ANTIBIOTIC SUSCEPTIBILITY AND PLASMID PROFILE OF *ESCHERICHIA COLI* FROM DOOR HANDLES IN TWO TERTIARY INSTITUTIONS IN NASARAWA STATE, NIGERIA

BY

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MSc MEDICAL MICROBIOLOGY

ADISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, NASARAWA STATE UNIVERSITY, KEFFIIN PARTIAL FULFULMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS OF SCIENCE (M.Sc.) DEGREE IN MEDICAL MICROBIOLOGY

NOVEMBER, 2016

DECLARATION

I hereby declare that this dissertation titled "Antibiotic Susceptibility and Plasmid Profile of *Escherichia coli* From Door Handles In Two Tertiary Institutions In Nasarawa State, Nigeria" was performed by me in the Department Biological Sciences(Microbiology unit), under the supervision of Prof. J.O.Ehinmidu. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any institution.

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Tsaku Paul Alumbugu

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Date

CERTIFICATION

This dissertation titled "Antibiotic Susceptibility and Plasmid Profile of *Escherichia coli* From Door Handles In Two Tertiary Institutions In Nasarawa State, Nigeria" by **Tsaku Paul Alumbugu** meets the regulations governing the award of the degree of Masters of Science in Medical Microbiology of the Nasarawa State University, Keffi, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To Jesus Christ, the King of kings and Lord of lords I dedicate this work for the abundant, inestimable Grace, Mercyand Love he lavished upon me boundlessly and endlessly.

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ABSTRACT

Contamination of door handles with antibiotic resistant bacteria can be a major threat to public health, as the antibiotic resistant determinants can be transferred to other pathogenic bacteria thus, compromising the treatment of severe bacterial infections. *Escherichiacoli*a harmless commensal and a versatile pathogen,has been known to develop or acquire resistance to a variety of antibiotics by different mechanisms. Thus this study therefore isolated*E. coli* from door handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa and determined their susceptibilities to currently prescribed antibiotics using standard microbiological procedures. A total of 62 *E. coli* were isolated out of 200 door handles sampled and their susceptibilities to ten different commonly used antibiotics were determined. All the isolates had 87 – 100% resistance to all tested antibiotics with the highest susceptibility (13%) exhibited to only Gentamicin and Imipenem. Thirty-two of the isolates have Multiple-Antibiotic Resistance (MAR) index of 1.0 and 21(65.6%) of them produced β-lactamase enzymes. Thirteen (59.09%) of the multiple antibiotics resistant *E. coli* isolates transferred resistance plasmid*Proteus mirabilis*via conjugation. Electrophoresis of plasmid DNA in the test multi-antibiotics resistant *E. coli* isolates showed varying number of plasmids with molecular weights ranging between 1200 and 3000 base pairs. This study has shown that multi-antibiotic resistance genes from test *E. coli* could be transmitted to pathogenic bacteria which can result in serious health hazard. Thus, improved hygiene practices should be encouraged and constant microbiological surveillance of door handles in these higher institutions should be encouraged to determine effective antibiotics to solve the health hazard that may arise from *E. coli* infections.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The spread of infectious diseases through hand contact has been an area of major concern. According to a study conducted by Itah and Ben (2004), Gram-positive *Staphylococcus aureus*, and Gram-negative enteric bacteria such as *Escherichia coli*, *Klebsiella* species, *Citrobacter* species, were found to contaminate various contact surfaces including chairs, tables, windows, door handles, and many other common household fixtures. Infectious diseases top the list for causes of death worldwide and contribution to morbidity and mortality cannot be readily quantified due to lack of data for most countries and it remains a global concern (Barbosa and Levy, 2000).

Diseases commonly spread by means of environmental surfaces such as computers, classroom walls, door handles, toilets, chairs, and so on include the common cold, cold sores, conjunctivitis, giardiasis, impetigo, meningitis, pin worm disease, diarrhoea and pneumonia, to mention but a few (WHO, 1980). Bacteria such as *Escherichia coli*, *Shigella dysenteriae*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Staphylococcus aureus* as well as *Corynebacterium diphtheriae* cause diarrhoea, dysentery, pneumonia, food poisoning and intoxication as well as whooping cough respectively (WHO, 1980).

Human hands have been implicated as the major transmitter of microorganisms to environmental surfaces. Curtis and Carncross(2003) reported that hands often act as vectors that carry disease-causing pathogens including bacteria and viruses from person to person either through direct contact or indirectly via surfaces. Defective

personal hygiene can facilitate the transmission of some of these pathogenic bacteria found in the environment to human hands (Mensah *et al.,* 2002).

Microorganisms carried on the human skin are of two types, that is, resident and transient (Talaro and Talaro, 2006).Pathogens that may be present on the hand as transient types includes *Escherichia coli, Salmonella spp., Shigella spp., Clostridium perfringens, Giardia lamblia,* Norwalk virus and Hepatitis A virus (Morton, 2006). Since human hands usually harbour microorganisms both as residents and transients (Lindberg *et al*., 2004; Williams, 1963), it is conceivable that transfer of pathogens could occur between people who access the same area or surfaces. The chance that other persons will acquire these organisms is dependent on how long the organism can survive in the environment. For example, *Listeria species* can survive for a while on the hand and in the environment (Snelling *et al*., 1996); also, a growing body of research has examined environmentally persistent *E. coli* which can survive for extended periods outside of a host (Ishii and Sadowsky, 2008).

Some research results indicate that surfaces that are routinely touched with hands have higher bacteria load as compared to restroom floor and toilet seats. One could expect the opposite to be true. This observation could be due to cumulative contamination of door handles as result of poor sanitary conditions (not washing and cleaning hands with disinfectants after using the toilets) (Augustino *et al*., 2014). Hand washing which is traditional was the first line of defense in preventing the spread of disease; it has been neglected and must be embraced vigorously by families, schools and healthcare professionals. However many people seem to run water over their hands without using soap and some fail to wash their hands at all after leaving the restroom (Barker and Bloomfield, 2000).

Bacteria are microscopic organism found everywhere in the Universe as pathogenic or non-pathogenic. They are found in the environment all around us and within each one of us, there are trillions and trillions of them. Majority of them are harmless to human and animals but those few which are harmful can lead to death of affected individuals (Hooper, 2001; Short *et al*., 2007). The time of survival depends on the type of pathogen, majority including S*higella* species , *Escherichia* species, *Clostridium* species, severe acute respiratory syndrome (SARS) coronavirus, and norovirus which can survive on surfaces for weeks or even months (Kramer *et al*., 2006).

*Escherichia coli*is a Gram-negative, facultatively anaerobic, rod-shapedbacterium of the genus*Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Singleton, 1999). Most *E. coli*strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (Vogt and Dippold, 2005). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2 (Bentley and Meganathan, 1982), and preventing colonization of the intestine with pathogenic bacteria (Hudault *et al*., 2001; Reid *et al*., 2001).

Escherichia coli and other facultative anaerobes constitute about 0.1% of gut flora (Eckburg *et al*., 2005), and faecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for faecal contamination (Feng *et al*., 2002; Thompson and Andrea, 2007).

1.2 Statement of the Problem

Sanitary conditions in public places have always been amajor problem, especially in schools. Health Departments are continually checking the cleanliness and safety of these bacteria breeding places to prevent thespread of sickness and disease (Chris *et al*., 2002).

The hands are the chief organs for physical manipulation of the environment. As a paired organ, the hand is controlled by the opposing brain hemisphere (Maria and Eliane, 2004) and enables one to do all manner of things. The hand serves as a medium for the propagation of microorganisms from place to place and from person to person. Although it is nearly impossible for the hand to be free of microorganisms, the presence of pathogenic bacteria may lead to chronic or acute illness. Human's hands usually harbour microorganisms both as part of body normal flora as well as transient microbes contacted from the environment (Augustino*et al*., 2014). The natural habitat of microorganisms like *Escherichia coli* is the human skin and can therefore be passed from one person to another. Many food borne diseases and pathogenic microorganisms are spread by contaminated hands. One common way by which organisms that are not resident in the hand are picked up is by contact with surfaces such as table tops, door knob or handles, banisters, toilet handles and taps in restrooms.

It is in view of the above that this research idea was conceived, to isolate *Escherichia coli* from door handles, and to study antibiotics resistance and the resistance factors.

1.3 Aim of the Study

This study is designed to isolate and to determine the antibiotics susceptibility and plasmid profile of *Escherichia coli* from door handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa, Nasarawa State, Nigeria.

1.4 Objectives

I. To isolate and identify*Escherichia coli* from door handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa, Nasarawa State, Nigeria.

II. To carryout antibiotic susceptibility test of the *Escherichia coli* isolates.

III. To determine the minimum inhibitory concentration of the test antibiotics on the isolates.

IV. To determine the presence of Beta-lactamase production in resistant *Escherichia coli* isolates.

V. To determine presence and sizes of resistant factor (plasmids) in the *Escherichia coli* isolates.

VI. To determine whether the resistant factors are transferable by conjugation method.

1.5 Justification/Relevance of the study

According to Salton, Gram negative bacteria show virulent characteristics due to the presence of endotoxin in their outer membranes (Talaro and Talaro, 2006). Endotoxin causes activation of immune system cells such as macrophages, neutrophils, and Blymphocytes, which may lead to tissue necrosis and endotoxic shock. It has been further demonstrated that Gram negative bacteria have higher antibiotic resistance, partially due to the presence of an outer membrane which may prevent the entry of some antibiotics(Thomson and Bonomo, 2005). These observations are important considerations that merited this study with door handles. The presence of these

pathogenic bacteria on environmental surfaces such as door handles poses a potential risk to vulnerable, immune-compromised individuals. It has been shown that hard, non-porous surfaces, such as door handles, have the highest bacterial transfer rates to hands (Rusin*et al*., 2002). In recent past a lot of effort has been invested in emphasized hand hygiene through hand wipes and hand sanitizers. According to a study by Stout *et al.*(2010), hand wipes with higher ethanol content are more effective in not only antimicrobial activity but also removal of endospores via the mechanical action. Even though people are commonly aware of such practices, the possibility of inaccessibility or lack of use of these practices does occur. According to Hansen and Knochel (2003), up to 60% of adults do not wash their hands when appropriate.

1.6 Scope and limitation of the study

This research work is limited to the isolation of *Escherichia coli* from one hundred (100) door handles, equally distributed between the five faculties in the main campus of Nasarawa State University, Keffi, and one hundred (100) other samples obtained from Federal Polytechnic, Nasarawa. Antibiotic susceptibility study, conjugation analysis and plasmid profiling were conducted on the isolates.

1.7 Statement of Hypothesis

Null Hypothesis

1. *Escherichia coli* cannot be isolated from door handles.

2. The isolated*E. coli*are not susceptible to antibiotics.

3. *Escherichia coli* do not produce β-Lactamases.

4. The resistance genes of the *E. coli* are not plasmid mediated.

Alternate hypothesis

1. *Escherichia coli* can be isolated from door handles.
2. The *E. coli*isolates are multiresistant.
3. *Escherichia coli*produces β-Lactamases.
4. The resistance genes of the *E. coli* are plasmid mediated.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Escherichia coli*

Escherichia coli (/ˌɛʃɪˈrɪkiə ˈkoʊlaɪ/; also known as *E. coli*) is a Gram-negative, facultatively anaerobic, rod-shapedbacterium of the genus*Escherichia* that is commonly reported found in the lower intestine of warm-blooded organisms (endotherms) (Singleton, 1999). Most *E. coli*strains are known to be harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (CDC, 2012; Vogt andDippold, 2005). Some of the harmless strains have been known to be part of the normal flora of the gut, and have been reported to benefit their hosts by producing vitamin K_2 (Bentley and Maganathan, 1982), and preventing colonization of the intestine with pathogenic bacteria (Hudault *et al*., 2001; Reid *et al*., 2001).

The scientific history of *Escherichiacoli* (*E. coli*) started with its first description in 1885 by Theodor von Escherich (Gross and Rowe, 1985), a paediatrician and scientist, who in a series of pioneering studies of the intestinal flora of infants, discovered a normal microbial inhabitant of healthy individuals that he named *Bacterium coli commune*. Escherich's name became officially accepted name for this bacterium in 1958, in his honour (Kuhnert *et al*., 2000).

Escherichia coli and other facultative anaerobes constitute about 0.1% of gut flora (Eckburg *et al.,* 2005), and faecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to

test environmental samples for faecal contamination (Feng *et al*., 2002; Thompson, 2007). A growing body of research has examined environmentally persistent *E. coli* which can survive for extended periods outside of a host (Ishii and Sadowski, 2000).

The bacterium can be grown and cultured easily and inexpensively in a laboratory setting, and has been intensively investigated for over 60 years. *E. coli* is the most widely studied prokaryoticmodel organism, and an important species in the fields of biotechnology and microbiology, where it has served as the host organism for the majority of work with recombinant DNA. Under favourable conditions, it takes only 20 minutes to reproduce.

2.1.1 Types and Morphology

Escherichia coli is a Gram-negative (bacteria which do not retain crystal violet dye), facultative anaerobic (that makes ATP by aerobic respiration if oxygen is present, but is capable of switching to fermentation or anaerobic respiration if oxygen is absent) and non-sporulating bacterium. Cells are typically rod-shaped, and are about 2.0 micrometres (μm) long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm^3 (*Kubitschek, 1990;* Yu *et al*., 2014; Britannica, 2015*).*

Strains that possess flagella are motile. The flagella have a peritrichous arrangement (Darnton *et al*., 2007).

2.1.2 Metabolism

Escherichia coli can live on a wide variety of substrates and uses mixed-acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate, and carbon dioxide. Since many pathways in mixed-acid fermentation produce hydrogen gas, these pathways require the levels of hydrogen to be low, as is the case

when *E. coli* lives together with hydrogen-consuming organisms, such as methanogens or sulphate-reducing bacteria (Madigan and Martinko, 2006).

2.1.3 Culture Growth

Optimum growth of *E. coli* occurs at 37°C (98.6 °F), but some laboratory strains can multiply at temperatures of up to 49°C (Fotadar *et al*., 2005). Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen, and amino acids, and the reduction of substrates such as oxygen, nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide (Ingledew and Poole, 1984).

2.1.4 The Cell Cycle

Unlike eukaryotes, prokaryotes do not rely upon either changes in gene expression (Arends and Weiss, 2004) or changes in protein synthesis (Rueda *et al*., 2003) to control the cell cycle. This probably explains why they do not have similar proteins to those used by eukaryotes to control their cell cycle, such as cdk1. This has led to research on what the control mechanism is in prokaryotes. Recent evidence suggests that it may be membrane- or lipid-based (Furse *et al*., 2015).

2.1.5 Genetic Adaptation

Escherichia coli and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population. This process led to the spread of the gene encoding Shiga toxin from *Shigella* to *E. coli* O157:H7, carried by a bacteriophage (*Brüssow et al., 2004).*

2.1.6 Pathogenicity of *Escherichia coli*

Pathogenic *E.coli*strains can be categorised based on elements that can elicit an immune response in animals, namely:

i. O-antigen: part of lipopolysaccharide layer
ii. K-antigen: capsule
iii. H-antigen: flagellin

O-antigen

The outer membraneof an *E. coli*cell contains millions oflipopolysaccharide (LPS) molecules, which consist of:

a. Antigen, a polymer of immunogenic repeating oligosaccharides(1–40 units).
b. Core regionof phosphorylated nonrepeating oligosaccharides.
c. Lipid A (endotoxin).

The O antigen is used for serotyping *E. coli* (Don *et al*., 2005).

It should be noted though that antibodies towards several O antigens cross-react with other O antigens and partially to K antigens not only from *E. coli*, but also fromother *Escherichia*species and *Enterobacteriaceae* species (Don *et al*., 2005).

The O antigen is encoded by the rfb gene cluster. rol (cld) geneencodes the regulator of lipopolysaccharide O-chain length.

K-antigen

The acidic capsular polysaccharide (CPS) is a thick, mucous-like, layerof polysaccharide that surrounds some pathogenic*E. coli*.

There are two separate groups of K-antigen groups, named group I and group II (while a small in-between subset (K3, K10, and K54/K96)has been classified as group III) (Don *et al*., 2005). The former (I) consist of 100 kDa (large) capsular polysaccharides, while the latter (II), associated with extra-intestinal diseases, are under 50 kDa in size (Don *et al*., 2005).

Group I K-antigens are only found with certain O-antigens (O8, O9,O20, and O101 groups), they are further subdivided on the basis ofabsence (IA, similar to that of *Klebsiella* species in structure) or presence (IB) of amino sugars and some group I K-antigens are attached to the lipid A-core of the lipopolysaccharide (KLPS), in a similar way to O antigens (and being structurally identical to O antigens in some instances are only considered as K antigens when co-expressed with another authentic O antigen) (Don *et al*., 2005).

Group II K-antigens closely resemble those in gram-positivebacteriaand greatly differ in composition and are further subdivided according to their acidic components, generally 20–50% of the CPS chains are bound to phospholipids (Don *et al*., 2005). In total there are 60 different K-antigens that have been recognized.

H-antigen

The flagellum allows *E. coli* to move. H-antigens groups go from H1 to H56 (with some exceptions (H13 and H22 were not *E. coli*antigensbut from *Citrobacterfreundii*, also a coliform, and H50 being the same as H10). These are encoded by the fliC gene.

2.1.6.1 Role in Disease

In humans and in domestic animals, virulent strains of *E. coli* can cause various diseases such as; gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for haemolytic-uremic syndrome, peritonitis, mastitis, septicaemia and gram-negative pneumonia (Todar, 2007).

2.1.6.2 Gastrointestinal Infection

Certain strains of *E. coli*, such as O157:H7, O104:H4, O121, O26, O103, O111, O145, and O104:H21, produce potentially lethal toxins. Food poisoning caused by *E. coli* can result from eating unwashed vegetables or poorly butchered and undercooked meat. O157:H7 is also notorious for causing serious and even life-threatening complications such as haemolytic-uremic syndrome. This particular strain is linked to the 2006 United States *E. coli* outbreak due to fresh spinach. The O104:H4strain is equally virulent. Antibiotic and supportive treatment protocols for it are not as well-developed (it has the ability to be very enterohaemorrhagic like O157:H7, causing bloody diarrhoea, but also is more enteroaggregative, meaning it adheres well and clumps to intestinal membranes). It is the strain behind the deadly June 2011 *E. coli* outbreakin Europe. Severity of the illness varies considerably; it can be fatal, particularly to young children, the elderly or the immunocompromised, but is more often mild. Earlier, poor hygienic methods of preparing meat in Scotland killed seven people in 1996 due to *E. coli* poisoning, and left hundreds more infected. *E. coli* can harbour both heat-stableand heat-labile enterotoxins. The latter, termed LT, contain one A subunit and five B subunits arranged into one holotoxin, and are highly similar in structure and function to cholera toxins. The B subunits assist in adherence and entry of the toxin into host intestinal cells, while the A subunit is cleaved and prevents

cells from absorbing water, causing diarrhoea. LT is secreted by the Type 2 secretion pathway (Tauschek *et al*., 2010).

If *E. coli* bacteria escape the intestinal tract through a perforation (for example from an ulcer, a ruptured appendix, or due to a surgical error) and enter the abdomen, they usually cause peritonitis that can be fatal without prompt treatment. However, *E. coli* are extremely sensitive to such antibiotics as Streptomycin or Gentamicin. Recent research suggests treatment of enteropathogenic *E. coli* with antibiotics may not improve the outcome of the disease, as it may significantly increase the chance of developing haemolytic-uremic syndrome (Wong *et al*., 2000).

Intestinal mucosa-associated *E. coli* are observed in increased numbers in the inflammatory bowel diseases, Crohn's disease and ulcerative colitis (Rolhion and Darfeuille, 2007). Invasive strains of *E. coli* exist in high numbers in the inflamed tissue, and the number of bacteria in the inflamed regions correlates to the severity of the bowel inflammation (Boumgart *et al*., 2007).

2.1.7 Virulence Properties

Enteric *E. coli* (EC) are classified on the basis of serological characteristics and virulence properties.Virotypes include: Enterotoxigenic *E. coli*(ETEC), Enteropathogenic*E. coli*(EPEC), Enteroinvasive*E. coli*(EIEC), Enterohemorrhagic*E. coli*(EHEC) and Enteroaggregative*E. coli* (EAEC) (WHO, 2015).

2.1.7.1 Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *Escherichia coli*(ETEC) is a type of *Escherichia coli*and the leading bacterial cause of diarrhoea in the developing world, as well as the most common cause of travellers' diarrhoea (WHO, 2015). Insufficientdata exist, but conservative estimates suggest that each year,approximately 210 million cases and 380,000 deaths occur, mostly inchildren, from ETEC (Gupta *et al*., 2008).

A number of pathogenic isolates are termed ETEC, but the main hallmarks of this type of bacteria are expression of one or more enterotoxinsand presence of fimbriae used for attachment to host intestinal cells.

Enterotoxins

Enterotoxins produced by ETEC include heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) (Qadri *et al*., 2005). Some strains of ETEC produce a heat-labile exotoxin (LT) that is under the genetic control of a plasmid. The strain also triggers fimbrial adhesion that extends from the surface of the *E. coli* cell and has a B subunit that attaches to the GM1 ganglioside at the brush border of the small intestine epithelium cell and stimulates the entry of the A subunit into the cell, where the latter activates adenylyl cyclase (WHO, 2015). This increases the amount of cAMP, and results in intense and prolonged hypersecretion of chlorides and water; while also inhibiting the reabsorption of sodium. The gut lumen contains large amount of fluid, and motility, leading to diarrhoea that can last for several days. LT is an antigen that can cross-react with the enterotoxin to stimulate the production of neutralizing immunoglobulin antibodies in the serum of previously infected people with enterotoxigenic *E coli*. People that reside in locations that have high prevalence of this strain are likely to possess antibodies to said strain and are less prone to presenting with diarrhoea if re-exposed to the strain (PATH, 2011). Some strains of ETEC produce another toxin called heat-stable enterotoxin (ST) which are regulated by a

heterogeneous group of plasmids. ST activates guanylyl cyclase in the signal pathway of the enteric epithelial cells and triggers fluid secretion. Also, some ST E. coli strains are capable of making LT; the strain that contains both ST and LT toxins produces a more severe diarrhoea. The plasmids carrying the genes for enterotoxins may also encode genes used for the colonization factors that initiate the attachment of *E. coli* strains to the intestinal epithelium.

[Mnemonic: Labile like the Air (cAMP), Stable like the Ground (cGMP)].

Presentation

Infection with ETEC can cause profuse watery diarrhoea with no blood nor leukocytes and abdominal cramping. Fever, nausea with or without vomiting, chills, loss of appetite, headache, muscle aches and bloating can also occur but are less common (CDC, 2015).

2.1.7.2 Enteropathogenic *Escherichia coli* (EPEC)

Enteropathogenic *Escherichia coli* (EPEC) were originally serogroup-defined *E. coli* associated with infantile diarrhoea. However, it became clear that serogroup/serotype designation over-diagnosed EPEC. Subsequently, they were defined by their characteristic localized adherence pattern in tissue cultured cells.

Currently, they are identified mainly based on the presence of specific virulence genes. A hallmark phenotype of EPEC is the ability to produce attaching and effacing (A/E) lesions (Nataro and Kapper, 1998). In the A/E lesion the bacteria attach tightly to the host cell membrane causing a disruption of the cell surface leading to effacement of microvilli. Intestinal cell attachment is mediated by an outer membrane protein called intimin, encoded by eae, which is currently used for the molecular

diagnosis of EPEC. All of the genetic elements required for the A/E lesion are encoded on a large genomic pathogenicity island called the locus of enterocyte effacement (LEE). Furthermore, EPEC are classified into typical and atypical strains based on the presence of the plasmid *E. coli* adherence factor (EAF). There are two important operons on this plasmid, bfp and per, the first encoding the type IV bundle-forming pilus (BFP), and the second encoding a transcriptional activator called plasmid encoded regulator (Per). By definition all EPEC lack genes to produce shigatoxin (stx).*E. coli* strains that areclassified as typical EPEC (tEPEC), most of these strains belong to classic O:H serotypes, and produce the localized adherence (LA) phenotype associated with the production of BFP (Trabulsi *et al*., 2002). On the other hand, *E. coli*strains eae+bfpA−stx−are classified as atypical EPEC (aEPEC). These strains display localized-like (LAL), diffuse (DA), or aggregative adherence (AA) patterns. The LAL pattern in aEPEC is associated with the *E. coli* common pilus and other known adhesins (Skaletski *et al*., 2010). More than 200 O-serogroups have been identify among aEPEC strains; most do not belong to the classic EPEC O-serogroups and many have been designated non-typeable (Schmidt, 2010).

EPEC Epidemiology

Overall, the significance of EPEC as pathogens has declined in published literature of the last several decades (Ochoa *et al*., 2008; Okeke, 2009). It is unclear whether EPEC infections have declined due to interventions, particularly breastfeeding promotion, or whether earlier studies that based diagnosis on O- or O:H-typing overestimated the relative contribution of these organisms compared to recent studies in which EPEC identification was based on molecular methods and/or adherence assays. In a systematic review of paediatric diarrhoea aetiology, using 266 studies published between 1990 and 2002, EPEC were still identified as being among the

most important pathogens, with a median prevalence of 8.8% (IQR, inter-quartile range, 6.6–13.2) in the community setting, 9.1% (IQR 4.5–19.4) in the outpatient setting and 15.6% (IQR 8.3–27.5) in the inpatient setting (Lanata *et al*., 2002). The distribution of pathogens in the inpatient setting represents the most severe cases and can be used as a proxy for cases associated with mortality. In this context, EPEC was the second most common cause of inpatient diarrhoea after rotavirus (25.4%). However, there are important regional and temporal variations. In a recent study of hospitalized diarrhoeal patients in India, EPEC was responsible for 3.2% of 648 diarrhoea samples in children younger than 5 year of age (Nair *et al*., 2010).

Virulence genes

EPEC is considered a non-invasive pathogen and relies upon a T3SS to deliver effector proteins directly into host cells to subvert numerous host cell functions ultimately leading to disease. The first EPEC effectors discovered are all encoded on the LEE pathogenicity island. More recently, effectors encoded outside the LEE region have been found in all A/E pathogens. EPEC strain E2348/69 (serotype O127:H6) has been used worldwide as a prototype strain to study EPEC biology, genetics, and virulence. The recent completion of the EPEC genome sequence of strain E2348/69 (Iguchi *et al*., 2009) enabled analysis of over 400 known/predicted effector sequences and identified only 21 putative effectors, providing a clear picture of the core LEE and non-LEE effector genes (Ochoa *et al*., 2011).

LEE contains genes encoding the outer membrane adhesin (intimin), T3SS machinery (Esc and Sep proteins), chaperones (Ces proteins), translocators (EspA, EspB, and EspD) and effector proteins (EspF, EspG, EspH, Map and EspZ), as well as the translocated intimin receptor (Tir), and the regulatory proteins Ler (LEE-encoded regulator), GrlR (global regulator of LEE proteins, repressor), and GrlA (global

regulator of LEE proteins, activator). EspA forms a needle-shaped structure projecting from the bacterial surface to the plasma membrane of the host cell. Following bacterial attachment, EspB and EspD interact and form a pore in the host membrane. EspB is a translocator and a translocated effector (Luo and Donnenberg, 2006). In general, many translocated effectors show overlapping functions and have cooperative activities, a theme that has been summarized as "multifunctional, cooperative and redundant" EPEC effector behaviour (Dean and Kenny, 2009).

The non-LEE-encode (Nle) (Deng *et al*., 2004) effectors genes are clustered in six pathogenicity islands scattered throughout the genome (Dean and Kenny, 2009). The Nle effectors known so far are: NleA-H, EspG2/ Orf3, Cif, EspJ and EspL. NleA (also called EspI) is reported to inhibit protein secretion; EspJ inhibits phagocytosis; NleE and NleH activate innate immune responses.

2.1.7.3 Enteroinvasive *Escherichia coli* (EIEC)

Enteroinvasive *Escherichia coli* (EIEC) are a type of pathogenic bacteria whose infection causes a syndrome that is identical to Shigellosis, with profuse diarrhoea and high fever. EIEC are highly invasive, and they use adhesin proteins to bind to and enter intestinal cells. They produce no toxins, but severely damage the intestinal wall through mechanical cell destruction. It is closely related to *Shigella*.

After the *E. coli* strain penetrates through the epithelial wall, the endocytosis vacuole gets lysed; the strain multiplies using the host cell machinery, and extends to the adjacent epithelial cell. In addition, the plasmid of the strain carries genes for a type III secretion system that is used as the virulent factor. Although it is an invasive disease, the invasion usually does not pass the sub mucosal layer. The similar pathology to shigellosis may be because both strains of bacteria share some virulent

factors. The invasion of the cells can trigger a mild form of diarrhoea or dysentery, often mistaken for dysentery caused by *Shigella* species. The illness is characterized by the appearance of blood and mucus in the stools of infected individuals or a condition called colitis.

Dysentery caused by EIEC usually occurs within 12 to 72 hours following the ingestion of contaminated food. The illness is characterized by abdominal cramps, diarrhoea, vomiting, fever, chills, and a generalized malaise. Dysentery caused by this organism is generally self-limiting with no known complications.

It is currently unknown what foods may harbour EIEC, but any food contaminated with human faeces from an ill individual, either directly or via contaminated water, could cause disease in others. Outbreaks have been associated with hamburger meat and unpasteurized milk.

2.1.7.4 Verotoxin-producing/Enterohaemorrhagic *Escherichia coli*(EHEC)

Verotoxin-producing *Escherichia coli*consists of strains of thebacterium *Escherichia coli*that, when infecting humans, have been linked with the severe complication haemolytic-uremic syndrome(HUS).They are known by a number of names, including enterohaemorrhagic*E. coli*(EHEC), shiga-like toxin-producing *E. coli*(STEC or SLTEC),hemolytic uremic syndrome–associated enterohaemorrhagic *E. coli*(HUSEC) and verocytotoxin-or verotoxin-producing *E. coli* (VTEC) (Karch *et al*., 2005). All these strains of pathogenic bacteriaproduce Shiga-like toxin (also known as verotoxin), a major cause of foodborne illness. These aredistinguished from other pathotypes of intestinal pathogenic *E. coli*including enterotoxigenic *E. coli*(ETEC), enteropathogenic *E. coli*(EPEC), enteroinvasive *E. coli*(EIEC), enteroaggregative *E. coli*(EAEC),and diffusely adherent *E. coli* (DAEC) (Bae *et al*., 2006). The best known

of these strains is O157:H7, but non-O157 strainscause an estimated 36,000 illnesses, 1,000 hospitalizations and 30 deaths in the United States yearly (Mallove, 2010).

Foodsafety specialists recognize "Big Six" strains; O26, O45, O103, O111, O121, and O145 (Mallove, 2010). A 2011 outbreak in Germanywas caused byanother STEC, O104:H4. This strain has both enteroaggregative andenterohemorrhagic properties. Both the O145 and O104 strains cancause hemolytic-uremic syndrome; the former strain shown to accountfor 2% to 51% of known HUS cases; an estimated 56% of such cases are caused by O145 and 14% by other EHEC strains.

EHECs that induce bloody diarrhoea lead to HUS in 10% of cases. Theclinical manifestations of post-diarrhoeal HUS include acute renal failure, microangiopathic haemolytic anaemia, and thrombocytopenia. Theverocytotoxin (shiga-like toxin) can directly damage renal andendothelial cells. Thrombocytopenia occurs as platelets are consumed by clotting. Haemolytic anaemia results from intravascular fibrindeposition, increased fragility of red blood cells, and fragmentation (Bae *et al*., 2006).

Antibiotics are of questionable value and have not shown to be of clear clinical benefit. Antibiotics that interfere with DNA synthesis, such as floroquinolones, have been shown to induce the Stx-bearingbacteriophage and cause increased production of toxins (Zhang *et al*., 2000). Attempts to block toxin production with antibacterials which target the ribosomal protein synthesis are conceptually more attractive. Plasma exchangeoffers a controversial but possibly helpful treatment. The use of antimotility agents(medications that suppress diarrhoea by slowing bowel transit) in children under 10 years of age or in elderly patientsshould be avoided, as they increase the risk of HUS with EHEC infections (Bae *et al*., 2006).

Generally, antibacterial are not recommended for therapy of STEC infections because they can lyse cell walls therefore releasing the toxins (Waterspiel *et al*., 1992; Wong *et al*., 2000). Additionally, they are usually avoided because they can also cause increased expression of the toxins in vivo (Zhang *et al*., 2000). Despite the general practice of not using antibacterial to treat STEC infections, there have been recent reports suggesting that antibiotics multi-resistance of STEC is on the rise (Galland *etal*., 2001; Willshaw *etal*., 2001; Schroeder *etal*., 2002).

Infectivity and Virulence

The infectivityor the virulenceof an EHEC strain depends on several factors, including the presence of fucose in the medium, the sensing of this sugar and the activation of EHEC Pathogenicity Island.

Pathogenicity Island

EHEC colonization depends on the locus of enterocyte effacement (LEE) Pathogenicity Island. This pathogenicity island encodes a regulator for its own expression called ler and a type III secretion system, a molecular syringe which injects effectors into the host cell, leading to the formation of attaching and effacing lesions on enterocytes. LEE expression is regulated by an inter-kingdom chemicalsignalling system involving the host hormones adrenaline and/or noradrenaline and a signal, autoinducer-3 (AI3), produced by the microbial flora. These signals are sensed by two histidine sensor kinases, QseC and QseE, which initiate a signalling cascade that promotes the expression of LEE genes thus activating virulence.

Regulation of Pathogenicity Island

When EHEC is not in a host the expression of the pathogenicity islandis a waste of energy and resources, so it is only activated if some molecules are sensed on the environment. When QseC or QseE bind with one of their interacting signalling molecule, they autophosphorylate and transfer its phosphate to the response regulator. QseC senses an Endonuclease I-SceIII, encoded by a mobile group I intron within the mitochondrial COX1 gene (AI3) and adrenalineand noradrenaline. QseE senses adrenaline, noradrenaline, SO4 and PO4. These signals are a clear indication to the bacteria that they are no longer free in the environment, but in the gut (Talaro and Talaro, 2006).

QseC phosphorylates QseB (which activates flagella), KpdE (activates the LEE) and QseF. QseE phosphorylates QseF. QseBC and QseEF repress the expression of FusK and FusR. FusK and FusR are the two components of a system to repress the transcription of the LEE genes. FusK is a sensor kinase which is able to sense many sugars among which fucose. When fucose is present in the medium FusK phosphorylates FusR which represses LEE expression. Thus when EHEC enters the gut there is a competition between the signals coming from QseC and QseF, and the signal coming from FusK. The first two would like to activate virulence, but Fusk stops it because the mucous layer, which is a source of fucose, isolates enterocytes from bacteria making the synthesis of the virulence factors useless. However, when fucose concentration decreases because bacterial cells find an unprotected area of the epitelium, then the expression of LEE genes will not be repressed by FusR, and KpdE will strongly activate them. In summary, the combined effect of the QseC/QseF and FusKR provide a fine-tuning system of LEE expression which saves energy and allow the mechanisms of virulence to be expressed only when the chances of success are higher (Talaro and Talaro, 2006).

2.1.7.5 Enteroaggregative *Escherichia coli* (EAEC)

Enteroaggregative *Escherichia coli*(often referred to as EAEC) are a pathotype of *Escherichia coli*often associated with diarrhoeal illness. The defining characteristic of EAEC compared to other pathotypes of *E.coli*is a "stacked brick" pattern of adhesion to the human epithelial cell line HEp-2. The pathogenesis of EAEC involves the bacteria aggregating and colonizing the intestinal mucosa, releasing enterotoxinsand cytotoxinsthat damage host cells and inducing inflammation- resulting in diarrhoea and other gastrointestinal symptoms (Talaro and Talaro, 2006).

EAEC is being increasingly recognised as an emerging enteric pathogen. In particular, EAEC is a common bacterial cause ofpaediatricdiarrhoea, especially in developing countries. It has also been associated with traveller's diarrhoea and infections in HIV-infected individuals, as well as a cause of sporadic food-poisoning outbreaks in the developed world (Huang, 2006).

Awareness of EAEC was increased by a serious outbreakin Germany during 2011, causing over 4000 cases and at least 50 fatalities. The pathogen responsible was found to be an EAEC O104:H4 strain which had acquired a Shiga toxin (usually associated with Verotoxin-producing *Escherichia coli*) (Nadia *et al*., 2015).

Strains of EAEC are highly genetically heterogeneous, but some genes have been associated with virulence. A transcription factor named AggR, part of the AraC family of transcription activators, may regulate some of the virulence factors for EAEC. Several toxins have been linked to EAEC virulence, including ShET1 (Shigella enterotoxin 1), Pet (plasmid-encoded toxin), and EAST-1. Other virulence factors include adhesins, with a family of bundle-forming fimbriae termed aggregative adherence fimbria (AAF) found to be important in many EAEC strains (Harrington *et al*., 2006).

2.1.8 Prevention of *E. coli* Infections

Control measures and good hygiene practices are important measures in the prevention of infections due to all types of *E. coli*. Measures given by World Health Organisation (2011) include:

i. Washing hands thoroughly before eating or preparing food, after using the toilet, changing nappies or cleaning up others with diarrhoea and after contact with domestic or farm animals.

ii. Hygienic handling, preparation and storage of raw meat and other animal products and making sure that raw meat and the implements used to handle them do not come into contact with cooked meat or ready-to-eat food.

iii. Peeling raw fruit and vegetables and washing thoroughly before eating and avoiding unpasteurised milk (which can become contaminated from contact with cow udders) and unpasteurised fruit juices.

iv. Avoiding swallowing water when swimming in untreated or public water facilities.

v. Having adequate fluid intake, especially water and avoiding contamination of the urinary tract by organisms from the anus by wiping 'front to back' after urinating (women).

vi. The risk of getting traveller's diarrhoea can be lowered by drinking only safe water (treated, boiled or sealed bottle), and by consuming ice known to be made from treated water. Food should be eaten freshly cooked and piping hot, salads should be avoided and fruit eaten only if it can be peeled (Public Health, 2011).

2.2 ANTIBIOTICS CLASSES STUDIED

2.2.1 β-Lactams

β-lactam antibiotics (**beta-lactam antibiotics**) are a broad class of antibiotics, consisting of all antibiotic agents that contain a β-lactam ring in their molecular structures. This includes penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems (Holten and Onusko, 2000). Most β-lactam antibiotics work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics. Until 2003, when measured by sales, more than half of all commercially available antibiotics in use were β-lactam compounds (Elander, 2003).

Bacteria often develop resistance to β-lactam antibiotics by synthesizing a β-lactamase, an enzyme that attacks the β-lactam ring. To overcome this resistance, β-lactam antibiotics are often given with β-lactamase inhibitors such as clavulanic acid.

A **β-lactam** (**beta-lactam**) ring is a four-membered lactam (Gilchrist, 1987). (A lactam is a cyclic amide). It is named as such because the nitrogen atom is attached to the β-carbon atom relative to the carbonyl. The simplest β-lactam possible is 2-azetidinone.

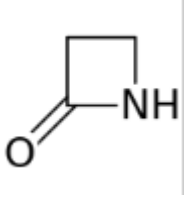


Figure 2.1: Structure of the simplest β-lactam; 2-azetidinone

Mode of Action of β-lactam

β-lactam antibiotics are bacteriocidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms, being the outermost and primary component of the wall. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by DD-transpeptidases which are penicillin-binding proteins (PBPs). PBPs vary in their affinity for binding penicillin or other β-lactam antibiotics. The amount of PBPs varies among bacterial species.

β-lactam antibiotics are analogues of D-alanyl-D-alanine—the terminal amino acid residues on the precursor NAM/NAG-peptide subunits of the nascent peptidoglycan layer. The structural similarity between β-lactam antibiotics and D-alanyl-D-alanine facilitates their binding to the active site of PBPs. The β-lactam nucleus of the molecule irreversibly binds to (acylates) the Ser_{403} residue of the PBP active site. This irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis (Fisher *et al*., 2005).

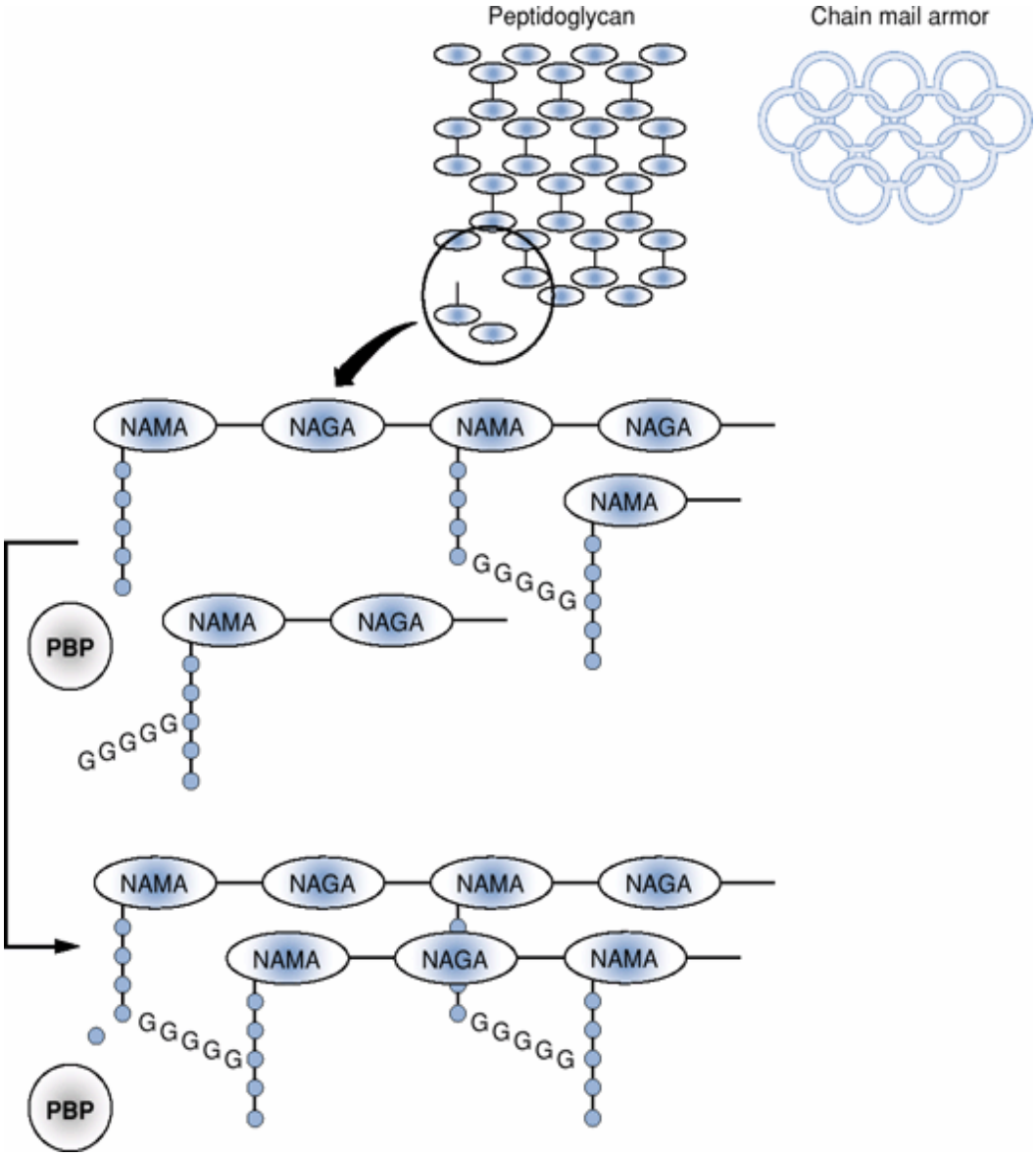


Figure 2.2: Structural representation of the peptidoglycan layer of the bacterial cell wall showing the point of interaction (PBP) with β-lactams.

Molecular Mechanism of Action of β-Lactams

The bacterial cell wall is a cross linked polymer called peptidoglycan which allows a bacteria to maintain its shape despite the internal turgor pressure caused by osmotic pressure differences.If the peptidoglycan fails to crosslink the cell wall will lose its strength which results in cell lysis. All β-lactams disrupt the synthesis of the bacterial cell wall by interfering with the transpeptidase which catalyzes the cross linking process.

Peptidoglycan is a carbohydrate composed of alternating units of N-Acetyl Muramic Acid (NAMA) and N-Acetyl Glucose Amine(NAGA). The NAMA units have a peptide side chain which can be cross linked from the L-Lys residue to the terminal D-Ala-D-Ala link on a neighboring NAMA unit.This is done directly in Gram (-) bacteria and via a pentaglycine bridge on the L-lysine residue in Gram (+) bacteria.

The cross linking reaction is catalyzed by a class of transpeptidases known as penicillin binding proteins. A critical part of the process is the recognition of the D-Ala-D-Ala sequence of the NAMA peptide side chain by the PBP. Interfering with this recognition disrupts the cell wall synthesis.

β-lactams mimic the structure of the D-Ala-D-Ala link and bind to the active site of PBPs, disrupting the cross-linking process.The amide of the β-lactam ring is unusually reactive due to ring strain and a conformational arrangement which does not allow the lone pair of the nitrogen to interact with the double bond of the carbonyl.β-Lactams acylate the hydroxyl group on the serine residue of PBP active site in an irreversible manner.This reaction is further aided by the oxyanion hole, which stabilizes the tetrahedral intermediate and thereby reduces the transition state energy.The reaction of β-lactams with target microbial cell is summarized in Figure 2.3below:

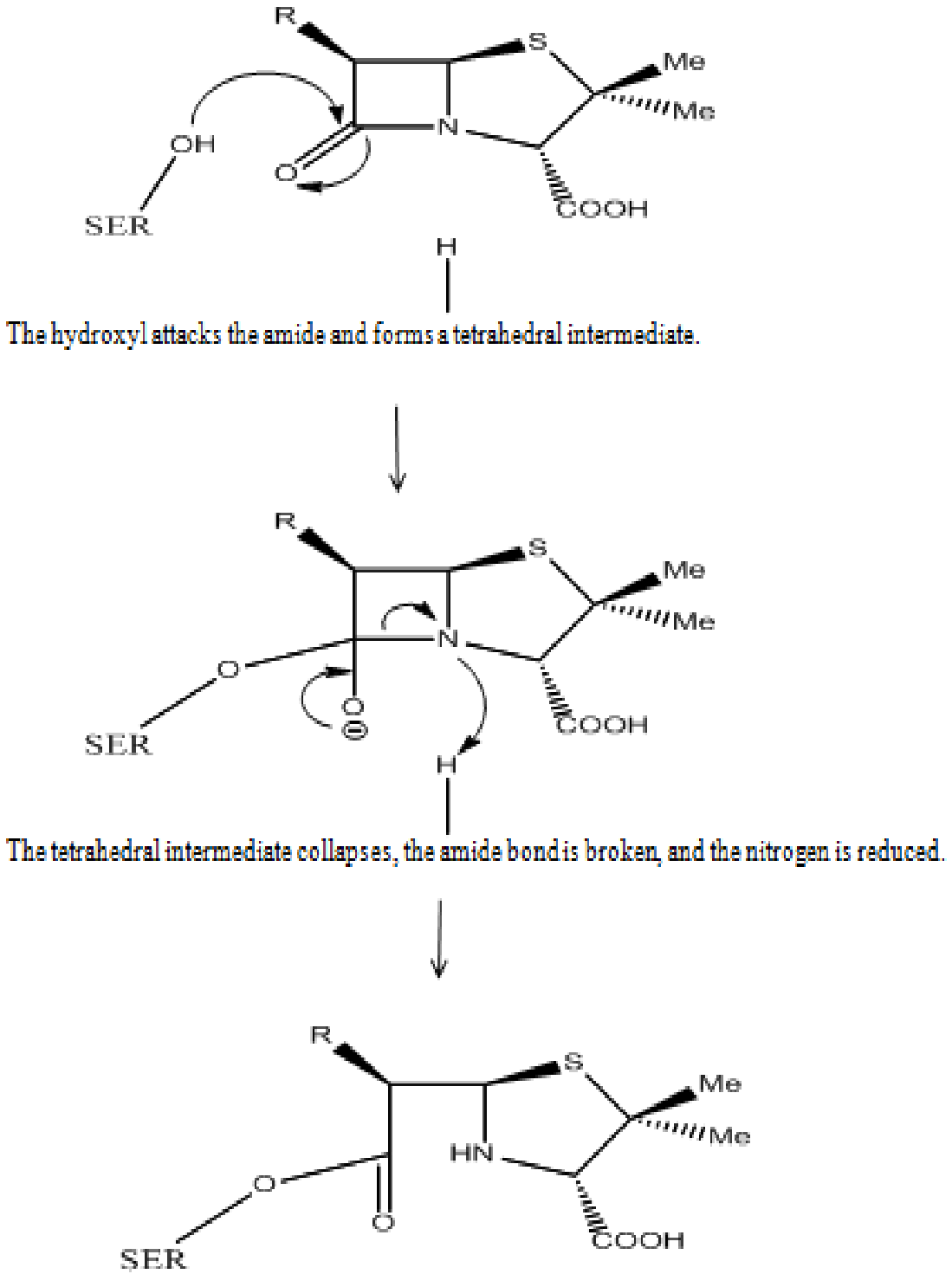


Figure 2.3: Structural representation of the molecular interaction between β-lactam molecule and bacterial peptidoglycan cell wall.

β-lactam antibiotics block not only the division of bacteria, including cyanobacteria, but also the division of cyanelles, the photosynthetic organelles of the glaucophytes, and the division of chloroplasts of bryophytes. In contrast, they have no effect on the plastids of the highly developed vascular plants. This is supporting the endosymbiotic theory and indicates an evolution of plastid division in land plants (Kasten and Reski, 1997).

Under normal circumstances, peptidoglycan precursors signal a reorganisation of the bacterial cell wall and, as a consequence, trigger the activation of autolytic cell wall hydrolases. Inhibition of cross-linkage by β-lactams causes a build-up of peptidoglycan precursors, which triggers the digestion of existing peptidoglycan by autolytic hydrolases without the production of new peptidoglycan. As a result, the bactericidal action of β-lactam antibiotics is further enhanced.

Resistance to β-Lactams

By definition, all β-lactam antibiotics have a β-lactam ring in their structure. The effectiveness of these antibiotics relies on their ability to reach the PBP intact and their ability to bind to the PBP. Hence, there are two main modes of bacterial resistance to β-lactams:

- **Enzymatic hydrolysis of the β-lactam ring:** If the bacterium produces the enzyme β-lactamase or the enzyme penicillinase, the enzyme will hydrolyse the β-lactam ring of the antibiotic, rendering the antibiotic ineffective (Drawz and Bonomo, 2010). (An example of such an enzyme is NDM-1, discovered in 2009). The genes encoding these enzymes may be inherently present on the bacterial chromosome or

may be acquired via plasmid transfer (plasmid mediated resistance), and β-lactamase gene expression may be induced by exposure to β-lactams.

The production of a β-lactamase by a bacterium does not necessarily rule out all treatment options with β-lactam antibiotics. In some instances, β-lactam antibiotics may be co-administered with a β-lactamase inhibitor. For example, Augmentin (FGP) is made of amoxicillin (a β-lactam antibiotic) and clavulanic acid (a β-lactamase inhibitor). The clavulanic acid is designed to overwhelm all β-lactamase enzymes, and effectively serve as an antagonist so that the amoxicillin is not affected by the β-lactamase enzymes.

Other β-Lactamase inhibitors such as boronic acids are being studied in which they irreversibly bind to the active site of β-lactamases. This is a benefit over clavulanic acid and similar beta-lactam competitors, because they cannot be hydrolysed, and therefore rendered useless. Extensive research is currently being done to develop tailored boronic acids to target different isozymes of beta-lactamases (Leonard *et al*., 2012).

However, in all cases where infection with β-lactamase-producing bacteria is suspected, the choice of a suitable β-lactam antibiotic should be carefully considered prior to treatment. In particular, choosing appropriate β-lactam antibiotic therapy is of utmost importance against organisms which harbour some level of β-lactamase expression. In this case, failure to use the most appropriate β-lactam antibiotic therapy at the onset of treatment could result in selection for bacteria with higher levels of β-lactamase expression, thereby

making further efforts with other β-lactam antibiotics more difficult (Anon, 2011).

- **Possession of altered penicillin-binding proteins:** As a response to increased efficacy of β-lactams, some bacteria have changed the proteins to which β-lactam antibiotics bind. β-lactams cannot bind as effectively to these altered PBPs, and, as a result, the β-lactams are less effective at disrupting cell wall synthesis. Notable examples of this mode of resistance include methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae*. Altered PBPs do not necessarily rule out all treatment options with β-lactam antibiotics.

2.2.1.1 Cephalosporins

The **cephalosporins** are a class of β-lactam antibiotics originally derived from the fungus *Acremonium*, which was previously known as "*Cephalosporium*" (Toda *et al*., 2008). Together with cephamycins, they constitute a subgroup of β-lactam antibiotics called cephems. Cephalosporins were discovered in 1945 and were first sold in 1964 (Oxford, 2009).

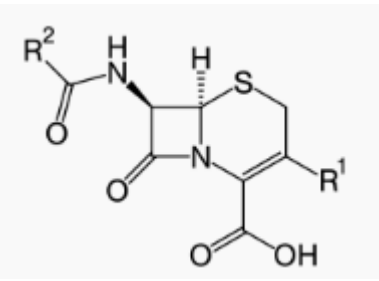


Figure 2.4: Core structure of the Cephalosporins

Mode of Action of Cephalosporins

Cephalosporins are bactericidal and have the same mode of action as other β-lactam (section 2.2.1 above) antibiotics (such as penicillins), but are less susceptible to β-lactamases.

2.2.1.1.1 Ceftazidime

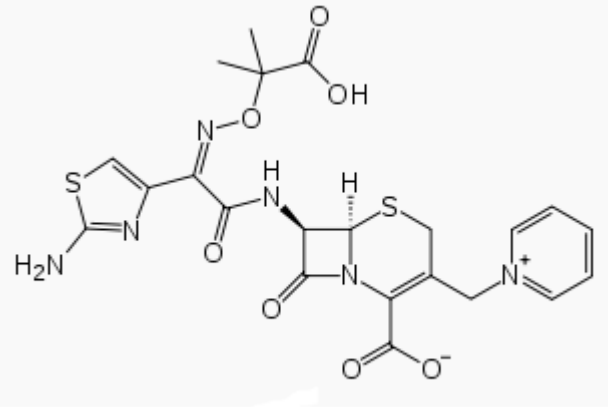


Figure 2.5: Structural formula of Ceftazidime

Ceftazidime, sold under the brand names **Fortaz** and **Tazicef** (Lexicomp, 2014), is an antibiotic useful for the treatment of a number of bacterial infections. It is a third-generation cephalosporin.

As a class, cephalosporins have activity against Gram-positive and Gram-negative bacteria. The balance of activity tips toward Gram-positive organisms for earlier generations; later generations of cephalosporins have more Gram-negative coverage. Ceftazidime is one of the few in this class with activity against *Pseudomonas*. It is not active against methicillin-resistant *Staphylococcus aureus*.

Third-generation cephalosporins differ from earlier generations in the presence of a C=N-OCH_3 group in their chemical structure (Cefuroxime and Cefuzonam also bear this functional group but are only listed as class II). This group provides improved

stability against certain beta-lactamase enzymes produced by Gram-negative bacteria. These bacterial enzymes rapidly destroy earlier-generation cephalosporins by breaking open the drug's beta-lactam chemical ring, leading to antibiotic resistance. Though initially active against these bacteria, with widespread use of third-generation cephalosporins, some Gram-negative bacteria known as extended-spectrum beta-lactamases (ESBLs) are even able to inactivate the third-generation cephalosporins. Infections caused by ESBL-producing Gram-negative bacteria are of particular concern in hospitals and other healthcare facilities (*Sharma et al., 2013).*

Ceftazidime is used to treat lower respiratory tract, skin, urinary tract, blood-stream, joint, and abdominal infections, and meningitis. The drug is given intravenously (IV) or intramuscularly (IM) every 8–12 hours (two or three times a day), with dose and frequencing varying by the type of infection, severity, and/or renal function of the patient. Ceftazidime is also commonly prescribed off-label for nebulization in Cystic Fibrosis patients for the suppression of *Pseudomonas* in the lungs as well as the treatment of pulmonary exacerbations. Those with kidney disease are dosed less frequently.

Ceftazidime is the first-line treatment for the tropical infection, melioidosis, an important cause of sepsis in Asia and Australia (White, 2013; White *et al.*, 1989).

Labeled indications include the treatment of patients with:

- *Pseudomonas aeruginosa* infections
- other Gram-negative, aerobic infections
- neutropenic fever (Lexicomp, 2014)

Spectrum of activity

Clinically relevant organisms against which ceftazidime has activity include:

- **Gram-negative aerobes**, such as *Enterobacter*, *E. coli*, *H. influenzae*, *Klebsiella*, *Proteus*, *Pseudomonas*, and *N. meningitidis*
- **Gram-positive aerobes**, such as group B streptococci, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*
- **Anaerobes**, such as *Bacteroides*

The following represents MIC susceptibility data for a few clinically significant pathogens:

- *Escherichia coli* - 0.015 µg/mL - 512 µg/mL
- *Pseudomonas aeruginosa* - ≤0.03 µg/mL - 1024 µg/mL

2.2.1.1.2 Cefuroxime

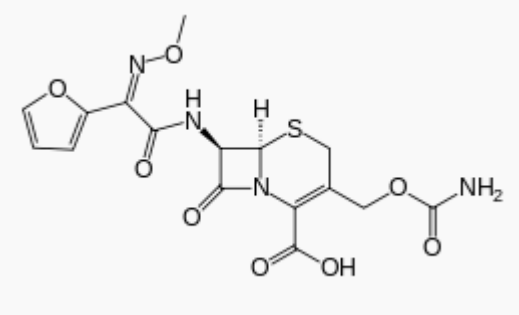


Figure 2.6: Structural formula of Cefuroxime

Cefuroxime is an enteral second-generation cephalosporinantibiotic. It was discovered by the Glaxo, now GlaxoSmithKline and first marketed in 1978 as Zinacef. It received approval from the U.S. Food and Drug Administration in October of 1983.

Cefuroxime axetil is an acetoxyetyl-ester-prodrug of cefuroxime which is effective orally. As with the other cephalosporins, although as a second-generation variety, it is less susceptible to beta-lactamase. Hence, it may have greater activity against *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and Lyme disease. Unlike most other second-generation cephalosporins, cefuroxime can cross the blood-brain barrier.

Cefuroxime is generally well-tolerated and its side effects are usually transient. If ingested after food, this antibiotic is both better absorbed and less likely to cause its most common side effects of diarrhoea, nausea, vomiting, headaches/migraines, dizziness, and abdominal pain compared to most antibiotics in its class.

Although a widely stated cross-allergic risk of about 10% exists between cephalosporins and penicillin, recent assessments have shown no increased risk for a cross-allergic reaction for cefuroxime and several other second-generation or later cephalosporins (Pichichero, 2002).

2.2.1.2 Carbapenems

Carbapenems are antibiotics used for the treatment of infections known or suspected to be caused by multidrug-resistant (MDR) bacteria. Their use is primarily in people who are hospitalized.

Like the penicillins and cephalosporins, they are members of the beta lactam class of antibiotics, which kill bacteria by binding to penicillin-binding proteins and inhibiting cell wall synthesis. They exhibit a broader spectrum of activity compared to cephalosporins and penicillins. Their effectiveness is less affected by many common mechanisms of antibiotic resistance than other beta lactams.

Carbapenem antibiotics were originally developed at Merck & Co. from the carbapenem thienamycin, a naturally derived product of *Streptomyces cattleya* (Sneader, 2006; Bimbaum *et al*., 1985). Concern has arisen in recent years over increasing rates of resistance to carbapenems, as there are few therapeutic options for treating infections caused by carbapenem-resistant bacteria (such as the carbapenem-resistant Enterobacteriaceae) (Breilh *et al*., 2013; Papp-Wallace *et al*., 2011; Livermore and Woodford, 2000).

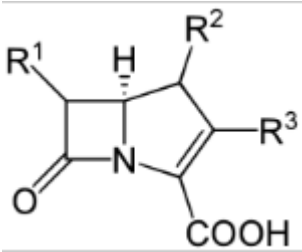


Figure 2.7: Core structure of the carbapenem molecule

Medical Uses of Carbapenems

- **Abdominal infections:** The carbapenem ertapenem is one of several first-line agents recommended by the Infectious Disease Society of America for the empiric treatment of community-acquired intra-abdominal infections of mild-to-moderate severity. Agents with anti-pseudomonal activity, including doripenem, imipenem, and meropenem are not recommended in this population. Doripenem, imipenem, and meropenem are recommended for high-risk community-acquired abdominal infections and for abdominal infections that are hospital-acquired (Solomkin *et al*., 2010).
- **Complicated urinary tract infections:** A 2015 systematic review found little evidence that would support the identification of a best antimicrobial regimen for complicated urinary tract infections, but identified three high-quality trials

supporting high cure rates with doripenem, including in patients with levofloxacin-resistant *E. coli* infections (Golan, 2015).

- **Pneumonia:** The carbapenems imipenem and meropenem are recommended by the American Thoracic Society and the Infectious Disease Society of America as one of several first-line therapy options for people with late-onset hospital-acquired or ventilator-associated pneumonia, especially when *Pseudomonas*, *Acinetobacter*, or extended spectrum beta-lactamase producing *Enterobactericeace* are suspected pathogens. Combination therapy, typically with an aminoglycoside, is recommended for *Pseudomonas* infections to avoid resistance development during treatment (Anon, 2005).

  Carbapenems are less commonly used in the treatment of community-acquired pneumonia, as community-acquired strains of the most common responsible pathogens (*Streptocuccus pneumoniae, Haemophilus influenazae*, atypical bacteria, and Enterobactericeace) are typically susceptible to narrower spectrum and/or orally administered agents such as fluoroquinolones, amoxicillin, or azithromycin. Imipenem and meropenem are useful in cases in which *P. aeruginosa* is a suspected pathogen (Woodhead *et al*., 2011).

- **Bloodstream Infections:** A 2015 meta–analysis concluded that the anti-pseudomonal penicillin-beta lactamase inhibitor combination piperacillin-tazobactam gives results equivalent to treatment with a carbapenem in patients with sepsis (Shiber *et al*., 2015). In 2015, the National Institute for Health and Care Excellence recommended piperacillin-tazobactam as first

line therapy for the treatment of bloodstream infections in neutropenic cancer patients.

For bloodstream infections known to be due to extended spectrum beta-lactamase producing *Enterobacteriaceace*, carbapenems are superior to alternative treatments (Vardakas *et al*., 2012).

Spectrum of activity of carbapenems

Carbapenems exhibit broad spectrum activity against Gram-(-) bacteria and somewhat narrower activity against Gram-(+) bacteria. For empiric therapy (treatment of infections prior to identification of the responsible pathogen) they are often combined with a second drug having broader spectrum Gram-(+) activity.

- Gram-(-) pathogens: The spectrum of activity of the carbapenems imipenem, doripenem, and meropenem includes most Enterobacteriaceace species, including *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Citrobacter freundii, Proteus mirabilis, and Serratia marcescens*. Activity is maintained against most strains of E. coli and K. pneumoniae that are resistant to cephalosporins due to the production of extended spectrum beta-lactamases. Imipenem, doripenem, and meropenem also exhibit good activity against most strains of Pseudomonas aeruginosa and Acinetobacter species. The observed activity against these pathogens is especially valued as they are intrinsically resistant to many other antibiotic classes (Breilh *etal*., 2013).
- **Gram-(+) pathogens**: The spectrum of activity of the carbapenems against Gram-(+) bacteria is fairly broad, but not as exceptionally so as in the case of Gram-(-) bacteria. Good activity is seen against methicillin-sensitive strains of *Staphylococcus* species, but many other antibiotics provide coverage for such infections. Good activity is also

observed for most *Streptococcus* species, including penicillin-resistant strains. Carbapenems are not highly active against methicillin-resistant *Staphyloccusaureus* or most enterococcal infections because carbapenems do not bind to the penicillin-binding protein used by these pathogens (Breilh *etal*., 2013).

- **Other:** Carbapenems generally exhibit good activity against anaerobes such as *Bacteriodesfragilis*. Like other beta lactam antibiotics, they lack activity against atypical bacteria, which do not have a cell wall and are thus not affected by cell wall synthesis inhibitors (Breilh *etal*., 2013).

Classes of Carbapenems

- ✓ **Approved for clinical use: Imipenem**, the first clinically used carbapenem, was developed at Merck and Co. It was approved for use in the United States in 1985. Imipenem is hydrolyzed in the mammalian kidney by a dehydropeptidase enzyme to a nephrotoxic intermediate, and thus is co-formulated with the dehydropeptidase inhibitor cilastatin (Papp-Wallace *et al*., 2011).

  Meropenem is stable to mammalian dehydropeptidases and does not require co-administration of cilastin. It was approved for use in the United States in 1996. In most indications it is somewhat more convenient to administer than imipenem, 3 times a day rather than 4. Doses of less than one gram may be administered as an IV bolus, whereas imipenem is usually administered as a 20-minute to one hour infusion. Meropenem is somewhat less potent than imipenem against Gram-(+) pathogens, and somewhat more potent against Gram-(-) infections. Unlike imipenem, which produced an unacceptable rate of seizures in a phase 2 trial, meropenem is effective for the treatment of

bacterial meningitis (Zhanel *et al*., 1998). A systematic review performed by an employee of the company that markets meropenem concluded that it provides a higher bacterial response and lower adverse event rates than imipenem in people with severe infections, but no difference in mortality rate (Edwards *et al*., 2005).

Ertapenem is administered once daily as an intravenous infusion or intramuscular injection. It lacks useful activity against the *P. aeruginosa* and *Acetinobacter* species, both of which are important causes of hospital-acquired infections.

Doripenem has a spectrum of activity very similar to that of meropenem. Its greater stability in solution allows the use of prolonged infusions and it is somewhat less likely to produce seizures than other carbapenems (Chahine *et al*., 2010).

Panipenem/betamipron (Japanese approval 1993)

Biapenem (Japanese approval 2001) exhibits similar efficacy and adverse event rates as other carbapenems (Pei *et al*., 2014).

✓ **Unapproved/experimental**

- Razupenem (PZ-601)
    - PZ-601 is a carbapenem antibiotic currently being tested as having a broad spectrum of activity including strains resistant to other carbapenems. Despite early Phase II promise, Novartis (who acquired PZ-601 in a merger deal with Protez Pharmaceuticals) recently

dropped PZ-601, citing a high rate of adverse events in testing (George, 2010).

- Tebipenem: Tebipenem is the first carbapenem whose prodrug form, the pivalyl ester, is orally available (Hazra *et al*., 2014).
- Lenapenem
- Tomopenem
- Thienamycin (thienpenem) the first discovered carbapenem

Bacterial Resistance to Carbapenems

- **Enterobacteriaceae:** Enterobacteriaceae are common pathogens responsible for urinary tract infections (Gupta *etal*., 2011; Hooton *etal*., 2010), abdominal infections (Solomkin *etal*., 2010), and hospital-acquired pneumonia (Anon, 2005). Beta lactam resistance in these pathogens is most commonly due to the expression of beta lactamase enzymes (Delgado *etal*., 2013).

  Between 2007 and 2011, the percentage of *Escherichia coli* isolates from Canadian hospitals that produce extended spectrum beta lactamases (ESBL) increased from 3.4% to 4.1%; among *Klebsiella pneumoniae* isolates ESBL producers increased from 1.5% to 4.0%. These strains are resistant to third generation cephalosporins.that were developed for the treatment of beta lactamase-producing ‘’Enterobacteriaceae’’ and carbapenems are generally regarded as the treatment of choice (Denisuik *et al*., 2013). More recently, many countries have experienced a dramatic upswing in the prevalence of *Enterobacteriaceace* that produce both ESBLs and carbapenemases such as the Klebsiella pneumoniae carbapenemase (KPC). As of 2013, 70% of Greek *Klebsiella pneumoniae* isolates are resistant to third generation and

cyclosporins and 60% are resistant to carbapenems. The growing prevalence and difficulty of treating such multi-drug resistant *Enterobacteriaceace* has led to a renaissance of the use of antibiotics such as colistin, which was discovered in the 1950s but rarely used until recently due to unattractive levels of toxicity (Giske, 2015).

- ***Pseudomonas aeruginosa* and *Acinetobacter baumannii:*** Infections caused by the non-fermenting Gram-(-) bacteria *Pseudomonasaeruginosa* and *Acetinobacterbaumanni* are most commonly encountered in hospitalized people. These bacteria exhibit an unusually high level of intrinsic resistance to antibiotics due to their expression of a wide range of resistance mechanisms. Antibiotics cross the outer membrane of *Pseudomonas* and *Acinetobacter* approximately 100 times more slowly than they cross the outer membrane of Enterobacteriaceae, due in part to their use of **porins** that can adopt a conformation having a very restricted entry channel. Further, the porin levels may be down-regulated in response to antibiotic exposure. Antibiotic molecule that successfully traverse the porin channels may be removed by efflux pumps. Downregulation of the porin OprD2 is an important contributor to imipenem resistance (Rice, 2006).

  Like the *Enterobacteriaceace*, *Pseudomonas* and *Acinetobacter* can express a wide range of antibiotic-deactivitating enzymes, including beta lactamases. *Pseudomonas* produces an inducible broad spectrum beta lactamase, AmpC, that is produced in response to beta lactam exposure. The combination of inducible AmpC expression, poor membrane permeability, and efflux pumps make *Pseudomonas* resistant to most beta lactams. The clinical efficacy of carbapenems in *Pseudomonas* infection arises in part because, while they are

strong inducers of AmpC, they are poor substrates. The identification of *Pseudomonas* strains that produce beta lactamases capable of cleaving carbapenems, such as the New Delhi metallo beta lactamase has raised increasing concern regarding the potential for an era of untreatable *Pseudomonas* infections (Morita *et al*., 2014).

2.2.1.3 Imipenem

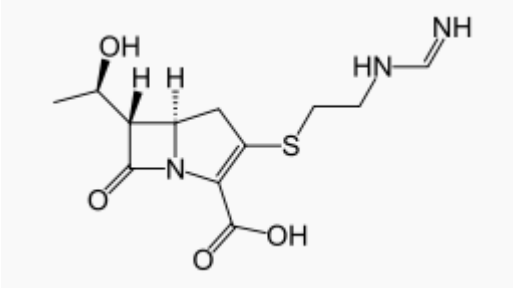


Figure 2.8: Molecular Structure of Imipenem

Imipenem (**Primaxin**) is an intravenousβ-lactamantibiotic discovered by Merck scientists Burton Christensen, William Leanza, and Kenneth Wildonger in 1980. It was the first member of the carbapenem class of antibiotics. Carbapenems are highly resistant to the β-lactamase enzymes produced by many multiple drug-resistant Gram-negative bacteria (Clissold *et al*., 1987), thus play a key role in the treatment of infections not readily treated with other antibiotics (Vardakas *et al*., 2012).

It was discovered via a lengthy trial-and-error search for a more stable version of the natural product thienamycin, which is produced by the bacterium *Streptomyces cattleya*. Thienamycin has antibacterial activity, but is unstable in aqueous solution, so impractical to administer to patients (Kahan *et al*., 1983). Imipenem has a broad spectrum of activity against aerobic and anaerobic, Gram-positive and Gram-negativebacteria (Kesado *et al*., 1980). It is particularly important for its activity

against *Pseudomonas aeruginosa* and the *Enterococcus* species. It is not active against MRSA, however.

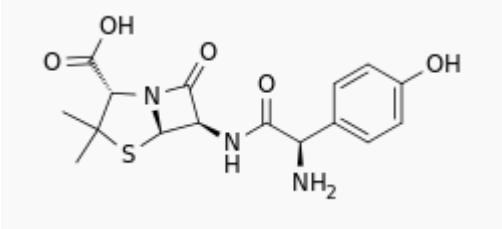
Mechanism of Action of Imipenem

Imipenem acts as an antimicrobial through inhibiting cell wall synthesis of various Gram-positive and Gram-negative bacteria. It remains very stable in the presence of β-lactamase (both penicillinase and cephalosporinase) produced by some bacteria, and is a strong inhibitor of β-lactamases from some Gram-negative bacteria that are resistant to most β-lactam antibiotics. The molecular basis of the interaction is similar to that of β-lactams as explain in section 2.2.1.

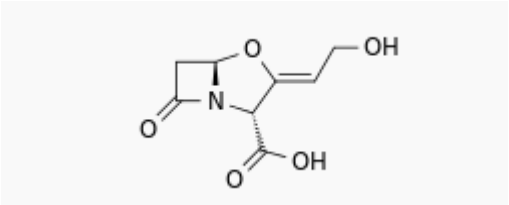
Spectrum of bacterial susceptibility and resistance to Imipenem

Acinetobacter anitratus, *Acinetobacter calcoaceticus*, *Actinomyces odontolyticus*, *Aeromonas hydrophila*, *Bacteroides distasonis*, *Bacteroides uniformis*, and *Clostridium perfringens* are generally susceptible to imipenem, while *Acinetobacter baumannii*, some*Acinetobacter* spp., *Bacteroides fragilis*, and *Enterococcus faecalis* have developed resistance to imipenem to varying degrees. Not many species are resistant to imipenem except *Pseudomonas aeruginosa* (Oman) and *Stenotrophomonas maltophilia*.

2.2.1.4 Amoxicillin/Clavulanic Acid



A



B

Figure 2.9: Molecular structures of: (A) Amoxicillin (B) Clavulanic Acid

Amoxicillin/clavulanic acid or **co-amoxiclav** is an antibiotic useful for the treatment of a number of bacterial infections. It is a combination antibiotic consisting of amoxicillin trihydrate, a β-lactam antibiotic, and potassium clavulanate, a β-lactamase inhibitor. This combination results in an antibiotic with an increased spectrum of action and restored efficacy against amoxicillin-resistant bacteria that produce β-lactamase.

Side effects include an increased risk of yeast infections and diarrhoea (Gillies *et al.*, 2014).

It is on the World Health Organization's List of Essential Medicines, a list of the most important medications needed in a basic health system (WHO, 2013). Amoxicillin/clavulanic acid was developed at Beecham Pharmaceuticals and marketed under the trade name **Augmentin** (Royal Pharmaceutical Society of Great

Britain, 2009). It is available as a generic and marketed under a variety of trade names worldwide.

Amoxicillin/clavulanic acid is widely used to treat or prevent many infections caused by susceptible bacteria, such as: urinary tract infections, respiratory tract infections, skin and soft tissue infections, sinus infections, cat scratches, infections caused by the bacterial flora of the mouth, such as; dental infections, infected animal bites, infected human bites (including uncomplicated "clenched-fist" or "reverse-bite" injuries) (Gordon, 2010).

Mechanism of action

The action of amoxicillin is same as that of β-lactams as described in section 2.2.1.

Clavunate potassium is an irreversible inhibitor of β-lactamases produced by both gram-positive and gram-negative bacteria. It prevents hydrolysis of amoxicillin resulting in extending the spectrum of amoxicillin to β-lactamase producing bacteria. Clavuate potassium does not possess any significant antimicrobial action itself.

2.2.1.5 Ampicillin

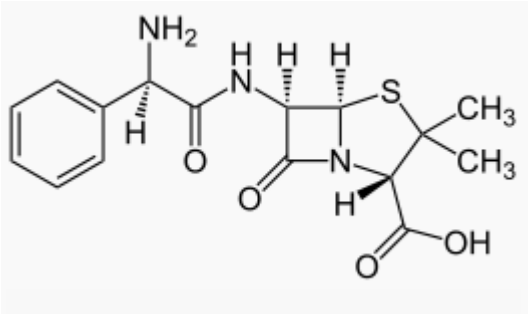


Figure 2.10: Molecular structure of Ampicillin

Ampicillin is an antibiotic used to prevent and treat a number of bacterial infections. This includes respiratory tract infections, urinary tract infections, meningitis, salmonella infections, and endocarditis. It may also be used to prevent group B streptococcal infection in new-borns. It is used by mouth, by injection into a muscle, or intravenously. It is not useful for the treatment of viral infections.

Common side effects include rash, nausea, and diarrhoea. It should not be used in people who are allergic to penicillin. Serious side effects may include *Clostridiumdifficile* colitis or anaphylaxis. While usable in those with kidney problems the dose may need to be decreased. Its use during pregnancy and breastfeeding appear to be okay.

Ampicillin was developed in 1961 (Ravina, 2011). It is on the World Health Organization's List of Essential Medicines, the most important medication needed in a basic health system (WHO, 2013).

Ampicillin is active against Gram-(+) bacteria including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, some isolates of *Staphylococcus aureus* (but not penicillin-resistant or methicillin-resistant strains), and some *Enterococci*. Activity against Gram-(-) bacteria includes *Neisseria meningitidis*, some *Haemophilus influenzae*, and some *Enterobacteriaceae*. Its spectrum of activity is enhanced by co-administration of sulbactam, a drug that inhibits beta lactamase, an enzyme produced by bacteria to inactivate ampicillin and related antibiotics (Hauser, 2013, Akova, 2008).

It is used for the treatment of infections known to be or highly likely to be caused by these bacteria. These include common respiratory infections including sinusitis, bronchitis, and pharyngitis, as well as otitis media. In combination with vancomycin (which provides coverage of ampicillin-resistant pneumococci), it is effective for the

treatment of bacterial meningitis. It is also used for gastrointestinal infections caused by consuming contaminated water or food, such as *Salmonella*, *Shigella*, and *Listeriosis* (Finberg and Fingeroth, 2012).

Ampicillin is a first-line agent for the treatment of infections caused by *Enterococci*. The bacteria are an important cause of healthcare-associated infections such as endocarditis, meningitis, and catheter-associated urinary tract infections that are typically resistant to other antibiotics (Finberg and Fingeroth, 2012).

Mechanism of Action of Ampicillin

Ampicillin is in the penicillin group of beta-lactam antibiotics and is part of the aminopenicillin family. It is roughly equivalent to amoxicillin in terms of activity (AHFS, 2006).

Ampicillin is able to penetrate Gram-positive and some Gram-negative bacteria. It differs from penicillin G, or benzylpenicillin, only by the presence of an amino group. That amino group helps the drug penetrate the outer membrane of Gram-negative bacteria.

Ampicillin acts as an irreversible inhibitor of the enzyme transpeptidase, which is needed by bacteria to make their cell walls (AHFS, 2006). It inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to cell lysis; therefore ampicillin is usually bacteriocidal (Petri *et al*., 2011; AHFS, 2006). The molecular basis of this interaction has been described in section 2.2.1.

2.2.2 Aminoglycosides

Aminoglycoside is a medicinal and bacteriologic category of traditional Gram-negativeantibacterial therapeutic agents that inhibit protein synthesis and contain as a portion of the molecule an amino-modified glycoside (sugar) (Mingeot *et al*., 2009); the term can also refer more generally to any organic molecule that contains amino sugar substructures. Aminoglycoside antibiotics display bactericidal activity against gram-negative aerobes and some anaerobic bacilli where resistance has not yet arisen, but generally not against Gram-positive and anaerobic Gram-negative bacteria (Levison, 2012). They include the first-in-class aminoglycoside antibioticstreptomycin (images at right) derived from *Streptomyces griseus*, the earliest modern agent used against tuberculosis, and an example that *lacks* the common 2-deoxystreptamine moiety (image right, below) present in many other class members. Other examples include the deoxystreptamine-containing agentskanamycin, tobramycin, gentamicin, and neomycin.

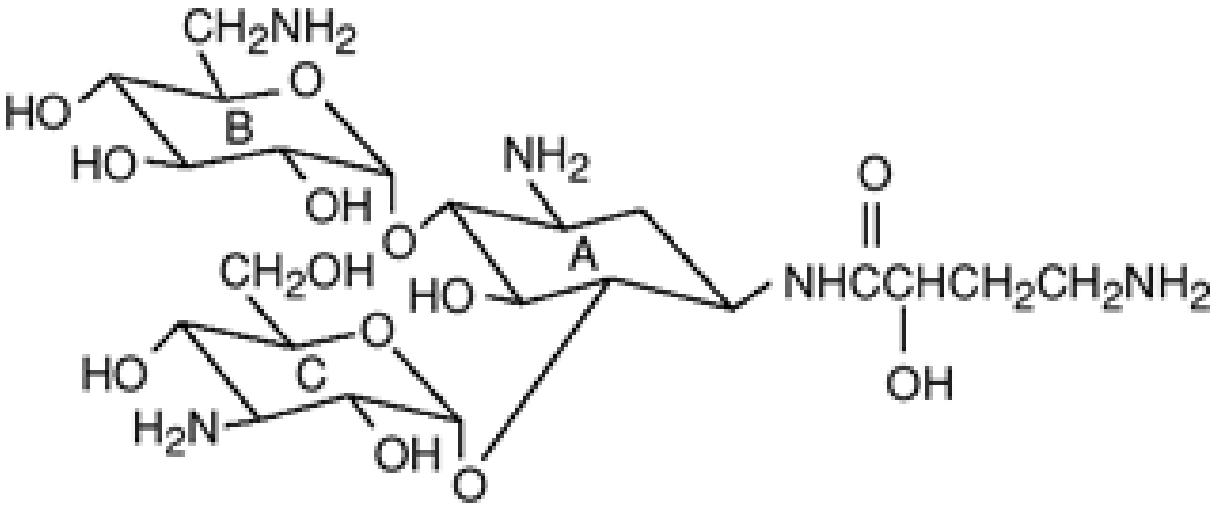


Figure 2.11: The structure of the aminoglycoside amikacin. Features of aminoglycosides include amino sugars bound by glycosidic linkages to a relatively conserved six-membered ring that itself contains amino group substituents.

Mechanism of Action of Aminoglycosides

Aminoglycosides display concentration-dependent bactericidal activity against "most gram-negative aerobic and facultative anaerobic bacilli" but not against gram-negative anaerobes and most gram-positive bacteria (Levison, 2012). They require only short contact time, and are most effective against susceptible bacterial populations that are rapidly multiplying (Boothe, 2012). These activities are attributed to a primary mode of action as protein synthesis inhibitors, though additional mechanisms are implicated for some specific agents, and/or thorough mechanistic descriptions are as yet unavailable (Mingeot *et al*., 1999; Levison, 2012; Boothe, 2012).

The inhibition of protein synthesis is mediated through aminoglycosides' energy-dependent, sometimes irreversible binding, to the cytosolic, membrane-associated bacterial ribosome (Mingeot *et al*., 1999). (Aminoglycosides first cross bacterial cell walls—lipopolysaccharide in gram-negative bacteria)—and cell membranes, where they are actively transported (Boothe, 2012). While specific steps in protein synthesis affected may vary somewhat between specific aminoglycoside agents, as can their affinity and degree of binding (Boothe, 2012), aminoglycoside presence in the cytosol generally disturbs peptide elongation at the 30S ribosomal subunit, giving rise to inaccurate mRNA translation and therefore biosynthesis of proteins that are truncated, or bear altered amino acid compositions at particular points (Mingeot *et al*., 1999). Specifically, binding impairs translational proofreading leading to misreading of the RNA message, premature termination, or both, and so to inaccuracy of the translated protein product. The subset of aberrant proteins that are incorporated into the bacterial cell membrane may then lead to changes in its permeability and then to "further stimulation of aminoglycoside transport" (Mingeot *et al*., 1999). The amino-sugar portion of this class of molecules (for example, the 2-deoxystreptamine in

kanamycins, gentamicins, and tobramycin) are implicated in the association of the small molecule with ribosomal structures that lead to the infidelities in translation. Inhibition of ribosomal translocation—that is, movement of the peptidyl-tRNA from the A- to the P-site—has also been suggested (Boothe, 2012). (Spectinomycin, a related but distinct chemical structure class often discussed with aminoglycosides, does not induce mRNA misreading and is generally not bactericidal) (Boothe, 2012).

Finally, a further "cell-membrane effect" also occurs with aminoglycosides; "functional integrity of the bacterial cell membrane" can be lost, later in time courses of aminoglycoside exposure and transport (Boothe, 2012).

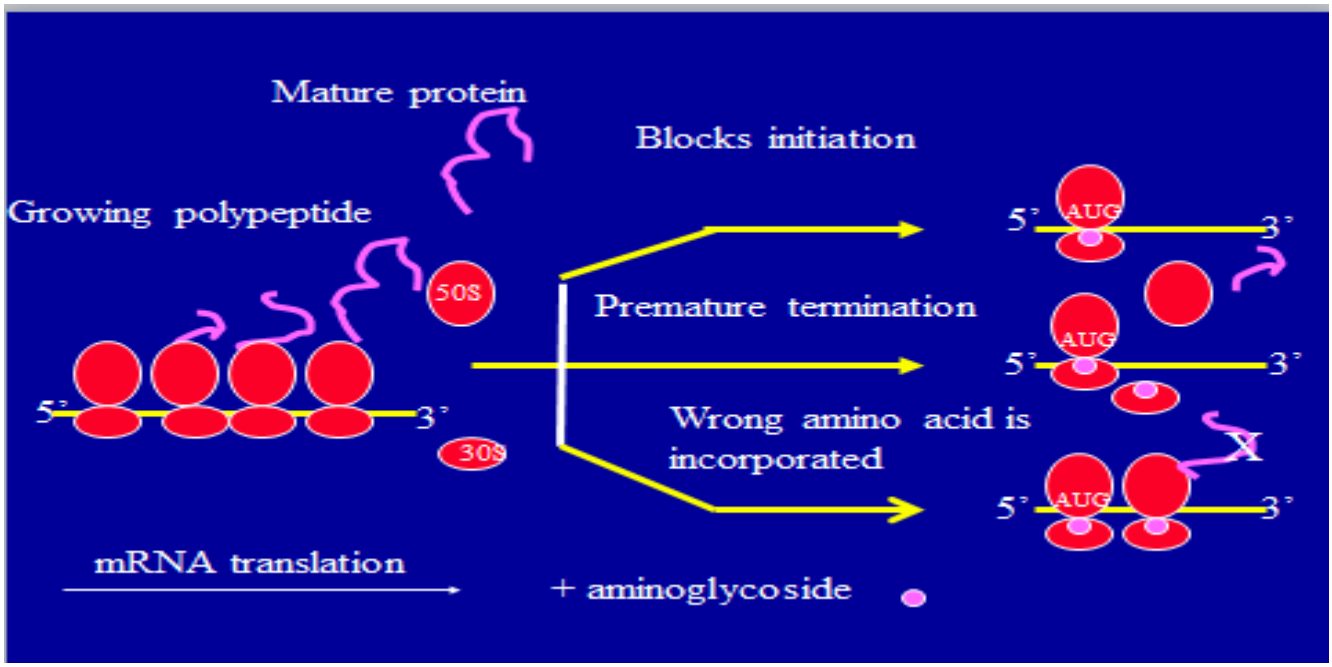


Figure 2.12: Schematic representation of the effect of aminoglycosides on bacterial protein synthesis

Spectrum of Activity of Aminoglycosides

Aminoglycosides are useful primarily in infections involving aerobic, Gram-negative bacteria, such as *Pseudomonas*, *Acinetobacter*, and *Enterobacter*. In addition, some *Mycobacteria*, including the bacteria that cause tuberculosis, are susceptible to aminoglycosides. Streptomycin was the first effective drug in the treatment of

tuberculosis, though the role of aminoglycosides such as streptomycin and amikacin has been eclipsed (because of their toxicity and inconvenient route of administration) except for multiple-drug-resistant strains. The most frequent use of aminoglycosides is empiric therapy for serious infections such as septicemia, complicated intra-abdominal infections, complicated urinary tract infections, and nosocomial respiratory tract infections. Usually, once cultures of the causal organism are grown and their susceptibilities tested, aminoglycosides are discontinued in favor of less toxic antibiotics.

As noted, aminoglycosides are mostly ineffective against anaerobic bacteria, fungi, and viruses (Mingeot *et al*., 1999). Infections caused by Gram-positive bacteria can also be treated with aminoglycosides, but other types of antibiotics are more potent and less damaging to the host. In the past, the aminoglycosides have been used in conjunction with beta-lactam antibiotics in streptococcal infections for their synergistic effects, in particular in endocarditis. One of the most frequent combinations is ampicillin (a beta-lactam, or penicillin-related antibiotic) and gentamicin. Often, hospital staff refer to this combination as "amp and gent" or more recently called "pen and gent" for penicillin and gentamicin.

Nonsense suppression: The interference with DNA proofreading has been exploited to treat genetic diseases that result from premature stop codons (leading to early termination of protein synthesis and truncated proteins). Aminoglycosides can cause the cell to overcome the stop codons, insert a random amino acid, and express a full-length protein (Feero *et al*., 2010). The aminoglycoside gentamicin has been used to treat cystic fibrosis (CF) cells in the laboratory to induce them to grow full-length proteins. CF is caused by a mutation in the gene coding for the *cystic fibrosis*

transmembrane conductance regulator (*CFTR*) protein. In approximately 10% of CF cases, the mutation in this gene causes its early termination during translation, leading to the formation of is truncated and non-functional CFTR protein. It is believed that gentamicin distorts the structure of the ribosome-RNA complex, leading to a mis-reading of the termination codon, causing the ribosome to "skip" over the stop sequence and to continue with the normal elongation and production of the CFTR protein (Wilschanski *et al*., 2003).

Medical Uses of Aminoglycosides

The recent emergence of infections due to Gram-negative bacterial strains with advanced patterns of antimicrobial resistance has prompted physicians to reevaluate the use of these antibacterial agents (Falagas *et al*., 2008). This revived interest in the use of aminoglycosides has brought back to light the debate on the two major issues related to these compounds, namely the spectrum of antimicrobial susceptibility and toxicity. Current evidence shows that aminoglycosides do retain activity against the majority of Gram-negative clinical bacterial isolates in many parts of the world. Still, the relatively frequent occurrence of nephrotoxicity and ototoxicity during aminoglycoside treatment makes physicians reluctant to use these compounds in everyday practice. Recent advances in the understanding of the effect of various dosage schedules of aminoglycosides on toxicity have provided a partial solution to this problem, although more research still needs to be done in order to overcome this problem entirely (Durante *et al*., 2009).

Aminoglycosides are in pregnancy category D, that is, there is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing

experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks (Levison, 2012).

Resistance to Aminoglycosides

The main mechanism for aminoglycoside resistance has been reported to be by enzymatic inactivation (Shaw *etal*., 1993; Mingeot-Leclercq *etal*., 1999), but reduced uptake and chromosomal mutations conferring high levels of resistance to streptomycin have also been described (Quintiliani *et al.,* 1999).

Aminoglycoside resistance has been reported mediated by more than 50 aminoglycoside modifying enzymes that are classified as either aminoglycoside n-acetyltransferases (aac), aminoglycoside adenyltransferases (aad or ant), and aminoglycoside phosphotransferases (aph) (Shaw *etal*., 1993, Mingeot-Leclercq*etal*., 1999, Aarts *etal*., 2006). Most aac, ant and aph genes are located on mobile genetic elements such as plasmids, transposons, or gene cassettes (Shaw *etal*., 1993; Recchia and Hall, 1995; Davies and Wright, 1997; Mingeot-Leclercq *etal*., 1999; Sandvang *et al*.,1997). The modifications of aminoglycosides and aminocyclitols by inactivating enzymes have been described in detail in various reviews (Shaw *etal*., 1993, Davies and Wright, 1997).

2.2.2.1 Gentamicin

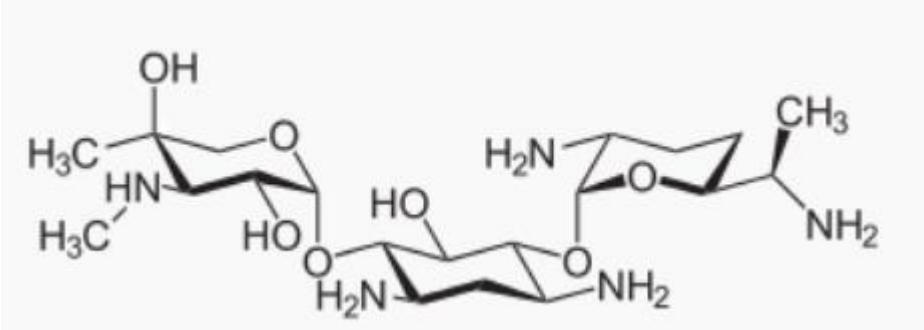


Figure 2.13: Molecular Structure of Gentamicin

Gentamicin, sold under the brand-name Garamycin among others, is an antibiotic used to treat many types of bacterial infections. This may include bone infections, endocarditis, pelvic inflammatory disease, meningitis, pneumonia, urinary tract infections, and sepsis among others. It is not effective for gonorrhoea or chlamydia infections. It can be given intravenously, by injection into a muscle, or topically. Topical formulations may be used in burns or for infections of the outside of the eye (*Bartlett, 2013).* In the developed world it is often only used for two days until bacterial cultures determine what antibiotics the infection is sensitive to. The dose required should be monitored by blood testing.

Gentamicin can cause inner ear problems and kidney problems. The inner ear problems can include problems with balance and problems with hearing. These problems may be permanent. If used during pregnancy it can cause harm to the baby. It appears to be safe for use during breastfeeding. Gentamicin is a type of aminoglycoside. It works by stopping the bacteria from making protein which typically kills the bacteria.

Gentamicin was discovered in 1963 (Pucci, 2011). It is made from the bacteria *Micromonospora purpurea*. Gentamicin is on the World Health Organization's List of Essential Medicines, the most important medication needed in a basic health system (WHO, 2013). It is available as a generic medication (Burchum, 2014).

Gentamicin is active against a wide range of bacterial infections, mostly Gram-negative bacteria including *Pseudomonas*, *Proteus*, *Serratia*, and the Gram-positive *Staphylococcus*.Gentamicin is also useful against *Yersinia pestis*, its relatives, and *Francisella tularensis* (the organism responsible for tularemia seen often in hunters and/or trappers) (Goljan, 2011). Some *Enterobacteriaceae*, *Pseudomonas spp.*, *enterococci*, *Staphylococcus aureus* and other *staphylococci* are resistant to gentamicin sulfate, to varying degrees.

Gentamicin is not used for *Neisseria gonorrhoeae*, *Neisseria meningitidis* or *Legionella pneumophila* bacterial infections (because of the risk of the person going into shock from lipid Aendotoxin found in certain Gram-negative organisms).

Mechanism of Action of Gentamicin

Gentamicin is a bactericidal antibiotic that works by interrupting microbial protein synthesis. This mechanism of action is similar to other aminoglycosides (Section 2.2.2).

2.2.3 Quinolones and Flouroquinolones

The **quinolones** are a family of syntheticbroad-spectrum antibiotic drugs (Anderson and MacGowan, 2003; Ivanov and Budanov, 2006). Quinolones, and derivatives, have also been isolated from natural sources (such as plants, animals and bacteria) and can act as natural antimicrobials and/or signalling molecules (Heeb, *et al.*, 2011).

Quinolones exert their antibacterial effect by preventing bacterial DNA from unwinding and duplicating (Hooper, 2001). The majority of quinolones in clinical use are **fluoroquinolones**, which have a fluorineatom attached to the central ring system, typically at the 6-position or C-7 position. Most of them are named with the *-oxacin* suffix.

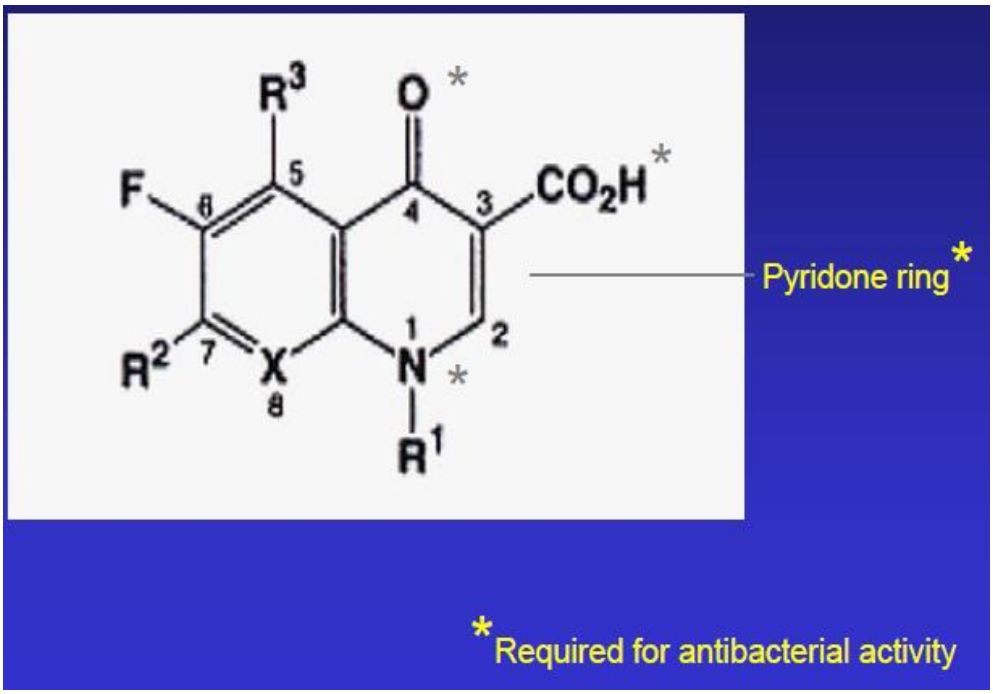


Figure 2.14: Essential structure of quinolone showing sites required for antibacterial activity

Medical Uses of Quinolones/Flouroquinolones

Fluoroquinolones are broad-spectrum antibiotics (effective for both gram-negative and gram-positive bacteria) that play an important role in treatment of serious bacterial infections, especially hospital-acquired infections and others in which resistance to older antibacterial classes is suspected. Because the use of broad-spectrum antibiotics encourages the spread of multidrug-resistant strains and the development of *Clostridium difficile* infections, treatment guidelines from the Infectious Diseases Society of America, the American Thoracic Society, and other

professional organizations recommend minimizing the use of fluoroquinolones and other broad-spectrum antibiotics in less severe infections and in those in which risk factors for multidrug resistance are not present.

Fluoroquinolones are featured prominently in The American Thoracic Society guidelines for the treatment of hospital-acquired pneumonia (Anon, 2005). The Society recommends fluoroquinolones not be used as a first-line agent for community-acquired pneumonia (Mandell *et al*., 2007), instead recommending macrolide or doxycycline as first-line agents. The Drug-Resistant *Streptococcus pneumoniae* Working Group recommends fluoroquinolones be used for the ambulatory treatment of community-acquired pneumonia only after other antibiotic classes have been tried and failed, or in cases with demonstrated drug-resistant *Streptococcus pneumoniae*(MacDougall *et al*., 2005).

Fluoroquinolones are often used for genitourinary infections, and are widely used in the treatment of hospital-acquired infections associated with urinary catheters. In community-acquired infections, they are recommended only when risk factors for multidrug resistance are present or after other antibiotic regimens have failed. However, for serious acute cases of pyelonephritis or bacterial prostatitis where the patient may need to be hospitalised, fluoroquinolones are recommended as first-line therapy (Liu and Mulholland, 2005).

Due to sickle-cell disease patients' being at increased risk for developing osteomyelitis from the *Salmonella* genus, fluoroquinolones are the "drugs of choice" due to their ability to enter bone tissue without chelating it, as tetracyclines are known to do.

Mechanism of Action of Quinolones/Flouroquinolones

First and second generation fluoroquinolones selectively inhibit the topoisomerase II ligase domain, leaving the two nuclease domains intact. This modification, coupled with the constant action of the topoisomerase II in the bacterial cell, leads to DNA fragmentation via the nucleasic activity of the intact enzyme domains. Third and fourth generation fluoroquinolones are more selective for the topoisomerase IV ligase domain, and thus have enhanced gram-positive coverage.

Fluoroquinolones can enter cells easily via porins and, therefore, are often used to treat intracellularpathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*. For many gram-negative bacteria, DNA gyrase is the target, whereas topoisomerase IV is the target for many gram-positive bacteria. Some compounds in this class have been shown to inhibit the synthesis of mitochondrial DNA (Bergan and Bayer, 1988; Bergan *et al*., 1985; Castora *et al*., 1983; Suto *et al*., 1992).

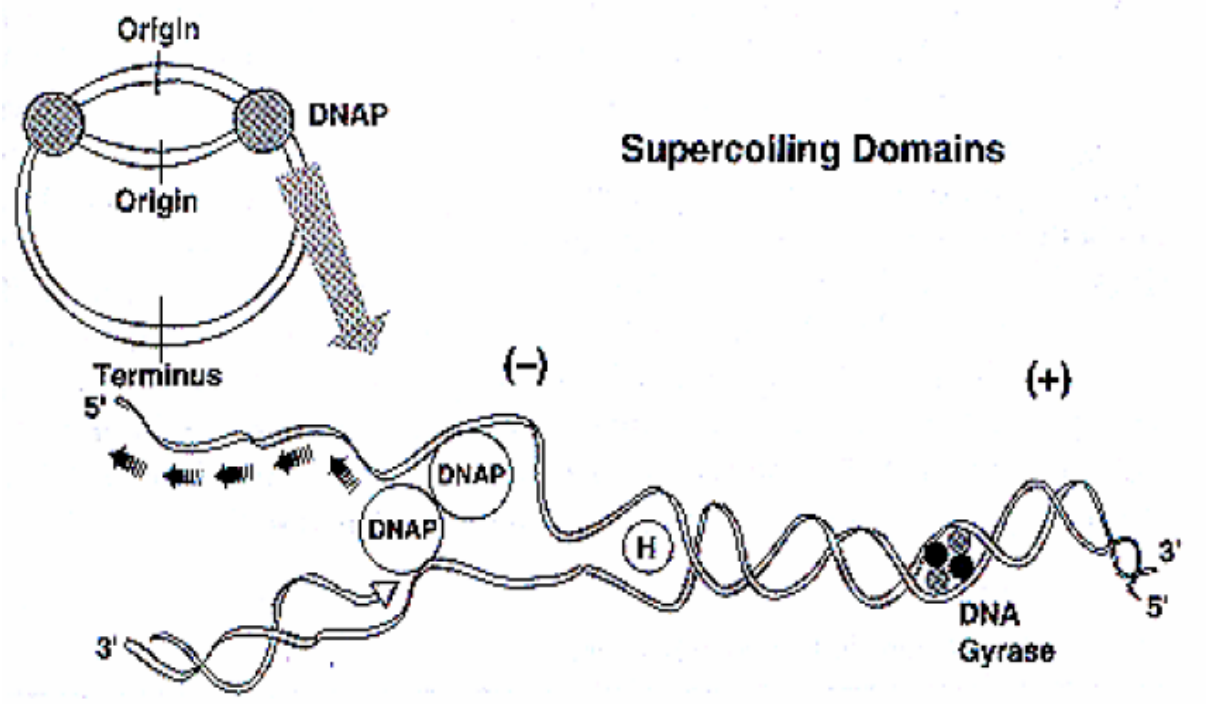


Figure 2.15: Mode of action of quinolones

Resistance to Quinolones and Fluoroquinolones

Quinolones and fluoroquinolones are potent inhibitors of bacterial DNA replication (Schwarz *etal*., 2006). The two major mechanisms of bacterial resistance development to fluoroquinolone antibiotics have been reported to be by point mutations and decreased intracellular accumulation (Schwarz *etal*., 2006). Severalrecent reviews deal with the molecular basis and epidemiology of quinolone resistance in *E. coli* and *Salmonella* species of animal origin (Drlica and Zhao, 1997; Everett and Piddock, 1998; Hooper, 1999; Cloeckaert and Chaslus-Dancla, 2001; Webber and Piddock, 2001; Ruiz, 2003).

Briefly, point mutations in the target genesgyrA and gyrB coding for DNA gyrase and or for parC and parE coding for DNA topoismerase IV are frequent in quinolone and fluoroquinolone resistance (Schwarz *etal*., 2006). Detection of these point mutations in the region of the gyrA, gyrB, or parC and parE genes can be accomplished through polymerase chain reaction (PCR) (Aarts *etal*., 2006), while microarrays have been used to assess multidrug efflux systems. Resistance genes associated with multidrug efflux pumps vary depending on the organism involved (Schwarz *etal*., 2006) and they may lead to high levels of resistance to quinolones and other antibiotics where multidrug efflux pumps and decreased membrane permeability are involved (Lee *etal*., 2000). Quinolone and fluoroquinolone resistance has also been reported to result from interaction between different resistance mechanisms, decreased drug uptake and DNA gyrase protection (Schwarz *etal*., 2006).

2.2.3.1 Ciprofloxacin

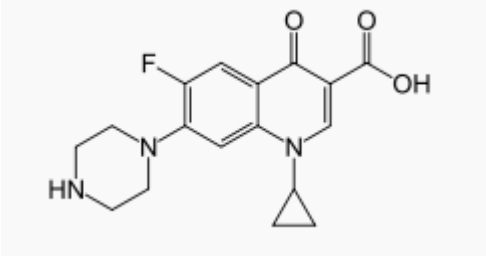


Figure 2.16: The Molecular Structure of Ciprofloxacin

Ciprofloxacin is an antibiotic used to treat a number of bacterial infections. This includes bone and joint infections, intra-abdominal infections, certain type of infectious diarrhoea, respiratory tract infections, skin infections, typhoid fever, and urinary tract infections, among others. For some infections it is used in addition to other antibiotics. It can be taken by mouth or used intravenously.

Common side effects include nausea, vomiting, diarrhoea, and rash. Ciprofloxacin increases the risk of tendon rupture and worsening muscle weakness in people with the neurological disorder myasthenia gravis. Rates of side effects appear to be higher than some groups of antibiotics such as cephalosporins but lower than others such as clindamycin (Heidelbaugh, 2013). Studies in other animals raise concerns regarding use in pregnancy. No problems were identified; however, in the children of a small number of women who took the medication. It appears to be safe during breastfeeding. It is a second-generation fluoroquinolone with a broad spectrum of activity (Ball, 2000; Oliphant *et al.*, 2002).

Ciprofloxacin was introduced in 1987. It is on the World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system (WHO, 2015). It is available as a generic medication and not very expensive (Hamilton, 2014).

Ciprofloxacin is used to treat a wide variety of infections, including infections of bones and joints, endocarditis, gastroenteritis, malignant otitis externa, respiratory tract infections, cellulitis, urinary tract infections, prostatitis, anthrax, and chancroid.

Ciprofloxacin only treats bacterial infections; it does not treat viral infections such as the common cold. For certain uses including acute sinusitis, lower respiratory tract infections and uncomplicated gonorrhoea, ciprofloxacin is not considered a first-line agent.

Ciprofloxacin occupies an important role in treatment guidelines issued by major medical societies for the treatment of serious infections, especially those likely to be caused by Gram-negative bacteria, including *Pseudomonas aeruginosa*. For example, ciprofloxacin in combination with metronidazole is one of several first-line antibiotic regimens recommended by the Infectious Diseases Society of America for the treatment of community-acquired abdominal infections in adults. It also features prominently in treatment guidelines for acute pyelonephritis, complicated or hospital-acquired urinary tract infection, acute or chronic prostatitis, certain types of endocarditis, certain skin infections, and prosthetic joint infections.

In other cases, treatment guidelines are more restrictive, recommending in most cases that older, narrower-spectrum drugs be used as first-line therapy for less severe infections to minimize fluoroquinolone-resistance development. For example, the Infectious Diseases Society of America recommends the use of ciprofloxacin and other fluoroquinolones in urinary tract infections be reserved to cases of proven or expected resistance to narrower-spectrum drugs such as nitrofurantoin or trimethoprim/sulfamethoxazole. The European Association of Urology recommends ciprofloxacin as an alternative regimen for the treatment of uncomplicated urinary

tract infections, but cautions that the potential for “adverse events have to be considered”.

Although approved by regulatory authorities for the treatment of respiratory infections, ciprofloxacin is not recommended for respiratory infections by most treatment guidelines due in part to its modest activity against the common respiratory pathogen *Streptococcus pneumoniae* (Zuger, 1998; Vardakas *et al*., 2008; Donaldson, 1994). "Respiratory quinolones" such as levofloxacin, having greater activity against this pathogen, are recommended as first line agents for the treatment of community-acquired pneumonia in patients with important co-morbidities and in patients requiring hospitalization. Similarly, ciprofloxacin is not recommended as a first-line treatment for acute sinusitis (*Karageorgopoulos et al., 2008; Chow et al., 2012).*

Ciprofloxacin is approved for the treatment of gonorrhoea in many countries, but this recommendation is widely regarded as obsolete due to resistance development (Hugh, 2003; CDC, 2007).

Spectrum of Activity of Ciprofloxacin

Its spectrum of activity includes most strains of bacterial pathogens responsible for respiratory, urinary tract, gastrointestinal, and abdominal infections, including Gram-negative (*Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*), and Gram-positive (methicillin-sensitive, but not methicillin-resistant *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Streptococcus pyogenes*) bacterial pathogens.

Bacterial Resistance to Ciprofloxacin

As a result of its widespread use to treat minor infections readily treatable with older, narrower spectrum antibiotics, many bacteria have developed resistance to this drug in recent years, leaving it significantly less effective than it would have been otherwise (*Vatopoulos and Kalapothaki, 1997).*

Resistance to ciprofloxacin and other fluoroquinolones may evolve rapidly, even during a course of treatment. Numerous pathogens, including enterococci, *Streptococcus pyogenes* and *Klebsiella pneumoniae* (quinolone-resistant) now exhibit resistance (Jacobs, 2005). Widespread veterinary usage of the fluoroquinolones, particularly in Europe, has been implicated. Meanwhile, some *Burkholderia cepacia*, *Clostridium innocuum* and *Enterococcus faecium* strains have developed resistance to ciprofloxacin to varying degrees.

Fluoroquinolones had become the class of antibiotics most commonly prescribed to adults in 2002 (Linder *et al*., 2005). Nearly half (42%) of those prescriptions in the U.S. were for conditions not approved by the FDA, such as acute bronchitis, otitis media, and acute upper respiratory tract infection, according to a study supported in part by the Agency for Healthcare Research and Quality (Linder *et al*., 2005). Additionally, they were commonly prescribed for medical conditions that were not even bacterial to begin with, such as viral infections, or those to which no proven benefit existed.

Mechanism of Action of Ciprofloxacin

Ciprofloxacin is a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II

topoisomerase, and topoisomerase IV (Drlica *et al*., 1997), enzymes (Pommier *et al*., 2010) necessary to separate bacterial DNA, thereby inhibiting cell division. Details of this process are explained in section 2.2.3.

2.2.3.2 Ofloxacin

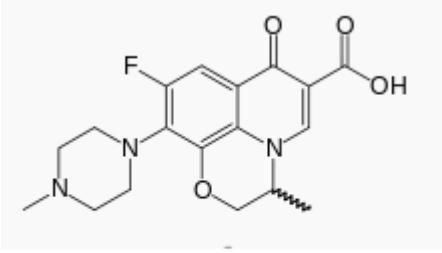


Figure 2.17: Molecular Structure of Ofloxacin

Ofloxacin is a synthetic antibiotic of the fluoroquinolone drug class considered to be a second-generation fluoroquinolone (Nelson *et al*., 2007; Kawahara, 1998).

Ofloxacin was first patented in 1982 (European Patent Daiichi) and received approval from the U.S. Food and Drug Administration (FDA) on December 28, 1990. Ofloxacin is sold under a wide variety of brand names as well as generic drug equivalents, for oral and intravenous administration. Ofloxacin is also available for topical use, as eye drops and ear drops (marketed as Ocuflox and Floxin Otic respectively in the United States and marketed as Optiflox, eylox respectively in Jordan and Saudi Arabia).

Ofloxacin is a racemic mixture, which consists of 50% levofloxacin (the biologically active component) and 50% of its "mirror image" or enantiomer dextrofloxacin.

Ofloxacin has been associated with adverse drug reactions, such as tendon damage (including spontaneous tendon ruptures) and peripheral neuropathy (which may be irreversible); tendon damage may manifest long after therapy had been completed, and, in severe cases, may result in lifelong disabilities.

Mechanism of Action of Ofloxacin

Ofloxacin is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV (Drlica *et al*., 1997), which is an enzyme necessary to separate (mostly in prokaryotes, in bacteria in particular) replicated DNA, thereby inhibiting bacterial cell division.Details of the process involved is explained in section 2.2.3.

Spectrum of Activity of Ofloxacin

According to the product package insert, ofloxacin is effective against the following microorganisms (Sato *et al*., 1982).

- Aerobic Gram-positive microorganisms: *Staphylococcus aureus* (methicillin-susceptible strains), *Streptococcus pneumoniae* (penicillin-susceptible strains), *Streptococcus pyogenes*
- Aerobic Gram-negative microorganisms: *Citrobacter koseri* (*Citrobacter diversus*), *Enterobacter aerogenes, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Neisseria gonorrhoeae, Proteus mirabilis, Pseudomonas aeruginosa.*
- Other microorganisms such as *Chlamydia trachomatis.*

Antibiotic abuse and bacterial resistance to ofloxacin

Resistance to ofloxacin and other fluoroquinolones may evolve rapidly, even during a course of treatment. Numerous pathogens, including *Staphylococcus aureus*, enterococci, and *Streptococcus pyogenes* now exhibit resistance worldwide (Jacobs, 2005).

Ofloxacin and other fluoroquinolones had become the most commonly prescribed class of antibiotics to adults in 2002. Nearly half (42%) of these prescriptions were for conditions not approved by the FDA, such as acute bronchitis, otitis media, and acute upper respiratory tract infection, according to a study that was supported in part by the Agency for Healthcare Research and Quality (Linder *et al*., 2005). Additionally they are commonly prescribed for medical conditions that are not even bacterial to begin with, such as viral infections, or those to which no proven benefit exists.

2.2.4 Sulphonamides and Trimethoprim

Antibacterial sulphonamides are first effective chemotherapeutic agents used for bacterial infection in humans. The term sulphonamide is usually employed as a generic name for the derivatives of para amino benzene sulphonamides. Sulphonamides inhibit Gram-positive and Gram-negative bacteria, *Nocardia*, *Chlamydiatrachomatis* and some Protozoa. Some enteric bacteria such as *E. coli*, *Kelbsiella*, *Salmonella*, *Shigella* and *Enterobacter* are inhibited. Sulphonamides are used in the treatment of tonsillitis, septicemia, meningococcal meningitis, bacillary dysentery and number of infections of urinary tract.

Classification of Sulphonamides

Sulphonamide can be classified in various ways as follows:

I. On the basis of site of action

a) Sulphonamides for general infections: example; Sulphanilamide, Sulphapyridine, Sulphadiazine, Sulphamethocine, Suphamethoxazole.
b) Sulphonamides for intestinal infections: example; Phthalyl sulphathiazole, Succinyl sulphathiazole, Sulphasalazine.
c) Sulphonamides for local infections: example; Sulphacetamide, Mafenamide, Silver sulphadiazine.
d) Sulphonamides for dermatitis: example; Dapsone, Solapsone.
e) Sulphonamide Combination: example; Sulphamethoxazole with Trimethoprim, Sulphadiazine with Trimethoprim, Sulphadoxime with Pyrimethamine, Sulphamoxole with Trimethoprim.

II. On the basis of pharmacokinetic properties

a) Poorly absorbed Sulphonamides: (Locally acting sulphonamides) these agents are poorly absorbed in GIT and mainly used to treat intestinal disease or to reduce luminal bacterial population prior to bowel surgery. Example; Sulphasalazine, Phthalyl sulphathiazole.
b) Rapidly absorbed and excreted Sulphonamides (Systemic sulphonamides) example; Sulphamethoxazole, Sulphisoxazole, Sulphadiazine.
c) Topically used Sulphonamides: They are mainly applied in burns, conjunctival sac, otic canal and vagina to treat bacterial infection. Example; Mafenide sodium, Sulphacetamide, Silver sulphadiazine.

III. On the basis of chemical classification

a) N_4– substituted Sulphonamides (pro drugs): example; Prontosil, Sulphaguanidine,

b) N_1 - substituted Sulphonamides: example; Sulphadiazine, Sulphacetamide, Sulphadimidine.

c) Both N_1 and N_4 - substituted Sulphonamides: example; Succinyl sulphathiazole, Phthalyl sulphathiazole.

d) Non-aniline Sulphonamides: example; Mefenide sodium.

IV. On the basis of pharmacological activity

a) Antibacterial agents: example; Sulphadiazine, Sulphisoxazole.

b) Oral hypoglycemic agent: example; Tolbutamide.

c) Diuretics: example; Furosemide, Bumetanide, Chlorthalidone.

d) Dermatitis: example; Dapsone.

V. On the basis of duration of action

a) Ultra long acting Sulphonamides: (half-life greater than 50 hours). Examples; Sulphasalazine, Sulphacetamide, Sulphalene.

b) Long acting Sulphonamides: (half-life greater than 24 hours). Examples; Sulphamethoxdiazine, Sulphadimethoxine.

c) Intermediate acting Sulphonamides: (half-life between 10-24 hours). Examples; Sulphasomizole, Sulphamethoxazole.

d) Short acting Sulphonamides (half-life less than 20 hours). Examples; Sulphamethizole, Sulphisoxazole.

2.2.4.1 Sulphamethoxazole (Gantanol)

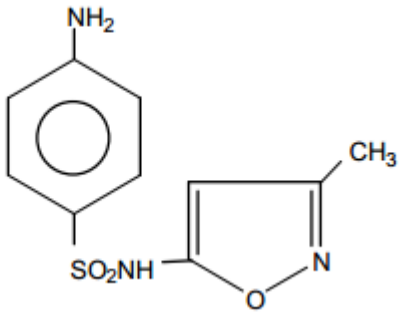


Figure 2.18: 4-Amino-N-(5-methyl -3 - isoxazolyl) benzene sulphonamide

2.2.4.2 Trimethoprim (TMP)

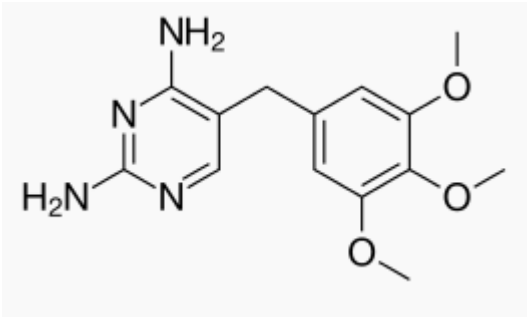


Figure 2.19: Molecular Structure of Trimethoprim

Trimethoprim (**TMP**) is an antibiotic used mainly in the treatment of bladder infections. Other uses include for middle ear infections and travelers' diarrhea. With sulfamethoxazole or dapsone it may be used for *Pneumocystis* pneumonia in people with HIV/AIDS (Masur *et al*., 2014).

Common side effects include nausea, changes in taste, and rash. Rarely it may result in blood problems such as not enough platelets or white blood cells. May cause sun sensitivity. There is evidence of potential harm during pregnancy in some animals but not humans. It works by blocking folate metabolism via dihydrofolate reductase in some bacteria which results in their death.

Trimethoprim was first used in 1962 (Huovinen, 2001). It is on the World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system (WHO, 2013).

Medical Uses of Trimethoprim

It is primarily used in the treatment of urinary tract infections, although it may be used against any susceptible aerobic bacterial species. It may also be used to treat and prevent *Pneumocystis jiroveci* pneumonia. It is generally not recommended for the treatment of anaerobic infections such as *Clostridium difficile* colitis (the leading cause of antibiotic-induced diarrhea) (Rossi, 2013).

2.2.4.3 Trimethoprim/Sulfamethoxazole Synergy

Trimethoprim/sulfamethoxazole (**TMP/SMX, or SXT**), also known as **co-trimoxazole** among other names, is an antibiotic used to treat a variety of bacterial infections. It consists of one part trimethoprim to five parts sulfamethoxazole (Hamilton, 2015). It is used for urinary tract infections, MRSA skin infections, travellers' diarrhoea, respiratory tract infections, and cholera, among others (Hamilton, 2015). It may be used both to treat and prevent pneumocystis pneumonia in people with HIV/AIDS. It can be given by mouth or intravenously.

Common side effects include nausea, vomiting, rash, and diarrhoea. Severe allergic reactions and *Clostridiumdifficile* diarrhoea may occasionally occur. Its use near the end of pregnancy is not recommended. It appears to be okay during breastfeeding as long as the baby is healthy. TMP/SMX generally results in bacterial death. It works by stopping the metabolism of folate.

TMP/SMX was first sold in 1974. It is on the World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system (WHO, 2013).

Co-trimoxazole was claimed to be more effective than either of its components individually in treating bacterial infections, although this was later disputed (Brumfitt and Hamilton-Miller, 1993). Because it has a higher incidence of adverse effects, including allergic responses, its use has been restricted in many countries to very specific circumstances where its improved efficacy has been demonstrated. It may be effective in a variety of upper and lower respiratory tract infections, renal and urinary tract infections, gastrointestinal tract infections, skin and wound infections, septicaemias, and other infections caused by sensitive organisms. The global problem of advancing antimicrobial resistance has led to a renewed interest in the use of co-trimoxazole more recently (Falagas *et al*., 2008).

Susceptibility to Trimethoprim/sulfamethoxazole

Organisms against which co-trimoxazole can be effective include (Wormser *et al*., 1982): ***Acinetobacter*** spp., ***Aeromonas hydrophila, Alcaligenes xylosoxidans, Bartonella henselae, Bordetella pertussis*** (pertussis), *Brucella* spp., *Burkholderia cepacia*, *Burkholderia mallei* (**glanders**), *Burkholderia pseudomallei* (**melioidosis**),

Chlamydia trachomatis (chlamydia), ***Chryseobacterium meningosepticum,*** *Citrobacter* spp., *Enterobacter* spp., ***Escherichia coli, Haemophilus influenzae, Hafnia alvei, Kingella*** spp., *Klebsiella granulomatis, Klebsiella pneumoniae, Legionella* spp., *Listeria monocytogenes* (**listeriosis**), *Moraxella catarrhalis, **Morganella morganii, Mycobacterium tuberculosis*** (**tuberculosis**), ***Neisseria gonorrhoeae*** (gonorrhoea), *Neisseria meningitidis* (meningococcal disease), ***Nocardia*** spp., ***Plesiomonas shigelloides,*** *Pneumocystis jirovecii, Proteus mirabilis, Proteus vulgaris, **Providencia rettgeri, Providencia stuartii,*** *Salmonella typhi* (typhoid fever), Non-typhi *Salmonella, **Serratia** spp., Shigella* spp., *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, **Stenotrophomonas maltophilia,*** *Streptococcus agalactiae, Streptococcus faecalis, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus viridans, Toxoplasma gondii* (toxoplasmosis), ***Tropheryma whippelii*** (Whipple's disease), *Vibrio cholerae* (cholera), *Yersinia enterocolitica, Yersinia pestis* (bubonic plague) and *Yersinia pseudotuberculosis.*

The only notable non-susceptible organisms are the mycoplasmae (Wormser *et al*., 1982) and *Francisella tularensis* (the causative organism of tularaemia) (Harik, 2013).

Mechanism of Action of Sulfamethoxazole/Trimethoprim

Sulphonamides are bacteriostatic in nature. The sulphonamide sensitive micro-organisms require p-Amino benzoic acid (PABA) for the synthesis of folic acid which is essential for the synthesis of DNA and RNA. Sulphonamides block the biosynthesis of this folate coenzyme resulting into the arrest of bacterial growth and cell division.

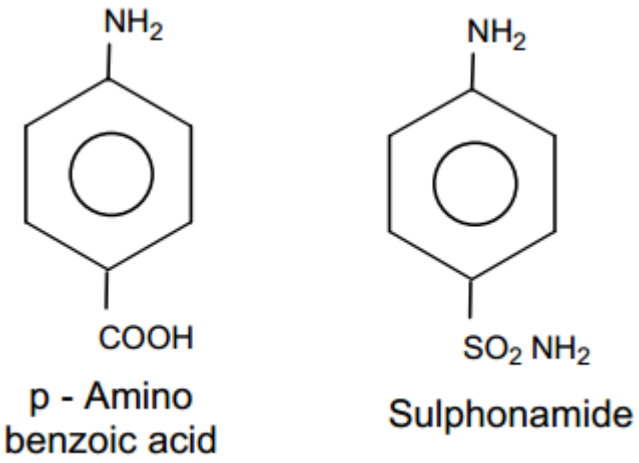


Figure 2.20: Molecular Structures of para-amino benzoic acid and Sulphonamide

Sulfonamides are structural analogues of paraamino benzoic acid (PABA), competitively inhibiting synthesis of dihydrofolic acid.

Trimethoprim is an analogue of the pteridine portion of dihydrofolic acid inhibiting synthesis of tetrahydrofolic acid.

Sequential interference with folic acid synthesis results in bacterial synergism often with bactericidal activity.

Sulfamethoxazole inhibits bacterial synthesis of dihydrofolic acid by competing with PABA. Trimethoprim blocks production of tetrahydrofolic acid by inhibiting the enzyme dihydrofolate reductase. This combination blocks 2 consecutive steps in bacterial biosynthesis of essential nucleic acids and proteins and is usually bactericidal.

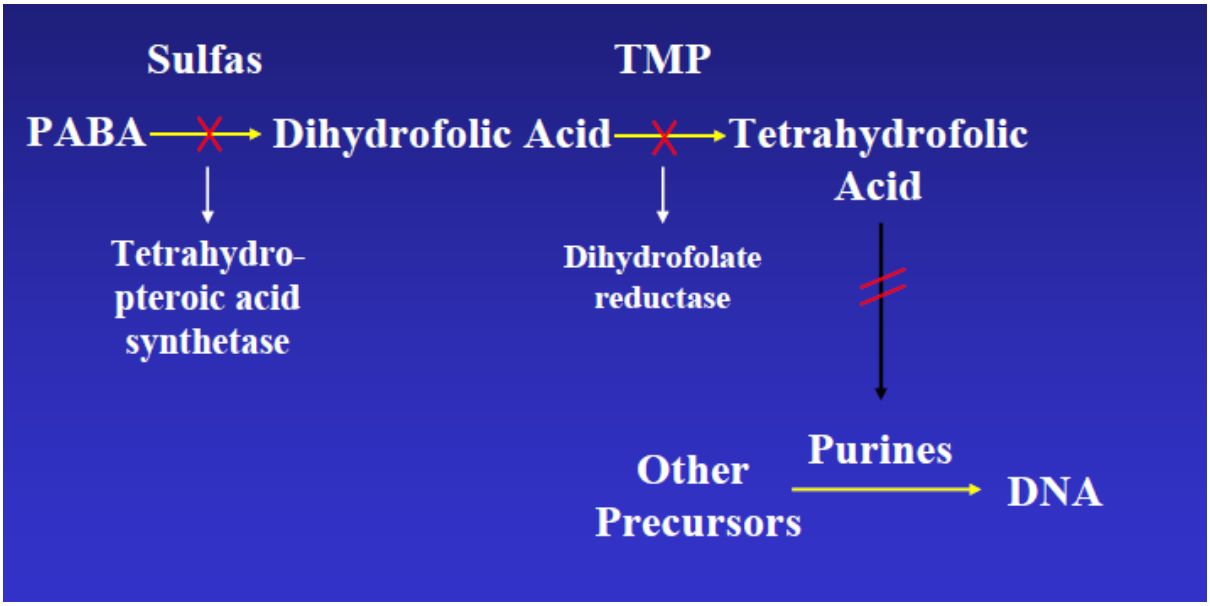


Figure 2.21: Structural representation of the interaction between trimethoprim and microbial nucleic acid

Trimethoprim binds to dihydrofolate reductase and inhibits the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF) (Brogden *et al*., 1982). THF is an essential precursor in the thymidine synthesis pathway and interference with this pathway inhibits bacterial DNA synthesis. Trimethoprim's affinity for bacterial dihydrofolate reductase is several thousand times greater than its affinity for human dihydrofolate reductase. Sulfamethoxazole inhibits dihydropteroate synthase, an enzyme involved further upstream in the same pathway. Trimethoprim and sulfamethoxazole are commonly used in combination due to possible synergistic effects, and reduced development of resistance (Bogden *et al*., 1982). This benefit has been questioned (Brumfitt and Hamilton-Miller, 1993).

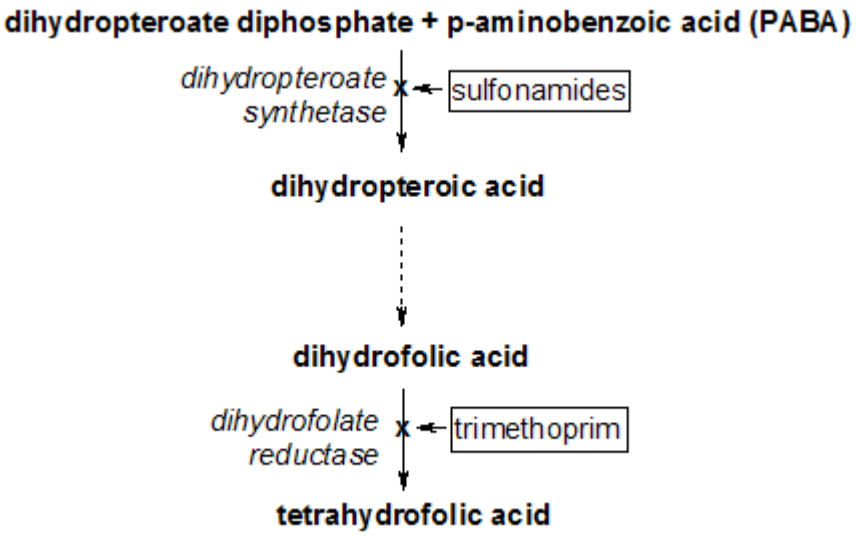


Figure 2.22: A flow chart of the interaction between sulphonamides, trimethoprim and PABA

Resistance to Sulphonamides and Trimethoprim

Sulphonamides and trimethoprim are competitive inhibitors of different enzymatic steps in folate metabolism (Schwarz *et al*., 2006). Sulphonamide resistance has been reported due to chromosomal mutations in the dihydropteroate synthase (folP) gene or by acquisition of resistant dihydropteroate synthase genes (sul genes) (Aarts *et al*., 2006; Schwarz *et al*., 2006). Three sul genes have been described in gram negative bacteria (Swedberg and Skold, 1980; Radstrom and Swedberg, 1988; Aarts *et al*., 2006). The sulI gene is associated with class 1 integrons and, therefore, often linked to other bacterial resistance genes. It has been reported to be common in gram negative bacterial species as part of transposons or as conjugative plasmids (Sundstrom *et al*., 1988). The sulII gene often occurs with streptomycin resistance genes strA and strB on conjugative or nonconjugative plasmids (Radstrom and Swedberg, 1988; Kehrenberg and Schwarz, 2004), while the sulIII gene has been found on conjugative plasmids (Perreten and Boerlin, 2003).

Trimethoprim resistance is primarily mediated by acquisition of dfr gene encoding resistant dihydrofolate reductase (Aarts *et al*., 2006; Schwarz *et al*., 2006). Transferable trimethoprim resistance has been identified in a variety of gram negative bacteria and several of these genes are part of plasmids, transposons, or gene cassettes (Recchia and Hall, 1995; Ito *et al*., 2007). Other potential mechanisms of trimethoprim resistance for some bacteria include permeability barriers and efflux pumps (Kohler *et al*., 1996; Huovinen, 2001) and dhfr and folate auxotrophy (Quintiliani *et al*., 1999). Mutations in chromosomal genes have also been observed (Huovinen, 2001).

2.2.5 Nitrofuran/Nitroimidazole

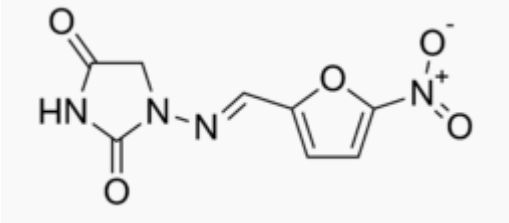


Figure 2.23: Molecular Structure of Nitrofurantoin

Nitrofurantoin, sold under the trade name **Macrobid** among others, is an antibiotic used to treat bladder infections. It is not effective for kidney infections. It is taken by mouth.

Common side effects include nausea, loss of appetite, diarrhoea, and headaches. Rarely numbness, lung problems, or liver problems may occur. It should not be used in people with kidney problems. While it looks like it is generally safe during pregnancy it should not be used near delivery. It works by slowing growth rather than killing bacteria.

Nitrofurantoin was first sold in 1953 (Blass, 2015). It is on the World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system (WHO, 2013). It is available as a generic medication. Nitrofurantoin has been available for the treatment of urinary tract infections (UTIs) since 1953. Its current uses include the treatment of uncomplicated UTIs and prophylaxis against UTIs in people prone to recurrent UTIs (Garau, 2008).

Increasing bacterial antibiotic resistance to other commonly used agents, such as fluoroquinolones and trimethoprim/sulfamethoxazole, has led to increased interest in using nitrofurantoin (Garau, 2008; McKinnell *et al*., 2011). Several trials comparing nitrofurantoin to other commonly used agents have shown this drug results in similar cure rates for uncomplicated UTIs (Christiaens *et al*., 2002; Gupta *et al*., 2007; Iravani *et al*., 1999; Steins, 1999). The efficacy of nitrofurantoin in treating UTIs combined with a low rate of bacterial resistance to this agent makes it one of the first-line agents for treating uncomplicated UTIs as recommended by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases (Gupta *et al*., 2011).

Nitrofurantoin is not recommended for the treatment of pyelonephritis (Gupta *et al*., 2011), prostatitis and intra-abdominal abscess (Solomki *et al*., 2010), because of extremely poor tissue penetration and low blood levels.

Antibacterial activity of Nitrofurantoin

Nitrofurantoin has been shown to have good activity against: *E. coli*, *Staphylococcus saprophyticus*, Coagulase negative staphylococci, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Citrobacter* species, *Klebsiella* species (Gupta *et al*., 1999).

Many or all strains of the following genera are resistant to nitrofurantoin (Gupta *et al*., 1999): *Enterobacter, Klebsiella, Proteus, Pseudomonas.*

Mechanism of Action of Nitrofurantoin

The mechanism of action of nitrofurantoin is unique and complex. The drug works by damaging bacterial DNA, since its reduced form is highly reactive. This is made possible by the rapid reduction of nitrofurantoin inside the bacterial cell by flavoproteins (nitrofuran reductase) to multiple reactive intermediates that attack ribosomal proteins, DNA (Tu and McCalla, 1975), respiration, pyruvate metabolism and other macromolecules within the cell. Nitrofurantoin exerts greater effects on bacterial cells than mammalian cells because bacterial cells activate the drug more rapidly. It is not known which of the actions of nitrofurantoin is primarily responsible for its bactericidal activity. The broad mechanism of action for this drug likely is responsible for the low development of resistance to its effects, as the drug affects many different processes important to the bacterial cell.

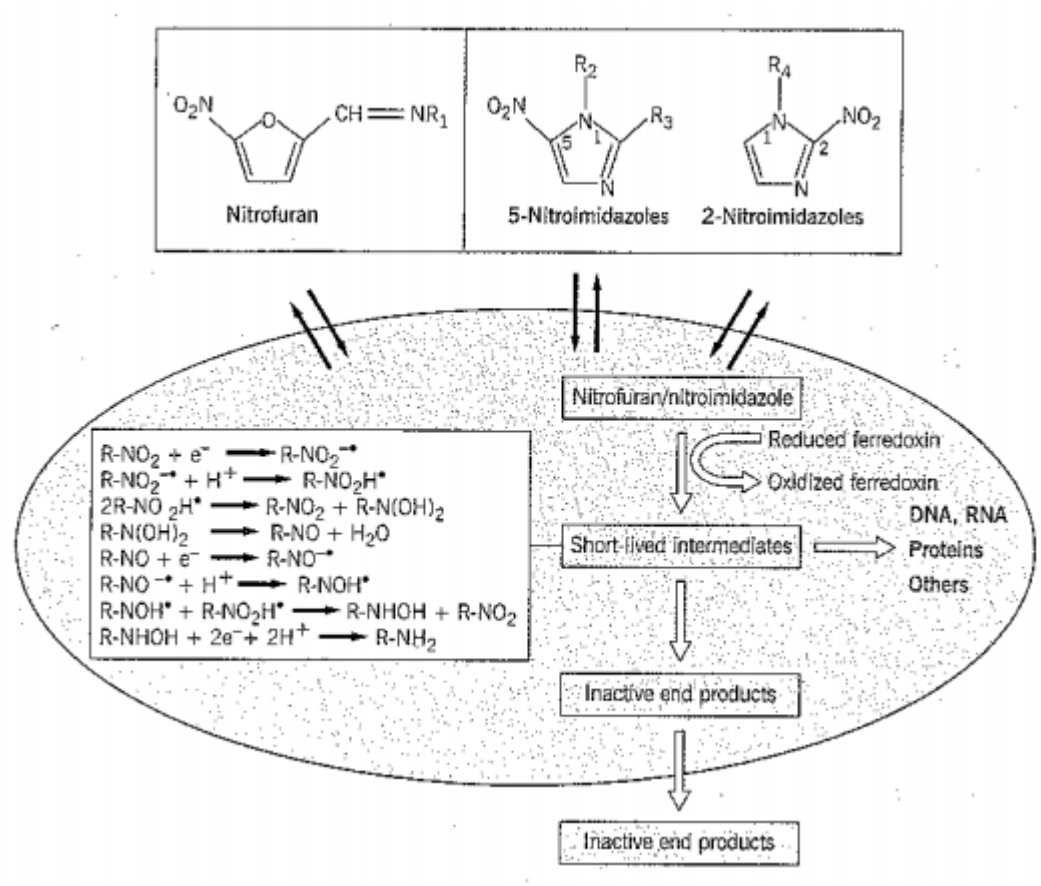


Figure 2.24: Modes of action of nitrofurans and nitroimidazoles. The modes of action include passage through the cell membrane, reduction to highly reactive products, interaction with intracellular targets and release of inactive end products.

2.3 DETERMINANTS OF RESISTANCE IN BACTERIA

Antibiotic resistance can be classified as either natural resistance or acquired resistance (Todar, 2002). The natural resistance refers to an organism which has the inherent ability for resisting an antibiotic. An example for this is the inherent resistance of a Gram-negative bacterium like *E. coli*to Penicillin G because there is no reaction site of penicillin G in its structure. The acquired resistance refers to a qualitative alteration of the genetic material of the organism as the result of microbes changing in some ways to eliminate the effectiveness of drugs through mutation (Rodriguez *etal*., 2005).

I. Mutation

Exposure of bacteria to sub-lethal concentrations of antibiotics results in the selection of resistant strains by the process of natural selection. Under continuous antibiotic pressure, the survivor bacteria, which have initial intrinsic resistance to antibiotics, reproduce, spread, rapidly dominate, or can even displace the antibiotic-susceptible population (Silbergeld *etal*., 2008). Over time, the survivor bacteria undergo mutations which may further enhance their resistance to antibiotics. Spontaneous mutation may lead to the development of antibiotic resistance inbacteria and favour survival under antibiotic pressure (Silbergeld *etal*., 2008; Conter *etal*., 2009). For example, resistance to fluoroquinolones (FQ) in some bacterial species has been reported to occur spontaneously due to mutations, particularly point mutations, in drug target genes. A single point mutation which occurs in the quinolone resistance-determining region (QRDR) of DNA gyrase A (GyrA), substantially develops resistance towards fluoroquinolones in Campylobacter, while in other enteric organisms (such as, *Salmonella* and*E.coli*), stepwise accumulation of pointmutations has been reported to acquire high-level fluoroquinolone resistance (Han *etal*., 2008; Luangtongkum*etal*., 2009).

II. Gene Transfer

The resistance genes may be acquired by horizontal gene transfer (HGT) which requires a donor of the resistance genes (Martinez, 2009). In bacteria, horizontal gene transfer has been reportedly mediated by three mechanisms (Matthew, 2007; Luangtongkum *etal*., 2009) namely:

a. **Transformation**: This is the incorporation of foreign (exogenous) DNA from the surroundings into the genome of a bacterial cell. Transformation may be a main

mechanism for acquiring chromosomally encoded resistance (for example, fluoroquinolone and macrolide resistance in *Campylobacter*) (Matthew, 2007; Luangtongkum *etal*., 2009). It is a critically important method of gene transfer (Prescott, 2000) in vitro but less important in vivo (Schwarz *etal*., 2006).

b. **Transduction**:This is the transfer of resistant genes via a bacterial virus or phage (Schwarz and Chaslus-Dancla, 2001; Matthew, 2007). It is thought to be a relatively unimportant method of resistance transfer because of the specificity of bacteriophages (Prescott, 2000) and the limited amount of space for DNA to be packaged into the phage (Schwarz *etal*., 2006). Occasionally,resistance plasmids can be accidentally packed up into phage heads during phage assembly and subsequently be able to infect new cells by injecting plasmid DNA into a recipient cell (Schwarz *etal*., 2006). Neither transformation nor transduction requires a viable donor cell or a link between donor and recipient (Guardabassi and Courvalin, 2006).

c. **Conjugation**: Conjugation is the transfer of resistance genes from a resistant organism to a sensitive organism through a protein channel (Bennett, 1995; Prescott, 2000; Schwarz and Chaslus-Dancla, 2001). Gene transfer in conjugation allows the spread of mobile genetic elements such as plasmids, transposons, or integron/gene cassettes (Bennett, 1995; Schwarz and ChaslusDancla, 2001). These elements can possess multiple antibiotic resistant genes and may be responsible for the rapid dissemination of genes among different bacteria (Kruse and Sorun, 1994; Salyers and Amiable-Cuevas, 1997; Sandvang *etal*., 1997). For example, the antibiotic resistant pattern of *Salmonella typhimurium* DT104 constitutes an integron coding for resistance to sulfonamides, ampicillin and streptomycin (Conter *et al*., 2009). Linked clusters of antibiotic resistance on a single mobile element can also aggregate in such a way that antibiotics of a different class or even non antimicrobial substances like heavy metals or disinfectants can select for antibiotic resistant bacteria (Recchia and

Hall, 1997, Salyers *et al*., 2004). Exchange of resistance genes between pathogens and non-pathogens or between gram-positive and negative bacteria has also been documented (Prescott, 2000; McDermott *et al*., 2002; Salyers *etal*., 2004).

2.3.1 Mobile Genetic Elements

The acquisition of genetic elements such as plasmids, transposons, or integrons/gene cassettes has been reported as a critical part of horizontal transfer of antibiotic resistance. These elementsvary considerably from each other in regard to their carriage of resistance, their replication andtransmission. It is estimated that mobile genetic elements accounts for more than 95 percent of antibiotic resistance acquired by gene transfer (Silbergeld*etal*., 2008). They transmit genetic resistance determinants for several different antibiotic resistant mechanisms and may result in rapid dissemination of resistance genes among different bacteria (McDermott *etal*., 2002).

I. Plasmids

These are extra-chromosomal circular DNA which can replicate independently, but synchronously with chromosomal DNA (Schwarz *etal*., 2006). When resistance is transferred as a result of plasmids, a copy of the plasmid is always retained by the parent (Cohen, 1993). It has been reported that most plasmids carry the gene required for conjugation, but some plasmids can be mobilized by using the conjugal apparatus for self-transmissible of plasmids within the cell (Marcelo *et al*., 1998).

Plasmids have been reported to code for resistance to one or up to ten different antimicrobials (multiple antibiotic resistance) (Prescott, 2000). Multi-resistant plasmids have been reported as a result of interplasmidic recombination, integration of transposons, or insertion of gene cassettes (Schwarz*etal*., 2006). All resistance

genes on a multi-resistant plasmid are transferred when the plasmid is transferred, whether there is selective pressure for all of the resistance genes on the plasmid or for just one of the resistance genes (Schwarz *etal*., 2006). Plasmids have also been reported to act as vectors for transposons and integrons/gene cassettes (Bennett, 1995).

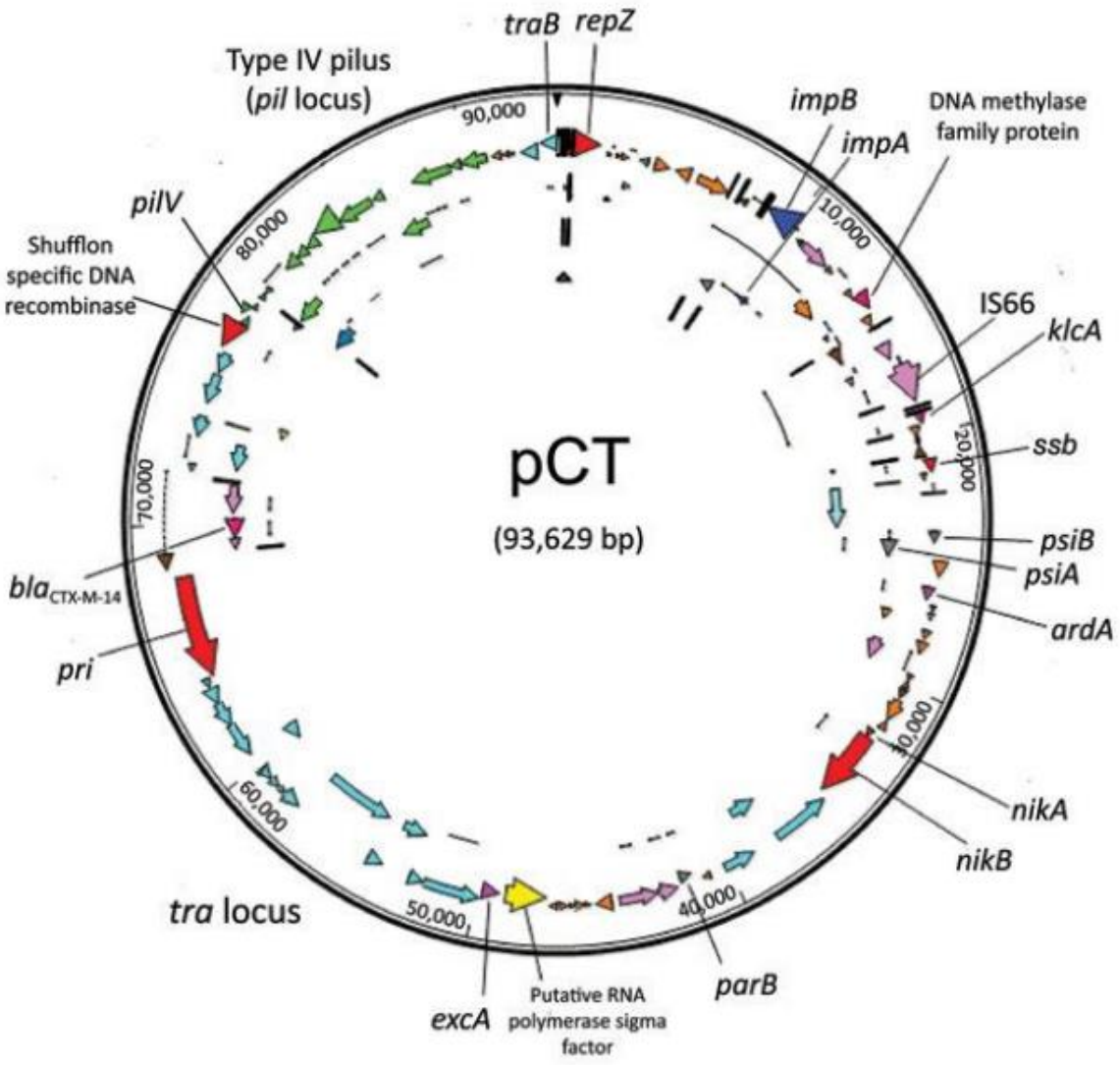


Figure 2.25: Circular map of plasmid pCT. Open reading frames are colour coded as follows: brown, pseudogenes; orange, hypothetic proteins; light pink, insertion sequences; light blue, tra locus; green, pil locus; dark pink, antimicrobial drug resistance gene; yellow, putative sigma factor; red, replication-associated genes. Arrows show the direction of transcription. pCT, IncK plasmid (Adapted from Cottel *et al*., 2011).

II. Transposons (jumping genes)

These are short sequences of DNA that has been reported to move from plasmid to plasmid, or from plasmid to chromosome and vice versa (Kidwell, 2005). Transposons do not possess replication systems and must be incorporated into chromosomal DNA or plasmids (Schwarz *et al*., 2006). Unlike plasmids, no copy of the transposon remains within the original cell as the transposon moves between the donor andrecipient (Slotkin and Martienssen, 2007). All transposons has been reported to move and integrate into foreign DNA by non-homologous recombination, which permits the same transposon to be found in the genome or plasmids of highly unrelated organisms (Kazazian, 2004).

III. Integrons

These have been described as mobile element often found on plasmids and are distinct from transposons (Roy, 1995). They are a site specific recombination system that contains an integrase enzyme, a gene-capture site, and a captured gene or genes. The genes are present as mobile gene cassettes that represent small mobile elements that contain only a single resistance gene and a specific recombination site (Recchia and Hall, 1995, Nandi *etal*., 2004). The recombination site allows mobility when they are recognized by site-specific integrases, which catalyze integration of the cassettes at specific sites within the integron thereby permitting integrons containing multiple resistance gene cassettes (Cambray *et al.*, 2010). Gene expression of an integron is dependent on various factors including promoter strength, gene copy number, the relative distance of the gene cassette from the promoter, and the presence of additional internal promotors (Martinez-Freijo *etal*., 1998, Martinez-Freijo *etal*., 1999). Expression has been reported to be usually mediated via a common promoter

situated upstream (5'-end) of the gene cassettes, rather than through individual promoter copies (Matinez-Freijo *etal*., 1998). Higher levels of gene expression have been reported achieved if a second promoter is included adjacent to the first, or if the gene in question is included as multiple copies (Matinez-Freijo *etal*., 1998).

2.3.3 Drivers of Antibiotic Resistance

There are several reported factors which accelerate bacterial antibiotic resistance. These includes but not limited to: use and misuse of antibiotics, environmental stresses, and socio-economic factors.

I. Use and Misuse of Antibiotics

Antibiotics are commonly used to treat infections in humans and animals. However, their use and misuse have been reported to exert selection pressure and accelerate selection of resistant bacterial populations. The use and misuse of antibiotics in animal production and human medicine are summarized below.

b. Antibiotics in Animal Production: Antibiotics have been reported used in animal production systems to treat and control bacterial infections as well as for growth promotion (McDermott *et al*., 2002; Conter *et al*., 2009). The prolonged use of antibiotics, particularly at low levels, promotes the selection of antibiotics resistance among commensal bacteria in the gastrointestinal tract of food animals. For example, Fluoroquinolone-resistant *Campylobacter* have emerged as a result of Fluoroquinolone use in poultry (Asai *et al*., 2007). The increasing resistance to quinolones observed in humans has been reported to be as a result of the use of the same class of antibiotics in animals (Neely and Holder, 1999). When contaminated food is consumed, the resistance gene from commensal bacteria has been reported

transferred to other bacteria, including foodborne pathogens, in the intestinal tract of humans. Several studies conducted by the Centers for Disease Control and Prevention (CDC) on antibiotic-resistant *Salmonella* showed that increased resistance in *Salmonella* strains was most likely due to the antibiotic use in food animals, and that most infections caused by resistant strains are acquired from the consumption of contaminated food such as milk and meat products (McDermott*etal*., 2002; Gilchrist*etal*., 2007). This increase was due to sequential acquisition of plasmids and transposons coding for drug resistance to a wide range of antibiotics such as ampicillin, chloramphenicol, gentamicin, kanamycin, sulphonamides, tetracycline and trimethoprim (giving rise to R-type ACGKSSuTTm) (Threlfall, 2002). It has been postulated that the higher the prevalence of bacterial resistance in animal production, the greater the extent of transfer of antibiotics resistance from animals to humans (Van den Bogaard and Stobberingh, 2000; Nel, 2002). In view of this, even in the presence of specific pressure amongst humans, development of bacterial resistance among human bacterial isolates has been reported due to transfer of resistance via members of say, enterobacteriaceae (Nel, 2002). This could possibly explain why persons exposed to farm animals and abattoir workers have a considerably higher percentage of antibiotic resistant *E. coli* in their intestinal flora (Van den Bogaard and Stobberingh, 2000; Ishihara *et al*., 2001; Nel, 2002).

c. Antibiotics in Human Medicine: Antibiotics have been reported commonly used in human medicine to treat bacterial infections. They are not meant to be used against viral infections like common cold, most sore throats, and flu (FDA, 2010; CDC, 2011). Both overuse, such as overprescribing of antibiotics for critically ill patients, and underuse, such as taking inadequate dose for inappropriate length of time, have been reported as the main cause of selection of antimicrobial-resistant bacteria populations (WHO, 2011; CDC, 2011). The role played by the spread of resistant

bacteria from farm animals to humans has been reported as a major factor in the development of resistance among human bacteria isolates (Bonten *et al*., 2001, White *et al*., 2002, Threlfall, 2002, Ungemach *et al*., 2006). The inappropriate use of antibiotics in the hospitals and close contact among sick patients creates an environment for the dissemination of antibiotic-resistant bacteria (Neely and Holder, 1999; NIAID, 2009). For example, methicillin-resistant *Staphylococccusaureus* (MRSA) and vancomycin-resistant enterococci (VRE) are mainly associated with hospital environments or those who have had prolonged stays in the hospital (Goodyear, 2002; Hawkey, 2008).

II. Environmental Stresses

Several environmental stresses, which are frequently applied in food preservation processes, have been linked to the increase in bacterial resistance towards antibiotics. For example, a study reported an increase in antibiotics resistance in foodborne pathogens, including *Staphylococcusaureus*, *Escherichiacoli*, and *Salmonellaenterica* serovar *Typhimurium*, under sub-lethal low pH or high sodium chloride stress. Another study showed that high osmolarity and starvation regulates the expression of bacterial lipocalin, a protein which helps bacterial adaptation to environmental stress and is responsible for the dissemination of antibiotic resistance genes. Environmental stress can enhance plasmid transfer and plasmid numbers, thereby increasing resistance (McMahon *et al*., 2007).

III. Socio-economic Factors

Socio-economic factors have been reported as drivers of bacterial resistance among human bacterial isolates both in developed and developing countries (Byarugaba, 2004). In the latter, antibiotics are available over the counter and hence easily accessible, leading to overuse (Okeke and Adebayo, 2003; Nys *etal*., 2004). This has been reported believed to account for resistance rates of 90% among human bacterial isolates to tetracycline in West Africa where misuse of this group of antibioticshas been practiced for many years (Okeke and Adebayo, 2003). In developing countries, under use has also been identified as an important cause of development of resistance (Byarugaba, 2004, Neely and Holder, 1999). This is because in poorer countries, patients are either unable to afford the full course of the medicines to be cured of their illness, can only purchase counterfeit drugs on black market, or receive sub-optimal doses. In view of this, bacterial resistance would therefore most likely be a problem in Africa where antibacterial use are unregulated and antibiotics are sold often of substandard quality (Richet *etal*., 2001; Okeke and Adebayo, 2003; Nys *etal*., 2004). The use of substandard antibiotics has been reported to select resistant pathogens during treatment even if the diagnosis is correct (Okeke and Adebayo, 2003; WHO, 2000).

In developed countries, overuse has been identified as the main concern as far as development of bacterial resistance is concerned. This includes subtler ways like prescribing broad spectrum antibiotics when bacteriological evidence indicates that a narrower spectrum drug would be sufficient, and prescribing antibiotics due to patient pressure, when the odds are that the infection is viral, rather than bacterial (Fidler, 1998; WHO, 2002; Okeke, 2005).

IV. Role of Antibiotic Residues in Foods of Animal Origin

Humans have been reported to acquire bacterial resistance enteric organisms by ingesting antibiotics treated animal products (Nel, 2002). Antibiotics resistance is a complex problem involving myriad interactions between humans, animals, drug and the environment (Byarugaba, 2004; Williams, 2005). However, out of this complexity a simple truth emerges: antibiotics breed bacterial resistance, no matter the access routes.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

The equipment used in this study includes:

- Incubator (HME Global, Model No. DNP-9022A, England)
- Hot air oven (Townson/Mercer Ltd. Croydon, England)
- Refrigerator/Freezer (Model PRN 1313 HCA, BEKO, Germany)
- Autoclave (Yamato, USA)
- Microscope (Wild Herbrug M11, Switzerland)
- Agarose gel unit (HE 33; Hoefer, San Francisco CA, USA)
- Laminar air flow cabinet (PCR-8 re-circulating laminar flow pre station Labcaire product 220/240v)
- Centrifuge (Model 5417R)
- Electronic weighing balance (Model QT 600)
- Vortex machine (Touch plate Super Mixer, CAT No 1291 Lab-line instrument Inc USA).
- UV Transilluminator (302 nm) (Vilber Lourmat, Germany).

3.1.2 Media

Bacteriological media such as Nutrient broth, Nutrient Agar, Eosin Methylene Blue Agar, MacConkey Agar, Methyl Red Voges Proskauer Medium, Simmon's Citrate Agar, Urea broth, and Peptone Water were products of Oxoid Ltd., England, Mueller-

Hinton Agar (MHA) is produced by Hi Media, Vadhani, India. Others such asLuria-Bertani broth was obtained from Difco Ltd., USA.

3.1.3 Reagents

Chemical reagents such as Lugol's iodine, Ethidium Bromide dye, TrisBorate EDTA (TBE), Tris-HCl (10mM) were obtained from Sigma chemical Ltd., England. Crystal Violet, Acetone and Carbon fuschin were from BDH Chemicals Ltd., England, whileAgarose gel was from Schwarz/Mann Biotech.

3.1.4 Antibiotic Discs

Antibiotic discs used in the study are: Ceftazidime - 10µg, Cefuroxime - 30µg, Gentamicin - 10µg,Ciprofloxacin - 5µg, Ofloxacin - 5µg, Amoxicillin/Clavulanic acid – 20-10µg, Nitrofurantoin - 100µg, Ampicillin - 10µg (RapidLab, Basingstoke, UK); Sulphametoxazole/Trimethoprim – 23.75-1.25µg and Imipenem - 10µg (Oxoid, England).

3.1.5 DNA Extraction Kit

Inqaba Biotec West Africa Ltd provided Bacterial DNA MiniPrep^{TM} Kits (Code No. ZR D6005, Made in USA by Zymo Research).

3.2. Methods

3.2.1 Study area

The study areas were Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa, Nasarawa State, Nigeria.

Keffi is approximately 68km from Abuja, the Federal Capital Territory and 128km from Lafia, the Capital of Nasarawa state. Keffi is located between latitude $8^{o}5$ N of

the equator and longitude $7^{o}8$ E and situated on an altitude of 850m above sea level (Akwa *et al*., 2007),and Nasarawa is approximately 35km South-west from Keffi.

3.2.2 Media Preparation and Sterilization

Each bacteriological medium used were prepared from commercially available powder according to the Manufacturer's instructions. Sterilization was by autoclaving at 121°C for 15 minutes.Solutions of Crystal violet, Lugol's iodine, dilute Carbol-fushin, and Kovac's reagent were prepared according to Cheesbough, (2006).

3.2.3 Sample collection

Sterile swab sticks were used for the collection of the samples as described by Opere *et al*. (2013) with some modification. The swab sticks were immersed in 0.85% sterile normal saline solution and each door handle was swabbed immediately with a single sterile swab stick, and replaced into its cover immediately. The samples were then transported to the microbiology laboratory of Nasarawa State University, Keffi for the analysis within six hours of sample collection.

3.2.4 Isolation of *Escherichia* spp

The isolation of *Escherichia coli* was carried out as described by Opere *et al*. (2013) with some modifications; the swab sticks were inoculated aseptically into Bijou bottles containing sterile Nutrient Broth medium and incubated at 37°c for 24 hours. Specimens from the Nutrient Broth medium were then sub-cultured by streaking on Levine Eosin Methylene Blue (EMB) Agar plates aseptically, using sterile wire loop and incubated at 37°c for 24 hours. The plates were observed after 24 hours incubation, greenish metallic sheen indicates the presence of Escherichia spp; further biochemical and immunological tests were performed to confirm the organism.

Identification of *Escherichiacoli*

3.2.5 Gram Staining Examination

The Gram staining technique was carried out as described by Cheesbrough(2006) with some modifications; A small portion of cultural organism was transferred onto a clean grease-free glass slide, and emulsified in a drop of distilled water until a thin homogeneous film is obtained, then the wire loop was re-sterilized and the thin homogeneous film was allowed to air-dry, and heat-fixed by passing through the flame. The slide was then flooded with crystal violet for 1 minute, and then rinsed with distilled water. The stain was again flooded with Lugol's iodine for 1 minute, and rinsed with distilled water and then decolourised, rapidly with acetone alcohol until no more colour appeared to flow from preparation and rinsed appropriately with distilled water. The stain was then counter-stained with neutral red for 1 minute, and rinsed with distilled water and allowed to air dry and viewed microscopically using x100 oil immersion objective. Gram positive organism retains the dark blue colour inferred by the iodine/crystal violet complex, while Gram negative organisms appears red; maintaining the colour of the secondary dye. *Escherichia coli* is a Gram negative cocco-bacilli.

3.2.6 Biochemical tests

The following biochemical tests were carried out on the suspected*Escherichia coli*isolates: Catalase test, Indole, Methyl red, Vorges-Proskauer tests, Nitrate reduction, Urease production, Citrate utilisation, and glucose fermentation tests.

3.2.6.1 Indole Test

The Indole Test for the suspected organism was carried out as described by Cheesbrough (2006). A colony of the organism from culture plate was inoculated unto 5ml tryptone broth and incubated at 37°C for 24 hours. After which a few drops of Kovac's reagent was added to the overnight tryptone broth culture, and shaken. A positive reaction is indicated by the development of red ring colour in the reagent layer above the broth within 10 minutes observed. *Escherichia coli*is positive for this reaction.

3.2.6.2 Methyl Red/Vorges-Proskauer Test

The Methyl Red Test for the suspected organism was carried out as described by Cheesbrough(2006). A pure culture of test organism was inoculated in to MR-VP medium and incubated at 37°C for 72 hrs after which the culture was divided in to two portion. To the first portion three drops of methyl red was added and formation of red colour was indicative of methyl red positive. To the second portion 10 drops of 40% KOH (Potassium hydroxide) was added, followed by four drops of alpha-napthol was added and observed for 30 minutes. Formation of pink/red colour indicates Voges-Proskauer positive and formation of yellow colour indicates Voges-Proskauer negative. *Escherichia coli* is positive for methyl red and negative for Vorges-Proskauer.

3.2.6.3 Citrate Utilisation Test

The Citrate Utilization Test for the suspected organism was carried out as described by Cheesbrough(2006). A pure culture of the organism was inoculated as a single streak on the slant surface of citrate agar and was incubated at 37°C for 24 hours, blue colour on the medium indicated the presence of alkaline products and it is therefore

positive, while green colour is negative.*Escherichia coli* is negative for citrate utilization.

3.2.6.4 Catalase test

Catalase test was performed as described by Cheesbough (2006). A pure colony of the organism was streaked aseptically on Nutrient agar slant and incubated at 37°C for 24hours. Three drops of Hydrogen peroxide (H_2O_2) was added to the slant and observed for bubbling gas. *Eschechia coli* is positive for this test.

3.2.6.5 Nitrate reduction test

This test was carried out as described by Cheesbrough (2006). A pure colony of the isolate was inoculated aseptically into Nitrate broth and incubated at 37°C for 24hours. Five drops of nitrate reagent A (Sulfanilic acid) and five drops of nitrate reagent B (dimethyl-alpha-naphthalamine) was added to the medium. Red colour indicates positive, otherwise, it is negative. A confirmatory test for nitrate reduction was performed by adding Zinc powder to the negative tubes. If the colour changes then the result is confirmed negative. If there is no change then, it is positive because the organism have reduced nitrate completely into ammonia and nitrogen gas. *Escherichia coli* are positive for nitrate reduction.

3.2.6.6 Urease test

This was performed by inoculating the organism into Urea broth and incubated at 37°C for 24hours. Intense pink colour indicates positive, otherwise, it is negative (Cheesbrough, 2006). *Escherichiacoli* are negative for urease production.

3.2.6.7 Sugar fermentation test

This uses Phenol Red Broth to test for the fermentation of different sugars. Phenol Red Broth is a general purpose fermentation media that includes the pH indicator Phenol Red and a series of tubes each with a different sugar. A wire loop was aseptically used to inoculate the test organism into the broth containing the test sugar and inverted Durham tubes. This was incubated at 37°C for 18 hours. A bright yellow colour indicated the production of enough acid products from fermentation of the sugar. Production of gas was determined with a Durham tube, a small inverted vial filled with the broth. Gas production during fermentation of the sugar, was seen trapped at the top of the Durham tube and appeared as a bubble.

3.3 Antibiotics Susceptibility Test

The antibiotics susceptibility test of the isolates was carried out usingKirby-Baeur disk diffusion method with some modifications as described in Clinical Laboratory Standard Institute manual (CLSI, 2012). The antibiotic disks were firmly placed on the sterile Muiller Hinton Agar (MHA) plates seeded with test organisms, standardised to 0.5 MacFarland's standard (equivalent to 10^5Cfu) and incubated at 37^oC for 24 hours. Diameter of zones of inhibition was then measured to the nearest millimetre and reported in accordance with the antimicrobial susceptibility breakpoint of EUCAST (2015).

3.3.1 Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR Index was determined according to the method of Krumperman (1983) and Paul*et al.* (1997). From the result of the antibiotic susceptibility test, MARI was calculated using the following formula:$$\text{MAR Index} = \frac{\text{No. of antibiotics to which isolate is resistant}}{\text{Total no.of antibiotics tested}}$$

3.4 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of six of the antibiotics used was studied using standard agar dilution method following the procedures described in the Clinical Laboratory Standard Institute Manual (CLSI, 2012).

The isolates obtained were standardised to 1 in 5000 dilution using a micro pipette to transfer the organism into a solution of Normal saline.

Double strength Mueller Hinton agar was prepared and 10mls was dispensed into each bottle and sterilised. Each molten agar bottle was diluted with antibiotics divided into 9 different concentrations; four concentrations below and four concentrations above the breakpoint value of European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015) were used by dilution with sterile distilled water. Antibiotics were not added to some agar bottles; this was to serve as control signifying viability of the organisms used. Antibiotic-agar mixtures were poured into sterilised petri dishes and allowed to solidify. The petri dishes were labelled accordingly and the standardised organisms were inoculated, this was allowed to stand for 1hour for proper diffusion of organism after which it was incubated for 24hours. The lowest concentration that inhibited growth was the MIC.

3.5 Detection of β-lactamase producing *Escherichiacoli*

Iodometric and acidometric methods were used in the detection of β-lactamase producing species of *Escherichia coli* from selected multiple antibiotics resistant isolates using standard procedures as described by Samant and PAI (2012).

3.5.1 Iodometric Method

100 µl of penicillin solution was dispensed into a well of microtitre plate. Several colonies of the organism to be tested were emulsified into the solution to get dense suspension. Two drops of starch were added and then the plate was kept at room temperature for 30-60 minutes. One drop of iodine was then added which turn the solution blue. If the blue colour disappeared in 10 minutes, the organism was considered as β lactamase positive. Negative control with penicillin alone was kept without any culture suspension. B-lactamase producing strain of *E. coli* ATCC 25922 was used as positive control (Catlin, 1975).

3.5.2 Acidometric Method

100 µl of penicillin phenol red solution was placed in the well of microtitre plate. Several colonies were suspended in the solution to get dense suspension. The solution turned yellow within 10-15 minutes if β Lactamase enzyme was produced.

Colour changes after 15 minutes is not of significance since it represents decomposition of substrate (Boxer and Everett, 1949; Rubin and Smith, 1973; Thornsberry and Kirvin, 1974).

3.6 Conjugation Experiment

The transfer of resistance traits by the resistant isolates of*Escherichiacoli* to ciprofloxacin sensitive *Proteusmirabilis* was investigated using the methods described by Onaolapo and Klemperer (1986) with some modifications.

The minimum inhibitory concentrations (MIC) of the test antibiotics against the sensitive *Proteusmirabilis*were determined as described under section 3.4 above. The resistant isolates of *E. coli* were each grown in sterile nutrient broth (5ml) each at

37°C for 18 hours. The ciprofloxacin sensitive *Proteusmirabilis* was subcultured into the sterile nutrient broth and incubated at 37°C for 18 hours. The overnight cultures of the potential donor (R^+)*E. coli*resistant isolates and the recipients (R^-) i.e. sensitive *Proteusmirabilis* was grown in a ratio of 10:1 respectively in 5ml volume of sterile nutrient broth and incubated in a static incubator at 37°C for 18 hours. One loopful of recombinants (transconjugants) from the admixtures (*E. coli* and *Proteusmirabilis*) bottles were subcultured in triplicate on MacConkey agar plates incorporated with the antibiotic of MIC strength (0.125 µg/ml of ciprofloxacin in double strength MacConkey agar) against sensitive *Proteusmirabilis* and incubated at 37°C for 24 hours. The plates were examined for the presence or absence of cultural characteristics of *Proteusmirabilis*and lactose fermenting properties. Original culture of *Proteusmirabilis* and *E. coli* separately was diluted in1:100 ratio and a drop was spread on MacConkey agar containing ciprofloxacin 0.125µg/ml for positive control. The colonies of *Proteusmirabilis* observed were aseptically picked and transferred into nutrient agar slant and subcultured, after which the MICs of transconjugants were determined as described by Lennette *et al*. (1990).

3.7 Curing Transconjugants

The curing of transconjugants*Proteusmirabilis* (Rf plasmid) was carried out by treating the *Proteusmirabilis* transconjugants with acridine orange dye as described by Onaolapo and Klemperer (1986), viz: Each of the transconjugants (PR) was grown overnight on sterile nutrient broth and incubated at 37°C for 18 hours in a static incubator. The overnight culture of *Proteusmirabilis* transconjugants were standardized (10^5 cfu/ml). A stock solution of acridine orange in sterile distilled water (10,000µg/ml), was prepared and 1.0ml of the solution was dispensed into test tubes

containing sterile nutrient broth (2ml). The contents of each tube was vortexed and mixed properly, mixtures were labelled and allowed to settle.

Twentymicrolitre (20µl) of standardized *Proteusmirabilis* transconjugants (10^5 cfu/ml) was inoculated into mixed solution of acridine orange and sterile nutrient broth and incubated at 37°C for 18hours. The growth from the overnight culture of *Proteusmirabilis* transconjugants were sub cultured. The colonies obtained from curing transconjugants experiment were assessed for their antibiotic sensitivity using the MIC method. This was an attempt to determine whether the resistant pattern had changed or not.

3.8 Plasmid DNA Analyses of Isolates

Plasmid DNA analyses was carried out at Sheda Sciences and Technology Complex (SHESTCO) Gwagwalada, Abuja.

3.8.1 Preparation and purification of total DNA using Spin-Column Protocol

The transconjugant strains and resistant isolates were subjected to plasmid DNA analyses following the protocol of Bimboim and Doly (1979) and Vogelstein and Gillespie (1979). Each isolate was inoculated into 10ml Luria-Bertani (LB) broth incorporated with appropriate selection antibiotic and incubated for 16hours at 37°C while shaking at 200-250rpm. The bacterial culture was harvested by centrifugation at 8000rpm in a microcentrifuge for two minutes at room temperature. The supernatant was decanted and all remaining medium removed. The pelleted cells were resuspended in 250µl of resuspension solution and transferred to microcentrifuge tube.

Exactly 250 µl of lysis solution was added and mixed thoroughly by inverting the tube 4-6 times until solution was viscous and slightly clear. This was followed by adding 350 µl of neutralization solution and mixed by inverting the tube. Centrifugation was carried out at 10000rpm for five minutes to pellet cell debris and chromosomal DNA. The supernatant was transferred to GeneJET spin column by decanting.

Centrifugation was carried out for one minute and the flow-through wasdiscarded. Wash solution of 500 µl was added to the column and centrifuged for 30 to 60 seconds, flow-through was discarded and column placed back into collection tube. The wash procedure was repeated to avoid residual ethanol in plasmid preps. The GeneJET spin column was transferred into fresh 1.5 ml microcentrifuge tube and 50µl of elution buffer was added to the centre of the column to elute plasmid DNA. This was incubated for 2 minutes at room temperature and centrifuged for 2 minutes. The purified plasmid DNA was stored at -20°C for further studies.

3.8.2 Detection of number and sizes of Plasmid DNA (Agarose Gel Electrophoresis)

One percent (1.0%) agarose gel was used to resolve DNA fragment. This was prepared by combining 1g agarose in ten timesconcentration of tris-borate ethylene diamine tetraacetate (10ml 10XTB-EDTA) buffer and 90ml sterile distilled water in 250ml beaker flask and heating in a microwave for 2 minutes until the agarose is dissolved (Moore *etal*., 2002).

Exactly 0.5 µl of Ethidium bromidewas added to the dissolved agarose solution with swirling to mix. The gel was then poured onto a mini horizontal gel electrophoresis tank and casting combs were inserted. This was allowed to gel for 30 minutes. The casting combs were carefully removed after the gel had solidified completely. One

times concentration (1X) TBE buffer was added to the reservoir until it covered the agarose gel.Precisely 0.5 µl of gel tracking dye (bromophenol blue) was added to 20 µl of each sample with gentle mixing. Twenty microliters volume of the sample was loaded onto the wells of the gel, the mini horizontal electrophoresis gel setup was covered and electrodes connected. Electrophoresis was carried out at 100-200mA for one hour. At the completion of electrophoresis, the gel was removed from the buffer and viewed under UV-transilluminator. The band pattern of DNA fragments was photographed with a Polaroid camera and documented using electrophoresis gel documentation system.

3.9 Statistical Analysis

Statistical computation of data obtained was performed using Microsoft excel^{TM} 2010 and Smith's Statistical Package (SSP) version 2.8 for analysis Chi-square test and analysis of variance (ANOVA) were used to compare the results.

CHAPTER FOUR

RESULTS

4.1 Isolation and Identification of *Escherichia coli*

A total of 200 specimens were obtained from door handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa. The specimen were cultured and 62(31.00%) of the specimens yielded growth of *Escherichia coli*; 36 from Nasarawa State University, Keffi and 26 from Federal Polytechnic, Nasarawa. Table 4.1 below shows distribution of *Escherichia coli* in the two institutions investigated.

Distribution of *Escherichia coli* in different location studied showed that; Faculty of Social Sciences in Nasarawa State University account for least prevalence of the bacteria with 4(20.00%) occurrence. Faculty of Arts 9(45.00%) has the highest prevalence of the bacteria. Faculty of Administration and Faculty of law are both tied with 8(40.00%), While Faculty of Natural and Applied Sciences Accounted for 7(35.00%) of the prevalence and the total prevalence from Federal Polytechnic, Nasarawa is 26.00% (Figure 4.1). Figure 4.2 below shows the distribution of *Escherichia coli* in different locations in Federal Polytechnic, Nasarawa, where School of Business Studies (50.00%) has the highest prevalence while School of Basic and Applied Sciences,School of Environmental Studies,School of Engineering Technologyand School of General Studies has the prevalence rate of 30.00%, 25.00%, 20.00% and 5.00% respectively.

Table 4.1: Distribution of *E. coli* in door handles of Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa

INSTITUTION	No. of Samples	No. of Isolates (%)
NSUK	100	36 (36.00)
FPN	100	26 (26.00)
TOTAL	200	62(31.00)

χ^2 = 0.5467
P = 0.4596
No statistical difference exist between the values from the two studied institutions, as the p-value is greater than 0.05.

Key:
NSUK – Nasarawa State University, Keffi
FPN – Federal Polytechnic, Nasarawa

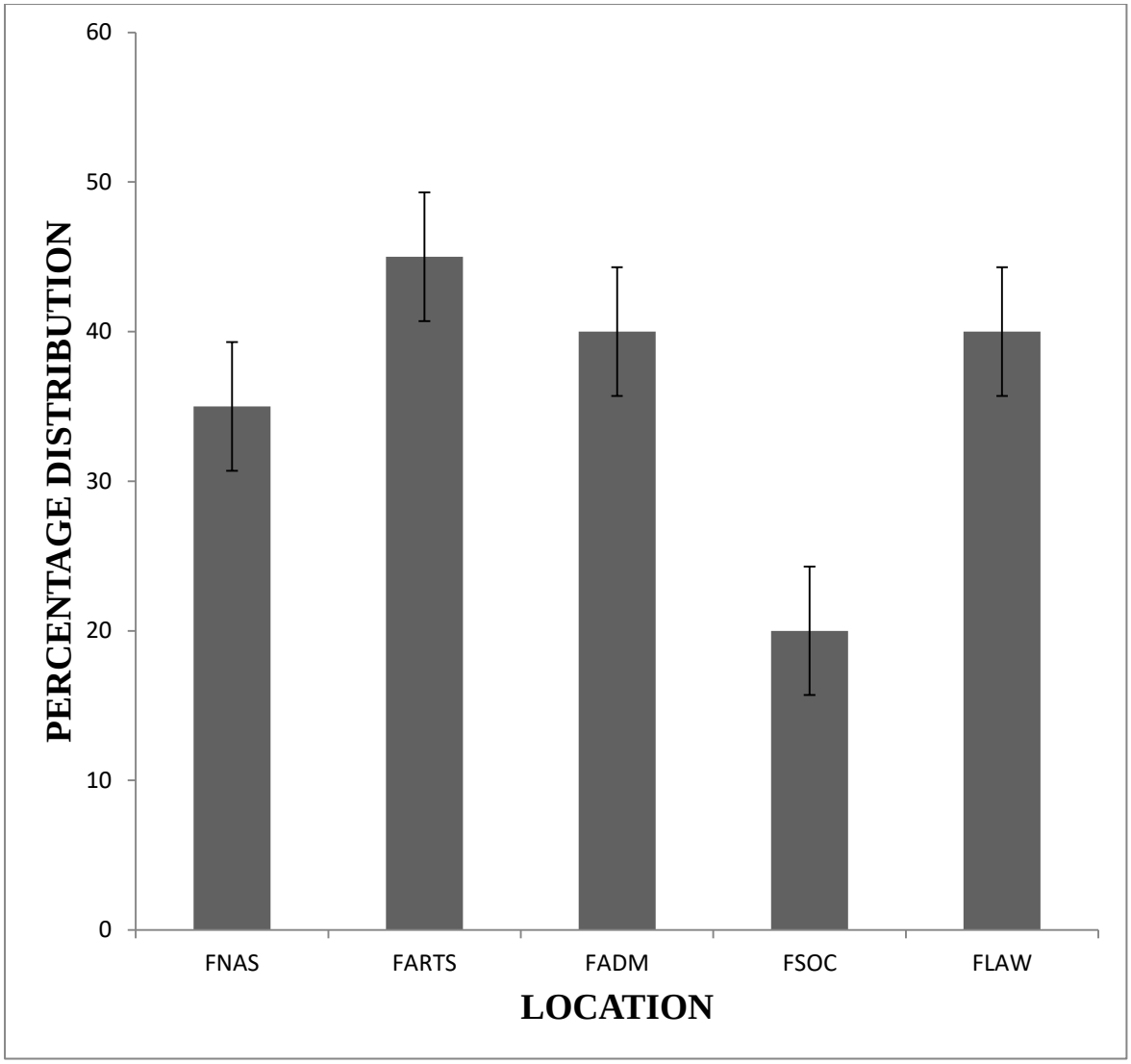


Figure 4.1: Distribution of *E. coli* in door handles from different locations in Nasarawa State University, Keffi

KEY:
FNAS – Faculty of Natural and Applied Sciences
FARTS – Faculty of Arts
FADM – Faculty of Administration
FSOC – Faculty of Social Sciences
FLAW – Faculty of Law

$\chi^2 = 1.2922$

P = 0.8627

No statistical difference exist between the values from the five study locations, as the p-value is all greater than 0.05.

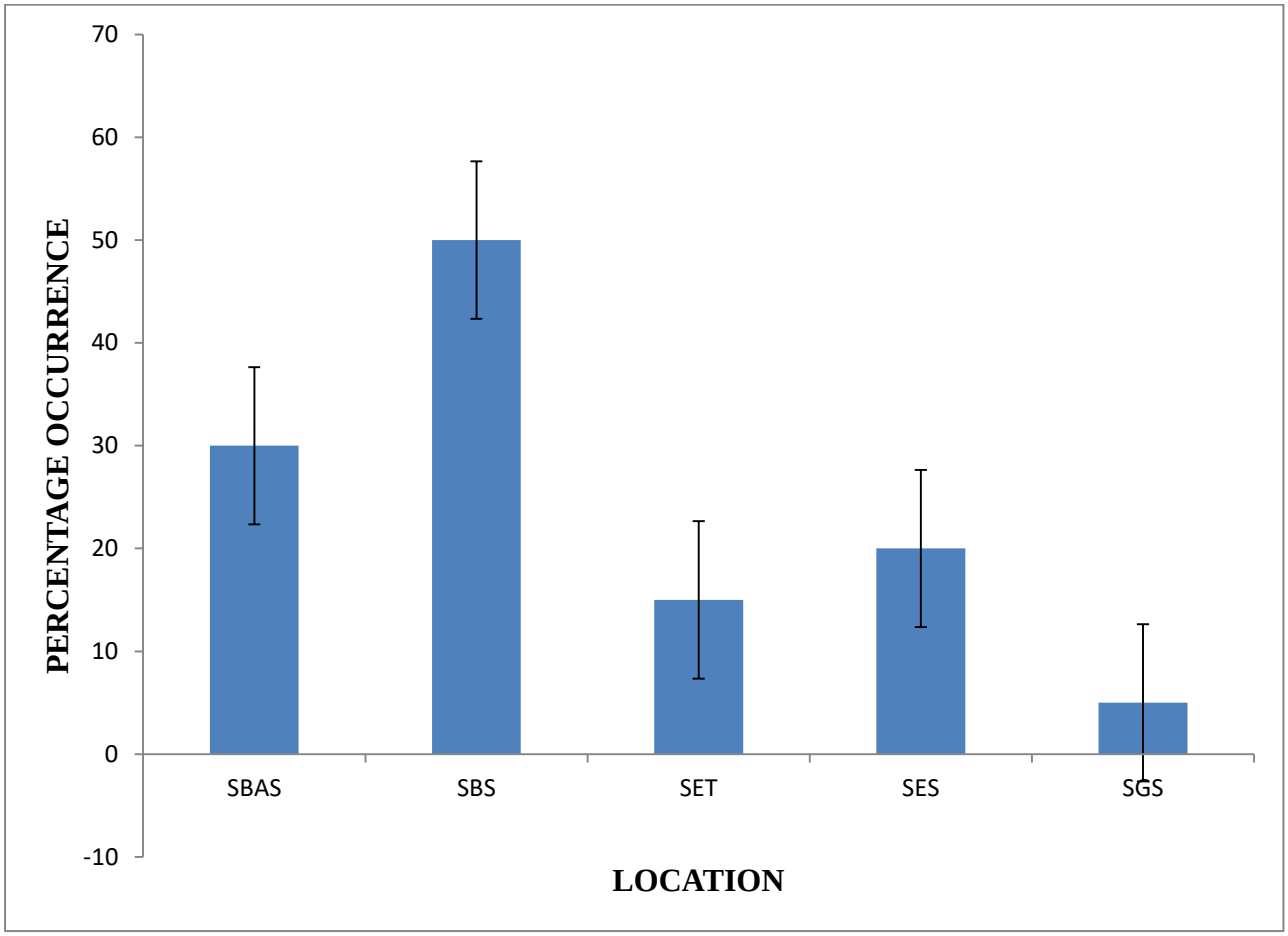


Figure 4.2: Distribution of *E. coli* in door handles from different locations in Federal Polytechnic, Nasarawa

KEY:
SBAS – School of Basic and Applied Sciences
SBS – School of Business Studies
SET – School of Engineering Technology
SES – School of Environmental Studies
SGS – School of General Studies

$\chi^2 = 0.8714$

P = 0.9286

No statistical difference exist between the values from the five study locations, as the p-value is all greater than 0.05.

4.2 Antibiotic Susceptibility Testing

The antibiotic susceptibility testing of *Escherichia coli* isolates showed that 8(12.90%) of the isolates were susceptible to Gentamicin and Imipenem, 6(9.68%) were susceptible to Co-trimoxazole and Nitrofurantoin. Ceftazidime was the least effective in all the antibiotics tested, with 100% resistance (Table 4.2).

Susceptibility test results on the isolates from the two different institutions showed that Nitrofurantoin 5(13.89%) was most effective at Nasarawa State University, Keffi, while Imipenem 6(23.08%) was the most effective antibiotics from Federal Polytechnic, Nasarawa. Ceftazidime, Cefuroxime and Augmentin were found to be 100% inactive against isolates from Nasarawa State University, Keffi; while, Ceftazidime and Ciprofloxacin were also 100% resistant against the isolates from Federal Polytechnic, Nasarawa (Table 4.3).

Table 4.4 below shows the resistance pattern in percentage of 10 selected antibiotics on *Escherichia coli* isolates from the five faculties in Nasarawa State University, Keffi and from Federal Polytechnic, Nasarawa.

The Multiple Antibiotics Resistance (MAR) Index was computed and it showed that most of the isolates had MAR Indices ≥0.2, which implies that they are resistant to two or more of the test antibiotics; whereby 35.48% of the isolates had MAR Index 0.1, meaning, they are susceptible to all but one of the test antibiotics; and 51.61% had MAR index 1.0 (Table 4.5).

Table 4.2: Susceptibility profile of *E. coli* from door handles in the two different institutions studied

ANTIBIOTICS	No. SUSCEPTIBLE (%) TOTAL (n=62)	NSUK (n=36)	FPN (n=26)
Ceftazidime	**0(0.00)**	**0(0.00)**	**0(0.00)**
Cefuroxime	**3(4.84)**	**0(0.00)**	**3(11.54)**
Gentamicin	**8(12.90)**	**3(8.33)**	**5(19.23)**
Ciprofloxacin	**1(1.61)**	**1(2.78)**	**0(0.00)**
Ofloxacin	**5(8.06)**	**2(5.56)**	**3(11.54)**
Augmentin	**1(1.61)**	**0(0.00)**	**1(3.85)**
Nitrofurantoin	**6(9.68)**	**5(13.89)**	**1(3.85)**
Ampicillin	**4(6.45)**	**3(8.33)**	**1(3.85)**
Sulphamethoxazole/Trimethoprim	**6(9.68)**	**3(8.33)**	**3(11.54)**
Imipenem	**8(12.90)**	**2(5.56)**	**6(23.08)**

P = 0.6801

Variation in susceptibility between the two institutions is statistically insignificant since P value is greater than 0.05

Key:
NSUK – Nasarawa State University, Keffi
FPN – Federal Polytechnic, Nasarawa

Table 4.3: Antibiotics Resistance Pattern of *Escherichia coli* from Door Handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa

	% OF RESISTANT ISOLATES				
ANTIBIOTICS	**FNAS n=7**	**FARTS n=9**	**FADM n=8**	**FSOC n=4**	**FLAW n=8**
Ceftazidime	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Cefuroxime	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Gentamicin	**100.00**	**100.00**	**75.00**	**75.00**	**100.00**
Ciprofloxacin	**100.00**	**88.89**	**100.00**	**100.00**	**100.00**
Ofloxacin	**100.00**	**88.89**	**87.50**	**100.00**	**100.00**
Augmentin	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Nitrofurantoin	**51.14**	**88.89**	**100.00**	**100.00**	**87.50**
Ampicillin	**51.14**	**100.00**	**100.00**	**100.00**	**100.00**
Co-trimoxazole	**100.00**	**100.00**	**62.50**	**100.00**	**100.00**
Imipenem	**100.00**	**100.00**	**87.50**	**100.00**	**87.50**

P = 0.4721

Antibiotics resistance across the five faculties is statistically insignificant since P value is greater than 0.05

KEY:
FNAS – Faculty of Natural and Applied Sciences
FARTS – Faculty of Arts
FADM – Faculty of Administration
FSOC – Faculty of Social Sciences
FLAW – Faculty of Law

Table 4.4: Antibiotics Resistance Pattern of *Escherichia coli* from Door Handles in Federal Polytechnic, Nasarawa

	% OF RESISTANT ISOLATES				
ANTIBIOTICS	**SBAS n=6**	**SBS n=10**	**SET n=4**	**SES n=5**	**SGS n=1**
Ceftazidime	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Cefuroxime	**100.00**	**20.00**	**100.00**	**100.00**	**00.00**
Gentamicin	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Ciprofloxacin	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Ofloxacin	**16.67**	**10.00**	**100.00**	**100.00**	**00.00**
Augmentin	**100.00**	**100.00**	**25.00**	**100.00**	**100.00**
Nitrofurantoin	**16.67**	**88.89**	**100.00**	**100.00**	**87.50**
Ampicillin	**16.67**	**100.00**	**100.00**	**100.00**	**100.00**
Co-trimoxazole	**100.00**	**100.00**	**100.00**	**60.00**	**100.00**
Imipenem	**16.67**	**20.00**	**100.00**	**40.00**	**00.00**

P = 0.3783

Antibiotics resistance across the five locations is statistically insignificant since P value is greater than 0.05

KEY:
SBAS – School of Basic and Applied Sciences
SBS – School of Business Studies
SET – School of Engineering Technology
SES – School of Environmental Studies
SGS – School of General Studies

Table 4.5: Multiple Antibiotics Resistance (MAR) Index of *Escherichia coli* from Door Handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa

MAR Index	No. of Isolates (n=62)	Percentage
0.10	22	35.48
0.20	6	9.68
0.30	2	3.23
1.00	32	51.61

4.3 Minimum Inhibitory Concentrations of Selected Antibiotics

Table 4.6 shows antibiotic susceptibility profile of 32 isolatesbased on Minimum Inhibitory Concentrations determined using EUCAST (2015). Peak plasma level of each tested antibiotics was used as breakpoint to determine resistance. Imipenem was observed to be the most effective test antibiotic with only 2(6.25%) resistant test *E. coli* isolates. However, the *E. coli* isolates were highly resistant to Augmentin 29(90.63%) followed by Nitrofurantoin 27(84.38%). This result shows that susceptibility to the antibiotics based on MIC and Peak plasma levels follows this order: Imipenem > Gentamicin > Ciprofloxacin > Co-trimoxazole > Nitrofurantoin > Augmentin.

Results from this study shows that Augmentin was 100% and 73% ineffective against the test bacterial isolates in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa respectively. There was no resistant *E. coli* isolate to imipenem in Nasarawa State University, Keffi while 18% Imipenem resistant *E. coli* isolates was observed from Federal Polytechnic, Nasarawa.

The resistance profile of test *E. coli* isolates from different Faculties in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa based on MIC using Peak Plasma Levels of each antibiotic as resistant breaking point, are shown in Table 4.7 and 4.8 respectively.

Table 4.6: Resistance profile of test *E. coli* isolates from the two institutions studied using antibiotic Peak Plasma Levels as breaking points

ANTIBIOTICS	PEAK PLASMA LEVEL (µg/mL)	No. (%) RESISTANT		
		TOTAL (n=32)	NSUK (n=21)	FPN (n=11)
Gentamicin	10.0	5(15.62)	2(9.52)	3(27.27)
Ciprofloxacin	4.4	10(31.25)	7(33.33)	3(27.27)
Augmentin	5.0	29(90.63)	21(100.00)	8(72.72)
Nitrofurantoin	64.0	27(84.38)	18(85.71)	9(81.81)
Co-trimoxazole	5.0	22(68.75)	14(66.67)	8(72.72)
Imipenem	14.0	2(6.25)	0(0.00)	2(18.18)

Key:
NSUK – Nasarawa State University, Keffi
FPN – Federal Polytechnic, Nasarawa

Table 4.7: Resistance profile of selected isolates from different faculties in Nasarawa State University, Keffi using antibiotic Peak Plasma Levels as break point

ANTIBIOTICS	P.P.L (µg/mL)	% RESISTANT					
		FNAS (n=1)	FARTS (n=7)	FADM (n=3)	FSOC (n=4)	FLAW (n=6)	TOTAL (n=21)
Gentamicin	**10.0**	**0.00**	**14.29**	**33.33**	**0.00**	**16.67**	**14.29**
Ciprofloxacin	**4.4**	**0.00**	**28.57**	**100.00**	**75.00**	**0.00**	**38.10**
Augmentin	**5.0**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
Nitrofurantoin	**64.0**	**100.00**	**71.43**	**100.00**	**75.00**	**100.00**	**85.71**
Co-trimoxazole	**5.0**	**0.00**	**71.43**	**66.67**	**100.00**	**50.00**	**66.67**
Imipenem	**14.0**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

KEY:
FNAS – Faculty of Natural and Applied Sciences
FARTS – Faculty of Arts
FADM – Faculty of Administration
FSOC – Faculty of Social Sciences
FLAW – Faculty of Law

Table 4.8: Resistance profile of test *E. coli* isolates from different locations in Federal Polytechnic, Nasarawa using antibiotic Peak Plasma Levels as breaking point

ANTIBIOTICS	PEAK PLASMA LEVEL (µg/mL)	% RESISTANT					
		SBAS (n=4)	SBS (n=3)	SET (n=1)	SES (n=3)	SGS (n=0)	TOTAL (n=11)
Gentamicin	**10.0**	**0.00**	**33.33**	**0.00**	**66.67**	**0.00**	**27.27**
Ciprofloxacin	**4.4**	**25.00**	**0.00**	**100.00**	**33.33**	**0.00**	**27.27**
Augmentin	**5.0**	**50.00**	**100.00**	**100.00**	**66.67**	**0.00**	**72.73**
Nitrofurantoin	**64.0**	**75.00**	**100.00**	**100.00**	**100.00**	**0.00**	**90.91**
Co-trimoxazole	**5.0**	**75.00**	**66.67**	**0.00**	**100.00**	**0.00**	**72.73**
Imipenem	**14.0**	**50.00**	**0.00**	**0.00**	**0.00**	**0.00**	**18.18**

KEY:
SBAS – School of Basic and Applied Sciences
SBS – School of Business Studies
SET – School of Engineering Technology
SES – School of Environmental Studies
SGS – School of General Studies

4.4 Beta-Lactamase Production

Beta-lactamase enzyme production test was performed on 32 of the selected resistant *Escherichia coli* isolates and 21 of the isolates shows positive for beta lactamase enzymes production. The result of this test is shown in table 4.9 below.

Table 4.9: Beta lactamase enzymes production in selected *E. coli* isolates

ISOLATES	ACIDOMETRIC	IODOMETRIC
FNAS 02	+ +	+ +
FARTS 01	+ +	+ +
FARTS 02	+ +	+ +
FARTS 08	+ +	+ +
FARTS 13	- -	- -
FARTS 17	+ +	+ +
FARTS 18	+ +	+ +
FARTS 19	+ +	+ +
FADM 12	+ +	+ +
FADM 14	- -	- -
FADM 19	+ +	+ +
FSOC 04	+ +	+ +
FSOC 07	+ +	+ +
FSOC 09	+ +	+ +
FSOC 12	- -	- -
FLAW 09	- -	- -
FLAW 10	- -	- -
FLAW 11	+ +	+ +
FLAW 12	- -	- -
FLAW 19	+ +	+ +
FLAW 20	+ +	+ +
FPN 03	- -	- -
FPN 15	- -	- -
FPN 17	- -	- -
FPN 18	+ +	+ +
FPN 26	+ +	+ +
FPN 38	+ +	+ +
FPN 49	- -	- -
FPN 50	+ +	+ +
FPN 57	+ +	+ +
FPN 79	+ +	+ +
FPN 86	- -	- -

4.6Conjugation Studies

The result of the conjugation studies on Table 4.10 showed that out of the twenty two (22) donor isolates, thirteen were observed to transfer resistance trait and they had the culture characteristics of the recipient *Proteusmirabilis*. The M.I.Cs of ciprofloxacin on transconjugants after conjugation was also observed to increase.

4.6 Transconjugants Curing

Changes were observed in the sensitivity pattern of tested transconjugants after curing. Table 4.11 shows that the Minimum Inhibitory Concentrations of ciprofloxacin for each transconjugants decreased when compared to those before curing.

Table 4.10: Minimum Inhibitory Concentration (M.I.C) of Ciprofloxacin before and after Conjugation

ISOLATES	MIC of Recipient test *P. mirabilis* Before Conjugation (µg/mL)	Recipient Characteristics	MIC of Transconjugants (µg/mL)
FARTS 02		+	4.00
FARTS 13		+	2.00
FARTS 17		+	8.00
FARTS 18		-	-
FADM 12		-	-
FADM 19		+	8.00
FSOC 04		-	-
FSOC 07		+	8.00
FSOC 09		+	16.00
FSOC 12		+	8.00
FLAW 09	0.125	-	-
FLAW 10		+	4.00
FLAW 11		+	2.00
FLAW 19		+	8.00
FLAW 20		-	-
FPN 18		+	8.00
FPN 26		+	4.00
FPN 49		-	-
FPN 50		-	-
FPN 57		-	-
FPN 79		+	8.00
FPN 86		-	-

+ Represents isolate showing pink on MacConkey agar plates.

- Represent isolates that did not transfer resistant traits.

Table 4.11: Minimum Inhibitory Concentrations (M.I.C.) of Ciprofloxacin on Transconjugants

DONOR *E. coli* ISOLATES CODE	RECIEPIENT *P. mirabilis* MIC BEFORE CONJUGATION(µg/mL)	MIC OF TRANSCONJUGANT *P. mirabilis* BEFORE CURING (µg/mL)	MIC OF TRANSCONJUGANT *P. mirabilis* AFTER CURING (µg/mL)
FARTS 02		4.00	2.00
FARTS 13		2.00	0.50
FARTS 17		8.00	4.00
FADM 19		8.00	8.00
FSOC 07		8.00	4.00
FSOC 09		16.00	8.00
FSOC 12	0.125	8.00	0.50
FLAW 10		4.00	1.00
FLAW 11		2.00	0.25
FLAW 19		8.00	4.00
FPN 18		8.00	8.00
FPN 26		4.00	0.25
FPN 79		8.00	4.00

4.7 Plasmid Profiling

Agarose gel electrophoresis was used to determine the number and molecular weight of plasmid DNA in some selected isolates that were observed to have transferred resistance to a non-resistant *Proteus mirabilis* in conjugation experiment. This analysis showed that all the resistant isolates harbours varying number of plasmids of various molecular sizes ranging from 1200 to 3000 base pairs (Plate 4.2 and Table 4.15).

The agarose gel electrophoresis resolution of plasmid DNA from multiple antibiotics resistant *Escherichia coli* isolates and the recipient *Proteus mirabilis* showed that Lanes 1, 2, 7 and 8 of test *Escherichia coli* isolates displayed one plasmid each with different molecular weights; however, lanes 3, 4, 5, 6, 9 and 10 indicated that the test *Escherichia coli* isolates has two plasmids each but Lane 12 shows the recipient *Proteus mirabilis* before conjugation carrying no plasmid (Plate 4.1). The plasmid profile of the test multiple antibiotics resistant isolates showed plasmids with different molecular weights (Tables 4.12 and 4.13).

The semi-logarithm plot of the standard marker, DNA ladder versus the distance moved by the marker gene was used to estimate molecular weight of isolated plasmid DNA (Figure 4.3).

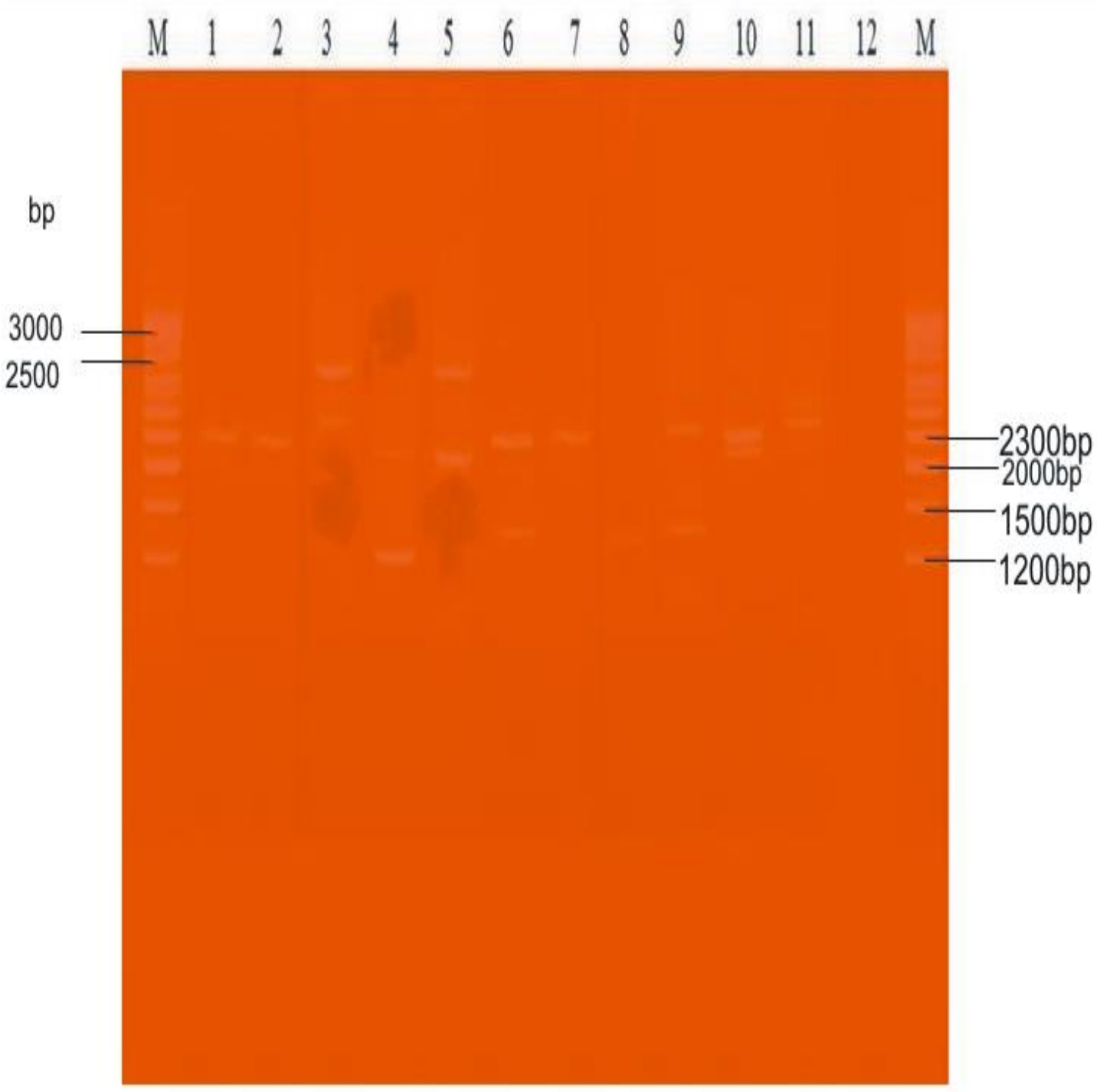


Plate 4.1: 1% Agarose Gel Electrophoresis of Plasmid DNA from Multiple Antibiotic Resistant *Escherichia coli* Isolates and the Recipient *Proteus mirabilis* Before Conjugation.

Lane M: 100bp Plus Ladder (Marker) composed of DNA fragments (in base pairs).

Lane 1 to 11: Resistant *Escherichia coli* (Lab numbers; FARTS02, FARTS17, FADM19, FSOC07, FSOC09, FSOC12, FLAW10, FLAW11, FLAW19, FPN18, FPN26 respectively).

Lane 12: Recipient *Proteus mirabilis* before conjugation.

The plasmid profiling of the examined resistant *E. coli* isolated showed that there was similarity in the plasmid base pair of 2300 from *E. coli* isolates from FARTS02, FARTS17, FSOC09 FLAW10, FLAW19 within the Nasarawa state university.

Similarly in resistant *E. coli* from Nasarawa state university, Keffi and federal polytechnic, Nasarawa has similar base pair plasmid of 2300 for example FARTS02, FARTS17, FSOC12, FLAW19 with FPN18 (Table 4.14). This observation probably indicates the mobility of this plasmid on this area of study.

Table 4.12: Plasmid profile of some tested resistant *Escherichia coli* isolates

ISOLATES	Number of Plasmids	Estimated Molecular sizes in base pairs (bp)
FARTS02	1	2300
FARTS17	1	2300
FADM19	2	2350, 2500
FSOC07	2	1200, 2000
FSOC09	2	2000, 2500
FSOC12	2	1500, 2300
FLAW10	1	2300
FLAW11	1	1300
FLAW19	2	1400, 2300
FPN18	2	2100, 2300
FPN26	2	2350, 2400
FPN79	2	2000, 2500

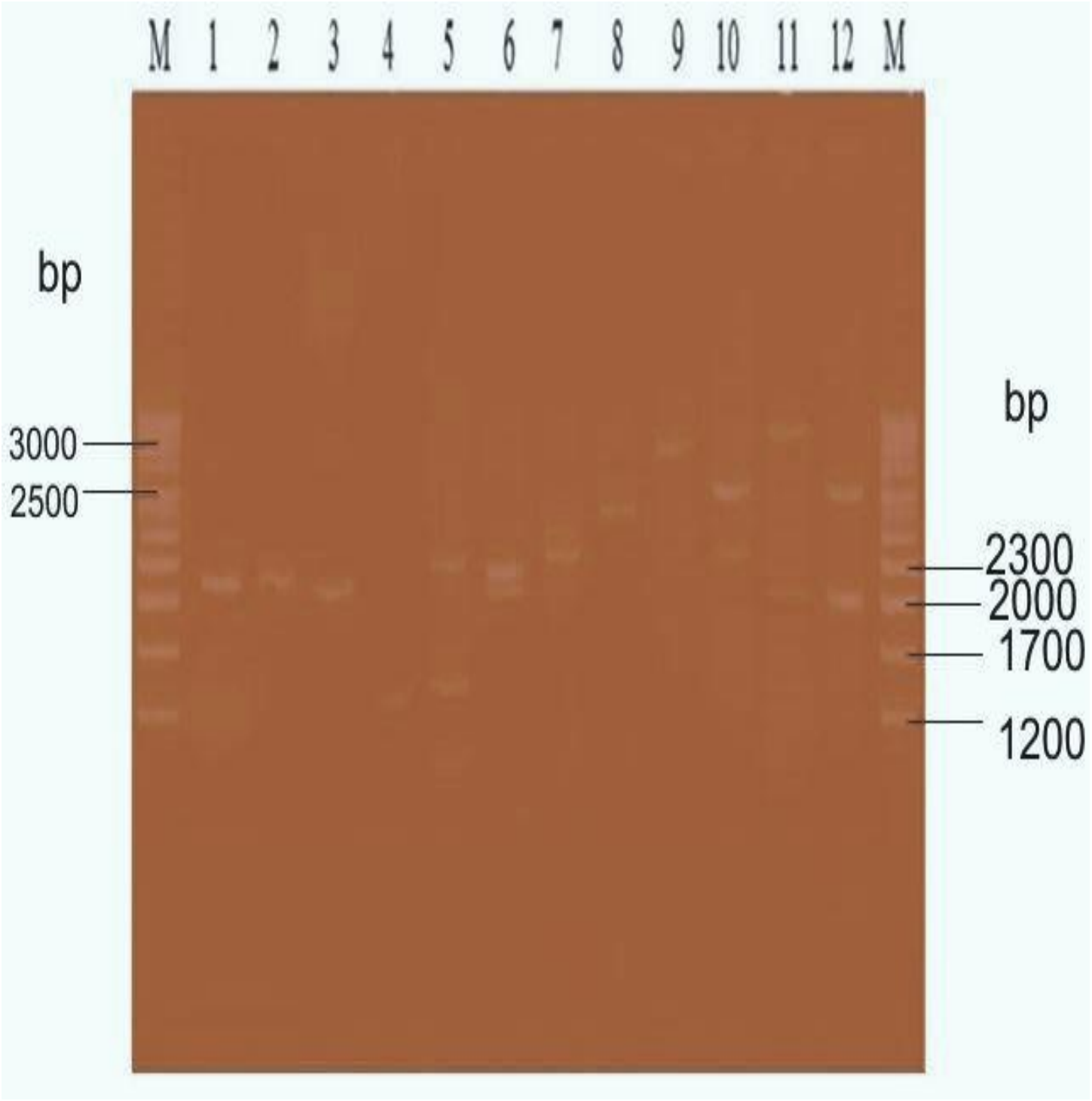


Plate 4.2: 1% Agarose Gel Electrophoresis of Plasmid DNAs from Transconjugant Isolates

Lane M: 100bp Plus Ladder (Marker) composed of DNA fragments (in base pairs).

Lane 1-11: Transconjugant recipients of; FARTS02, FARTS17, FADM19, FSOC07, FSOC09, FSOC12, FLAW10, FLAW11, FLAW19, FPN18, FPN26 respectively.

Lane 12: Resistant *E. coli* isolate FPN79.

Table 4.13: Plasmid profile of some transconjugants bacteria

ISOLATES	Number of Plasmids	Estimated Molecular sizes in base pairs (bp)
FARTS02	1	2100
FARTS17	1	2150
FADM19	1	2000
FSOC07	1	1300
FSOC09	2	1500, 2300
FSOC12	2	2000, 2300
FLAW10	1	2050
FLAW11	1	2350
FLAW19	1	2500
FPN18	2	2350, 2500
FPN26	2	2000, 3000

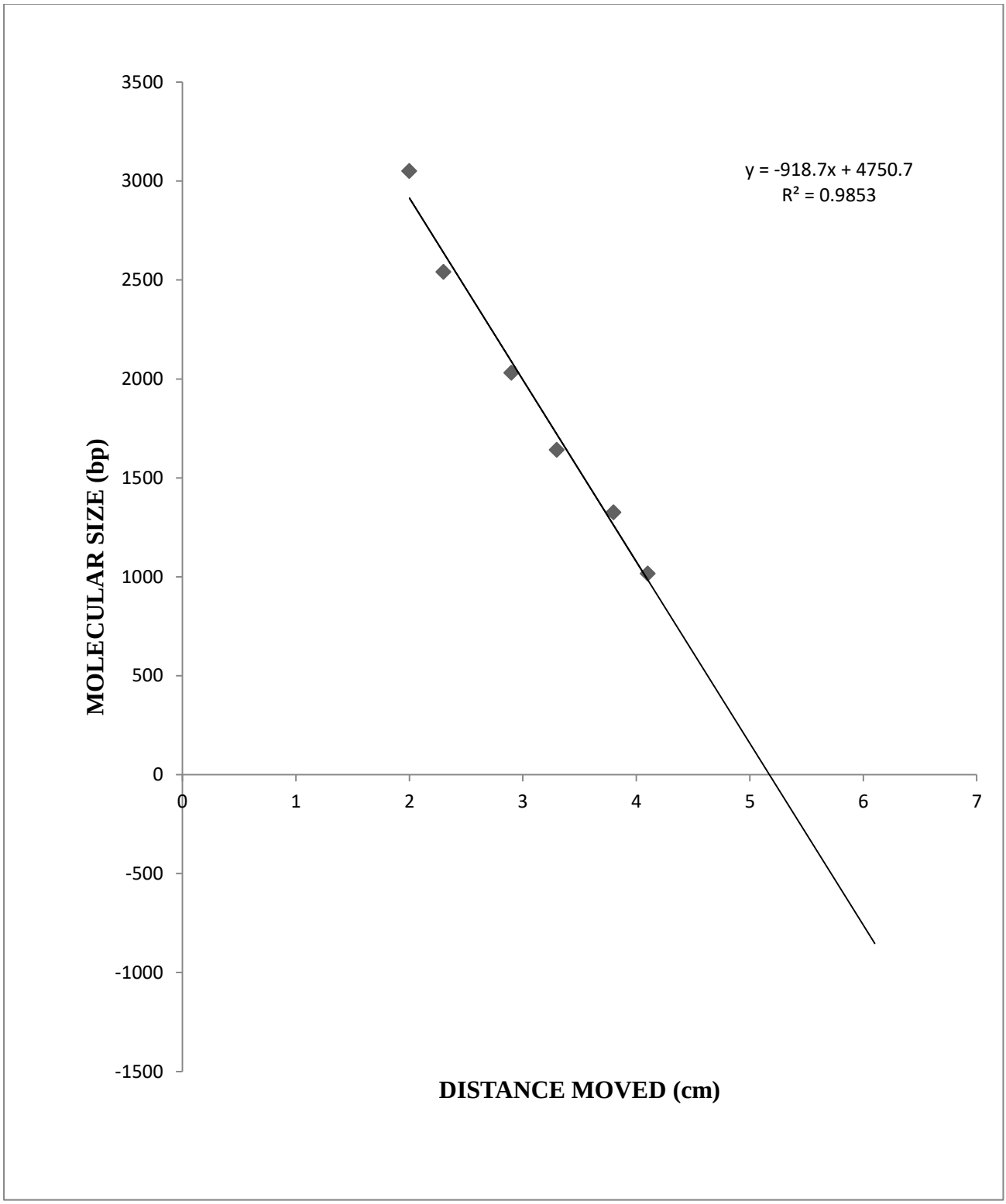


Figure 4.3: Semi-logarithm graph of the molecular weight of the standard DNA ladder versus the distance travelled.

CHAPTER FIVE

DISCUSSION, CONCLUSION, RECOMMENDATION

5.1 DISCUSSION

Microbiological assessment of door handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa showed 31.00% contamination rate of the door handles with *Escherichia coli*. This high level of bacterial contamination of door handles could be a consequence of poor hygiene practices within the institutions.

The number of *Escherichia coli* isolated in this study is in consonance with other studies involving isolation of *Escherichia coli* from door handles, crevices, bannisters, toilet knobs and other handy surfaces both internationally and locally (Samy and Hamdy, 2012; Lynn *et al*., 2013; Oranusi *et al*., 2013; Ajayi and Ekozien, 2014; Augustino *et al*., 2014).

The presence of *Escherichia coli* isolated in door handles studied indicates possible faecal contamination. There is a possibility of contamination with other enterobacteriaceae such as *Enterobacter* spp, *Salmonella* spp, *Proteus* spp, *Klebsilla* spp, *Citrobacter* spp, *Yersinia* spp and *Providencia* spp (Talaro and Talaro, 2006). Organisms from the enterobacteriaceae group have been isolated from door handle and other surfaces (Oranusi *et al.*, 2007; Samy and Hamdy, 2012; Ajayi and Ekozien 2014). *Escherichia coli* have been reportedly linked to diarrhoeal diseases, urethrocystitis, prostatitis and pyelonephritis (Kurt and Wolfgang, 2000; Leflon-Guibouta *et al*., 2002).

Being enteric bacteria, the presence of *Escherichia coli* indicates poor hygiene practices among students and staff of the institutions such as, not washing and cleaning hands with disinfectant after using toilets (Augustino *et al*., 2014). Hand

washing has been traditionally reported first line defense in preventing diseases (Barker and Bloomfield, 2000).

Antibiotics susceptibility assessment of *Escherichia coli* isolates from door handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa, Nigeria in this study shows varying degrees of antimicrobial resistance. Result of antibiotic susceptibility tests on*E. coli* isolatesrevealed that 34(54.39) of the isolates wereresistant to more than three of the test antibiotics which agreed with some reported studies on door handles and other public surfaces in Nigeria (Opere *et al*., 2013; Ajayi and Ekozien, 2014). This observation suggests that the *E. coli* isolates in this study may probably have originated from an environment where antibiotics were often used indiscriminately (Okonko *etal*., 2009). Broad-spectrum antibiotics are sometimes reported to be given in place of narrow-spectrum antibiotics as a substitute for culture and sensitivity testing, with the consequent risk of selection of antibiotic-resistant mutants (Krumperman, 1983; Nahum *etal*., 2006; Norwitz and Greenberg, 2009).

The order of antibacterial ineffectiveness of the studied antibiotics generally was Ceftazidime (100%) > Augmentin (98.39%) = Ciprofloxacin (98.39%) > Cefuroxime (95.16%) > Ampicillin (93.55%) > Ofloxacin (91.94%) > Co-trimoxazole (90.32%) = Nitrofurantoin (90.32%) > Gentamicin (87.10%) = Imipenem (87.10%). Gentamicin 8(12.90%) and Imipenem 8(12.90%) were relatively more effective than all the tested antibiotics, this could be as a result of accessibility to the antibiotics and the parenteral routes of administering them.

The findings from this present study agrees with the work reported by Okonko*etal*. (2009), who reported high bacterial isolates resistance to ampicillin, augmentin and co-trimoxazole (60 to 100%). Other researchers have reported bacterial isolates

obtained from public surfaces to be resistant to co-trimoxazole (Ajayi and Ekozien, 2014; Augustino *et al*., 2014; Lynn *et al*., 2013; Oranusi *et al*., 2013; Samy and Hamdy, 2012). This study highlights a highly diverse antibiotics resistance rates among the *E. coli* isolates.

Antibiotic resistance of *E. coli* isolated from door handles in this study may be a reflection of the prevailing resultant effects of self-medication of antibiotics in the schools investigated and the surrounding environments.

The multiple antibiotic resistance indices (MARI) give an indirect suggestion of the probable sources of an organism. According to previous researchers, Krumperman (1983) and Paul *etal*. (1997), MAR index greater than 0.2 indicates that an organism must have originated from an environment whereantibiotics were frequently used. *E. coli* isolates with resistance to three to eight antibiotics were frequently observed in this study. Out of sixty two *Escherichia coli*isolates studied, 32(51.61%) had MAR index of 1.0, while none was completely susceptible. Such multi-antibiotic resistance has important implications for the empiric therapy of infections caused by *Escherichiacoli* and other enterobacteriaceae (Sherley *etal*., 2004; Oteo *etal*., 2002). It has been well documented that Gram negative bacilli harbour series of antibiotic resistance genes like transposons or integrons and Rplasmids which can be transferred to other bacteria horizontally (Piddock, 2006; Depardieu *etal*., 2007; Leavitt *etal*., 2007; Lockhart *etal*., 2007).

Beta-lactamase production investigation revealed that twenty-one out of the thirty-two resistant *E. coli* isolates from door handles tested for ß-lactamase enzyme in this study produced beta-lactamase enzyme capable of hydrolysing beta lactam antibiotics. Akpan (1992)also reported similar result in Nigeria. This observation confirmed the high beta-lactam antibiotics resistance that was observed against ampicillin,

augmentin, ceftazidime and cefuroxime. Theimplication of these resistances is that many bacterial diseases that could be treated with inexpensive antibiotics, has recently been made more expensiveand less successful by the emergence and spread of resistant organisms (Okeke *etal*., 2007;Okonko *etal*., 2009). However, these multi-antibiotic resistances observed among some of the test *E. coli*isolates from door handles in this study has increased the growing therapeutic failure problem of infectious bacteria that account for most of Africa's disease burden, including respiratory and diarrhoeal diseases (Okeke *etal*., 2007).

Resistance genes are often located on extra-chromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes (Carattoli, 2003; Yah *etal*., 2007). The acquisition of a new gene may occur by genetic transformation or through mobilization by conjugative transfer. The latter may occur at high frequency and efficiency, and several resistance genes can be acquired simultaneously (Carattoli, 2003). Plasmid profiles have been reported to be useful in tracing the epidemiology of antibiotic resistance. The result of the conjugation studies in this work has shown possible acquisition of R-plasmids by sensitive *Proteusmirabilis* from multiple antibiotic resistant test*E. coli* isolates. It was observed that out of twenty two donor *E. coli* isolates, thirteen transferred resistance traits to ciprofloxacin sensitive*Proteusmirabilis* (Table 4.10). The result of antibiotics susceptibility of the transconjugants using M.I.C method was seen to have changed after conjugation. There was an increase in the M.I.Cs of the *P. mirabilis* transconjugants cells. Drastic changes were observed in the MICof ciprofloxacin against the cured tested transconjugants with acridine dye. Decrease in minimum inhibitory concentration of *P. mirabilis* transconjugants after curing as compared to those before curing revealed that acridine dye has significant curing effect on the *P.*

mirabilis transconjugants. However, conjugation analysis revealed that apart from plasmids that were transferable by conjugation, other resistance determinants were transferable to sensitive recipient strain of test *P. mirabilis* hence high MICs observed beyond the recipient's initial MIC with conjugation. This suggests that these resistance determinants were carried extra-chromosomally on R-plasmids.Similar resistance determinants movement have been attributed to the selection pressure created by uncontrolled use of antibiotics in feed-stuff for animals, in addition to the unregulated use of antibiotics by humans (Aarestrup, 1999; Bogaard and Stobberingh, 2000; Teuber, 2001; Van den). Indiscriminate use and sale of antibiotics (for example, sale of antibiotics without prescription, sale of substandard antibiotics) have been reportedto contribute to the development of antibiotic resistance among pathogenic bacteria (Indalo, 1997). In developed countries, the main reservoirs for antibiotic resistance in enteric bacteria has been attributed to farm animals such as cattle, sheep, pigs and poultry (CDC, 1996; Pohl *etal*., 1999). Contact with these animals or consumption of food products from them such as milk has been the main route of dissemination of resistance into the human populations.

Agarose gel electrophoresis analysis showed the presence of plasmid of various sizes among the multiple antibiotics resistant test *E. coli* isolates ranging from 1200 – 3000 base pairs. All the corresponding transconjugants contained similar plasmid sizes.

The plasmid profiles observed in this study indicated that the plasmids are distributed at random in these isolates. In most of the cases, bacterial isolates having similar antibiotic sensitivity patterns had different plasmid patterns. According to some researchers such as Carattoli (2003) and Yah *etal*. (2007), antibiotic resistance in some bacterial isolates which seem not to possess plasmids was associated with chromosome and/or transposons. In determining whether the plasmids resistance

markers could be transferred to sensitive isolates, the results showed that all the transconjugants expressed plasmid DNA that migrated approximately on agarose gels. All the test *Escherichia coli* isolates examined in this study had MAR Index of 1.0, meaning, they are resistant to all the 10 antibiotics tested. This shows that there is a relationship between possession of plasmid and resistance to antibiotics.

Multiple resistance genes clusters in large plasmidsare usually associated with transposons and insertion sequences (Miriagou *etal*., 2006). Plasmid profiles revealed thattest *E. coli* isolates with the same resistance profile may differ in their plasmid profiles. This suggests diversity in plasmid contents of bacterial isolates and the contribution of different plasmids in the resistance to a certain antibiotic. The exchange of plasmids between bacterial cells and the integration of resistance genes into specialized genetic elements play a major role in acquisition and dissemination of antibiotic resistance genes among bacteria isolates (Winokur *etal*., 2000; Carattoli, 2003; Helms *etal*., 2004; Osman *etal*., 2006; Yah *etal*., 2007).

5.2 CONCLUSION

a. Findings from this research showed that up to 32% of door handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa were contaminated with *Escherichia coli*.

b. The *Escherichia coli* isolates were generally resistant to test antibiotics in the order of Ceftazidime (100%) > Augmentin (98%) = Ciprofloxacin (98%) > Cefuroxime (95%) > Ampicillin (94%) > Ofloxacin (92%) > Co-trimoxazole (90%) = Nitrofurantoin (90%) > Gentamicin (87%) = Imipenem (87%).

c. Fourteen out of sixty-two isolates were shown to have transferred resistance factors to non-resistant bacteria (*Proteus mirabilis*) through conjugation experiment.

d. Agarose gel electrophoresis reveals that all the donors and the transconjugants tested harbours at least one or two plasmids of different molecular weights between 1200 to 3000 base pairs.

5.3 RECOMMENDATIONS

Based on the findings of this study, the following are recommended for improved hygiene and improved public wellbeing of the people of Keffi, Nasarawa, and its environs:

- Proper hand washing preferably with antiseptics should be encouraged and be made a common practice among the populace.
- Hand sanitizers or antiseptics should be placed at conspicuous places and its use should be encouraged.
- Since antimicrobial resistant patterns are constantly evolving, and present global public health problems, there is the need for constant antimicrobial susceptibility surveillance. This will help clinicians provide safe and effective empiric therapies.
- There is the need for controlling the spread of multidrug-resistant organisms by providing sufficient personnel and resources for infection control in all healthcare facilities.
- Judicious use of antimicrobials i.e. using the appropriate antibiotics at the appropriate dosage and for the appropriate duration through the appropriate route of administration is an important means of reducing the selective pressure that helps resistant organisms emerge.
- It is difficult to out-smart organisms that have had several billion years to learn how toadapt to hostile environments, such as those containing antimicrobial agents. Yet, with sufficient efforts to use antimicrobial agents wisely both in the human and veterinary fields, in order to prevent the emergence of resistant organisms, and strict attention to infection control guidelines to limit the spread of resistant organisms

when they develop, we should be able to stay at least one step ahead of the next resistant plague.

- New antibacterial agents with different mechanisms of action are needed.

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APPENDIX

APPENDIX I

Isolation and Identification of *Escherichia coli*

COLONY APPEARANCE ON EMB AGAR	GRAM'S STAIN REACTION	BIOCHEMICAL REACTIONS						
		IND	MR	VP	CIT	CAT	NIT	UR
Green metallic sheen	Gram negative rod	+	+	-	-	+	+	-

Key: EMB – Eosin Methylene Blue, IND – Indole, MR – Methyl-Red, VP – Vorges-Proskauer, CIT – Citrate, CAT – Catalase, NIT – Nitrate, UR – Urease.

APPENDIX II

European Committee on Antimicrobial Susceptibility Testing (EUCAST) Breakpoint tables for interpretation of MICs and zone diameters Version 5.0, valid from 2015-01-01 (EUCAST, 2015)

	MIC BREAKPOINT (mg/L)		DISK CONTENT (µg)	ZONE DIAMETRE BREAKPOINT	
	S ≤	R >		S ≥	R <
Ceftazidime	1	4	10	22	19
Cefuroxime	8	8	30	18	18
Gentamicin	2	4	10	17	14
Ciprofloxacin	0.5	1	5	22	19
Ofloxacin	0.5	1	5	22	19
Amoxycillin/Clavulanate	8	8	20-10	19	19
Nitrofurantoin	64	64	100	11	11
Ampicillin	8	8	10	14	14
Trimethoprim/Sulphamethoxazole	2	4	1.25-23.75	16	13
Imipenem	2	8	10	22	16

APPENDIX III

Antibiotics Susceptibility Test

S/N	ISOLATES	CAZ	CRX	GEN	CPR	OFL	AUG	NIT	AMP	SXT	IMP
1.	FNAS 02	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
2.	FNAS 03	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**
3.	FNAS 05	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**R**	**I**
4.	FNAS 06	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**I**
5.	FNAS 12	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
6.	FNAS 16	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
7.	FNAS 17	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
8.	FARTS 01	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
9.	FARTS 02	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
10.	FARTS 06	**R**	**R**	**I**	**R**	**R**	**R**	**S**	**R**	**R**	**R**
11.	FARTS 08	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
12.	FARTS 10	**R**	**R**	**R**	**S**	**S**	**R**	**R**	**I**	**R**	**I**

13.	FARTS 13	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**I**	**R**	**R**
14.	FARTS 17	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
15.	FARTS 18	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
16.	FARTS 19	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
17.	FADM 08	**R**	**R**	**S**	**I**	**R**	**R**	**R**	**R**	**S**	**I**
18.	FADM 09	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**I**	**I**
19.	FADM 10	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
20.	FADM 12	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
21.	FADM 14	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**I**	**I**
22.	FADM 15	**R**	**R**	**I**	**I**	**R**	**R**	**R**	**R**	**S**	**S**
23.	FADM 16	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**S**	**I**
24.	FADM 19	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
25.	FSOC 04	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
26.	FSOC 07	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
27.	FSOC 09	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
28.	FSOC 12	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
29.	FLAW 01	**R**	**R**	**S**	**R**	**R**	**R**	**S**	**R**	**R**	**I**
30.	FLAW 04	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**S**
31.	FLAW 09	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
32.	FLAW 10	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
33.	FLAW 11	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**I**
34.	FLAW 12	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
35.	FLAW 19	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
36.	FLAW 20	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
37.	FPN 01	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**S**	**S**

38.	FPN 02	**R**	**R**	**I**	**I**	**R**	**R**	**R**	**R**	**I**	**S**
39.	FPN 03	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
40.	FPN 04	**R**	**S**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
41.	FPN 05	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**I**	**I**
42.	FPN 06	**R**	**R**	**R**	**I**	**S**	**R**	**R**	**R**	**R**	**I**
43.	FPN 13	**R**	**R**	**S**	**R**	**S**	**R**	**R**	**R**	**S**	**R**
44.	FPN 14	**R**	**R**	**S**	**I**	**R**	**R**	**R**	**R**	**S**	**I**
45.	FPN 15	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
46.	FPN 17	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
47.	FPN 18	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
48.	FPN 26	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
49.	FPN 36	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**S**	**R**	**I**
50.	FPN 37	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**R**	**R**
51.	FPN 38	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
52.	FPN 49	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
53.	FPN 50	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
54.	FPN 55	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
55.	FPN 57	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
56.	FPN 66	**R**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**S**
57.	FPN 71	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**R**	**S**
58.	FPN 79	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
59.	FPN 83	**R**	**R**	**I**	**R**	**S**	**R**	**R**	**R**	**R**	**S**
60.	FPN 86	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
61.	FPN 91	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**I**
62.	FPN 93	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**S**

KEY: **GEN** – Gentamicin; **CPR** – Ciprofloxacin; **AUG** – Augmentin; **NIT** – Nitrofurantoin; **SXT** – Sulphamethoxazole/Trimethoprim; **IMP** – Imipenem; **FNAS** – Faculty of Natural and Applied Sciences; **FARTS** – Faculty of Arts; **FADM** – Faculty of Administration; **FSOC** – Faculty of Social Sciences; **FLAW** – Faculty of Law; **FPN** – Federal Polytechnic, Nasarawa**;** **I –** Intermediate; **R** – Resistant; **S -** Susceptible

APPENDIX IV

Minimum Inhibitory Concentrations (MICs) of Test Antibiotics Against*Escherichia coli* Isolates From Door Handles in Nasarawa State University, Keffi and Federal Polytechnic, Nasarawa

S/No	ISOLATES	GEN (µg/mL)	CPR (µg/mL)	AUG (µg/mL)	NIT (µg/mL)	SXT (µg/mL)	IMP (µg/mL)

1.	FNAS 02	0.50	2.00	64.0	128.0	1.00	1.00
2.	FARTS 01	0.50	1.00	512.0	64.0	16.0	1.00
3.	FARTS 02	2.00	4.00	128.0	1024.0	>32.0	2.00
4.	FARTS 08	0.25	0.13	>512.0	128.0	1.00	0.13
5.	FARTS 13	0.50	0.25	128.0	>1024.0	32.0	0.50
6.	FARTS 17	16.0	8.00	512.0	512.0	32.0	2.00
7.	FARTS 18	8.00	8.00	>512.0	64.0	>32.0	0.25
8.	FARTS 19	4.00	4.00	>512.0	1024.0	4.00	8.00
9.	FADM 12	0.13	8.00	256.0	>1024.0	16.0	2.00
10.	FADM 14	1.00	0.06	16.0	256.0	2.00	1.00
11.	FADM 19	16.0	4.00	256.0	>1024.0	8.00	1.00
12.	FSOC 04	4.00	8.00	64.0	512.0	32.0	2.00
13.	FSOC 07	8.00	8.00	512.0	>1024.0	8.00	4.00
14.	FSOC 09	8.00	8.00	512.0	64.0	>32.0	2.00
15.	FSOC 12	1.00	0.50	128.0	512.0	32.0	2.00
16.	FLAW 09	8.00	8.00	256.0	>1024.0	4.00	2.00
17.	FLAW 10	4.00	0.25	8.00	512.0	8.00	2.00
18.	FLAW 11	32.0	0.50	32.0	256.0	0.25	0.25
19.	FLAW 12	4.00	0.13	32.0	128.0	4.00	0.25
20.	FLAW 19	2.00	4.00	128.0	1024.0	>32.0	1.00
21.	FLAW 20	8.00	2.00	512.0	256.0	32.0	4.00
22.	FPN 03	0.13	0.03	4.00	512.0	8.00	0.13
23.	FPN 15	0.13	0.13	8.00	64.0	32.0	1.00
24.	FPN 17	8.00	2.00	16.0	>1024.0	2.00	1.00
25.	FPN 18	0.50	8.00	>512.0	256.0	2.00	16.0
26.	FPN 26	8.00	4.00	256.0	512.0	2.00	>32.0
27.	FPN 38	16.0	0.13	4.00	128.0	16.0	8.00
28.	FPN 49	16.0	1.00	2.00	>1024.0	>32.0	4.00
29.	FPN 50	>32.0	8.00	8.00	32.0	8.00	1.00
30.	FPN 57	2.00	8.00	32.0	128.0	32.0	0.13
31.	FPN 79	8.00	4.00	64.0	512.0	>32.0	0.13
32.	FPN 86	2.00	0.50	16.0	>1024.0	>32.0	0.13
	Peak Plasma Conc	10.00	4.40	5.00	64.00	5.00	14.00

KEY: **GEN** – Gentamicin; **CPR** – Ciprofloxacin; **AUG** – Augmentin; **NIT** – Nitrofurantoin; **SXT** – Sulphamethoxazole/Trimethoprim; **IMP** – Imipenem; **FNAS** – Faculty of Natural and Applied Sciences; **FARTS** – Faculty of Arts; **FADM** – Faculty of Administration; **FSOC** – Faculty of Social Sciences; **FLAW** – Faculty of Law; **FPN** – Federal Polytechnic, Nasarawa

APPENDIX V: Computations of Statistical Analyses

a) Chi-Square analysis of isolation frequency from the two institutions studied

Chi-Square Tests

The observed values:

	OBSERVED	EXPECTED	total
NSUK	36	42	78
FPN	26	39	65
total	62	81	143

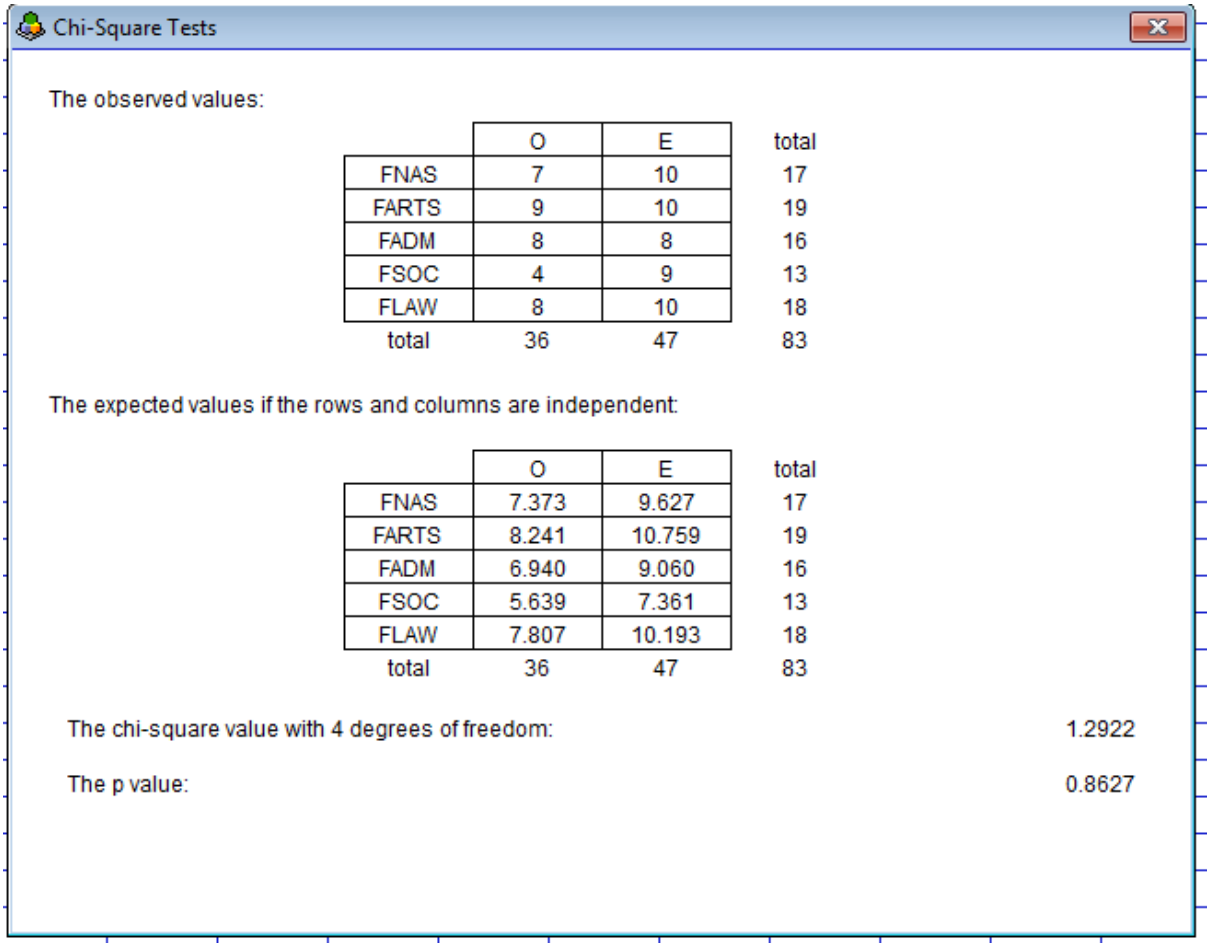
The expected values if the rows and columns are independent:

	OBSERVED	EXPECTED	total
NSUK	33.818	44.182	78
FPN	28.182	36.818	65
total	62	81	143

The chi-square value with 1 degree of freedom: 0.5467

The p value: 0.4596

b) Chi-square analysis of the isolation frequency from the five locations in Nasarawa state university, Keffi.



Chi-Square Tests

The observed values:

	O	E	total
FNAS	7	10	17
FARTS	9	10	19
FADM	8	8	16
FSOC	4	9	13
FLAW	8	10	18
total	36	47	83

The expected values if the rows and columns are independent:

	O	E	total
FNAS	7.373	9.627	17
FARTS	8.241	10.759	19
FADM	6.940	9.060	16
FSOC	5.639	7.361	13
FLAW	7.807	10.193	18
total	36	47	83

The chi-square value with 4 degrees of freedom: 1.2922

The p value: 0.8627

c) Chi-square analysis of the isolation differences in the five locations at Federal Polytechnic, Nasarawa

Chi-Square Tests

The observed values:

	OBSERVED	EXPECTED	total
SBAS	6	7	13
SBS	10	8	18
SET	4	6	10
SES	5	7	12
SGS	1	1	2
total	26	29	55

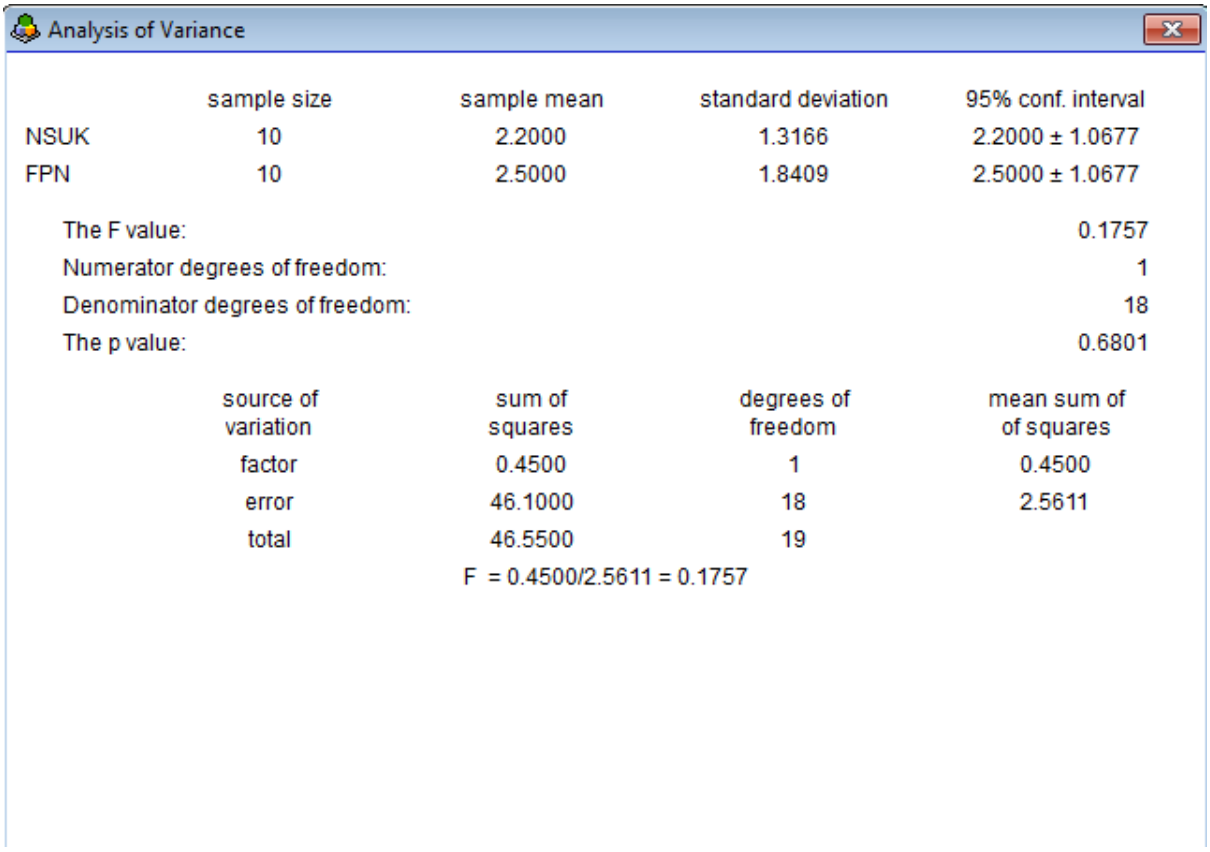
The expected values if the rows and columns are independent:

	OBSERVED	EXPECTED	total
SBAS	6.145	6.855	13
SBS	8.509	9.491	18
SET	4.727	5.273	10
SES	5.673	6.327	12
SGS	0.945	1.055	2
total	26	29	55

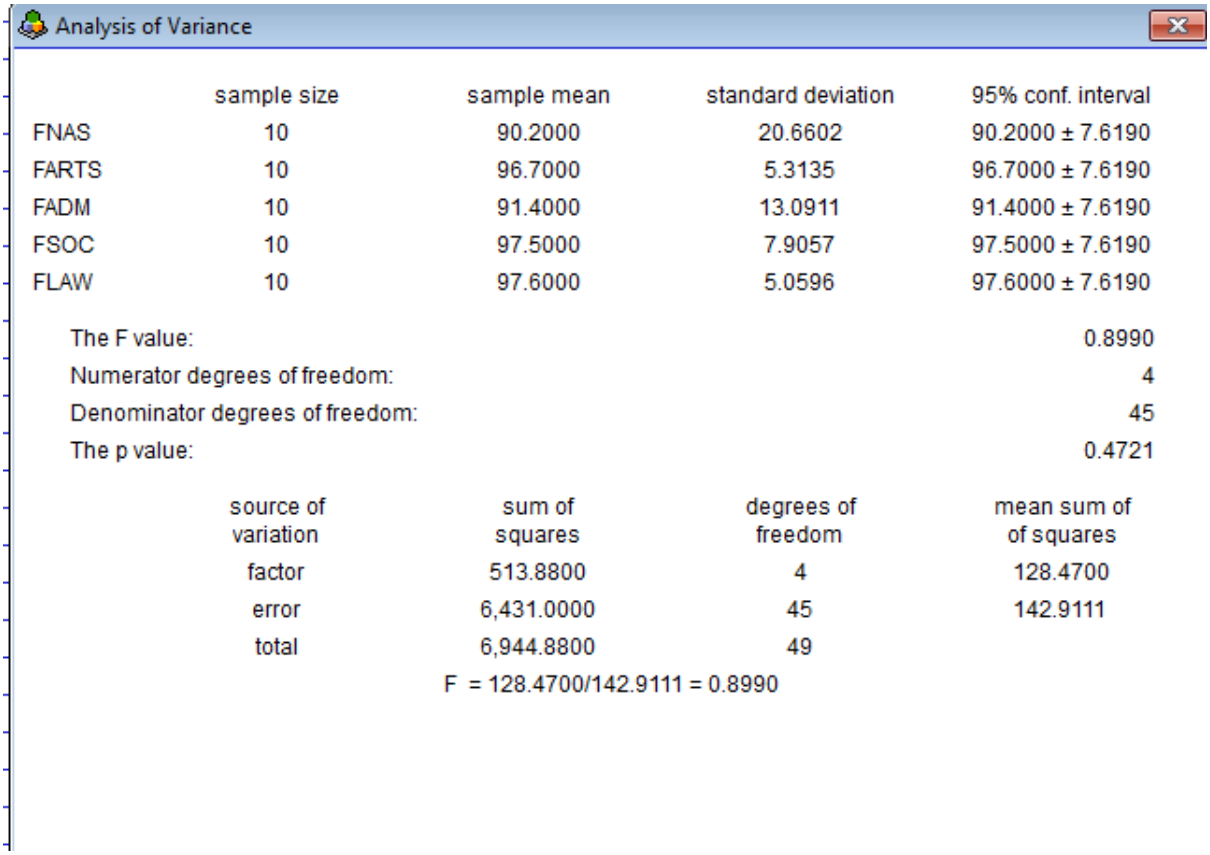
The chi-square value with 4 degrees of freedom: 0.8714

The p value: 0.9286

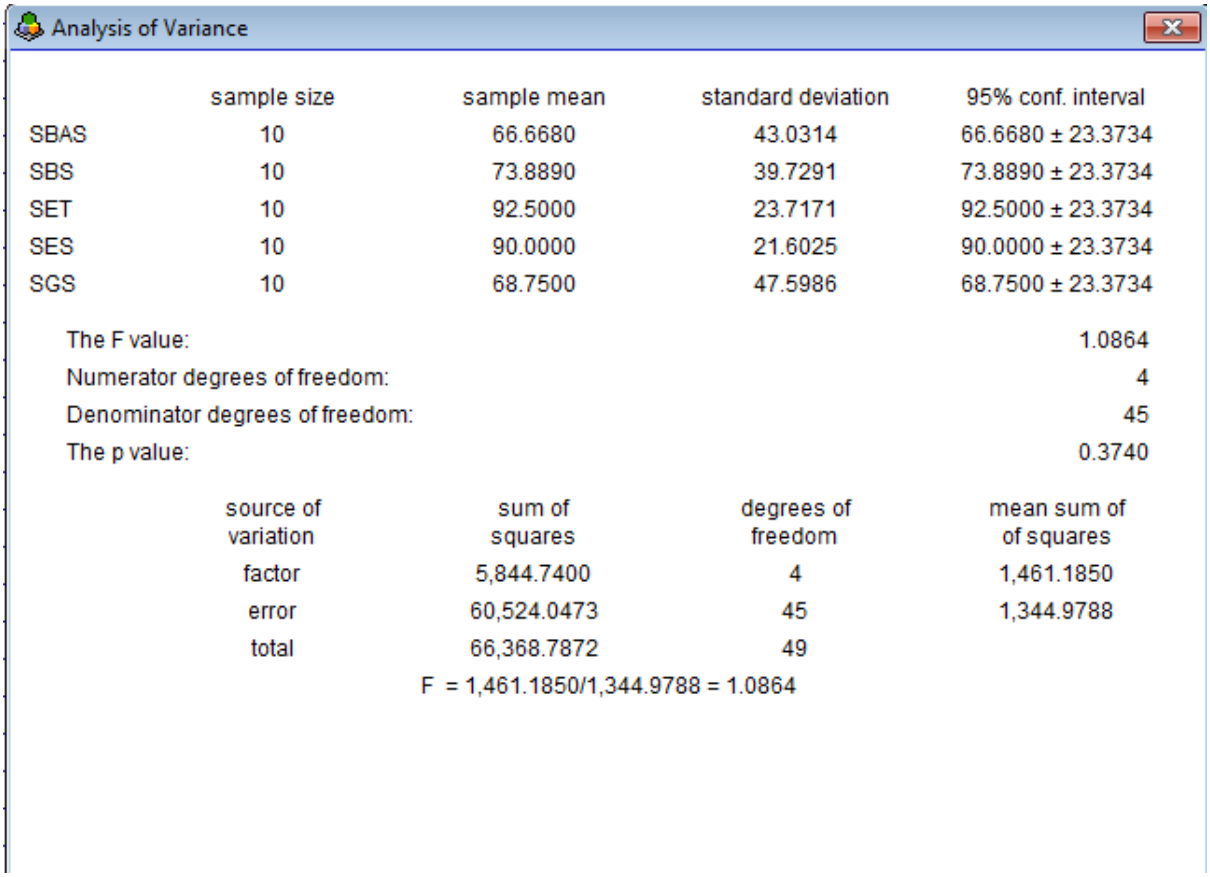
d) One-way ANOVA of the susceptibility of isolates from the two institutions



e) One-way ANOVA of the resistance profile of isolates from five locations in Nasarawa state university, Keffi



f) One-way ANOVA of the resistance profile of isolates from five locations in federal polytechnic, Nasarawa


Analysis of Variance
sample size
sample mean
standard deviation
95% conf. interval
SBAS 10 66.6680 43.0314 66.6680 ± 23.3734
SBS 10 73.8890 39.7291 73.8890 ± 23.3734
SET 10 92.5000 23.7171 92.5000 ± 23.3734
SES 10 90.0000 21.6025 90.0000 ± 23.3734
SGS 10 68.7500 47.5986 68.7500 ± 23.3734
The F value: 1.0864
Numerator degrees of freedom: 4
Denominator degrees of freedom: 45
The p value: 0.3740
source of variation
sum of squares
degrees of freedom
mean sum of of squares
factor 5,844.7400 4 1,461.1850
error 60,524.0473 45 1,344.9788
total 66,368.7872 49
F = 1,461.1850/1,344.9788 = 1.0864

APPENDIX VI

Africa's Genomics Company

Inqaba Biotec West Africa Ltd.

Co. Reg. No: RC1232028
VAT No: 17949735-0001

Quotation

Prepared for:	Quotation number: NG2016/160 Quotation Date: 14 February 2016 Sales Contact: Hazeez Durosomo	Delivery address:
NASARAWA STATE UNIVERSITY Tsaku Paul Microbiology Abuja Road Keffi 961 Nigeria Phone: +2347035425305		NASARAWA STATE UNIVERSITY Tsaku Paul Microbiology Abuja Road Keffi 961 Nigeria Phone: +2347035425305

Code	Description	In Stock	Qty	Price Per Unit	% Disc	Subtotal Excluding VAT
ZR D6005	ZR Fungal/Bacterial DNA MiniPrep™ Kit (50 Preps)	Yes	1	60,469.57 ₦		60,469.57 ₦
Del Non SA	Delivery Non SA	No	1	7,000.00 ₦		7,000.00 ₦

Total Excluding VAT	67,469.57 ₦
VAT	3,373.48 ₦
Total Including VAT	70,843.05 ₦

All quotes are valid for 14 days unless otherwise noted.

Origin of products: IB = inqaba biotec / FE, F, AB, DH, OBS and ND = Thermo Scientific / NEB = New England BioLabs / ZR = Zymo Research / SG= Seegene / BC = Biocom / CAPP = CAPP / CCR = Clare Chemical Research / GV = Genevac / LU = Lucigen / CLC = CLC bio / MIR =Mirus Bio / SC = ScienCell / Bioo = Bioo Scientific / BL =BioLytix /DV = Devyser /AM = Amresco / BPS = BPS Bioscience / ED = Edvotek / EL = Elchrom / RA = Rapigen / VB = Vilber