugants. Lane 10 is the negative control, 11 and 12 are the positive control while lane 13 is the no template control containing water.

4.5.2 PCR verification of plasmid transfer- results for OXA-48

All the isolates that were analysed in this part of the study tested positive for $bla_{\text{OXA-48}}$ genes on agarose gel. This was confirmed after the formation of 438bp bands in the gel. Figure 14 shows the results

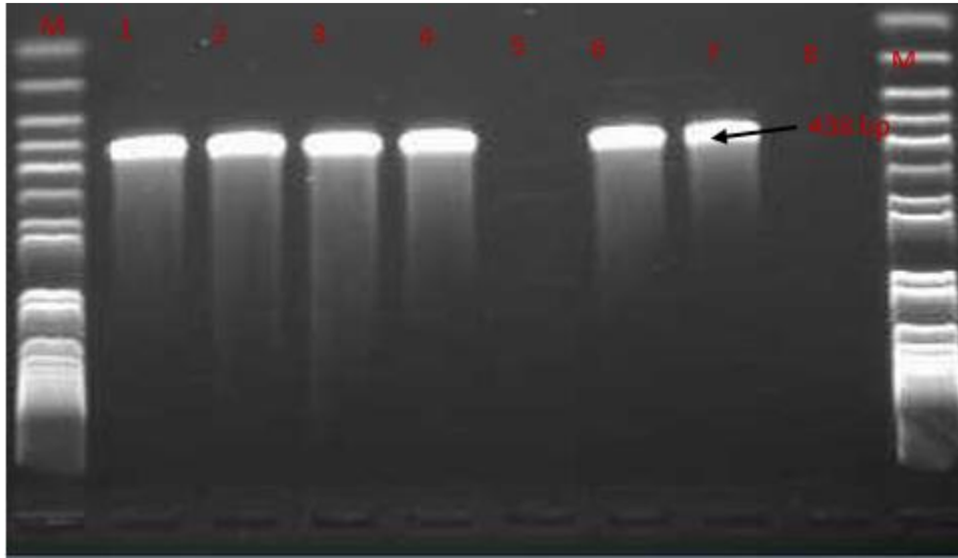

Figure 14. Agarose gel electrophoresis of PCR products. Lane M is molecular size marker. Lanes 1 and 2 are OXA-48 (438 Bp) positive transconjugants these were from K56-50-1 mutants. Lanes 3 and 4 are also OXA-48 positive transconjugants from K56-75-1 mutants. Lane 5 is the negative control. Lane 6 and 8 are the positive controls. Lane 8 is the no template control.

4.6 Antimicrobial susceptibility profile

In this study gradient strips were used to investigate the effect of the transferred plasmids in the different genetic backgrounds. the susceptibility profile of the isolates. Using this method the individual isolates MIC`s toward the various antimicrobial agents were determined.

The susceptibility profile determination was done on the transconjugants, and comparing them with the profiles of the donors (K71-77 and 50579417) and the recipients (G1-38, G1-40, K56-50-1 and K56-75-1).

The susceptibility profile of the isolates were interpreted according to EUCAST clinical breakpoints (Appendix B. Table for EUCAST breakpoints).

As expected all the recipient isolates used in this study were sensitive to all the tested antimicrobial agents except rifampicin (Table 9).

4.6.1 Transconjugants carrying *bla*$_{NDM-1}$ genes

The transconjugants carrying *bla*$_{NDM-1}$ genes showed high resistance to piperacillin-tazobactam and were intermediate for temocillin.

All the transconjugants showed high resistance to aminoglycosides (gentamicin, tobramycin and amikacin) and cephalosporins (cefotaxime, cefoxitin and ceftazidime).

Some isolates were intermediate to the monobactam aztreonam (TC-1 to TC-3), while others were resistant (TC-4 and TC-11). They however showed susceptibility to colistin and trimet-sulfamethoxazole.

Some of the isolates were resistant to ertapenem (TC-1, TC-4 and TC-11), while others were intermediate (TC-2 and TC-3).

Some of transconjugants showed sensitivity to imipenem (TC-2 to TC-4), while others fell into the intermediate category (TC-1 and TC-11).

All the transconjugants were however susceptible to meropenem.

The antimicrobial susceptibility profile of the transconjugants were similar to the donor. However, unlike the donor the transconjugants were sensitive to ciprofloxacin.

Table 9 shows the MIC profiles of the donors, while Table 10 shows the profile of transconjugants.

4.6.2 Transconjugants carrying *bla*$_{OXA-48}$ genes

Transconjugants carrying *bla*$_{OXA-48}$ genes showed high resistance to the penicillins, a profile similar to the donor. However unlike the donor the transconjugants were sensitive to the cephalosporins and aztreonam.

The transconjugants were all sensitive to colistin and all the carbapenems used in this study. This is similar to the donor, except only that it (donor) was resistant to ertapenem.

Furthermore the transconjugants were susceptible to ciprofloxacin and trimet-sulfamethoxazole. This is unlike the donor that was resistant to these agents.

The transconjugants were all sensitive to aminoglycosides. This differs from the donor which was resistant to all the aminoglycosides except to amikacin (see Table 9 and 10 for the full MIC profile of the donors, recipients and the transconjugants).

Table 9. MIC profile of the donors and recipients

	Donors		Recipients			
Ref. no	K71-77	50579417	G1-38	G1-40	K56-50-1	K56-75-1
Sequence type	ST410	ST405	ST73	ST95	ST100	ST69
Piperacillin-tazobactam	≥256	≥256	2	8	2	4
Temocillin	32	≥1024	4	8	4	4
Cefotaxime	≥256	≥256	0.06	0.06	0.03	0.03
Cefoxitin	≥256	128	4	2	2	1
Ceftazidime	≥256	≥256	0.25	0.25	0.25	0.125
Aztreonam	8	≥256	0.06	0.125	0.03	0.03
Meropenem	0.5	0.5	0.03	0.06	0.03	0.03
Imipenem	1	1	0.25	0.5	0.25	0.25
Ertapenem	4	8	0.03	0.125	0.125	0.03
Gentamicin	≥1024	16	0.25	1	1	1
Tobramycin	≥1024	8	1	1	2	2
Amikacin	≥256	4	2	2	2	2
Ciprofloxacin	≥32	≥32	0.03	0.03	0.03	0.03
Colistin	1	1	0.5	2	2	2
Trimet-sulphameth	0.5	≥32	0.125	0.06	0.03	0.03
Rifampicin	8	16	≥256	≥256	≥256	≥256

Table 10. MIC profile of transconjugants

	Transconjugants										
Ref. no	TC-1	TC-2	TC-3	TC-4	TC-5	TC-6	TC-7	TC-8	TC-9	TC-10	TC-11
ST	ST100	ST100	ST69	ST69	ST95	ST95	ST100	ST100	ST69	ST69	ST73
TZP	≥256	≥256	≥256	≥256	≥256	≥256	128	128	128	64	≥256
TMO	16	32	16	32	256	256	128	128	32	64	32
CTX	64	128	≥256	≥256	0.5	0.5	0.5	0.5	0.25	0.25	≥256
FOX	128	128	128	≥256	2	2	2	2	1	2	≥256
CAZ	≥256	≥256	≥256	≥256	0.25	0.5	0.25	0.25	0.125	0.125	≥256
ATM	2	2	2	8	0.06	0.5	0.03	0.06	<0.16	<0.16	16
MRP	1	1	1	1	0.5	0.125	0.125	0.125	0.03	0.03	0.5
IMI	8	2	1	1	2	1	0.5	1	0.125	0.25	4
ERT	2	0.5	1	2	1	0.125	0.25	0.25	0.06	0.125	2
CN	≥1024	≥1024	≥1024	≥1024	1	0.5	1	1	1	1	≥1024
TOB	≥1024	≥1024	≥1024	≥1024	1	1	0.5	0.5	0.5	1	≥1024
AK	≥256	≥256	≥256	≥256	2	4	2	2	2	2	≥256
CIP	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
CS	2	1	1	1	1	2	1	2	1	1	1
SXT	0.125	0.125	0.125	0.125	0.03	0.03	0.03	0.03	0.03	0.03	0.125
RIF	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256	≥256

TZP = Piperacillin-tazobactam, TMO = Temocillin, CTX = Cefotaxime, FOX = Cefoxitin, CAZ = Ceftazidime, ATM = Aztreonam, MRP = Meropenem, IMI = Imipenem, ERT = Ertapenem, CN = Gentamicin, TOB = Tobramycin, AK = Amikacin, CIP = Ciprofloxacin, CS = Colistin, SXT = Trimet-sulfamethoxazole, RIF =Rifampicin. TC-1 (K71-77 × K56-50-1), TC-2 (K71-77 × K56-50-1), TC-3 (K71-77 × K56-75-1), TC-4 (K71-77 × K56-75-1), TC-5 (50579417 × G1-40), TC-6 (50579417 × G1-40), TC-7 (50579417 × K56-50-1),TC-8 (50579417 × K56-50-1), TC-9 (50579417 × K56-75-1), TC-10 (50579417 × K56-75-1),TC-11 (71-77 × G1-38).

4.7 Pulsed-field gel electrophoresis results

Pulsed-field gel electrophoresis is a technique used in molecular biology labs. In this technique an electric field that changes direction is used to separate large separate large DNA fragments.

This enables scientists to generate DNA fingerprints of bacterial isolates.

4.7.1 Rifampicin resistant mutants and susceptible isolates

To verify if the susceptible isolates and the rifampicin resistant mutants had the same genetic background and that no genetic changes took place in the chromosome after the mutants were

generated pulsed-field gel electrophoresis was done on the isolates. 18 isolates comprising of 9 rifampicin resistant mutants and 9 corresponding rifampicin susceptible isolates were selected for analysis as shown in Figure 15 below. From this analysis all the susceptible isolates and their corresponding mutants had similar genetic backgrounds. However some isolates had extra bands which might be due to the fact that these isolates have been cultured a number of times might also explain the presence of the extra bands as evidence has shown that some strains develop bands differences over time after repeated culture [64].

However despite the few bands differences between the rifampicin resistant mutants and their susceptible counterparts the results indicates no genetic change.

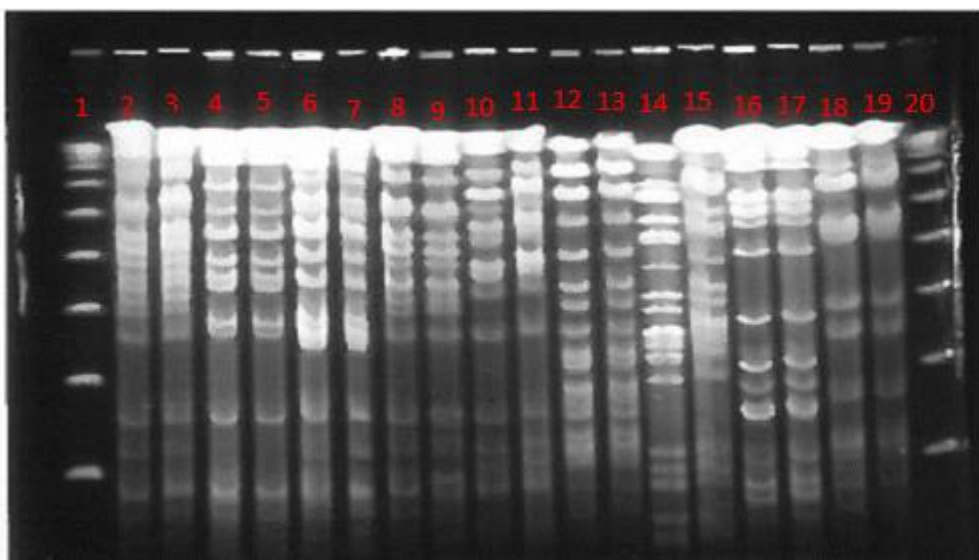

Figure 15. PFGE results of the susceptible isolates and their rifampicin mutants counterparts. Lane 1 and 20 PFG marker, 2 = K56-2(ST73[S]), 3 = G1-36 (ST73[M]), 4 = K56-17 (ST73[S]), 5 = G1-37(ST73[M]), 6 = 56-22 (ST73[S]), 7 = G1-38 (ST73[M]), 8 = K56-35 (ST73[S]), 9 = G1-39 (ST73[M]), 10=K56-68(ST95[S]), 11= G1-40 (ST95[M]), 12 = K56-50 (ST100[S]), 13 = G1-78 (ST100[M]), 14 = K56-65 (ST215[S]), 15 = G1-79(ST215[M]), 16 = K56-75(ST69[S]), 17 = G1-80 (ST69[M]), 18 = K56-78 (ST1235[S]), 19 = G1-81 (ST1235[M]). S = susceptible isolate, M = Rifampicin mutant. Each susceptible isolate is next to a rifampicin mutant that was generated from it.

4.7.2 Rifampicin resistant mutants and transconjugants

Similarly a comparative pulsed-field gel electrophoresis was done on the transconjugants and the rifampicin resistant mutants from which they were generated in order to verify if there were any genetic differences between them. If two or more isolates have a similar banding

pattern it is an indication that they are clonally related. The donor isolates K71-77 and 50579417 were compared with rifampicin resistant mutants (G1-38, G1-40, G1-78 and G1-80) and the transconjugants (G2-01, G2-02, G2-03, G2-04, G2-05, G2-06, G2-07, G2-08, G2-09, G2-10 and G2-11) as shown in figure 16.

From the Pulsed-field gel electrophoresis results K71-77 and 50579417 were distinct, while the G2-11 transconjugant (K71-77 × G1-38) was similar to G1-38 rifampicin resistant mutant. The G1-40 rifampicin resistant mutant was similar to G2-09 (50579417 × G1-40) and G2-10 transconjugants (50579417 × G1-40). The G1-78 (K56-50-1) rifampicin resistant mutant was similar to G2-01 (K71-77 × K56-50-1), G2-02 (K71-77 × K56-50-1), G2-03 (50579417 × K56-50-1) and G2-04 (50579417 × K56-50-1).

The G1-80 (K56-75-1) rifampicin resistant mutant was similar to G2-05 (K71-77 × K56-75-1), G2-06 (K71-77 × K56-75-1), G2-07 (50579417 × K56-75-1) and G2-08 (50579417 ×K56-75-1). However despite the similarities there were some band differences between some transconjugants and the rifampicin resistant mutants. The bands might have been due to plasmid DNA [65].

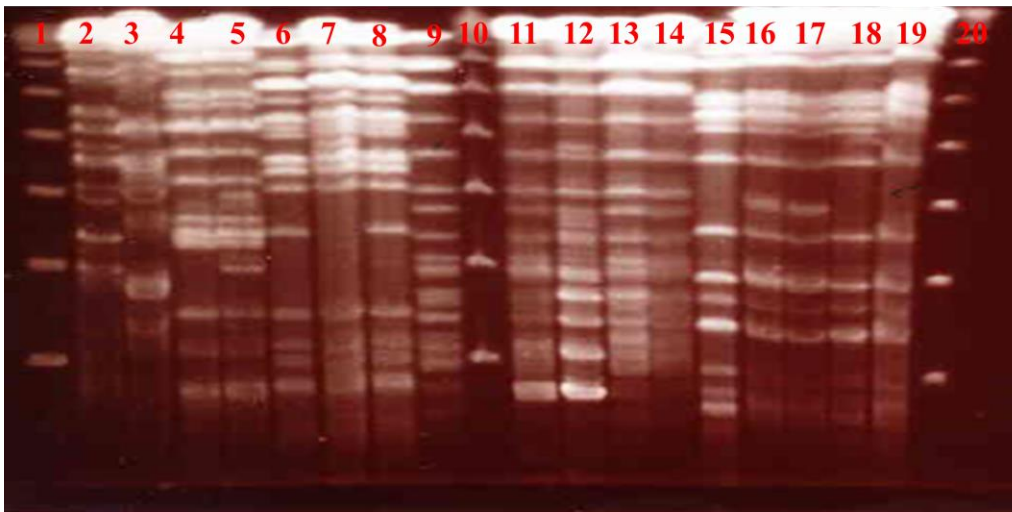

Figure 16. PFGE results of rifampicin resistant mutants and transconjugants. Lane 1,10 and 20 = PFG marker, 2 = K71-77 (NDM-1), 3 = 50579417 (OXA-48), 4 = G1-38 (ST73[M]), 5= G2-11 (G1-38 × K71-77), 6= G1-40 (ST95[M]), 7 = G2-09 (G1-40 × 50579417), 8= G2-10 (G1-40 × 50579417), 9 = G1-78 (ST100[M]), 11= G2-01 (K56-50-1 × K71-77), 12 = G2-02 (K56-50-1 × K71-77), 13 = G2-03 (K56-50-1 ×50579417), 14 = G2-04 (K56-50-1 ×50579417), 15 = G1-80 (ST69[M]), 16 = G2-05 (K56-75-1 × K71-77), 17 = G2-06 (K56-75-1 × K71-77), 18 = G2-07 (K56-75-1 × 50579417), 19 = G2-08 (K56-75-1 × 50579417. M = Rifampicin resistant mutant.

4.8 Plasmid relative fitness test

To ascertain if plasmids had any effect on the fitness of their host competition experiments were done. Competition experiments measures the relative fitness of two individual bacterial isolates grown in the same media. The densities of the competing isolates were measured just after they are mixed and after 24 hours incubation on selective and non-selective media. In this way the growth rate of the competing isolates can be measured. If one competitor grows slower than the other the reduced growth can be attributed to reduced fitness [30].

In this study two transconjugants, G2-06 (K71-77 × K56-75-1) and G2-07 (50579417 × K56-75-1) carrying $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes respectively were competed against a K56-75-1 rifampicin resistant mutant. The results (Figure 17) were obtained from three different independent experiments run in triplicates.

The relative fitness cost for G2-06 at 95% confidence interval was 0.83 (± 0.017). This indicates a 17 % reduction in fitness. The null hypothesis was rejected on a $P \leq 0.001$ value, making us to conclude that the means are statistically different

Similarly the relative fitness for G2-07 was 0.92 (± 0.01). This indicates a reduction in fitness of 8%. On a P value of 0.013, we rejected the null hypothesis and concluded that the means are statistically different. Figure 17 illustrates the results.

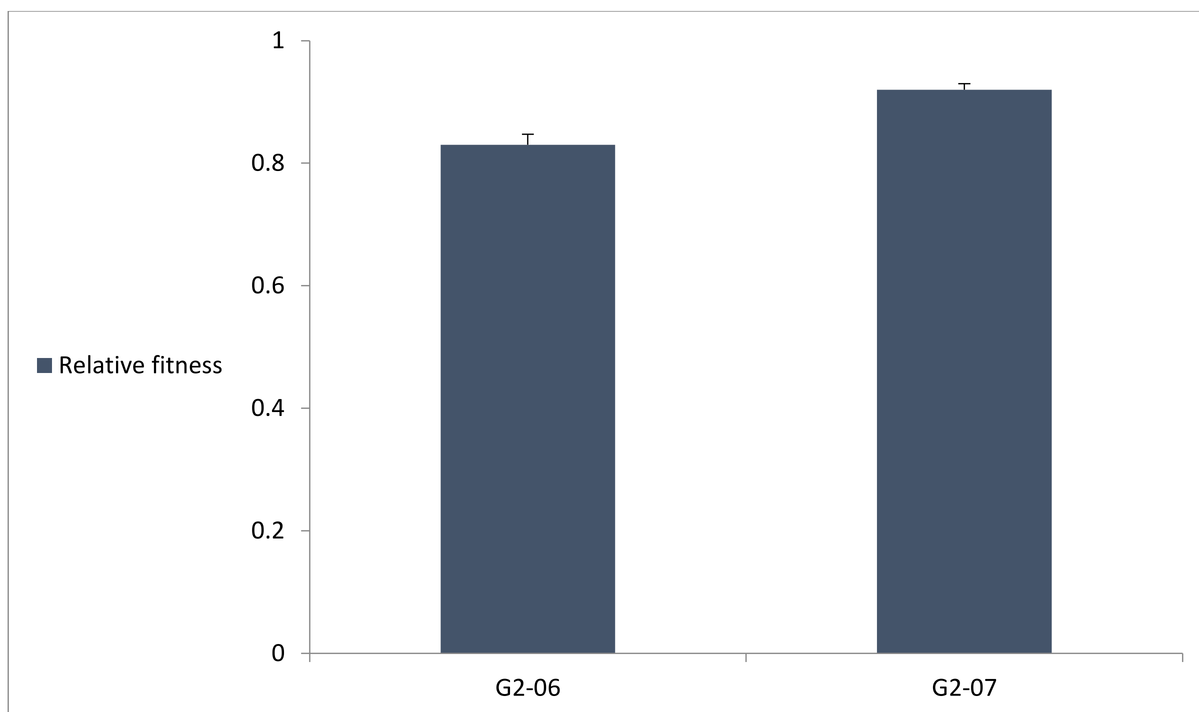

Figure 17. A graphical representation of the relative fitness costs bla_{NDM-1} and bla_{OXA-48} genes in K56-75-1.

4.9 Plasmid stability test

The inoculation loop pick method was used to perform this study. The loop was used to pick single colonies from a non-selective to a selective plate (see procedure 3.13). In this part of the study the resistant bacteria were grown in in the absence of antimicrobial agents to ascertain the stability of the plasmids without the selective influence of antimicrobial agents. This was done over a duration of 10 days. G2-06 and G2-07 harbouring $\textit{bla}_{NDM-1}$ and $\textit{bla}_{OXA-48}$ genes respectively were chosen for this study. After 10 days the isolates were able to grow on selective media. This demonstrates that the plasmids were 100% stable. Figure 18 and 19 gives the graphical illustration of the results.

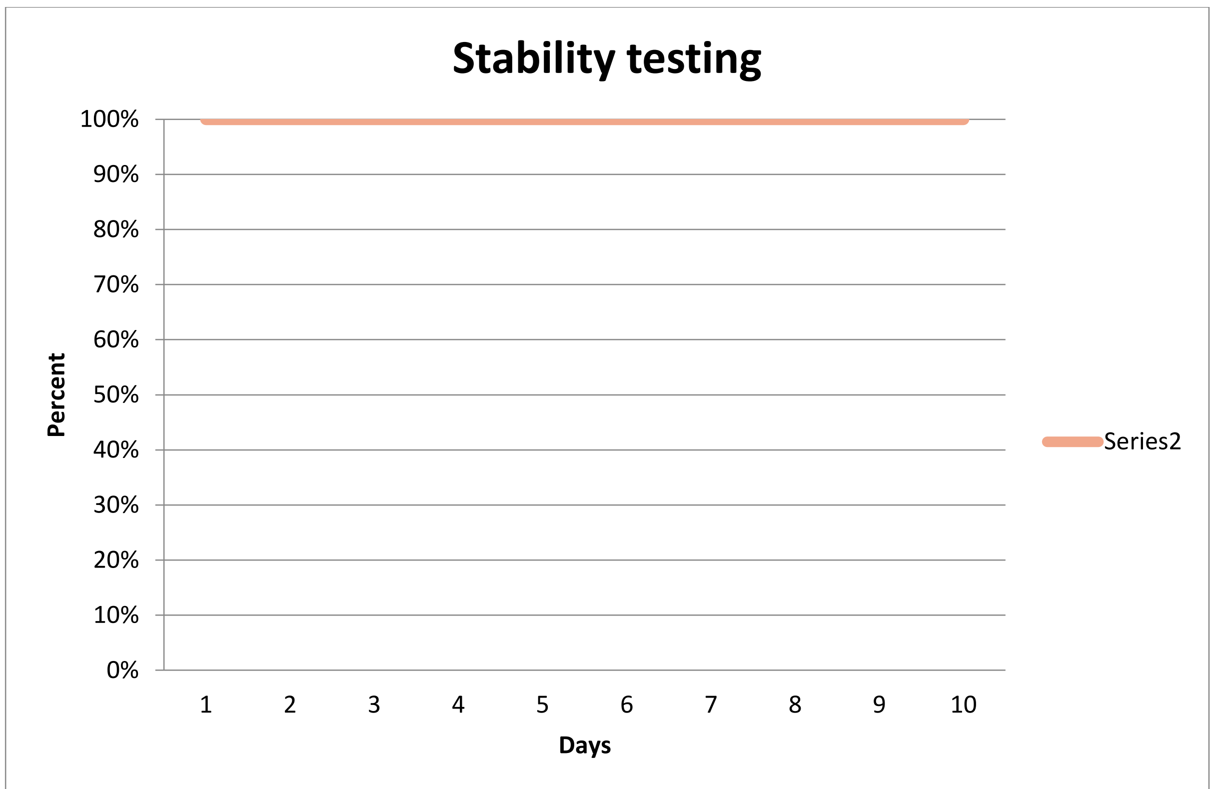

Figure 18. Graph illustrating the stability of the plasmids encoding *bla*$_{OXA-48}$ genes in K56-75-1

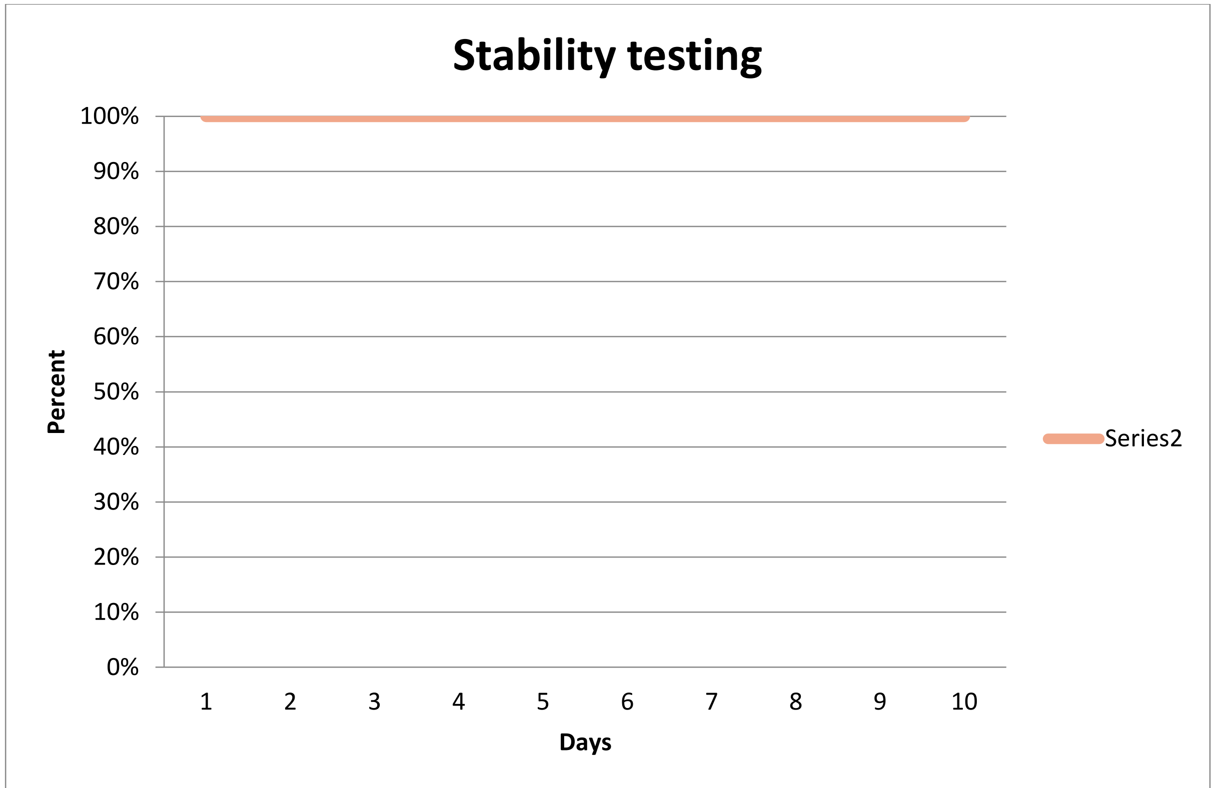

Figure 19. Graph illustrating the stability of the plasmids encoding *bla*$_{\text{NDM-1}}$ genes in K56-75-1

5. Discussion

In the recent decades there has been an increase in the spread of resistance genes. One of the major concerns among public health officials is the spread of the carbapenemases among Gram-negative bacteria. Carbapenemases are β-lactamases enzymes that has an ability to hydrolyse most β-lactams agents including carbapenems. Carbapenemases particularly NDM-1 and OXA-48 are now widely spread and have been reported on virtually all the continents [21-23].

Perhaps their spread has largely been helped by medical tourism and the ease in which people are travelling between different countries nowadays [7, 22].

The increase in the spread of these resistance genes has been mainly attributed to plasmids. Plasmids are extrachromosomal DNA molecules between 1-1000Kbp. Plasmids have an ability to carry several resistance genes and have a high ability to be spread from one bacterial to another through conjugative transfer and as a result have heavily contributed toward the increased disseminations of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes [1, 22].

This study focuses on how the sub-lethal concentration of antimicrobial agents can increase the frequency of plasmid transfer and how the absence of sub-lethal concentrations of antimicrobial agents can affect plasmid fitness and stability.

5.1 Generation of rifampicin resistant mutants

Scientists generates mutants in the laboratory for counter selection. This is achieved by taking advantage of the intrinsic properties of the individual bacteria. This process helps them to get rid of unwanted cells or bacteria [66].

In this study 100 µg/ml rifampicin was used to generate the rifampicin mutants. The resulting isolates. This further shows how antimicrobial agents can select for resistance.

5.2 Plasmid transfer studies

The widespread use of antimicrobial agents in humans, poultry and animal husbandry has led to an increased release of sub-lethal concentration of the agents in the environment. This has as a result led to continuous exposure of bacteria in the environment to sub-lethal concentration of antimicrobial agents. Results obtained from some recent experiments now indicate that minimal concentration of the agents can select for antimicrobial resistance. This is a departure from the widely held notion that only lethal concentration of antibiotics selects

for resistance. Sub-lethal concentrations have been found to affect the horizontal gene transfer rate. [67].

Sub-lethal concentration of antimicrobial agents have also been found to influence the rate at which mobile genetic elements are transferred. These mobile genetic elements includes transposons, pathogenicity islands, bacteriophages and plasmids. These mobile genetic elements plays a big role in the spread of resistance genes [68].

Furthermore it has been shown that sub-lethal concentration of β-lactam agents increases plasmid transfers in *Staphylococcus aureus* [69].

Sub-lethal concentration of antimicrobial agents might lead to an induction of a SOS response (stress response) in some bacteria. This response can promote expression of genes that are important in horizontal gene transfer. A good example of this has been found in the transfer of SXT integrating conjugative elements (ICE) of *Vibrio cholera.* ICE are mobile genetics elements that spreads from one cell to another through direct contact. It has been discovered that by growing the donor *E. coli* cells in sub-lethal concentration of ciprofloxacin the ICE transfer is increased by a 300 fold. Ciprofloxacin induces a SOS response in the bacteria that leads to increased SXT transfer [70].

Another study found that sub-lethal concentration of flavophospholipol reduced the transfer frequency of conjugative plasmids carrying vancomycin resistance genes by a 70-fold [71].

Our study shows that there was a marked increase in the transfer frequency of the $bla_{\text{NDM-1}}$ carrying plasmids in the presence of 0.02 µg/ml ciprofloxacin. The fact that there was a reduction in the transfer frequency of plasmids harbouring $bla_{\text{OXA-48}}$ genes in the presence of 0.02 µg/ml ciprofloxacin indicates that the antimicrobial agent has no effect on the transfer frequency of OXA-48 carrying plasmids. Furthermore the fact that G1-38 only took up *bla*NDM-1 carrying plasmids in the presence of 0.02 µg/ml ciprofloxacin albeit at a lower frequency further indicates that sub-lethal concentration of ciprofloxacin only has an effect on certain plasmids types, like $bla_{\text{NDM-1}}$ harbouring plasmids in our case. This might also have something to do with the genetic background of the host. The fact that two out of our three comparative experiments showed increased plasmid transfer is an indication that sub-lethal concentration of antimicrobial agents increases the frequency of plasmid transfer depending on the profile of the plasmid and the genetic background of the host.

However because our experiment was not very conclusive and we were limited in the number of isolates we worked with due to the limited time frame of this study, we acknowledge the fact that more studies are needed to be done on this in order to come to a firm conclusion.

5.3 Antimicrobial susceptibility profile

5.3.1 OXA-48

There have been a number of reported hospital outbreaks of OXA-48 in the Western European countries like France and Spain in recent years ever since it was first in 2001 reported in *K. pneumoniae* [20, 21].

The first isolates were found to have high level resistance to cephalosporins, cephamycins, monobactams and carbapenems. It was later revealed that this isolate co-expressed other β-lactamases like ESBLs, SHV-2, TEM-1 and OXA-47 [20-22].

OXA-48 can inactivate penicillins, first generations cephalosporins, but is infective against extended-spectrum cephalosporins and carbapenems. Multi-antimicrobial resistance in OXA-48-producing isolates is mainly due to co-production with other lactamases especially ESBLs [20, 22].

In this study the transconjugants showed high resistance to penicillins but were susceptible to the extended-spectrum cephalosporins, aminoglycosides and carbapenems.

The donor were however resistant to the penicillins, cephalosporins, aminoglycosides (except amikacin). The donor's multi resistant profile can be due to the presence of other ESBLs genes, most likely *bla*$_{CTX-M-15}$. There has been a strong association between isolates belonging to the donor sequence type (ST405) and *bla*$_{CTX-M-15}$ [40, 72].

This indicates that the plasmids that was transferred during this study only contained *bla*$_{OXA-48}$ genes and not ESBLs or aminoglycoside resistance genes, which is usual for OXA-48 plasmids not to carry additional resistance genes [20-22].

5.3.2 NDM-1

Like OXA-48, NDM-1 has widely spread ever since it was first reported in 2009. India has been singled out as being the epicentre of the spread of the producers. The presence of NDM-1 in Gram-negative bacteria makes them to become resistant to penicillins and cephalosporins. Some producers may have resistance to carbapenems. However many producers are susceptible to colistin. The level of resistance may vary between producers because some plasmids carrying $bla_{\text{NDM-1}}$ genes may harbour other resistance genes like those for macrolide resistance, aminoglycosides and ESBLs [22, 23].

In this study the transconjugants carrying $bla_{\text{NDM-1}}$ genes showed a high resistance to penicillins, aminoglycosides and the cephalosporins and there was a change in the susceptible profile toward the carbapenems. The variation in the susceptible profile of the transconjugants toward aztreonam and the carbapenems maybe an indication that different plasmids were transferred to the different recipients. However the susceptibility profile of the transconjugants is generally similar with the donor. The previous study which was done on the donor isolate showed that it also co-expressed $bla_{\text{CMY-6}}$, an AmpC plasmid mediated gene although it was negative for other ESBLs which might partly explain the susceptibility profile [33].

5.4. Verification of plasmids

5.4.1 Pulsed-field gel electrophoresis results

Pulsed-field gel electrophoresis gel-electrophoresis is often used by scientists for genotyping bacteria. It has helped in epidemiology and comparative studies and identify (fingerprinting) bacteria belonging to the same or different sequence types. This technique has further helped to identify successful clones (widely spread clones) [72].

In this study pulsed-field gel electrophoresis was used to verify clonal relativity of susceptible and rifampicin resistant isolates. It was also used to verify the clonal relativity between rifampicin resistant mutants and transconjugants. In both cases the isolates had similar banding patterns, although there were some additional bands in some isolates. The bands might have been artefacts formed due to incomplete digestion or as a result of the

isolates being subjected to multiple cultures in the case of susceptible and their corresponding rifampicin resistant isolates [64].

The bands differences between some rifampicin resistant mutants and transconjugants might be as a result of the restriction enzyme cutting through the plasmid DNA. However despite these bands variation there is a clear clonal relativity among the isolates suggesting that there were no chromosomal changes that took place.

5.4.2 PCR

PCR is the most reliable and accurate way of verifying the transfer of plasmids compared to the phenotypic way by looking at the susceptibility profile. PCR enables us to get results within a space of 4-6 hours. The only disadvantage of this technique is the high cost involved and the need to have properly trained personal [22].

PCR was used in this study to confirm transfer of plasmids carrying NDM-1 and OXA-48 genes. In both cases successful transfer was confirmed by the formation of 621bp and 438bp bands for $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes respectively.

5.4.3 Ciprofloxacin disc diffusion test

In an aim to harmonize the order in which results by different labs in Europe are reported EUCAST has introduced a disk diffusion test. It is based on MH media and uses paper discs impregnated with antimicrobial agents. The results are then calibrated according to clinical breakpoint set up by EUCAST [48].

In this study ciprofloxacin disc diffusion were used as the first verification for transconjugants. The interpretation of the results were done according to EUCAST recommendations. The formation of zones of inhibition which were between 34-42mm for both transconjugants transferred in the absence and presence of 0.02µg/ml ciprofloxacin indicated that the isolates were true transconjugants. This is a very important step prior to PCR confirmation. This test minimized out the chance of mistaking a donor for a transconjugants as donors have high resistance to ciprofloxacin.

5.4.4 Vitek 2 and MALDI-TOF

Vitek 2 and MALDI-TOF are among the automated methods used in the identification of bacterial species in medical and research laboratories. The Vitek identifies bacteria using cards inserted with chemicals. The biochemical pattern of the chemical determines the species [44].

The MALDI-TOF identifies the species by identifying their unique spectrums. Individual species have unique spectrums [60, 61].

Using these two systems we were able to identify and verify that all the isolates in this study were *E. coli* isolates.

5.5 Relative fitness

Studies have shown the acquisition of plasmids by host bacteria can place an energy burden on the host bacteria. This energy is required for maintenance of the newly acquired DNA. The newly acquired DNA may also encode proteins that may be detrimental to the host. All this can as a result lead to reduced fitness of the host bacteria [28, 30].

Relative fitness tests done in our study indicates that acquisition of NDM-1 plasmids imposes a fitness cost of 17% on the host (K56-75-1).

Similarly our study found that plasmids carrying OXA-48 had a fitness cost of 8% on the host. This suggests that plasmids acquisition have a large fitness effect on the host.

The difference in the fitness reduction between OXA-48 and NDM-1 may be due to that fact that the additional genes the NDM-1 plasmids carried might have led to a lot of energy demands on the host leading to more fitness effects, unlike OXA-48 whose results indicates that it carries no additional genes.

5.6 Plasmid stability

The magnitude of the biological costs imposed on the host by the plasmids determines how long they can be stably maintained in subsequent generations [30].

Studies have been done to verify if bacteria loses its plasmids without the selection pressure of antimicrobial agents. Some scientists suggested that reduction in the use of antimicrobial agents might help reduce the frequency of resistant isolates through natural selection.

However bacteria has been found to employ compensatory mechanisms (evolution) that allows them to stably maintain the plasmids in the absence of antimicrobial agents in the environment. This reduction in fitness caused by resistance may be overcome when the bacteria undergoes a compensatory mutations process, this can allow them to subsequently maintain the resistant genes.

Similarly in our study two transconjugants that were grown in the absence of antimicrobial agents over a duration of 10 days showed 100% plasmids stability (Figure 18 and 19). This suggests that these plasmids can be maintained in the absence of antimicrobial agents. This further raises doubts over the theory that reducing or discontinuing the use of antimicrobials agents can help reduce the frequency of resistant genes.

6. Conclusion

- Sub-lethal concentration of antimicrobial agents might increase plasmid transfer frequency depending on the profile of the plasmid.
- Plasmid imposes a fitness cost on the host and the magnitude of the cost depends on the plasmid type.
- Reducing the use of antimicrobial agents does not guarantee reduction in the frequency of resistance genes.
- The susceptible profile of transconjugants harbouring the same resistance genes may differ depending on the genetic background of the host.

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Appendices

Appendix A: Table 1. Buffers, media and reagents

Green agar plates Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Bromthymol blue solution 0,2 % (Merck) ddH2O	Green agar plates w/ 100 µg/ml rifampicin Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Bromthymol blue solution 0,2 % (Merck) 100 µg/ml rifampicin (Bristol-Myers Squibb) ddH2O
LB-broth 25 g Difco LB Broth (BD) ddH2O to 1 litre	LB-agar w/ 100 µg/ml rifampicin 40 g Difco LB Agar (BD) Rifampicin 100 µg/ml (Sigma-Aldrich) ddH2O to 1 litre
Freeze broth Glycerol (Merck) Brain Heart Infusion (Oxoid) ddH2O	0.45 % sodium chloride 4.5 g Natrium chloride (Merck) ddH2O to 1 litre
Green agar plates w/ 100 mg/ml ampicillin Bromthymol blue solution 0,2 % (Merck) Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Ampicillin (Bristol-Myers Squibb) ddH2O	Mueller-Hinton (MH) agar Mueller-Hinton agar base (BD) ddH2O
0.85 % NaCl 8.5 g Natrium chloride (Merck) ddH2O to 1 litre	10X TBE buffer 108 g Tris Base (Sigma-Aldrich) 55 g Boric acid (Sigma-Aldrich) 40 ml 0,5 M EDTA pH 8.0 (Sigma-Aldrich) ddH2O to 1 litre

BHI medium	PIV-buffer
52 g Brain Heart Infusion (Oxoid) 15 g Bacto agar (Difco) ddH2O to 1 litre	10 mM Tris-HCl pH 7.6 (Sigma-Aldrich) 1 M NaCl (Merck)
Lysis-buffer (for 5 plugs)	TE-buffer
10 ml basic buffer 0.02 g sodium deoxycholate (Sigma-Aldrich) 0.5 g N-laurolsylsarcosine (Sigma-Aldrich) 0.01 g lysozyme (Sigma-Aldrich) 1 µl RNase One (10U/ µl) (Promega)	10 mM Tris-HCl pH 8 (Sigma-Aldrich) 0.1 mM EDTA (Sigma-Aldrich)

Appendix A: Table 2. Buffers, media and reagents

3X GelRed staining solution	
15 µl GelRed 10,000 stock solution 50 ml ddH$_2$O	

Appendix B.

Table 1. General recipe for master mix used in this study

JumpStart REDTaq	500 µl
RNAse/DNAse free ddH_20	384 µl
Forward primer (OXA-48/NDM-1) 1.50 µl (pmole/µl)	8 µl
Reverse primer (OXA-48/NDM-1) 2.50 µl (pmole/µl),	8 µl
Total volume	**900 µl**

Table 2. How the PCR programs were run in this study (applies for both OXA-48 and NDM-1)

Process	Temperature	Time	Cycles
Denaturing	95^0C	5min	
Denaturing	95^0C	30 seconds	X 36 cycles
Annealing	56^0C	30 seconds	
Elongation	72^0C	50 seconds	
Elongation	72^0C	5 min	
Hold	4^0C	10 min	
Hold	10^0C	∞	

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Table 3.Eucast zone diameter breakpoint for ciprofloxacin

	Eucast zone diameter for ciprofloxacin	
Reporting	S ≥	R <
Diameter	22	19

Table 4. EUCAST Clinical Breakpoint Table for *Enterobacteriaceae*

	MIC breakpoint (mg/L)	
Antimicrobial agent	S ≤	R >
Piperacillin-tazobactam	8	16
Cefotaxime	1	2
Ceftazidime	1	4
Aztreonam	1	4
Meropenem	2	8
Imipenem	2	8
Ertapenem	0.5	1
Gentamicin	2	4
Tobramycin	2	4
Amikacin	8	16
Ciprofloxacin	0.5	1
Colistin	2	2
Trimethoprim-sulfamethoxazole	2	4