ANTIBIOTICRESISTANCE IN FAECAL *ESCHERICHIA COLI* AMONG UNDERGRADUATE STUDENTS OF OBAFEMI AWOLOWO UNIVERSITY, ILE-IFE

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ABSTRACT

The study isolated and identified faecal*Escherichia coli* from undergraduate students, determined the resistance pattern of the isolates, ascertained the production of extended spectrum beta lactamases (ESBL) and carbapenemase by the isolates and identified the possible risk factor that predispose to the carriage of drug- resistant organisms.These were with a view to determining burden of antibacterial resistance as a basis for resistance control.

The cross-sectionalstudy was approved by the Ethics and Research Committee of the Institute of Public Health,ObafemiAwolowo University, Ile-Ife,and the study was conducted at the Medical Microbiology Laboratory, Department of Medical Microbiology and Parasitology, ObafemiAwolowoUniversity. Two hundred faecal samples were collected from volunteer fresh students who have not been on antibiotics within the 30 days prior to sample collection and inoculated onto eosin methylene blue agar plates(EMB). Up to five discrete colonies with green metallic sheen, typical of *E. coli* were picked and streaked onto nutrient agar plates and incubated at 37^{o} C for 24 hours. Identification was carried out by microscopy and conventional biochemical tests. The Kirby-Bauer disc diffusion test was used for the antimicrobial susceptibility testing for all the isolates using 11 antibiotics and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines of 2014. All isolates resistant to third generation cephalosporin were tested for ESBL production by using the combined disc diffusion technique, involving the use of clavunate combinations of the third-generation cephalosporinscefotaxime and ceftazidimeaccording to the CLSI guidelines. All isolates resistant to meropenem were screened for both*Klebsiellapnuemoniae*carbapenemases and metallobeta-lactamases using modified Hodge test and combination synergy disk respectively. Molecular detection of ESBL encoding genes (*bla TEM, bla SHV,* and *bla CTX-M*)and carbapenemase encoding genes (*bla*

*NDM, bla KPC*and *bla VIM*)weredone using polymerase chain reaction. Risk factors which include demographic details, self-medication behaviour, hygiene, previous exposure to antibiotics and source of drinking water were analysed for association with carriage of multidrug resistant organisms. Data generated was analysed using descriptive and inferential statistics.

A total of 350 *E. coli* were isolated from 186 subjects, distributed as 100 females and 86 males. Rates of resistance in isolates ranged from 2.8% (n=10) for meropenem to 90.6% (n=317) for tetracycline. The percentage of isolates resistant to at least six drugs is 32.5%. Twenty (33%) of the 60 isolates resistant to third generation cephalosporinswere found to be ESBLs producersphenotypically and only 18 (30%) were positive to genotypic screening of the tested genes (*bla TEM, bla SHV,* and *bla CTX-M*). Phenotypically, five of 10 meropenem-resistant isolates were carbapenemase- producing. Self-treatment (χ^2=*4.*505; *p= 0.*034*)*, knowledge about causes of antibiotic resistance (χ^2=4.791;*p= 0.029)* and previous exposure to antibiotics (χ^2= 3.898;*p= 0.*048) were found to be associated with carriage of multidrug resistance.Self-treatment (χ^2= 4.382; *p= 0.036*) and source of drinking water (χ^2= 9.571; *p= 0.008*) were found to be associated with carriage of ESBL producing isolates.

In conclusion, the rate of antibiotic resistance is high showing the unrelenting effort of microorganism at developing resistance to commonly used antibiotics.

Keywords : Anticbioticresistance, faecal *Escherichia,* spectrum beta lactamases (ESBL) , carbapenemase, the combined disc diffusion technique,
both*Klebsiellapnuemoniae*carbapenemases

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CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Antimicrobial resistance, as defined by the World Health Organization (WHO), includes all forms of resistance by viral, protozoan, fungal, or bacterial pathogens to antimicrobial medicines. It can occur as an innate phenomenon because resistance genes have been found in samples that are millions of years old or in animals which have never been associated with humans (WHO, 2016).Bacteria are said to be multidrug resistant when there is an accumulation of natural and acquired resistance to commonly used antibiotics of different families during active treatment (WHO, 2012; Ventola, 2015). Globally two million patients are infected by bacterial agents' resistant to one or more antibiotics as estimated by Centre for Diseases Control and Prevention in 2009, and 23,000 patients die from antimicrobial resistance every year. The European Centre for Diseases Prevention and Control (ECDC) found mortality rate of 25,000 per year;meanwhile, the British government suggests that more than half a million people die worldwide from resistant infections (ECDC and EMEA, 2009; Davies *et al.*,2013; CDC, 2015).

According to Tikhomirov *et al.,* (2007) over 1.4 million people worldwide suffer from diseases resulting from infections with antibiotic-resistantmicro-organisms. According to O' Neil, (2014) simulation,diseases resulting fromresistant micro-organisms could cause 10 million people worldwide to diein 2050, which is more than deathsfrom cancer in 2016, and that US$ 100 trillion could be the cumulative costs from now to 2050.

Antimicrobial resistance has long been an issue of public health concern such that there are now reports of untreatable clinical infections even in the countries with strong health systems.

Antimicrobial resistance has increased dramatically in the last 20 years, and very small numbers of new antimicrobial products have been discovered, with almost no drug with any new mechanisms of action. Resistance to antibiotics has driven updisease severity which has in turn resulted in prolonged hospitalization, high morbidity, high mortality, and increasing treatment costs, including a need for use of alternative drugs(Cars *et al.*, 2008; Laxminarayan *et al.*, 2013).

Resistance affects both Gram-positive andGram-negative bacteria. Resistance inGram-negative bacteria is a big challenge as approximately no new antibiotics active against them have been made available in the last few years, representing a dramatic public health threat (Jean and Garance, 2015). Bacteria can resist antibiotics using any of the following mechanisms: reduced permeability, rapid expulsion, enzymatic inactivation, and chemical modification of target site (Willey *et al.,* 2008). According to Hatcher *et al*. (2012), inactivation of antimicrobial agent byGram-negative bacteria is usually through the production of enzymes. This can hydrolyse the antibiotics.

The misuse of antibiotics, counterfeit drugs, unhygienic living conditions, inadequate surveillance in developing countries, and low socioeconomic status can lead to the selective spread of resistant organisms (Ogeer-Gyles, 2006; Ventola, 2015). The consumption of antibiotics by human, animal and livestock populations is associated with antibiotic resistance. Consequently, introduction of different classes of antimicrobial agents has been followed closely by emergence of resistance in microorganisms. Antibiotics have been heavily relied upon, though different countries hold opposing views in the way they use antibiotics (Spellberg *et al.,* 2008; Finley *et al*., 2013). For example, in Europe, antibiotic usages by the Scandinavian countries are low relative to other parts of Europe, and consequently those countries have very low levels of resistance. However, countries like Greece, France, and Italy are heavy users with

Italy and Greece recording a high level of carbapenem-resistant *Enterobacteriaceae* (CRE).The Africans are heavy users, and still use antibiotics as growth promoters in livestock (Albrich *et al.,* 2004; Jean and Garance, 2015). Misuse of antibiotics can be by under dosage, over-dosage and self-treatment. In Nigeria, according to study conducted by Yah *et al.* (2008),53% of those who take antibiotics take incomplete regimen of antibiotics, a significant proportion of which were self-prescribed for unspecified ailments.

According to Obeng-Nkrumah *et al.* (2013), beta-lactam class of antibiotics are heavily relied upon because of their high therapeutic index, broad spectrum activities and low toxicity. There is selection pressure on the use of this class of antibiotics, and thus, emergence of organisms producing beta-lactamase so as to survive in the presence of the drug. This is a method used by *Escherichia coli* to inactivate beta-lactam antibiotics. Extended spectrum beta-lactamases (ESBLs) is an example of beta-lactamases, which allow organisms producing it to be resistant to penicillins, first-, second-, and third generation cephalosporins and aztreonam, but susceptible to the cephamycins and carbapenems (Willey *et al.,* 2008).

ESBL-producing *Enterobacteriaceae* are nowadays some of the world's greatest public healththreats becauseESBLs are carried on extra chromosomal DNA (plasmids) thus can be transferred horizontally. The prevalence of ESBLs in some countries can be up to 80% (Willey *et al.,* 2008; Khanfar *et al*., 2009;Rogers *et al*., 2011; Jean and Garance, 2015).The rate at which carbapenems(another group of beta-lactam antibiotics) are being consumed is increasing sharply worldwide, which increases antibiotic selectionpressure on this group. The prevalence of carbapenem resistant Enterobacteriaceae(CRE) is increasing in many countries and this form of antimicrobial resistance poses problems both in the community and in health facilities (Jean and Garance, 2015).

The carriage of multidrug-resistant commensal Enterobacteriaceae particularly *Escherichia coli* in colonized individuals or carriers represent a potential source forthe spread of antibiotic resistance and an indication of the burden of antibiotic misuse (Caprioli *et al.,*2000; Byarugaba, 2004; Onanuga and Berefgha, 2014). The acquisition of resistance by commensal bacteria is a serious concern, because intestinal flora that had been exposed to sub-therapeutic doses of antibiotics can act as potential reservoir of resistance genes which may be transferred to pathogenic bacteria within the host, leading to the general increase of bacterial resistance worldwide (Tauxe *et al.,* 1989). Commensal *E. coli*strains can efficiently exchange genetic materials with pathogens such as *Salmonella, Shigella, Vibrio cholerae* and other pathogenic *E. coli*(Okeke *et al.,* 2000; Witte, 2000; Catry *et al.,* 2003).

1.1. Justification

Surveillance is pivotal in the control of antibiotic- resistant organism and it helps in formulating appropriate intervention strategies. However, there is paucity of systematically collected data in developing countries especially in sub-Saharan Africa and even most of the available data are cross sectional based study and not longitudinal study. A group of researchers have been conducting periodic surveillance of antimicrobial resistance at Obafemi Awolowo University, Ile-Ife for three decades among undergraduate students. The studies havebeen looking at the carriage of antibiotic resistance by commensal faecal *E. coli*.Report from the group showed that the rates of the resistances have increased rapidly, so that the usefulness of drug moderately effective in 1986 has been severely compromised (Okeke *et al.,* 2000, Lamikanra *et al.,* 2011). Given that the last surveillance was in 2009, there is now a seven-year lacuna in the information pool on antibiotic resistance carriage by human intestinal commensals among the undergraduate students' population.

Assessment of intestinal flora particularly *E. coli* acquiring resistance due to exposure to sub-therapeutic doses of antibiotics which can acts as potential reservoir for antibiotic resistance because they can easily transfer genetic materials (to include resistance genes) to pathogenic strains or if they have virulence factor which make them potential pathogen. Hence, the study of the resistance profile of faecal *E. coli* is very important since it is the commonest commensal of the gut and a predominant antimicrobial resistance carrier among the intestinal enterobacteria (Okeke *et al.,* 2000; Witte, 2000; Catry *et al.,* 2003).

It is desirable to investigate current level of resistance and possible emergence of different multidrug resistant types.Apparently healthy people may be asymptomatic carriers of drug resistant-organisms and these microorganisms can easily spread among family members, through unhygienic practices, overcrowdingetc. Such factors as unhygienic practices and overcrowding are common among peer groups and in such closed settings as university campuses and hospitals (Okeke *et al.,* 2000).

1.2. Objectives of the study

The main objective of this study is to determine the present burden of antibiotic resistance among Obafemi Awolowo University undergraduate students.

The specific objectives of the study are to:

1. isolate and characterize *E. coli*using phenotypic methods
2. determine the antibiotic resistance pattern of the isolates
3. screen for ESBL producing *E. coli*phenotypically.

4. evaluate risk factors associated with carriage of antibiotic- resistance*E. coli.*

1.3. Hypothesis

Commensal faecal *Escherichia coli* are not increasingly resistant to common antibiotics

CHAPTER TWO

LITERATURE REVIEW

2.1. ANTIBIOTIC RESISTANCE

Microorganisms have existedon the earth millions of years prior to the evolution of man. A Quite number of them are beneficial, whereas some are pathogenic. Hope to do away with the pathogenic microorganisms of concern to man aroused at the discovery of antibiotics during the 19th century, however, microbial resistance to antibiotics continue to be a serious global health threat (Abdul Ghafur, 2010).

The discovery and development of penicillin as therapy by Alexander Fleming and Howard Florey respectively are generally accepted as the start of the modern antimicrobial revolution. With the advent of antimicrobial agents, the chances of recovering from an infection have increased considerably.Nevertheless, penicillinase had been discovered by Abraham and his colleague (Olsson *et al.,* 1983)prior to the clinical exploit of penicillin.Alexander Fleming had foretold the development of antimicrobial resistance when he was accepting his Nobel Prize for the discovery of penicillin in 1945 (WHO, 2001)stating thus:

"I would like to sound one note of warning…It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily under-dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

Penicillin was widely marketed as a wonder drug in tablets, syrups and throat lozenges starting from 1943 onwards, despite the warning. Resistant strains were soon noted in hospitals and by 1950; half of the *Staphylococcus aureus* isolates developed resistance to penicillin. Resistance to penicillin led to the development of a modified penicillin called methicillin in 1959. Before long, in 1961, methicillin resistant *Staphylococcus aureus*was discovered (Lindberg *et al.,* 1988; Levy, 2002). Ina bid to seek for solution;vancomycinwas introducedinto clinical use and used to treat methicillin resistant *Staphylococcus aureus* (MRSA). In 1997, vancomycin-intermediate resistant *Staphylococcus aureus* (VISA) emerged and complete resistant strain (>16µg/ml) termed vancomycin resistant *Staphylococcusaureus* (VRSA) was isolated in the United States in 2002. This was the first ever documented VRSA. Prevalence of resistance went on in other organisms, includingGram-negative organisms (Levy, 2002; Sancak et al., 2005).

A by-product of chloroquine synthesis called nalidixic acid, as the first quinolone was introduced in 1962,and structural modification brought about different generations of quinolones. The fourth generation fluoroquinolone (gatifloxacin, moxifloxacin) has broad spectrum of activities and is active against Gram positive organisms (Oliphant and Green, 2002; Scoper, 2008). This group of antimicrobial agent is a synthetic drug hence there is a delay in the development of resistance. But twenty years later, resistance to this group of antibiotics by mutation of *GyrA* and *ParC*in the quinolones-resistance determining region (QRDR) was seen(Yolisa, 2011).Astonishingly, in 1998, there was the discovery of plasmid-mediated quinolones resistance (PMQR) because of wide use of fluoroquinolones and this led to wide spread fluoroquinolone resistance, because the plasmid- carrying resistance gene were easily transferred horizontally to other bacteria (Yolisa, 2011).Since then, there has been considerably increase in the resistance of bacteria to different classes of antibiotics. In view of the fact that

bacteria are developing resistance against nearly all antimicrobial agent developed, the confidence in antimicrobial agent has receded and antimicrobial resistance is a global health threat.

Generally, infections caused by *E. coli*can be treated using different antibiotics including carbapenems, penicillins, cephalosporins, aztreonam, trimethoprim-sulfamethoxazole, amoxicillin, nitrofurantoin and the aminoglycosides. Different strains of *E. coli* vary in their susceptibility to these antibiotics. The misuse of these antimicrobials among people, in animals and for crops as well as the innate adaptability of micro-organism havemade these drugs less effective against *E. coli* (CDC, 2013). The incidence of resistance to fluoroquinolones by *E. coli*has increased globally and this has been attributed to the global use of fluoroquinolones particularly ciprofloxacin (Hopkins *et al.,* 2005;Kohanski *et al.*, 2007; Kohanski *et al.*, 2010; Lamikanra *et al.,* 2011). Antibiotic- resistant organisms are the major causes of hospital - acquired infection and other infections which are often fatal.According to the CDC,more than 90% of the bacteria now causing hospitalacquiredinfections are resistant to at least one of the drugsmost commonly used to treat them(Salgado, and Farr, 2003;CDC, 2013).

Antibiotic resistance in *E. coli*is a big problem because of the innate ability of this organism to form biofilms and to horizontally transfer resistance genes to other organisms (Hopkins *et al.,* 2005).

2.2. DEVELOPMENT OF RESISTANCE

Bacteria developed resistance as a means of adaptation or protection against toxic substances available in the environment or introduced into environment by humans, thus, helping them to tolerate and thrive under toxic and unfavourable conditions (Mitema *et al.,* 2004). Consequently,

the use of antimicrobial agents (appropriate or otherwise) has the potential of leading to antibiotic resistance in the bacteria present in the environment where such antimicrobial is being used. Although, proper use of narrow spectrum antibiotics can cause resistance, this will take some time since, the antibiotics are used only when there are some particular infections, but the superfluous use of broad spectrum antibiotic affects negatively the ecology of microorganisms, and thus allow the colonization of resistant bacteria (Livermore, 2005).Antibiotics are among the most commonly prescribed drugs in human medicine and their use continues to rise, partly driven by wrong prescriptions for minor viral infections, such as coughs or colds, where they confer no benefit. In some countries, the availability of antibiotics over the counter,falsified and counterfeit drugs and inadequate dosing as aresult of prescription of wrong dose, wrong duration or the wrong drug, all select for the development of resistance. Inanimals, antimicrobials are used to prevent, control and treatdisease, and in some countries antibiotics are used as growthpromoters (Okeke *et al.,* 2000). This practice has been banned in Europe anddiscouraged in the United States. However, more is needed to be done on appropriate use and stewardship of antimicrobials across both animal and humanhealth sectors.

2.3 ACQUISITION OF RESISTANCE

Resistance is the ability of microorganisms to circumvent or inactivate effective antibiotics. This property of drug resistance can be natural (intrinsic) or acquired. Evolutionary adaptation of bacteria to the environment, the inability of the antimicrobial to penetrate a bacterial cell, the lack of a target for the antimicrobial agent to act on, and the production of inactivating enzymes are all natural properties which result in antibiotic resistance (Heinemann, 2001; Mitema *et al.,* 2004; Alekshun and Levy,2007). Acquired resistanceon the other hand, is through genetic change which can be by specific gene mutation, or which may result from the horizontal transfer

of resistance gene between bacteria of the same or different species, or acquired from the environment by a process known as transformation (McManus, 2000;Apley*et al.,* 2003; Schwarz*et al.*, 2004; Witte, 2004; Perry and Wright, 2013).Spontaneous mutation occurs at a frequency of 10^{-12} to 10^{-7} and thus is an infrequent cause of the emergence of clinical drug resistance in a given patient(Sykes, 2010). Exposure of microorganism to one antimicrobial possibly will select for resistance to other antimicrobials, because of cross- or co-resistance. Cross-resistance refers to single resistance genes or mutations conferring resistance to more than one antimicrobial class (Schwarz *et al.*, 2004; Guardabassi and Kruse, 2008). Co-resistance is the co-existence of several genes conferring resistance to different antimicrobials (Schwarz *et al.*, 2004; Guardabassi and Kruse, 2008; Hatchner *et al.,* 2012).However, if mechanism of resistance is the efflux system then, resistance is demonstrated against different classes of drugs (Schaecter, 2009; Seki *et al.,* 2013).

2.3.1. Genetics Elements Important for Acquisition of Resistance

2.3.1.1.Bacterial Plasmid

The resistance gene can be carried on plasmids (called R- factor), double stranded, extra chromosomal genetic elements, bearing genes coding for adaptability and capable of independent replication with support of replication provided by the host cell(Mayer, 2000; Bennett, 2005). Many resistance plasmids are conjugative plasmids because they are able to promote their own transfer from one strain to another. Conjugative plasmids have a narrow (with transfer restricted to similar bacterial species) or broad host range (Akiba *et al.,* 2005).

Courtney *et al.* (2003) reported that R- factor was responsible for multidrug resistance (MDR) in *Klebsiella pneumoniae* isolates causing nosocomial infections in the USA. Epidemics of

dysentery in Burundi since 1979 were caused by multiple drug- resistant *Shigella dysentery* type 1 (Ries *et al.,* 2000). In 2002, Ezaki and colleague reported that R factor in *E. coli* is responsible for the lactose fermentation and antibiotic resistance (Ezaki *et al.,* 2002).

Mobilizable plasmids are other form of plasmids, which lacks gene that codes for cell to cell pair but have genes that are specifically for transfer of their own DNA. They are mobilized by a conjugative plasmid co-resident in the cell thus; mobilizable plasmid is smaller compared to conjugative plasmid. Therefore, plasmids carry considerably varieties of genes to include those coding for antibiotic resistance, virulence gene,and toxic heavy metal adaptability (Bennett, 2008).

2.3.1.2.Transposon

Resistance can also be transferred by transposon. These are certain sequences of DNA that can move from one point to another point along the length of the chromosome or from the chromosome to a plasmid and vice versa within same cell.The end sequence (insertion sequence) and a protein termed the transposase are the two critical functions necessary for the process. They differ from phage DNA in lacking viral life cycle and from plasmids in being unable to reproduce independently or exist apart from the chromosome (Bennette, 2004). Compositetransposable elementsare transposons that contain resistance genes.These types of transposons play an important role in generating plasmids that carrythis resistance gene. Plasmids can contain several different transposon target sites, which mean that many transposons can insert themselves into plasmid, giving rise to a multiple resistance- encoding plasmid. Tn5 is an example of transposon, encoding resistance to aminoglycosides (Stokes and Gillings, 2011).

2.3.1.3.Integron

Antibiotic resistance genes can be held within mobile elements called integrons. These elements capture and organize the expression of resistance genes. Several classes of integron exist, and they include classes 1, 2, 3, 4, 5, 6, 7, 8 and an unknown class (Clark *et al.,* 2000; Wiktor *et al.,* 2007).Within the integron, more than 60 gene cassettes have been found that confer resistance to a variety of agents (White *et al.,* 2001). Of these, the most prevalent genes are those coding for aminoglycosides and trimethoprim resistance (Fluit and Schmitz, 2000; White *et al.,* 2001).

Integrons consists of three different components: the integrase (which is responsible for site-specific insertion or excision of gene cassette) coded by gene *intI*, the cassette (a small non replicating double stranded DNA, containing only a single gene which may be resistant gene) and the promoter (this facilitate expression of cassette-associated genes) (Hall *et al.,* 1999; Hall, and Collins, 2001).Integron mediated antimicrobial resistance is one of the major mechanisms for transfer of resistancegene within Gram-negative bacteria (Leverstein-van Hall *et al.,* 2002a; O'Brien, 2002; Roe *et al.,* 2003b; Mathai *et al.,* 2004). Transfer of resistance gene from commensal to pathogenic organisms can be through integron (Goldstein *et al.,* 2001; Maguire *et al.,* 2001; Zhao *et al.,* 2001).

2.3.2. Mechanisms of Genetic Transfer

Horizontal transfer of DNA among bacteria can occurs through any of the following processes: conjugation, transduction and transformation.

2.3.2.1. Conjugation

Conjugation is the independent replication and transfer of a conjugative plasmid or chromosomally integrated conjugative element such as conjugative transposon between two bacteria by direct contact of the two cells. In conjugation, bacteria exhibit a kind of sexuality, as a certain strain is found to be the donor of genetic material and the other is the recipient. The donor cell attaches to the recipient cell with the aid of sex pili.The F-plasmid is an episome (aplasmid that can integrate itself into the bacterial chromosome by homologous recombination) with a length of about 100 kb. It carries its own origin of replication, the *oriV*, and an origin of transfer, or *oriT*.There can only be one copy of the F-plasmid in a given bacterium, either free or integrated, and bacteria that possess a copy are called *F-positive* or *F-plus* (denoted F+). Cells that lack F plasmids are called *F-negative* or *F-minus* (F−) and as such can function as recipient cells (Chopra *et al.,* 2001; Burrus and Waldor, 2004).

Among other genetic information, the F-plasmid carries a *tra* and *trb* locus, which together is about 33 kb long and consist of about 40 genes. The *tra* locus includes the *pilin* gene and regulatory genes,which together form pili on the cell surface. The locus also includes thegenes for the proteins that attach themselves to the surface of F−bacteria and initiate conjugation. Several proteins coded for in the *tra* or *trb* locus seem to open achannel between the bacteria and it is thought that the traD enzymelocated at the base of the pilus, initiates membrane fusion.Relaxaseenzyme createsa nick in one of the strands of the conjugative plasmid at the *oriT*to initiate conjugation. Relaxase may work alone or in a complex of over a dozen proteins collectively known as a relaxosome. In the F-plasmid system therelaxase enzyme is called TraI and the relaxosome consists of TraI,TraY, TraM and the integrated host factor IHF. The nicked strand, or*T-strand*, is then unwound from the unbroken strand and transferred tothe recipient cell in a 5'-terminus to 3'-terminus direction. The remaining strand is replicated either independent of

conjugative action (vegetative replication beginning at the *oriV*) or in concert withconjugation (conjugative replication similar to the rolling circle replication of lambda phage). Conjugative replication may require a second nick before successful transfer can occur (Lujan *et al.,* 2007). If the F-plasmid that is transferred has previously been integrated into the donor's genome (producing an Hfr strain [High Frequency of Recombination]). Some of the donor's chromosomal DNA may also be transferred with the plasmid DNA. The amount of chromosomal DNAthat is transferred depends on how long the two conjugating bacteria remain in contact. It takes about 100 minutes for common laboratory strains of *E.coli* to transfer the entire chromosome. The transferred DNA can then be integrated into the recipient genome via homologous recombination (Ryan and Ray, 2004).

A cell culture that contains in its population cells with non-integrated F-plasmids usually also contains a few cells that have accidentally integrated their plasmids. It is these cells that are responsible for the low-frequency chromosomal gene transfers that occur in such cultures. Some strains of bacteria with an integrated F-plasmid can be isolated and grown in pure culture. Because such strains transfer chromosomal genes very efficiently they are called Hfr (Michod *et al.,* 2008).

2.3.2.2.Transduction

Transduction is the transfer of foreign DNA via bacteriophage (phage), which are viruses that attack bacteria for instance phage T4 and phage lambda infect *E. coli.* Phages are obligatory intracellular parasite and must invade a host cell in order to replicate. Phage T4 multiply by lytic cycle,whereas Phage lambda replicates by lysogeny cycle.

2.3.2.2.1. Lytic Cycle: This involves the multiplication of the virus within bacteria that leads to the lysis of the host. The phage attached to the cell wall of the bacterium and it penetrates the cell wall by tail core. The viral DNA is injected and then integrated into the host genome and thus, directs the biosynthesis of viral parts using the host cell's machinery. Maturation of the phage take place as it assembles it part and the phage is being released by the lysis of the bacterium.

2.3.2.2.2. Lysogeny (temperate) cycle: It is a little bit different from lytic cycle in that the phage DNA remains latent in the host until it breaks out in a lytic cycle. The phage attaches to a bacterium and inject its DNA into the bacterium. The phage circularizes and can either enter into lytic or lysogenic cycle. The lytic cycle would occur as described previously but in the lysogenic cycle, the circular phage recombines with *E. coli* DNA (prophage). As the bacterium undergoes cell division and pass prophage to daughter cells, the prophage may exit the chromosome and enter into lytic cycle.

2.3.2.2.3. Generalized transduction: It is the process by which any bacterial gene may be transferred to another bacterium via a bacteriophage, and typically carries only bacterial DNA and no viral DNA. In essence, this is the packaging of bacterial DNA into a viral envelope. This may occur in two main ways; recombination and headful packaging.If bacteriophages undertake the lytic cycle of infection upon entering a bacterium, the virus will take control of the cell's machinery for use in replicating its own viral DNA. If by chance bacterial chromosomal DNA is inserted into the viral capsid which is usually used to encapsulate the viral DNA, the mistake will lead to generalized transduction.If the virus replicates using "headful packaging," it attempts to fill the nucleocapsid with genetic material. If the viral genome results in spare capacity, viral packaging mechanisms may incorporate bacterial genetic material into the new virion.The new

virus capsule, now loaded with part bacterial DNA, continues to infect another bacterial cell. This bacterial material may become recombined into another bacterium upon infection. When the new DNA is inserted into this recipientcell, it can fall to one of three fates:

1. The DNA may be absorbed by the cell and be recycled for spare parts.
2. If the DNA was originally a plasmid, it will re-circularize inside the new cell and become a plasmid again.
3. If the new DNA matches with a homologous region of the recipient cell's chromosome, it will exchange DNA material similar to the actions in bacterial recombination.

2.3.2.2.4. Specialized transduction: This is the process by which a restricted set of bacterial genes is transferred to another bacterium. The genes that get transferred (donor genes) depend on where the phage genome is located on the chromosome. Specialized transduction occurs when the prophage excises imprecisely from the chromosome so that bacterial genes lying adjacent to the prophage are included in the excised DNA. The excised DNA is then packaged into a new virus particle, which then delivers the DNA to a new bacterium, where the donor genes can be inserted into the recipient chromosome or remain in the cytoplasm, depending on the nature of the bacteriophage.When the partially encapsulated phage material infects another cell and becomes a "prophage" (is covalently bonded into the infected cell's chromosome), the partially coded prophage DNA is called a "heterogenote".Example of specialized transduction is λ phages in *Escherichia coli.*

Transduction does not require physical contact between the donating cell and the recipient. It is DNAse resistant.Transduction is especially important because it explains one mechanism by

which antibiotic drugs become ineffective due to the transfer of antibiotic-resistance genes between bacteria. They also have a narrow host range, and as a result transduction is a less important mechanism for resistance gene transfer(Zinder and Lederberg, 1952 Jones *et al.,* 1998; Chopra *et al.,* 2001; Todar, 2008).

2.3.2.3.Transformation

Transformation occurs when bacteria pick up from the environment free DNA made available through cell lysis. Transformation is common in about 1% of bacterial species; it works best with DNA from closely related species (Frost *et al.,* 2005).For transformation to take place, the recipient bacteria must be in a state of competence, which might occur in nature as a time-limited response to environmental conditions such as starvation and cell density, and may also be induced in a laboratory. As at 2014 about 80 species of bacteria were known to be capable of transformation, about evenly divided between Gram-positive andGram-negative bacteria (Johnston *et al.,* 2014)

2.3.2.3.1. Competency: Naturally competent bacteria carry sets of genes that provide the protein machinery to bring DNA across the cell membrane(s). The transport of the exogenous DNA into the cells may require proteins that are involved in the assembly of type IV pili and type II secretion system, as well as DNA translocase complex at the cytoplasmic membrane. Due to the differences in structure of the cell envelope between Gram-positive andGram-negative bacteria, there are some differences in the mechanisms of DNA uptake in these cells, however most of them share common features that involve related proteins. The DNA first binds to the surface of the competent cells on a DNA receptor, and passes through the cytoplasmic membrane via DNA translocase. Only single-stranded DNA may pass through, the other strand being degraded by

nucleases in the process. The translocated single-stranded DNA may then be integrated into the bacterial chromosomes by a RecA-dependent process. InGram-negative cells, due to the presence of an extra membrane, the DNA requires the presence of a channel formed by secretins on the outer membrane. Pilin may be required for competence, but its role is uncertain. The uptake of DNA is generally non-sequence specific, although in some species the presence of specific DNA uptake sequences may facilitate efficient DNA uptake (Sisco and Smith 1979; Long *et al.,* 2003; Chen and Dubnau, 2004).

Bacterial adaptation for DNA transfer that depends on the expression of numerous bacterial genes whose products appear to be responsible for this process is termed natural transformation.

Bacterial transformation has a few challenges. Firstly, the compatibility between the free DNA and the intact recipient is narrow (McManus, 2000). Additionally, free DNA in the environment would be highly susceptible to digestion by nuclease. As a result, transformation is not thought to contribute significantly to the dissemination of antibiotic resistance (Chopra *et al.,* 2001).

2.4. FACTORS THAT ENHANCE GLOBAL DISSEMINATION OF ANTIMICROBIAL RESISTANCE

In general, the acquisition of foreign DNA or mutations provides little advantage for a bacterial cell unless there is express need for the phenotype encoded by the new genes. Therefore, bacteria harbouring resistance genes are given selective advantage over those that do not only in the presence of the antibiotic in question. If a population of bacteria comprises predominantly of susceptible strains, these are killed off on exposure to an antibiotic. The resistant ones are left to flourish, replacing the old population (Alonge *et al.,* 2002; Guardabassi and Kruse, 2008). This simple fact has been responsible for the success of resistant bacteria in a world where antibiotics

are used for a broad range of purposes such as plants growth promoters and the prohibitive cost of newer antimicrobials, when available, places them out of the reach of majority of patients most especially in most part of African countries.

Studies in Nigeria (Okeke *et al.,* 2000; Aminov and Mackie, 2007; Guardabassi and Kruse, 2008;Ajayi *et al.,* 2011; Lamikanra *et al.,* 2011; Ogundipe *et al.,* 2013; Onanuga and Berefgha, 2014) show that commensal organisms from different environment carry many types of resistance genes conferring resistance to older first-, second-, and third- generation antimicrobials. In these studies, the rate of antibiotic resistance to first-, second- and third-generation antimicrobials are on the increase. Despites these studies, subsequent studies are needed to tackle the menace of antibiotic resistance because the extent of the burden of resistant commensals locally and even on the continent of Africa remains insufficient. In addition to providing selection pressure of antibiotic use of antibiotics, humans have encouraged spread of resistance among bacteria by creating conditions suitable for bacterial multiplication and the exchange of genetic material (Alonge *et al.,* 2002; Larson, 2008; WHO, 2010). Warm, moist and unclean environments are conducive for the spread of pathogens; they also encourage the spread of resistant organisms that may not be pathogenic (Wasfy *et al.,* 2002). These organisms often carry resistance genes that can be spread to pathogens and therefore constitute a hidden reservoir of antibiotic resistance. For example, surveys of healthy studentsin Nigeria have shown that they often carry large proportions of resistant *Escherichia coli* in their gastro-intestinal (Okeke *et al.,* 2000; Lamikanra *et al.,* 2011; Ogundipe *et al.,* 2013; Onanuga and Berefgha, 2014).Resistant bacteria or their genes are not limited by ecological, phylogenetic or geographical borders and thus, the epidemiology of resistance must be seen from a holistic and global point of view (Guardabassi and Kruse, 2008).

Bacteria that have acquiredresistance may spread between hosts by skin to skin contact, via excreta or saliva containing the resistant bacteria, clothing materials or by exposure to contaminated food,feed, air, or water (Schwarz *et al.*, 2001). Human or animal excreta that containresistant bacteria may contaminate the environment directly, or via theapplication of sludge or manure or slurry on lands (Marshall *et al.*, 2009; Wellington*et al.*, 2013). Spread to humans and animals then occurs throughcontact with soil, irrigation of crops, water, or wildlife (Wellington *et al.*, 2013).

The movement of animals, food, and humans is a factor in theglobal dissemination of antimicrobial resistance (Laxminarayan *et al.*, 2013).When resistant bacteria have reached the new host, they can colonise,infect, or reside only transiently (Schwarz *et al.*, 2001). In the new host, theresistant bacteria can spread their resistance genes to other bacteria, and alsoacquire other resistance genes from them (Schwarz *et al.*, 2001).Also, antimicrobial treatment reduces the competition from the residing microbiota in the individual undergoing treatment, thus exposing the individual to the risk of being colonized with a resistant strain from the environment (Samore and Lipsitch, 2002).Furthermore, the co-expression of extra -chromosomal marker of resistance to different classes of antibiotics at once is another factor that has increased antibiotic resistance. The co-expression can be through co-resistance or cross-resistance.

2.5. MECHANISMS OF ANTIMICROBIAL RESISTANCE

Bacteria resist antibiotics using several mechanisms. These can be broadly divided into three: (1) change or alteration in drug target site with decreased affinity for antibiotic (2) enzymatic inactivation of the antimicrobial agents (3) reduced access of antibiotics to the target organism either by permeability or by efflux pump (Sundsfjord *et al.,* 2004).

2.5.1. Alteration in Drug Target Site

Most antibiotics are designed to act on specific target site and bacteria can develop resistance to these antibiotics by altering the target site of such antibiotic. For instance,erythromycin-resistant organisms have an altered receptor on the 50S subunit of the ribosome, resulting from methylation of a 23S ribosomal RNA. Furthermore, resistance to some penicillins and cephalosporins may be a function of the loss or alteration of penicillin binding proteins (PBPs).

Target site of the antibiotics can be a particular enzyme, of which the bacteria alter.The altered enzyme can still perform its metabolic function but is much less affected by the drug such as the case seenin trimethoprim-resistant bacteria; the dihydrofolic acid reductase is inhibited far less efficiently than in trimethoprim-susceptible bacteria. Some microorganisms develop an altered metabolic pathway that bypasses the reaction inhibited by the drug, for example, sulfonamide-resistant bacteria do not require extracellular p- aminobenzoic acid (PABA) but, like mammalian cells, can utilize preformed folic acid (Lowy, 1998; Lowy, 2003; Deresinski, 2005; Sievert *et al.,* 2008).

2.5.2. Enzymatic Inactivation of the Antimicrobial Agents

Some microorganisms are to able produce enzymes that are capable of destroying the drug.Classical example of such enzymes is extended spectrum beta-lactamases produced byGram-negativebacteriarods, which hydrolyse beta-lactam class of antibiotics. Gram-negative bacteria resistant to aminoglycosides (by virtue of a plasmid) produce adenylating, phosphorylating, or acetylating enzymes that destroy the drug. Production of these enzymes can be mediated by chromosome or plasmid (Pratt and Taylor, 2003; Rice *et al.,* 2003).

2.5.3. Reduced Influx- Increased Efflux System of Resistance

Some bacteria have natural property that prevents the entry of some small molecules such as antibiotics. This is more common inGram-negative bacteria, because of their outer membrane that function as selective membrane, thus constituting the first line of defense. Some bacteria also produce an efflux pump system which helps the microorganism to pump out the antibiotics from the microorganism thus preventing intracellular accumulation. Resistance to tetracyclines can occur by efflux pump system located in the bacterial cell cytoplasmic membrane, which are responsible for pumping the drug out of the cell(Nikaido, 2001; Pratt and Taylor, 2003).

Burns *et al.* (2003) suggested that continuous exposureto low concentration of chloramphenicol by micro-organism has led to decrease sensitivity to chloramphenicol by the organism. Decreased membrane permeability involves alteration of the outer membrane porins.Structural modification of the major porinof *Enterobacter aerogenes* resulted in antibiotic resistance as established by Emmanuelle *et al.,* (2001).The rate at which membrane is permeable may be decreased and this may also enhance other mechanisms of antibiotic resistance (Nikaido, 2001; Pratt and Taylor, 2003).

Table 2.1: Modes of action and resistance mechanisms of commonly used antibiotics

MECHANISM	ANTIBIOTIC GROUP	EXAMPLES
Enzymatic inactivation	β –Lactams	β-Lactamases: penicillinases; cephalosporinases; carbapenemases
	Aminoglycosides	Aminoglycoside-modifying enzymes of Gram-negative and Gram-positive bacteria

Altered receptors such as Ribosomal alterations, DNA gyrase alteration, Altered bacterial enzymes, Altered cell wall	β – lactams, Tetracyclines, erythromycin, aminoglycosides, Quinolones, Trimethoprim, Sulfamethoxazole, Vancomycin	Altered penicillin-binding proteins of Gram-negative and Gram-positive bacteria
Altered antibiotic transport such as Alteration in outer membrane protein (porins). Reduced proton motive force.	Tetracyclines; erythromycins; aminoglycosides	Decreased influx of Aminoglycosides.

(Davies and Davies, 2010)

2.6 COMMENSALS AS RESERVOIRS OF RESISTANCE GENES

The occurrence of resistant genes in microbes inhabiting an environment free from antibiotics proposes that resistance occur naturally, even before, production and distribution of such drugs(Allen *et al.,* 2010). Interestingly, diverse soil bacteria carry resistance to virtually all antibiotics, some at relevant clinical concentration(D' Costa*et al.,* 2006). These soil bacteria are unrelated phylogenetically, and even some of them are astonishingly related to human pathogens genetically. With the resistant potential of soil bacteria, they could be contributing to the

mounting echelon of multidrug resistance seen among pathogens that infect human. For instance, *Escherichia coli* and *Enterococcus* spp., which colonize humans and many other mammalian species, also are widely distributed throughout soil and water environments. Ubiquitous and resistant to a host of antibiotics, these species deserve serious attention (D' Costa*et al.,* 2006; Bonnie *et al.,* 2009; Allen *et al.,* 2010).

E. coli often carry plasmids that code for multidrug resistance and under stress readily transfer these plasmids to other species. Indeed,*E. coli* is a frequent member of biofilms where many species of bacteria exist in close proximity to one another. This mixing of species allows *E. coli* that are piliated to accept from other as well as transfer to other bacteria various genes with different characteristics that include resistance factors, which confer resistance of organism to one or more class (es) of antibiotics (Anderson *et al.,* 2003; Spellberg *et al.,* 2008; Wellington *et al.*, 2013).

2.7. BETA-LACTAMS AND BETA-LACTAMASES

Over the last 60 years, the beta-lactam class of antibiotics were widely used in both community and hospital settings, and represent about 60% of all of the antibiotics used (by weight) in human and animal medicine (Anderson *et al.,* 2003; Marshall *et al.*, 2009;Perez-Llarena *et al.,* 2009). This is primarily because in general they work well against bacteria and are safe for human and animal consumption with few side effects observed. Antibiotics have targets, which are usually functional proteins such as enzymes and ribosomal proteins. The interaction between an antibiotic and target moiety is often quite specific. Alteration of target protein through mutations renders the bacterium resistant to drugs. For example, penicillin binding protein (PBP) is the target moiety for beta-lactam drug. Modification of this target moiety through mutation can

affect the affinities of these molecules for ß-lactam antibiotics and thus prevent transpeptidation (Anderson *et al.,* 2003; Marshall *et al.*, 2009).

In 1940, Abraham and Chain (1940) identified the first β-lactamase from *E. coli*isolate. In clinical settings, theintroduction of new classes of β-lactams has invariably been followed by theemergence of new β-lactamases capable of degrading them, as a paradigmaticexample of rapid bacterial evolution under a rapidly changing selective environment.Distribution and increased incidence of β-lactamases globally have dangerously disputed their clinical effectiveness. β-lactamases are enzymes produced by bacteria that breakdown (and thus inactivate) β-lactams.

2.7.1. Classification of β-lactamases

β-lactamasescan fall into any of the following classes based on the type of enzymes present: penicillinases, cephalosporinases, extended-spectrum beta-lactamases, AmpC-type β-lactamases and carbapenemases. The classification can also be based on functional classification (specific inhibitor of the enzymes and specific substrate) called Bush–Jacoby–Medeiros functional classification and Molecular classification (amino acid sequences) called Ambler molecular classification (Ambler, 1980; Bush *et al.,* 1995).According to the Bush, Jacoby and Medeiros scheme, beta-lactamases are divided into four groups: 1, 2, 3 and 4. Based on the differences among the enzymes in these groups, they were further divided into several subgroups. Among these groups, group 2 has the most number of subgroups.

In spite of being based on physiological properties, this classification is in agreement with Ambler's molecular classification. AmpC beta-lactamases fall under Bush's group 1 and Ambler's class C, whereas metallo-beta-lactamase fall under Bush's group 3 and Ambler's class B. Rest of the serine bate-lactamases were included in Ambler class A, whereas Bush divided

them into subgroups under group 2. Bush's functional classification in relation to Ambler's molecular classification is displayed in Table 2.2

2.7.1.1. Group 1 (Ambler Class C) beta-lactamases

Group 1 beta-lactamases also known as AmpC enzymes are cephalosporinases that are resistant to clavulanic acid, penicillins, cephamycins, as well as first, second and third generation cephalosporins, belonging to molecular class C. They are mostly encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms however; studies have also shown the shift of enzymes from chromosome to plasmid in some strains such as *E.coli* and *Klebsiella* spp (Sanders and Sanders, 1992). They are sensitive to cefepime and carbapenems (Sanders *et al*., 1996).

2.7.1.2. Group 2 (Ambler Class A) enzymes

Beta-lactamases in group 2 includes penicillinase and cephalosporinase which correspond to molecular classes A and D. The original group 2 enzymes (TEM and SHV) are susceptible to beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. They are harbored by plasmid and they could easily be transmitted into different bacterial cells, initiating rapid resistance to such enzymes. Group 2 comprises of six subgroups. Group 2a contains penicillinases only, 2b are broad spectrum beta-lactamases which are capable of hydrolyzing penicillins and cephalosporins; divided into 2be, consisting of the ESBLs and 2br, consisting of betalactamases with reduced binding to betalactamase inhibitors, 2c are carbenicillinases, inhibited by clavulanic acid, 2d are oxacillinases; molecular class D or A; poorly inhibited by clavulanic acid, 2e are chephalosporinases; hydrolyse monobactams, susceptible to clavulanic

acid and 2f are carbapenemases (serine based in contrast to group 3 zinc based carbapenemases), and are resistant to clavulanic acid.

2.7.1.3 Group 3 (Ambler Class B) enzymes

These are the zinc-based or metallo-enzymes capable of hydrolyzing penicillins, cephalosporins, and carbapenems. They correspond to molecular class B, which are the only betalactamases acting by the metal ion zinc. These enzymes are frequently found in *Pseudomonas aeruginosa*, *Bacteroides fragilis* and *Stenotrophomonas maltophilia*. They are not inhibited by clavulanic acid.

2.7.1.4 Group 4 beta-lactamases

Group 4 beta-lactamases encompasses those unusual penicillinases that are not inhibited by clavulanic acid and they do not yet have a corresponding molecular class. Some of these enzymes exhibit high rates of hydrolysis with carbenicillin and/or cloxacillin. Also a number of them show unusual behavior with respect to metal ion involvement. It is yet to be known if these enzymes represent another molecular class of beta-lactamase (Ghafourian *et al.,* 2014).

Table 2.2: Classification schemes for bacterial β-lactamases

Bush-Jacoby-Medeiros Grou	198 Bush Grou	Richmond-Sykes Clas	Mitsuhashi-Inoue Type[a]	Molecular Class	Preferred Substrate	Inhibited by: CA[b]	Inhibited by: EDTA	Representative Enzymes
1	1	Ia, Ib, Id	CSase	C	Cephalosporins	2	2	AmpC enzymes from Gram-negative bacteria; MIR-1
2a	2a	Not included	PCase V	A	Penicillins	1	2	Penicillinases from Gram-positive bacteria
2b	2b	III	PCase I	A	Penicillins, cephalosporins	1	2	TEM-1, TEM-2, SHV-1
2be	2b9	Not included except K1 in class IV	CXase	A	Penicillins, narrow-spectrum and extendedspectrum cephalosporins, monobactams	1	2	TEM-3 to TEM-26, SHV-2 to SHV-6, *Klebsiella oxytoca* K1
2br	Not included	Not included	Not included	A	Penicillins	6	2	TEM-30 to TEM-36, TRC-1
2c	2c	II, V	PCase IV	A	Penicillins, carbenicillin	1	2	PSE-1, PSE-3, PSE-4
2d	2d	V	PCase II,D PCase III		Penicillins, cloxacillin	6	2	OXA-1 to OXA-11, PSE-2 (OXA -10)
2e	2e	Ic	CXase	A	Cephalosporins	1	2	Inducible cephalosporinases from *Proteus vulgaris*
2f	Not included	Not included	Not included	A	Penicillins, cephalosporins, carbapenems	1	2	NMC-A from *Enterobacter cloacae*, Sme-1 from *Serratia marcescens*

3	3	Not included	Not included	B	Most β-lactams, including carbapenems	2	1	L1 from *Xanthomonas maltophilia*, CcrA from *Bacteroides fragilis*
4	4	Not included	Not included	ND[c]	Penicillins	2	?	Penicillinase from *Pseudomonas cepacia*

[a]Csase, cephalosporinase; PCase, penicillinase; CXase, cefuroxime-hydrolyzing b- lactamase. *b*CA, clavulanic acid. *c*ND, not determined.

Source: Bush *et al. (1995)*

Resistance to beta-lactam antibiotics has become a problem in recent decades, as strains of bacteria that produce extended-spectrum beta-lactamses (ESBLs) are common. These β-lactamase enzymes render most of the penicillins and third generation cephalosporins ineffective (Livermore and Woodford, 2006; Livermore, 2009; Nordmann *et al.,* 2011a). According to Bush *et al.* (1995), ESBLs belong to Group 2 of the Bush-Jacoby-Medeiros classification scheme of bacterial β-lactamases (alongside with cephalosporinases and penicillinases from Gram positive organism) and group 1 contains AmpC enzymes fromGram-negative bacteria, Group 3 carbapenemase.

2.7.2. Extended- Spectrum Beta-Lactamases (ESBLs)

ESBLs are group of enzymes that can hydrolyse the oxymino-cephalosporins and cause resistance to the third generation cephalosporin i.e. cefotaxime, ceftazidime, ceftriaxone, and monobactams (i.e. aztreonam) but not to the cephamycins (e.g. cefoxitin and cefotetan) and carbapenems (e.g. imipenem, meropenem, and ertapenem). ESBLs are usually inhibited by beta-lactamase-inhibitors like clavulanic acid and tazobactam (Bradford, 2001; Jacoby and Munoz-Price, 2005; Livermore, 2008; Hatchner *etal*., 2012).Based on their inhibition by clavulanic acid they are differentiated from AmpC-type of beta-lactamases (which is also able to hydrolyse third generation cephalosporins but not inhibited by clavulanic acid).

Generally, ESBLs are thought not to be carried on the bacterial chromosome, rather they are found on an independent element of DNA called a plasmid but have been shown to be

chromosomally mobilized (Canton *et al.,* 2012). Plasmids that carry ESBLs gene can carry many other different genes on them and have the ability to transfer replica of themselves to other bacteria. This can be very serious for a number of reasons.

Firstly, the other genes could include genes conferring resistance to other classes of antibiotics. These genes make the recipient bacteria resistant to multiple antibiotics, turning them into what are sometimes reported as a "superbug". Also, these plasmids can be carried by strains that, though primarily non- pathogenic (commensal) could transfer the plasmids to pathogenic strains (Pfaller and Segreti, 2006).

Studies have shown that isolates that produced ESBLs were found out to be resistant to other class of antibiotics such as phenicols, fluoroquinolone and aminoglycosides. (Jacoby and Medeiros, 1991; Ramphal and Ambrose, 2006; Khanfar, *et al.,* 2009).

Plasmid transferable ESBLs was first time showed in the 1960s and named TEM-1 after the name of a Greek girl Temoniera, who carried *E. coli*from which the TEM-1 enzyme was discovered. A large number of plasmid-transferable ESBL have been discovered since the 1980s (Day *et al.,*2016). Presently, different variants of ESBL (TEM-, SHV-, OXA- and CTX-M-) are known but the most prevalent one is CTX-M among *Enterobacteriaceae*(Bush, 2010;Day *et al.,* 2016).

More than 300 different CTX-M enzymes have been discovered and dividedinto five groups depending on their amino acid sequence namely CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 (http://www.lahey.org/studies/other.asp). ESBLs-producing organisms have been seen to be a cause of long hospitalization such as seen in the case report of Aboderin *et al.* (2012) and revealed by other studies (Moore *et al.,* 2005; Mougkou *et al.*, 2014).More recently,

studies have shown more CTX-M phenotypes in commensal *E. coli*(Pallecchi *et al.,* 2007; Ewers *et al.,* 2011; D'Andrea *et al.,* 2013;Franz *et al.,* 2015) which indicates commensal *E. coli* has a reservoir for dissemination of these ESBLs. The most common ESBL-producing bacteria are some strains of *Escherichia coli* and *Klebsiella pneumoniae* (Ramphal and Ambrose, 2006).

ESBLs are spread via direct and indirect contact with colonized or infected patients and contaminated environmental surfaces.

2.7.3. Carbapenemase

A unique quality of carbapenems is their resistance to hydrolysis byESBLs (Martin and Kaye, 2004; Poirel *et al*., 2016). They are group of powerful antibiotics that can only be given in hospital directly into bloodstream intravenously. Drug such as ertapenem, imipenem, meropenem and doripenem,belong to this class of antibiotics. Imipenem is susceptible to degradation by the enzyme dehydropeptidase-1 (DHP-1) and therefore requires co-administration with a DPH-1 inhibitor cilastatin. The later carbapenem have increased stability to DPH-1 and do not require DPH-1 inhibitor. Meropenem and doripenem are thought to be more potent in vitro agent againstGram-negative organisms. Meropenem has a niche in its spectrum of coverage because of its binding capabilities (Hrabak *et al.,* 2014; Poirel *et al*., 2016).

Carbapenems are bacteriocidal in activity by entering periplasmic space through porins, where they then inhibit transpeptidases.Until now, they were antibiotics that could always be relied upon when other antibiotics failed.

However,carbapenemase-producing *Enterobacteriaceae*(CPE) have become a challenge to clinical therapy because of the rapid worldwide dissemination of multi-drug resistance (Tangdem and Giske, 2015; Poirel*et al.*, 2016). Most organisms with the enzyme carbapenemase usually live harmlessly in the gut of humans or that of animals and helps in food digestion. CPE refers to bacteria such as *Klebsiella pneumonia, Escherichia coli, Acinetobacter,* and *Pseudomonas* (Vaux *et al.,* 2011).

Hospitals have been identified as primary transmission sites for CPE-based infections. Study by Perez and Van, (2013), have shown that up to 75% of hospital admissions attributed to CPE were from longterm-care facilities or transferred from another hospital.CDC (2013) hadmade estimation that carbapenem-resistant*E. coli*causes9,300health care-associatedinfections annually in United State (US). Dissemination and emergence of CPE is likely to be as a result of increased reliance on the class of antibiotics(Webb *et al.,*2016).Infections with CPE were associated with organ or stem cell transplantation, mechanical ventilation, exposure to antimicrobials, and overall longer length of stay in hospitals (Gupta *et al.,* 2011; Krishnaraju *et al.,* 2015).There are primarily two mechanisms of carbapenem resistance (i) production of enzymes, carbapenemase that is capable of hydrolysing virtually all β lactam (e.g. KPC, OXA, and VIM) or (ii) Porin modification and, or production of efflux pump leading to decreased cell membrane permeability; this often found in combination with ESBLs and AmpC (Nordmann *et al.,* 2012). The Ambler classes of carbapenemase based on mechanisms of hydrolyzing carbapenems.*Klebsiella pneumoniae* carbapenemases (KPC, class A); metallo- β -lactamases (MBLs,classB);and oxacillin- hydrolyzing metallo-β-lactamases (OXA, classD) are the most prevalent and clinically relevant ones and haverecurrently been seen in diversestrains of *E. coli* (Urban*etal*., 2008; Kumarasamy*etal*., 2010; Beyrouthy*etal*., 2013). Study of Kumarasamy *et al.,*

(2010) revealed that strains of *E. coli*are the major producer of metallo-b-lactamaseknownastheNew Delhi metallo-b-lactamase(NDM-1) and it agrees with the study carried out by Nordmann *et al*. (2011b). The resultingpossibilityof high environmentalcontaminationand spreadofthe bla_{NDM-1} gene hasbeensupportedsince *E. coli*can readily exchange genetic materials.(Kumarasamy*etal*., 2010; Poirel *etal.*, 2010; Peirano *etal.*, 2011).

2.8.*ESCHERICHIA COLI*

Theodore Escherich, a pediatrician, in the late 19th century was the first person to describe *Escherichia coli* (*E. coli*). He described *E. coli*as a normal flora of the gut and named it,*Bacterium coli* commune. This was later renamed as *E. coli*in his honour (Kaper *et al.,* 2004).

*E. coli*belongs to the family Enterobacteriaceae,and it is facultativeanaerobic, non- sporulating, andGram-negative rods that live in the intestinal tracts of animals and humans.The harmless strains are beneficial to their host by preventing the establishment of pathogenic bacteria within the gut and production of menaquinone (vitamin K2). In microbiological studies, *E. coli* is frequently used as a model organism. Cultivated strains (e.g. *E. coli* K12) are well-adapted to the laboratory environment. *E. coli*usually produces dry, pink (lactose positive) colonies with a surrounding pink area of precipitated bile salts on MacConkey agar and shining metallic green sheen colour on eosin methylene blue (EMB) agar; the colonies are occasionally mucoid. *E. coli* are amongst the most frequently isolated from clinical specimens (Kaper *et al.,* 2004; Smith *et al.,* 2005; Todar, 2008).

2.8.1. Biology and Biochemistry of *E. coli*

*E. coli*can have peritrichous flagella for motility. It hassex pili (type 1 pili), which are expressed in phases to include switch on and off for conjugation. *E. coli*cells are typically rod-like in shape and are about 1-3µm in length and 0.5µm in diameter having a layer of peptidoglycan in its periplasm. Peptidoglycan consist, for the purposes of description, of three parts: a backbone, composed of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid; a set of identical tetrapeptide side chains attached to *N*-acetylmuramic acid; and a set of identical peptide cross-bridges which consists of a direct peptide linkage between the diaminopimelic acid (DAP) amino group of one side chain and the carboxyl group of the terminal D-alanine of a second side chain. They are able to grow on simple nitrogen and carbon compounds. They also possess capsules and fimbriae (Smith *et al.,*2005). Biochemically, *E. coli*forms gas from glucose, ferments lactose, and are indole positive, methyl red positive, Voges proskauer negative, and oxidase negative (Ewing, 2006).

They do not utilize citrate, are urease negative and do not liquefy gelatin (Farmer *et al.,*2004; Brenner, 2005; Ewing, 2006). *E. coli* have complex antigenic structure consisting of somatic (O), capsular (K) and flagellar (H) antigens. There are more than 170 different heat-stable somatic O (lipopolysaccharide) antigens, more than 100 heat-labile K (capsular) antigens, and more than 50 H (flagellar) antigens which are used in their classification (Ewing, 2006).

This bacterium possesses an extra-cytoplasmic outer membrane that consists of a lipid bilayer, lipoproteins, and a capsule of lipopolysaccharide (LPS). The outer membrane interfaces with the bacterial and host environment. A variety of components of the outer membrane are critical determinants in antimicrobial resistance and pathogenesis. *E. coli*, a very adaptive organism, and it can relativelysynthesize excessive constituents, often from a single organic compound and a few minerals. They respond to changes in temperature and available nutrients by making rapid

adjustment in the synthesis of regulatory molecules. Most *E. coli* strains are capable of growing over a wide range in temperature (approximately 15-48^{o}C). The growth rate is maximal in the narrow range of 37-49^{o}C. *E. coli*can grow within a pH range of approximately 5.5-8.0 with best growth occurring at neutrality (Ewing, 2006; Darnton *et al.,* 2007).

2.8.2. Habitat

E. coli are common inhabitants of the gut of mammals most especially the caecum and the colon, though the number of anaerobic bacteria in the bowel outnumber *E. coli,* it is the most predominant facultative anaerobic organism in the gut. They are found in the mucous layer which covers the epithelial cells throughout the tract and are released into lumen with the degraded mucus component and excreted in the faeces. The human colon maintains a microbial density approaching 10^{12} organisms per gram of faeces of infant but it gradually decreases in the elderly, representing a perfectly balanced ecosystem. *E. coli*are the second species of bacteria to colonize the intestine during infancy after Gram positive rod though increased hygiene can reduced early colonization by *E. coli* (Jaureguy *et al.,* 2008).The presence of *E. coli* in the environment is usually considered to reflect faecal contamination and not the ability to replicate freely outside the intestine. There is evidence, however, to suggest that *E. coli* may freely replicate in tropical fresh water (Todar, 2008). Under certain conditions the number of these organisms in the intestines undergoes a marked and rapid increase, and this may be associated with definite signs of ill health and sometimes death. The prevalence and density varies from one host species to other because of distinct body size, gut morphologies, diets, having its prevalence to be more than 90% in human (Buxton and Fraser, 1988).

Usually, *E. coli* and its human host coexist in good health and with mutual benefit for decades. These commensal *E. coli* strains rarely cause disease except in immunocompromised hosts or where the normal gastrointestinal barriers are breached. The niche of commensal*E.coli* is the mucous layer of the mammalian colon. The bacterium is a highly successful competitor at this crowded site, comprising the most abundant facultative anaerobe of the human intestinal micro flora (Kelly *et al.,* 2002;Kaper*et al.,* 2004).

2.8.3. Economic Importance of *E. coli*

The harmless strains are part of the micobiota of the gut, and they can be advantageous to their host by competitively inhibiting the growth of pathogenic bacteria.Apart from the harmless strains that are part of the micoflora of the gut which are beneficial, some specific strains of *E. coli*are pathogenic. These pathogenic strains have the potential to cause infections ranging from gastrointestinal infections to extra-intestinal infections (urinary tract infection, neonatal meningitis, and sepsis) after acquiring some virulence factors such as biofilm, mannose resistant haemagglutinin(MRHA), hemolysis,gelatin liquefaction, and antibiotic resistance. These virulence attributes are normally encoded on genetic elements that can be exchanged between different strains. Specific combinations of virulence factors form different pathotypes i.e. a group of strains of a single species that cause a related disease using the same set of virulence factors (Kaper *et al.,* 2004; Vagarali *et al.,* 2008; Baker, 2015).

2.8.3.1.Diarrhoeagenic *E. coli*(DEC)

When Theodore Esherich first discovered *E. coli*in 1885, it was considered a part of the normal flora. Since then it has accounted for 80% of significantenterobacterial isolates from stool specimens received in clinical microbiology laboratories (Kelly *et al.,* 2002). One of the clinical

infections includes gastroenteritis. Inflammation of the gastrointestinal tract involving both stomach and small intestine resulting in acute diarrhoea and vomiting is termed gastroenteritis. It can be acquired by contact with food and water that has been contaminated by certain type of viruses or less often these strains of diarrhoeagenic *E. coli.*

There are six major categories of diarrhoeagenic *E. coli*(DEC) known to cause gastroenteritis in human beings.These include: entero-toxigenic *E. coli*(ETEC) that produces a toxin (heat labile and heat stable) resulting in travellers' diarrhoea, entero-invasive *E. coli*(EIEC) that penetrates the epithelial cells lining the intestinal mucosa hence diarrhoea, entero-pathogenic *E. coli*(EPEC) that causes infantile diarrhoea, entero-haemorrhagic *E. coli*(EHEC) that causes diarrhoea as a result of haemolysin production , entero-aggregative *E. coli*(EAEC) that causes prolonged diarrhoea and diffusely adherent *E. coli* (DAEC) causes childhood diarrhoea (Levin *et al.,* 2000; Patterson, 2002; Berge *et al.,* 2003; Palaniappan *et al.,* 2006; CDC, 2016). Others include cell detaching *E. coli*and cytolethal distending toxin producing *E. coli* (CLDT) (Okeke, 2009).

According to the literature review by Okeke, (2009) *E. coli*may cause diarrhoea using any of these model (i) toxin production(ETEC, EHEC, CLTD and CDEC); (ii) invasion (EIEC); (iii) adherence (EPEC, EHEC, EAEC, and DAEC).

2.8.3.2.Uropathogenic *E. coli* (UPEC)

In the United States every year, according to Welch *et al.,* (2002) uropathogenic *E. coli*(UPEC) cause an estimated 6-7 million urinary tract infections (UTIs) ($2.5 billion in medical expenses) and UPEC are the number one cause of UTIs in humans. A UTI is diagnosed in 50% - 60% of women in their lifetime while they are only diagnosed in about 20% of men.

When UPEC are introduced into the urethra from the colon it ascends to colonise the bladder and then the kidney using P fimbrae (pyelonephritis- associated pili) to bind the urinary tract mucosal cells and caused a localized infection such as urethritis, oracute cystitis, or pyelonephritis. UPEC can also form K antigen which contribute to the formation of biofilm. Symptoms of UTIs are diverse and can include acute painful urination, dysuria, urinary frequency and urgency, nocturia, and suprapubic discomfort. Acute cystitis is the most common clinical presentation of a UTI, and is usually treated with an antibiotic like trimethoprim, sulfamethoxazole or a fluoroquinolone, such as ciprofloxacin. However, if left untreated, a UTI can eventually lead to renal failure or bacteremia (Welch *et al.,* 2002).

2.8.3.3.Neonatal meningitis and bacteremia

Meningitis is inflammationof the meninges (membranesthat surround the brain and spinal cord protecting the brain from injury and infection).Bacteremia is a severe infection of the blood.Bacteriamultiply in the blood, releasing toxinsthat cause widespread damage to the body (Poolman and Wacker, 2016). Neonatal meningitis is the term used to describemeningitis that occurs in the first 28 days of life.

Many different organisms can cause neonatalmeningitis, broadly grouped as bacteria, virusesand fungi (Ginsberg, 2004). However, the most common causesare bacteria; in particular group B streptococcus(GBS) and*E. coli*.In developing countries like Nigeria, *E. coli*is an important cause of meningitis than other bacteria (Todar, 2008; Poolman and Wacker, 2016).*E. coli*may be passed to a baby during delivery (van de Beek *et al.,* 2006). Serious infections may occur if thebacteria invade blood stream or meninges. Occasionally, bacteria overcome the body'sdefences and cause infection. In the process thebacteria may spread through the

bloodstream tothe meninges and cause meningitis. When thebacteria infect the meninges, tiny blood vesselsin the membranes are damaged. This allowsthe bacteria to break through and infect thecerebrospinalfluid(CSF); therefore, the condition is classified as medical emergency. The meninges then become inflamed, increasing pressure aroundthe brain which can cause nerve damage andspecific symptoms associated with meningitis. Meningitis can be a life threatening because it can lead to serious long-term consequences such as deafness, epilepsy, hydrocephalus and cognitive deficits especially if not treated quickly (van de Beek *et al.,* 2006).

It is estimated that *E. coli*causes about 20% of cases of neonatal meningitis, but less than 2% of cases of meningitis at all other ages. However, 85% of babies infected recover but chance of having total recovery is not as high as other forms of meningitis (Todar, 2008).

Infection of the bloodstream can itself belife-threatening. The bacteria multiply rapidly,doubling in number around every 30 minutes,causing bacteremia. As the bacteria multiply,they begin to release toxins. The body's naturaldefences have little effect on these poisons. Asbacteremia advances, it affects the whole bodyand can cause organ damage or failure.The signs and symptoms of meningitis orbacteremia are often non-specific at first andcan be difficult to recognise in very young babies. Study by Diekema *et al.,* (2000) has shown that of all the microorganisms that are implicated in sepsis, *E. coli*has been shown to be the most common cause of septicemia in infant and the elderly that above 65 years of age.

Other than the above, *E. coli* are implicated as opportunistic pathogens in a wide array of human infections such as wound sepsis.

CHAPTER THREE

MATERIALS AND METHODS

3.1. STUDY AREA

The study was carried out at the Medical Microbiology Research laboratory, Department of Medical Microbiology and Parasitology of Obafemi Awolowo University, Ile-Ife.

3.2. STUDY DESIGN AND PERIOD OF STUDY

The study is a cross sectional study among undergraduate students of Obafemi Awolowo University, Ile-Ife. The study spanned the period of seven months, from November, 2015 to May, 2016.

3.3. SUBJECT AND ETHICAL APPROVAL

First-year students of Obafemi Awolowo University were recruited in the study. Information about them and informed consent (written) were obtained.

Approval was obtained from the Ethics and Research Committee of the Institute of Public Health Obafemi Awolowo University. Confidentiality of all the participants and their data was strictly maintained using codes so that no database will have volunteer identifiers.

3.3.1. Case Selection

Both male and female students of the institutionwere recruited with the following criteria used in selecting the subjects:

Inclusion criteria: Subject who have not been using antibiotics in the last 30 days prior to sample collection and those who are interest and willing to participate

Exclusion criteria**:**Subjectwho are presently on antibiotics and those who are not interested

3.4. SAMPLE SIZE DETERMINATION

Two hundred samples were obtained. This was determined by means of the formula:

$$n = \frac{p(1-p)s^2}{d^2}$$ (Daniel, 1999)

Where

n= Desired sample size

p= Prevalence value of antimicrobial resistant Commensal isolates of *E. coli* from apparently healthy people from previous study(84.6%) (Onanuga and Berefgha, 2014)

S= Confidence limit of the results which is 1.96

d= Degree of accuracy which is 5% or 0.05

3.5. SAMPLING TECHNIQUE

Sampling technique used in recruiting the subjects was volunteer sampling method.

3.6. SAMPLE COLLECTION

Stool sample of apparently healthy student was collected. Collection was done using clean, sterile universal bottles.

3.7. CULTURE OF SPECIMEN

All samples were inoculated onto eosin methylene blue (EMB) agar plates (Mast Group Ltd., Bottle, United Kingdom) incubated overnight aerobically at 37^{o} C and observed for discrete colonies.Discrete colonies typical of *E. coli* (greenish metallic sheen/purple) on EMB agar plates

were picked and streaked on nutrient agar (NA)(Mast Group Ltd., Bottle, United Kingdom) plates which were, in turn,incubatedat 37^{o} C for 24 hours and observed for discrete colonies. Confirmatory identification wasmade by Gram staining and biochemical testing. Afterward, all *E. coli* isolates were maintained in the laboratory in 16% Glycerol broth cryovials at 4^{o} C.

3.8. BIOCHEMICAL TESTS

The presumptive *E. coli*isolates were subjected to further biochemical tests which included Gram staining, citrateutilization, and indole.

3.8.1. Gram's staining

This was be done by fixing a smear of each isolate onto a clear glass microscope slide and flooded with 0.5% crystal violet stain for 60 seconds. The stain was rinsed off with distilled water and thereafter flooded with Lugol's iodine for 60 seconds after which it was decolourized with acetone for a few seconds, rinsed with distilled water and counter stained with aqueous safranin for yet another 60 seconds. The smear was finally rinsed with distilled water and allowed to dry. The stained smear was examined microscopically and the shape, arrangement and colour of the organism at x100 magnification under oil immersion were noted. Organisms that stain red were considered Gram negative while those which stain purple/blue as Gram positive (Cheesbrough, 2010). *E. coli* ATCC 29522, was be used as control strain.

3.8.2 Indole test

Indole production was tested on all the isolates and detected with Kovac's reagent (4-dimethyl amino benzaldehyde, isoamyl alcohol and hydrochloric acid). The test organism was cultured in sterilized peptone water and incubated at 37^{0}C overnight. Three drops (0.5ml) of Kovac's reagent

was added. The development of a red colour ring in the organic layer within 10 minutes was indicative that the organism was able to convert tryptophan to indole (Cheesbrough, 2010). *E. coli* ATCC 29522 was used as control strain.

3.8.3 Citrate utilization test

Simmon's citrate agar medium (Oxoid Ltd., Basingstoke, Hampshire, England), prepared in Bijou bottle and sterilized, was used to differentiate organisms on the basis of citrate utilization. A few colonies from a pure culture were picked with a straight flamed wire and inoculated onto the medium by stabbing the butt of the tube all the way to the bottom and streaking the surface of the slant slope in a zigzag pattern. The bottle was then be inoculated at 37^0C for 24hours. A change in colour of the slants from light green to blue was considered as an indication of the organism's ability to utilize citrate as a sole source of carbon (Cheesbrough, 2010). *E. coli* ATCC 29522 was used as control strain.

3.9. ANTIMICROBIAL SUSCEPTIBILITY TESTING BY DISC DIFFUSION METHOD

Kirby-Bauer disk diffusion techniques was used to determine the resistance pattern of all the *E. coli*isolates according to the Clinical and Laboratory Standards Institute guidelines (CLSI, formerly NCCLS). The test was performed on Muller-Hinton agar (MAST Group Ltd., Bootle, United Kingdom) plate. Prior to inoculation, the swab stick was dipped into bacterial suspension which had been earlier prepared by dislodging a few colonies of pure culture of isolates in saline and adjusted to visual equivalent turbidity of 0.5 McFarland standards.The surface was lightly and uniformly inoculated making lawn with test organisms by sterile cotton swab. The plates

were incubated overnight at 37^{0}C for 18-20 hours and zones of inhibition measured to the nearest millimetre. *E. coli* ATCC 25922 was used as control strain.

3.10. ANTIMICROBIAL AGENTS USED

A total of 11 antibioticswere usedto determine antibiogram of the isolates according to the Gram-negative panel recommended by CLSI (2014). The antibiotics included: meropenem (10µg), nalixidic acid (NAL) (30µg), ciprofloxacin (5 µg), cefotaxime (CTX) (30µg), ceftazidime (CAZ) (30µg), chloramphenicol (CLO) (30µg), ampicillin (AMP) (10µg), streptomycin (SIO) (10µg), sulphonamides (SUL) (300µg), trimethoprim (W) (5µg), tetracycline (TET) (30µg)(MAST Group Ltd., Bootle, United Kingdom). Interpretation of the diameters of zones of inhibition was done according to CLSI (2014).

3.11. Screening for ESBL production (phenotypically)

All isolates were tested for the detection of ESBL production by the confirmatory method of Clinical and Laboratory Standards Institute (2014) guidelines using cefotaxime (30 µg) and ceftazidime (30 µg) and a disc of cefotaxime plus clavulanic acid (30/10 µg) and ceftazidime plus clavulanic acid (30/10 µg) (MAST Group Ltd., Bootle, United Kingdom) placed at a distance of 20mm on a lawn culture (0.5 McFarland inoculum size) of suspected ESBL producing clinical isolate on Mueller-Hinton Agar (MAST Group Ltd., Bootle, United Kingdom). *Escherichia coli* ATCC 25922 were used as the negative control and *Klebsiella pneumoniae* ATCC 700603 was used as the ESBL positive control. ESBL production was inferred if the inhibition zone was ≥5 mm towards the cefotaxime plus clavulanic acid disc or ceftazidime plus clavulanic acid disc in comparison to the third generation cephalosporin disc

alone. Only those isolates identified phenotypically as ESBL producer were selected for genotypic detection of ESBL encoding genes.

3.12. CARBAPENEMASES SCREENING

All isolates that were resistant to meropenem were screened for the production of carbapenemases

3.12.1. *Klebsiella pneumoniae*Carbapenemase Confirmatory Screening Using Modified Hodge Test (MHT)

A susceptible, standardiseddilution of the *E. coli*ATCC 25922 in 5 ml of peptone water was prepared. Lawn streak of the suspension was made onto a Mueller Hinton agar plate and allowed to dry for 3–5 minutes. A 10 µg meropenem discwasplaced at the centre of the plate.Then a streak of suspension of the test organism (organism that has ≤ 22mm zone of inhibition to meropenem during sensitivity testing) from the edge of the disk to the edge of the plate was made in a straight line and incubated overnight at 37^{o}C for 16–24 hours, the plate was examined for a clover leaf-type indentation at the intersection of the test organism and the *E. coli*25922, within the zone of inhibition of the carbapenem susceptibility disk.

MHT Positive test has a clover leaf-like indentation of the *E. coli*25922 growing along the test organism growth streak within the disk diffusion zone.

MHT Negative test has no growth of the *E. coli*25922 along the test organism growthstreak within the disc diffusion.

3.12.2. Phenotypic Detection of Metallo-Beta-Lacmases (MBLs)

In addition, all isolates with resistance to meropenem were tested for the MBLs production by streaking the test organisms on Mueller Hinton plate.Two 10µg meropenem discs (Oxoid, UK) were placed on the plate, and 10µl of EDTA solution (0.5 M) was added to one of them. A positive strain have zone of inhibition of meropenem with EDTA (MEM + EDTA) greater than meropenem alone by 7 mm after 24 hours of incubation at 37˚C. (Lucena *et al*., 2014).

3.13. GENOTYPIC DETECTION

3.13.1 DNA Extraction

Boiling method was used to extract bacterial DNA according to the steps used by Bali*et al.* (2010). Isolates that were positive to phenotypic confirmatory test were grown on Nutrient agar for 12 hours. A single colony growth was picked and transferred to 0.1 ml of sterile water, stirred and boiled for 10 minutes so as to lyse the cells. The lysate was centrifuged briefly (10 seconds at 10,000 rpm), and 15 µl of the supernatant was used as the DNA sample for the PCR reaction.

3.13.2. DNA Amplification in Thermal Cycler

PCR analysis for beta-lactamase and carbapenemase genes of the family TEM, SHV and CTX-M (for ESBLs) KPC, VIM and NDM (for carbapenemases) were carried out. Primers obtained from South Africa used for TEM, SHV, and CTX-M as shown in (Table 3.1) was supplied by inqaba biotech.

3.13.3. Preparation of Reaction Mixture

For PCR amplification, a total volume of 25µl reaction mixture was prepared according to the following composition. 3 µl of template DNA was added to 12.5 µl of master mixture (containing

Dntp mixture, buffer, Taq polymerase), 0.5 µl of each primer stock solution, and remaining 6.5 µl volume was fulfilled by neuclease free water.

3.13.4. Amplification

The prepared PCR tubes with master mix were placed in the eppendorf thermal cycler. Amplification was carried out according to the following thermal and cycling condition illustrated in table 3.2.

3.13.5. Gel electrophoresis and Visualization under UV lights by trans-illuminator

3.13.5.1. Agarose gel electrophoresis

The PCR products was analyzed after electrophoresis in 1.5% agarose gel to detect specific amplified product by comparing with standard molecular weight marker. The strength (% Agarose) of the gel depends on the sizes of DNA fragments to be separated and the volume used depends on the size of the electrophoresis tray.

3.13.5.2. Preparation of agarose gel

A 1.5% agarose gel was prepared by melting 1.5mg agarose in 100 ml of diluted Tris Acetate EDTA (TAE) buffer using an electrical hotplate until the mixture is clear. The melted agarose was allowed to cool to about 45ºC and poured into the casting chamber with combs in place. After the gel has set, it was then placed in the electrophoresis chamber and covered (essentially "drown") with enough TAE buffer.

3.13.5.3. Loading and electrophoresis of the sample

10µl of amplified PCR product was slowly loaded into the well using disposable micropipette tips. A 100bp molecular weight DNA marker/ladder was loaded in one well to determine the size of the amplified PCR products. Electrophoresis was carried out at 100 volts for 45 minutes.

3.13.5.4. Visualization of the gel

The gel, after electrophoresis, was stained with ethidium bromide for 15minutes and de-stained for 30minuutes before it was visualized under the UV light for DNA bands and photographed with a video copy processor for documentation.

3.15. PCR PRECAUTIONS

1. Aerosol-free pipette tips were used at all stages of testing to prevent contamination.
2. Powder- free gloves were used, as powder can cause unwanted fluorescence in this essay.
3. Thorough primer constitution was ensured.
4. All the processes were done under aseptic condition.

3.16. QUESTIONNAIRE

Data collection was done using questionnaire. Both closed and open-ended questions were included in the questionnaires. The information sought included bio data characteristics (age, sex), self-

medication behaviour, Hygiene habits, previous exposure to antibiotics, and Adherence to antibiotics. Data gathered were analysed using Statistical Package for Social Science (SPSS) version 22.

Table 3.1: Primers used for sequencing of resistance genes by polymerase chain reaction (PCR).

Gene	Primer	Sequence (5´- 3´)	Size(bp)	T(ºC)	Reference
SHV	SHV-F	CGCCTGTGTATTATCTCCCT	293	60	1
	SHV-R	CGAGTAGTCCACCAGATCCT			
TEM	TEM-F	TTTCGTGTCGCCCTTATTCC	403	60	1
	TEM-R	ATCGTTGTCAGAAGTAAGTTGG			
CTX-M	CTX-M-F	CGCTGTTGTTAGGAAGTGTG	569	60	1
	CTX-M-R	GGCTGGGTGAAGTAAGTGAC			
KPC	KPC-F	ATGTCACTGTATCGCCGTCT	893	55	2
	KPC-R	TTTTCAGAGCCTTACTGCCC			
NDM	NDM-F	GACAACGCATTGGCATAAG	447	60	2
	NDM-R	AAAGGAAAACTTGATGGAATTG			
VIM	VIM-F	ATTCCGGTCGGMGAGGTCCG	633	60	2
	VIM-R	GAGCAAGTCTAGACCGCCCG			

1 = Bali *et al.* (2010)

2 = Pasanen*et al.* (2014)

Table 3.2. Thermal and cycling conditions for polymerase chain reaction

NO	STEP	PCR Temperature	and duration
1	Hot start	94^{o}C	3-minutes
2	Denaturation	94^{o}C	30-seconds
3	Annealing	60^{o}C	30-seconds
4	Extension	72^{o}C	1-minute
5	Cycling	Steps2-4 repeated 35 times	
6	Terminal extension	72^{o}C	7-minutes

CHAPTER FOUR

RESULTS

4.1.RATE OF ISOLATION OF *E. COLI*

A total of 186 (93%) faecal samples were positive for *E. coli* out of 200 faecal samples. Up to 5 colonies were picked, (where possible) from each sample based on their different morphologies and 501 isolates were identified as *E. coli* using biochemical test. Out of 501 *E. coli*only 350 were unique based on antibiogram pattern. Isolates from a sample with the same sensitivity pattern are taken to be same strain but if their sensitivity pattern differs then it is considered as different strains (unique isolates). The age of the subjects range from 16 years to 27 years and the mean age was 20.7. The sex distribution of the subjects were as follow:53.8% of the subjects were female and 46.2% were male.

4.2.RESISTANCE PROFILE OF ISOLATES

All *E. coli* isolates (350) were tested against 11 antibiotics. The highest resistance rate was demonstrated totetracycline (n= 317, 90.6%) and least resistance to meropenem (n= 10, 2.8%) as shown in Figure 4.1.

Generally, 347 (99.1%) of all 350 isolates were resistant to at least one of the antimicrobial agents tested. Only three isolates were susceptible to all antimicrobial agents. The percentage of isolates that is resistance to at least six drug is 32.5% as shown in Figure 4.2.

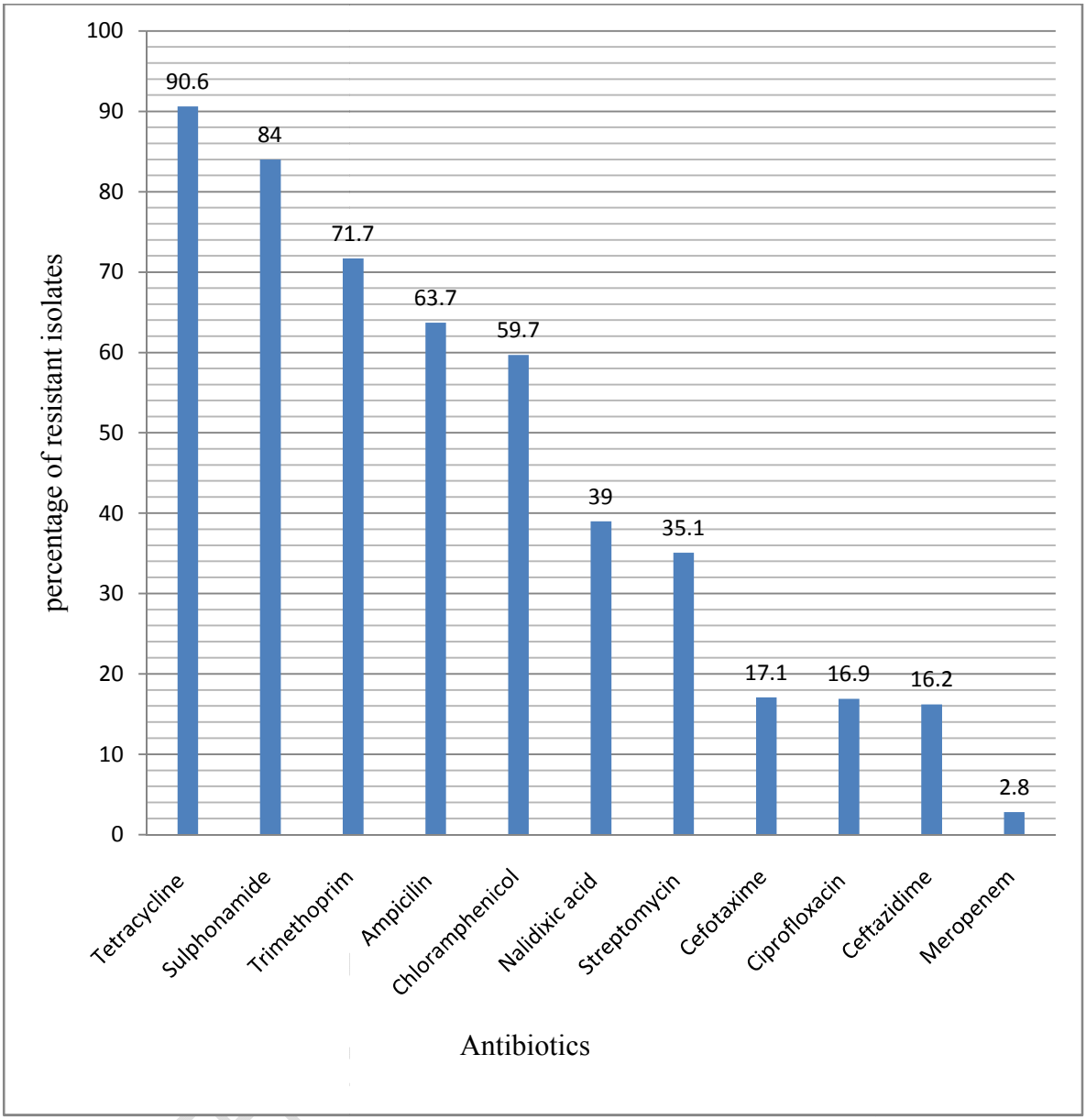

Fig 4.1.: Percentage Resistance of *E. coli* Isolates to Antibacterial Agents

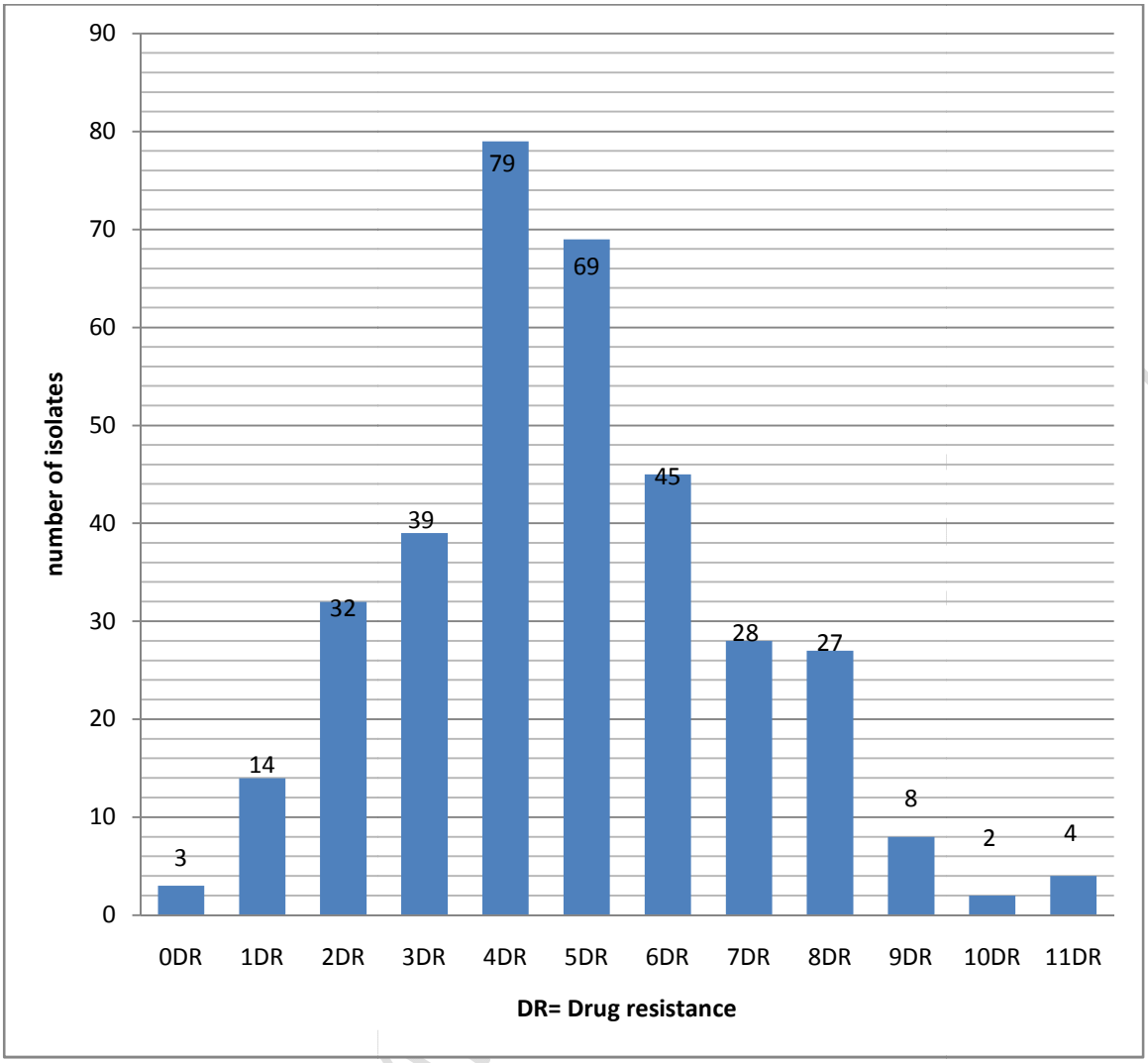

Fig 4.2.: Resistance to multiple classes of antibiotics

4.3. EXTENDED SPECTRUM BETA LACTAMASES (ESBL) PRODUCTION IN THE ISOLATES

Out of 350 isolates, 60(17.1%) isolates were found to be resistant to one or both of the third generation cephalosporin. Of the 60 isolates, only 20 (33%) isolates were found to be producers of extended spectrum beta lactamase (ESBL) and of these, only 18 isolates were from 18 different persons. This means 18 isolates out of 350 isolates (5.71%).

4.4. *KLEBSIELLA PNEUMONIAE* CARBAPENEMASES (KPC) AND METALLO BETA LACTAMASES (MBL) PRODUCTION IN ISOLATES

Out of the 350 isolates, only 10 (2.9%) isolates from 10 different persons were resistant to meropenem and out of these isolates, it was found out that 3 isolates(from three persons) were producer of MBL (NDM) and KPC. An isolate was a producer of NDM.

4.5.GENOTYPIC CONFIRMATION OF RESISTANCE GENES

Among the 20 isolates positive to ESBLs phenotypic screening, 18 were positive with multiplex PCR to one or more tested genes. 3 of the isolates harbored bla_{TEM} only , 1 of the isolates harbored *bla* SHV only, 3 of the isolates harbored bla_{TEM} an d bla_{SHV}, 4 of the isolates harbored bla_{TEM} and $bla_{\text{CTX-M}}$, 1 of the isolates harbored *bla* SHV and $bla_{\text{CTX-M}}$ and 6 of the isolates harbored bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$.Out of the five isolates positive to carbapenemases, 1of the isolates harbored bla_{NDM} while 3 of the isolates harbored bla_{KPC} and bla_{NDM}(Plates 4.1.and 4.2.)

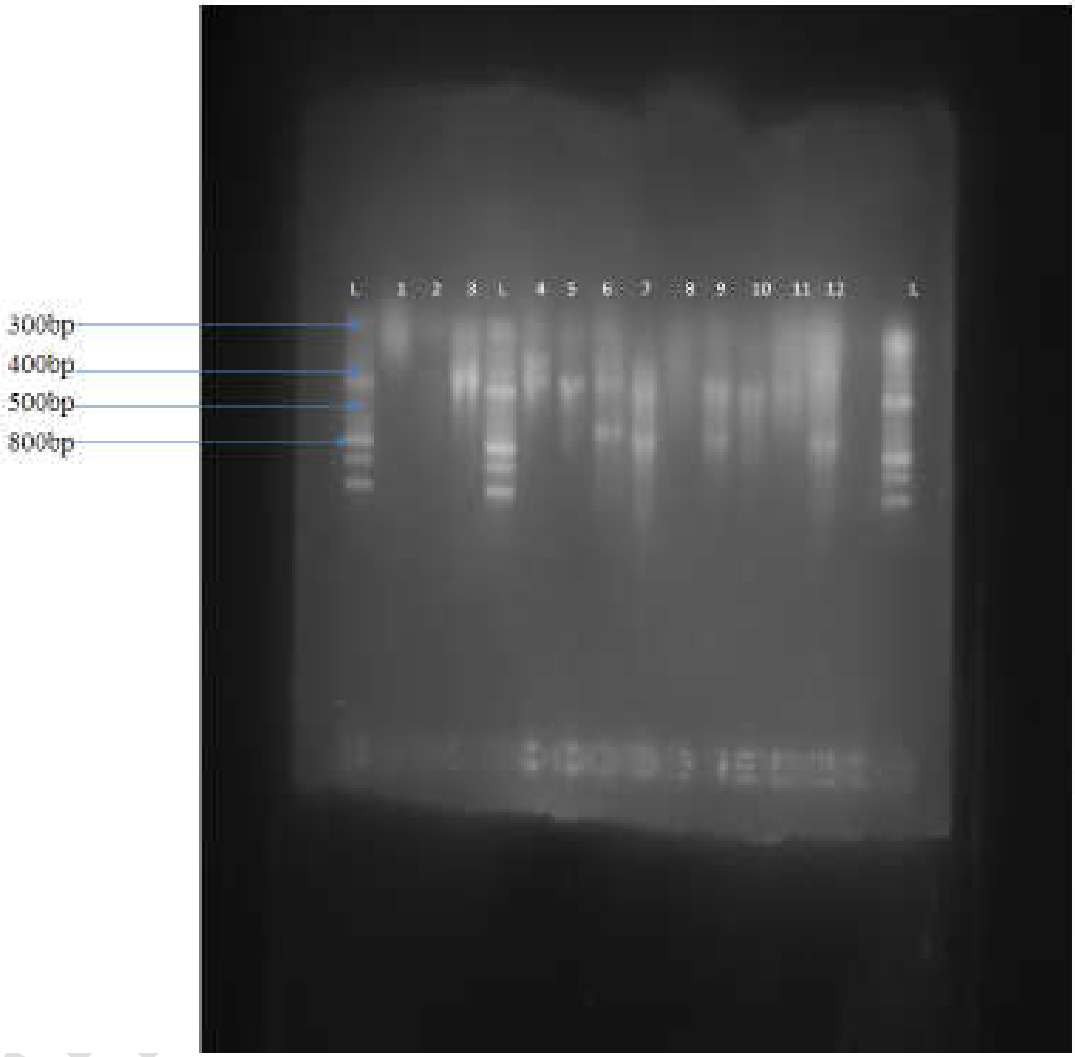

Plate 4.1.: Detection of ESBL genes by PCR: Lane 1 is SHV; Lanes 3, 11 are SHV &TEM; Lanes 4,5 &10 are TEM;Lanes 6 & 12 are CTX-M, TEM, SHV; Lanes 7 & 9 are TEM, CTX-M

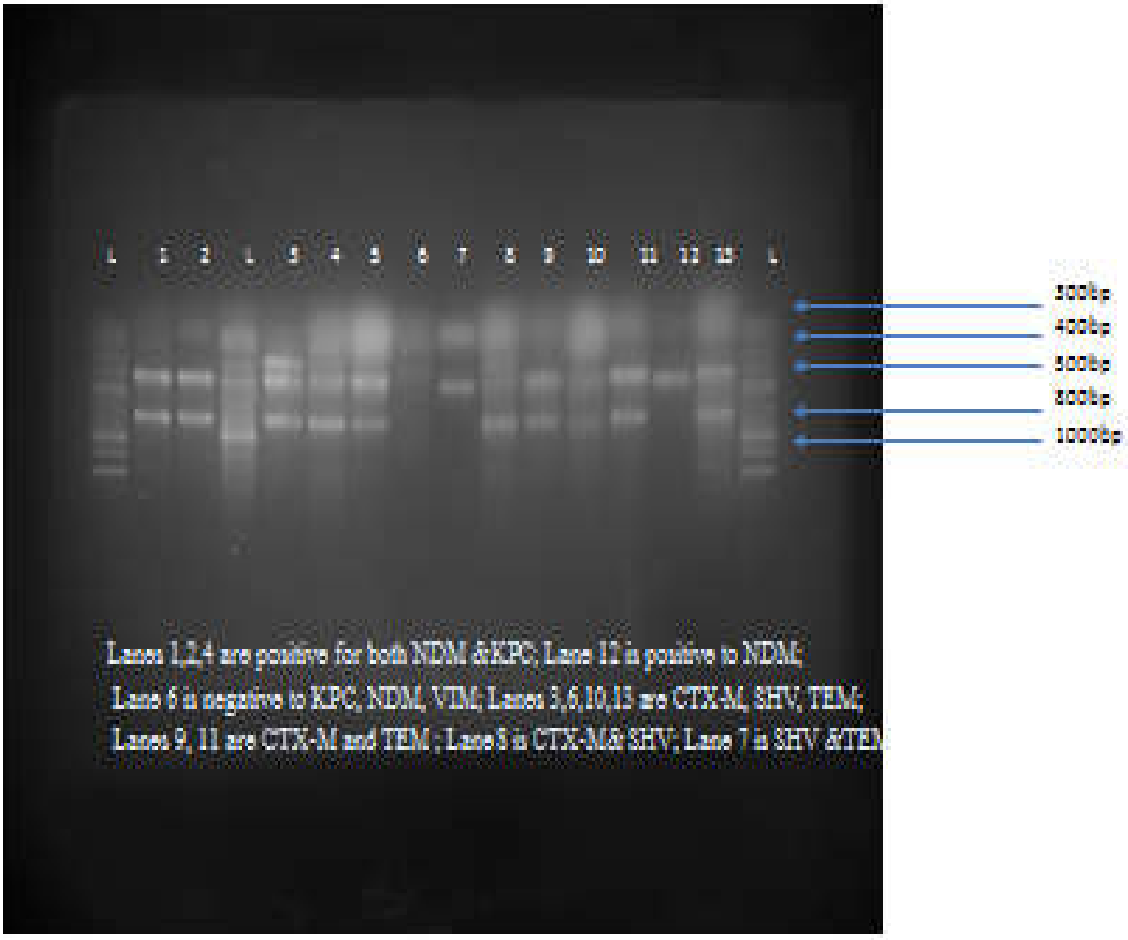

Plate 4. 2: Detection of carbapenemase genes by PCR: Lanes 1,2,4 are positive for both NDM &KPC; Lane 12 is positive to NDM; Lane 6 is negative to KPC, NDM, VIM; Lanes 3,6,10,13 are CTX-M, SHV, TEM; Lanes 9, 11 are CTX-M and TEM; Lane 8 is CTX-M& SHV; Lane 7 is SHV &TEM

4.6. RISK FACTOR ASSOCIATED WITH CARRIAGE OF ANTIBIOTIC RESISTANCE.

Certain risk factors were assessed for association with the carriage of resistant faecal *E. coli*. These factors include: self-medication behaviour of respondent, adherence to antibiotics, hygiene habits and knowledge about what causes antibiotic resistance.

Self-treatment and source of drinking water were significantly associated (p=0.036, 0.008 respectively) with the carriage ESBL producing *E. coli* as shown in Table 4.1.

From Table 4.2., it was shown that none of the risk factors was associated with the carriage of carbapenemases producing *E. coli*

Table 4.3.Showedthat self-treatment, knowledge about causes of antimicrobial resistance, previous exposure to antibiotics were significantly associated with the carriage of multi-drug resistant *E. coli.*

4.6. Limitations of the Study

Resource limitations hindered identification of the isolates to subspecies level and beyond. Also, all resistance genes were systematically not amplified. In spite of these limitations, the results of the study imply that it has yielded necessary information on the local ESBL and carbapenemase prevalence.

Table 4.1.: Risk factor associated with carriage of ESBL producing *E. coli.*

Risk factors	ESBL	Non ESBL	X^2	P
Sex			0.704	0.401
F	9	92		
M	11	76		
Self-treatment			4.382	0.036
Yes	16	107		
No	4	61		
Knowledge about causes AMR			0.735	0.391
	8	80		
Yes	12	88		
No				
Change of A.B dose			1.767	0.413
Yes	15	111		
No	5	57		
Previous exposure to AB			1.043	0.307
Yes	14	112		
No	6	56		
Frequent handwash			0.389	0.533
Yes	13	126		
No	7	42		
Source of water at home			9.571	0.008
well	8	30		
sachet/bottle	7	121		
rain	5	17		

Key

A.B = Antibiotics

AMR = Amtimicrobial resistance

Table 4.2.: Risk factor associated with carriage of carbapenemases producing *E. coli.*

Risk Factor	carbapenemase	Non carbapenemase	P
Sex			1.000
F	3(60)	97(54)	
M	2(40)	84(46)	
Self-treatment			0.876
Yes	4(80)	118(65)	
No	1(20)	163(35)	
Knowledge about causes AMR			0.329
Yes	4(80)	86(47)	
No	1(20)	95(53)	
Previous exposure to AB			
Yes	4(80)	123(68)	
No	1(20)	58(32)	
Frequent hand wash			0.769
Yes	3(60)	135(75)	
No	2(40)	46(25)	
Source of water at home			1.000
Well	4(80)	121(67)	
sachet/ bottle	1(20)	24(22)	
rain	0	20(11)	

Key

A.B = Antibiotics

Table 4.3.: Risk factor associated with carriage of MDR *E. coli.*

Risk Factor	MDR	Non MDR	X^2	P
Sex			1.124	0.289
F	84	16		
M	67	19		
Self-treatment			4.505	0.034
Yes	106	18		
No	45	17		
Knowledge about causes AMR			4.791	0.029
Yes	64	22		
No	87	13		
Change of A.B dose			3.809	0.149
Yes	110	20		
No	41	15		
Previous exposure to AB			3.898	0.048
Yes	108	19		
No	43	16		
Frequent hand wash			0.196	0.658
Yes	111	27		
No	40	8		
Source of water at home			2.687	0.261
well	35	5		
sachet/ bottle	102	24		
rain	14	6		

Key

A.B = Antibiotics

AMR = Amtimicrobial resistance

MDR = Multi-drug resistance

CHAPTER FIVE

DISCUSSION

This study determined the prevalence of antibiotic-resistant *E. coli,* their ESBL and carbapenamase production and the risk factors associated with the carriage of drug resistant *Escherichia coli* among apparently healthy, undergraduate student of Obafemi Awolowo University, Ile Ife, Osun State.

The normal faecal flora particularly, *E. coli,* are beneficial but can be problematic if they carry antibiotic resistance genes. They have been shown to be a potential reservoir of antibiotic resistance genes which can be transferred from non-pathogenic commensals to virulent microorganisms. *E. coli*, an important part of the faecal flora is associated with varying humans' infections ranging from urinary tract infection to bacteremia and it has been shown that it is the main carrier of antimicrobial resistance genes among the faecal flora (Infante *et al.,* 2005).

This study revealed that the commensal*E. coli* isolates in the healthy students were highly resistant to tetracycline, sulphonamides, trimethoprim and ampicilin (90.6%, 84%, 71.7%, and

63.7% respectively). The high rates of resistance to these drugs, which are among the older generations of antibiotics, might be due to selective pressure from their excessive and inappropriate uses in environmental setting (often due to the fact that they are inexpensive and readily available as first-line antibiotics for treatment of genitourinary tract, wound, and bloodstream infections); and this is principally among other factors for the ever increasing prevalence of antibiotic resistantbacteria. In relation to study carried out by Opintan *et al*. (2015), the resistance rate of the isolates in this study were similarly high. The generally high rates of resistance lend credence to the widely shared finding that the range of therapeutic options is growing narrower.

The isolates were least resistant to meropenem (2.8%) which is drug of last resort, and not easily available to patients, also, it is only prescribed when there is pronounced treatment failure, thus its effectiveness. With this low rate of resistance seen to meropenem in apparently healthy individual is nothing but a reflection that carbapenems are gradually losing their effectiveness against microorganism. These faecal strains can serve as reservoir to pathogens in the spread of resistance. Also, *E. coli* are known to be one of the leading cause of primary and opportunistic infections in human. Hence, they can be incriminated in virtually any type of infectious disease.

The results showed that the prevalence of isolates resistant to at least 6 drugs out of all the drugs tested is within the high range reported previously by Oyedeji *et al.* (2011). In a previous study carried out by Okeke *et al.* (2000), prevalence of isolatesresistant to at least six different antibiotics has been increasing among healthy undergraduate students. In their studies, the percentage of isolates that were resistant to at least six drugs as at 1986, 1994, 1996 and 1998 were 1.6%, 0%, 3.2% and 15.9%respectively and it is 32.5% in this present study. There was a little increase in the multidrug resistance from 1986 to 1996, but within 1996 to 1998 there was a

sharp increase. Data were not available for the studies carried out in 2005 and 2009. However, in the last two decades the rates of multidrug resistance have increased dramatically to 32.5 % (χ^2= 23.591; p=0.001).

Of 350 isolates, 20 (5.71 %) isolates were found to be producing ESBL.This does not fall within the report from countries where incidence of ESBL-producers varies between 30 and 55% (Daset *et al.,* 1999). Also, studies carried out in different part of Nigeria have shown that there is variation in the prevalence of ESBL (Aibinu*et al*., 2003; Omoregie *et al*., 2010 and Ogbolu *et al*., 2011). However, it agreeswith the studies of Bartoloni *et al.* (2006), Andre Birgy *et al.* (2012); Nicolas-Chanoine *et al.* (2012); Ogbolu *et al.,* 2013 and Angela *et al.* (2015). According to the study of Garrido *et al.* (2014) the prevalence of ESBL was found to be 0.6% in outpatient where as it was 5% in-patient. This low rate of isolates producing ESBL observed in this study may not be unconnected with the fact that the strains are commensals in view of their origin, though they may cause various types of infection in other sites.

Of the 20 isolates producing ESBL phenotypically, only 18 (90%) were able to be typed to a particular ESBL gene.The results of this study disagree with many studies all over the world that reported *bla*$_{\text{CTX-M}}$ as the most predominant gene among the ESBL-producing *E. coli* isolates from apparently healthy subjects and clinical isolates (Bonnet, 2004; Vaida *et al.,* 2010; Isendahl *et al.,* 2012; Lonchel *et al.,* 2012; Nicholas *et al.,* 2013). Livermore *et al*. (2007) also reported that several studies in Europe and Asia revealed that CTX-M has taken precedence from SHV and TEM. In this study TEM (88.9%) is the most prevalent followed by equal dissemination percentage of SHV and CTX-M. This agrees with the study done in South- West, Nigeria by Egbebi and Famurewa (2011). Aibunu *et al.* (2003) found only SHV and TEM with no CTX-M in their study. Although, within the same locality, Aboderin *et al.* (2012) found CTX-M-15 in

*Klebsiella pneumoniae isolated from an*eight-year-old girl with prolonged and uncontrolled fever.

In this study, 77.8% of the isolates positive to ESBL genes possessed multiple ESBL genes which is similar to the findings of Goyal *et al.* (2009); Bali *et al.* (2010) and Mohammed *et al.* (2016). Other isolates that had none of the genes tested showed the presence of other ESBL genes other than the tested genes and this is also similar to the findings of Mohammed *et al.* (2016).

The result of this study revealed that isolates producing ESBL are also multidrug resistant organism being resistant to four different classes of antibiotics (chloramphenicol, streptomycin, ciprofloxacin, cefotaxime and ceftazidime). This is similar to the study of Chayakulkeeree *et al.* (2003). Hence, this could pose a serious treatment failure, if the isolate becomes an opportunistic pathogen as these drugs are no longer effective in the treatment of infection caused by ESBL producing *E. coli*. The existence of faecal carriage of ESBL genes has implication clinically, as intestinal tract colonization is pre-requisite for infection by ESBLs- producing organisms. However, the route by which community infections arise is less clear. According to Soge *et al.* (2006), many patients with 'community' infection caused by ESBL producing *E. coli* have history of hospitalization, where they may have been colonized. Not all colonized individuals have a history of hospitalization and this may implies that low-level gut colonization occurs in the community, via food chain.

This study shows that out of 350 isolates only five (1.4%) isolates were carbapenemase producing *E. coli* which is similar to the findings of Mariana *et al.* (2011) and Webb *et al.* (2016). This low percentage might be due to low exposure to the carbapenems class of

antibiotics as it is not readily available. Out of the five (5) isolates, only four (80%) were positive genotypically to the carbapenemase genes tested. Interestingly, three (60%) out of the five isolates have both NDM and KPC co-existing which is similar to the findings of Okoche *et al* (2015). However, the presence of the carbapenemase in healthy individual forecast a grave outlook for antimicrobial options and access in this resources-constraint setting. If caution is not taken the meropenem will lose its effectiveness just as other first generation antibiotics did and there will be no antibiotic left to treat infections. Interestingly, all the isolates that are producing either ESBLs or carbapenemases are multidrug resistance..

The presence of ESBL- and carbapenemase- producing *E. coli* in the apparently healthy subject will probably represent a major health problem in the nearest future because the community is the immediate environment for the hospital. Large unrecognized reserviour of apparently healthy individual especially, the study population may spread antibiotic resistance to their peers.

In the risk factor analysis, students that do self-treat themselves were more likely to have multidrug resistant and ESBL producing *E. coli* (χ^2=4.505; p =0.034 and χ^2=4.382; p =0.036 respectively). Also, knowledge about causes of antibiotic resistance and previous exposure to antibiotics showed significant association with the carriage of multidrug resistant isolates (χ^2= 4.791; p =0.029 and χ^2= 3.898; p =0.048 respectively). Whereas, source of drinking water back at home (χ^2= 9.571; 0.008): Well, Bottle/ Sachet rain (p= 0.02, 0.01, 0.05 respectively). The result of this study infers that irrespective of source of potable water, carriage of ESBL producing isolates is possible.There was no association found between ESBL producing isolates and sex of the subjects (χ^2=0.704; p =0.401). This shows that the carriage of ESBL is not gender based and both genders should be screened for ESBL production if the condition warrants that.

There was no association found between carbapenemase producing isolates and any of the tested possible risk factors

CONCLUSION AND RECOMMENDATIONS

In conclusion, high rate of antibiotic resistance among commensal *E. coli* to most of the older, less expensive antimicrobial drugs used in the management of infections in Nigeria has been observed. Not only are these strains potential causes of infection, but they are also potential reservoirs of resistance genes that could be transferred to pathogens. For this reason, the rate of resistance seen with commensal *E. coli* may also occur with pathogenic organisms. The data suggest that meropenem and possibly ciprofloxacin and third generation cephalosporin may be useful in treating infections caused by pathogenic *E. coli* and other related bacteria in Nigeria. The future usefulness of these drugs will, however, depend on effective interventions to halt the selection and spread of resistance among enteric organisms.

RECOMMENDATION

- Tomonitor commensal organisms as well as pathogens by susceptibility testing to guide treatment.
- To inform the study area of the importance of personal hygiene to the spread of antibiotic resistance microorganisms; as well as to influence the proper attitude to the consumption of antimicrobials.
- To advocate for the role of antibiotics restriction in control of antibiotic resistance
- To inform the study area about what aggravate the emergence and spread of antibiotic resistance and the outcome of this menace
- To establish routine antibiotic resistance surveillance which is needed to conserve the usefulness of the remaining drugs.

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APPENDIX

A.1 QUESTIONNAIRE FOR RESEARCH

OBAFEMI AWOLOWO UNIVERSITY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND PARASITOLOGY

Note: All information will be treated with high confidentiality

A BIODATA

1. Age …….

2. Sex A. Male B. Female

B SELF-MEDICATION BEHAVIORS

3. Have you ever taken antibiotics before? A. yes B No

4. Have you ever treated yourself (self-medicated) with antibiotics? A. Yes B. No

5. How many times did you treat yourself with antibiotics in the past one year? …….

6. Your selection of antibiotics was based on… *(Check more than one if applicable)*

A. Recommendation by Doctor B. Opinion of family members C. Opinion of friends D. My own experience E. Recommendation by road side drug seller

F. Previous doctor's prescription G. The advertisement

7. Where did you usually obtain antibiotics from? *(Check more than one if applicable)*

A. pharmacies B. road side seller C. Leftover from previous prescription

D. left over from friend

F. Others (specify)……..

8. Did you ever change the dosage of antibiotics deliberately during the course of self-treatment? A. Yes, always B. Yes, sometimes C. Never

9. Why did you change the dosage of antibiotics during the course of treatment? *(Check more than one if applicable)* A. Improving conditions B. Worsening conditions

C. To reduce adverse reactions D. Drug insufficient for complete treatment E. Others (specify)

10. Did you ever stop or switch antibiotics during the course of treatment? A. Yes, I stop B. Yes, I switch C. Never

11. Why did you switch antibiotics during the course of treatment? *(Check more than one if applicable)* A. The former antibiotics did not work B. The former antibiotics ran out C. The latter one was cheaper D. To reduce adverse reactions

E. Others (specify)

If option A of question 11 above holds true in your case, please answer questions 12-14

12. Who advised you to switch antibiotics? A. A Doctor B. Self

13. How many antibiotics were you given and against what disease? …….

14. Mention the antibiotics

15. Do you know the factors that causes antibiotic resistance A Yes B No

16. List the factors

C. HABITS

14. Do you share utensils? A Yes B No

15. Do you often wash your hand? A yes B No

16. What is your source of drinking water A. pure water B dam water C rainwater?

17. Do you normally visit restaurant? A yes B No

A2. NUTRIENT AGAR

Beef extract	10.0
Peptone	5.0
Sodium chloride	8.0
Agar	12.0

Preparation

28 grams of powder was weighed and suspended in 1000ml of distilled water. The resulting suspension was swirled and heated to attain homogeneity and sterilized by autoclaving for 15 minutes at 121ºC (and 15 psi). The medium was allowed to cooled to about 45ºC and mixed well before pouring into sterile Petri dishes.

A3. Mueller Hinton Agar

Beef extract	2.0
Acid hydrolysate of casein	17.5
Starch	1.5

Agar 17.0

pH 7.3+0.1

Preparation

38 grams of powder was weighed and suspended in 1 litre of sterilized water. The resulting suspension was swirled gently to mix well and sterilized by autoclaving for 15 minutes at 121ºC (and 15 psi). The mixture was allowed to cool and mixed well before pouring into sterile Petri dishes.

A4. Eosin Methylene Blue Agar

Peptone 10.0

Lactose	5.0
Sucrose	5.0
Dipotassium phosphate	2.0
Agar	13.5
Eosin Y	0.4
Methylene Blue	0.065
Distilled water to	1L

Final pH = 7.2

Preparation

40 grams of powder was weighed and suspended in 1 litre of sterilized water. The resulting suspension was swirled gently to mix well and sterilized by autoclaving for 15 minutes at 121ºC (and 15 psi). The mixture was allowed to cool and mixed well before pouring into sterile Petri dishes.