MOLECULAR CHARACTERISATION OF MULTIDRUG-RESISTANT *Pseudomonas aeruginosa* IN SOUTHWESTERN NIGERIA.

BY

BAMIDELE TOLULOPE, ODUMOSU

B.Sc Microbiology (OOU), M.Sc. Pharmaceutical Microbiology (Ibadan)

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CERTIFICATION

I certify that this project was carried out by Mr B.T. Odumosu under my supervision in the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria.

Supervisor
Bola A. Adeniyi,
B.Sc, M.Sc (Ilorin), Ph.D. (Ibadan)
Professor, Department of Pharmaceutical Microbiology
University of Ibadan, Ibadan, Nigeria.

DEDICATION

This research work is dedicated to Almighty God, the God of Jacob who answers me in times of my distress and has been with me wherever I have gone. To you alone is glory.

And to the loving memory of my beloved mother, Florence Titilayo Adunni Odumosu, continue to rest in the bosom of our dear Lord (amen). Mummy how I wish you were here!

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ABSTRACT

Multidrug-Resistant (MDR) associated nosocomial infection is a global problem resulting in treatment failure particularly with respect to *Pseudomonas aeruginosa*. There is paucity of information on the molecular mechanisms of multidrug-resistant *P. aeruginosa* in Nigeria. Knowledge of the genetic basis of resistance of the organism to available antimicrobial agents will further improve empirical treatment. This study was undertaken to genetically characterise the multiple antibiotic resistance determinants in *P. aeruginosa* and describe the genetic locations of the resistance genes.

Antimicrobial susceptibility of 54 clinical isolates of *P. aeruginosa* obtained from 5 hospitals in 3 southwestern states of Nigeria, to 21 antibiotics representing nine classes of antimicrobial agents was determined using the antibiotic disk-diffusion method. Minimum inhibitory concentrations were determined by Etest. Plasmid DNA for the isolates were extracted by alkaline lysis while plasmid curing was carried out using acridine orange, ethidium bromide and sodium dodecyl sulphate. The Extended-Spectrum Beta-Lactamase (ESBL) phenotypic detection was carried out using double-disk synergy method. Twenty clinical isolates with resistance to more than three "anti-pseudomonas" drugs were randomly selected for molecular studies. Genetic characterisation of ESBL and other drug resistant genes were achieved by polymerase chain reaction with specifically designed primers and direct sequencing of the amplicons. Significant trends in the association of plasmid counts and antimicrobial resistance among *P. aeruginosa* strains was achieved using Fisher's Exact Test.

All the strains of *P. aeruginosa* were found to be resistant to ampicillin, tetracycline and amoxicillin-clavulanic acid, while 53.7%, 63.0%, 79.6% and 87.0% were resistant to ceftriaxone, carbenicillin, kanamycin, and ticarcillin-clavulanic acid respectively. Plasmid profile of the 54 isolates revealed the presence of 1-4 resistance plasmids varying in sizes from 2.3 to 210.0 kb. Highest curing activity was achieved with ethidium bromide on 81% of the isolates at 40 µg/mL. The MDR *P. aeruginosa* strains harboured significantly more plasmids (≥3) compared to their non-MDR counterparts, which carried < 2 plasmids ($p<0.01$). Out of the 20 isolates randomly selected for molecular studies, 80% harboured $bla_{\text{OXA-10}}$ that were plasmid encoded. Chromosomally encoded AmpC *β*-lactamase was found in 85%, while bla_{SHV} and $bla_{\text{CTXM-1}}$ were detected in one isolate each. Efflux pump regulators: *mexR* and *nfxB* were found in 45%, *aac (6') – I* was detected in 50% and *ant (2") – IV* in 45% while both genes coding for aminoglycoside modifying enzymes were harboured

in 35%. The class 1 integrons harbouring gene cassette array *aaA6-orfD* and *aaA13*, were also detected in the chromosomes of the isolates.

The presence of resistance plasmids, class 1 integrons, extended-spectrum beta-lactamase, aminoglycoside modifying enzymes and efflux pump regulator genes among the population of *P. aeruginosa* tested indicated a high prevalence of multidrug resistance.

Keywords: Multidrug-resistance *Pseudomonas aeruginosa*, Extended-spectrum beta-lactamase, Class 1 integrons.

Word count: 433

LIST OF MAIN ABBREVIATIONS

Abbreviation	Full meaning
AACs	Aminoglycoside acetyltransferases
AG	Aminoglycosides
AME	Aminoglycoside modifying enzymes
AmpC	AmpC cephalosporinase beta lactamase
ANTs	Aminoglycoside nucleotidytransferases
APHs	Aminoglycoside phosphotransferases
AST	Antibiotic sensitivity test.
ATCC	American Type Culture Collection
attI	Receptor site
BSA	Bovine serum albumin
CAUTI	Catheter associated urinary tract infection
CDC	Centre for Disease Control and Prevention
CFU	Colony forming unit
CLSI	Clinical and laboratory Standards Institute
CTX-M	Cefotaximase
CV-I	Crystal violet complex
dATP	2'-deoxyadenosine 5'-triphosphate
DDH_2O	Double distilled water
DDST	Double disk synergy test
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
E.coli	Escherichia coli
EDTA	Ethylenediametetraacetic acid.
ESBL	Extended Spectrum β-lactamase
Etbr	Ethidium bromide
FQ	Fluoroquinolone
H_2O	Water

ICU	Intensive care unit
intI	Integrase gene
IS	Insertion sequence
Kb	Kilo base
KOH	Potassium hydroxide
LB	Luria Bertani
MDR	multidrug resistance
MDR	Multiple drug resistant
mexR	Efflux pump regulator gene
$MgCl_2$	Magnessium hydroxide
MHA	Mueller Hinton agar
MIC	Minumum Inhibitory Concentration
MLSK	Macrolides-Lincosamides-Streptogramin-Ketolides
MTCC	Microbial type culture collection.
NAOH	Sodium hydroxide
NDM	New Delhi metallo-β-lactamase
NER	Non enzymatic resistance
nfxB	Efflux pump regulator gene
Omp	Outer membrane protein
OprD	Outer membrane porin
ORF	Open reading frame
ori	Origin
OXA	Oxacillinase
OXA	Oxacillinase
P. aeruginosa	*Pseudomonas aeruginosa*
parC,ParE	Topoisomerase genes
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate.
PER	Pseudomonas extended resistance
Qnr	Plasmid-mediated quinolone resistance
QRDR	Quinolone resistance determining region
QRDR	Quinolone resistant determining region

R Plasmid	Resistance plasmid
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic Acid/
SDS	Sodium Dodecyl Sulphate
SHV	Sulfhydryl variable
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
TEM	Temoniera
Tm	Temperature
UTIs	Unrinary tract infections
UV	Ultra violet
VEB	Vietnamese extended-spectrum beta-lactamase
WHO	World health organization.

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CHAPTER ONE

INTRODUCTION

1.1. Nosocomial Infections

Nosocomial infections are one of the increasing and persistent wide spread disease condition causing a significant problem throughout the world (Weinstein, 1998; Alvarado, 2000). Nosocomial infection is an infection that becomes clinically evident after 48 hours of admission in the hospital or after discharge and is also referred to as hospital-acquired infections (Coffin and Zaoutis, 2008). Nosocomial infection can manifest in several types of disease irrespective of age or sex and it include urinary tract infections, pneumonia, skin and mucous membrane infections, respiratory infections, surgical site infections, blood stream infection (Polack, 2010). The Centers for Disease Control and Prevention (CDC) and other reports estimated a roughly 1.7 million hospital-associated infections, from all types of bacteria combined, causing or contributing to 99,000 deaths each year with the annual cost ranging from $4.5 to $11 billion in the United States alone (Polack, 2010). In United Kingdom estimate was 10% infection rate (Aodhán , 2005), 5.6% was reported from France in 2001(Lepoutre *et al*., 2005), an estimate of 8.5% in Finland (Lyytikainen *et al*.,2005), while a survey in Italy gave a 4.9% rate in 2000 (Liziolia *et al*., 2003). The reported rates of nosocomial infections in developing countries are even higher (Hughes *et al*; 2005; Joshi *et al* 2006). In Nigeria, nosocomial infections due to pathogenic bacteria have also been reported (Oguntibeju and Nwobu, 2004; Adeniyi *et al*., 2006; Nwachukwu *et al*., 2009; Ohieku *et al*., 2010) but incidence rate and outbreaks has not been documented. The impact of nosocomial infections with clinical implications is more glaring in developing countries and most especially among those infected with HIV/AIDS due to transmission of the infection through unsafe medical equipment and treatments (Nyamogoba and Obala , 2002).

1.2 Antimicrobial Resistance

Infections caused by microorganisms have always been the major reason for disease conditions in human history. With the introduction of antibiotics in 1940s there seems to be hope for the treatment of common microbial infections until an evolution in the resistance of bacteria to antibiotics became prominent (CDC, 2010). This rapid emergence of antimicrobial resistance among bacteria has not only become a public health concern but has also resulted

into increase in morbidity and mortality rate with increase in cost of health care and treatments (Holmberg *et al*., 1987; Cosgrove and Carmeli, 2003; Maragakis *et al*., 2008).

The eventual appearance of strains simultaneously resistant to multiple antibiotics significantly worsened the problem (Clewell, 2008). There was complacency in the early '80s because pharmaceutical companies were not working on improvement and introduction of new antibacterial agents. They were concentrating on other aspect, such as viral infections, in the meantime, resistance by bacteria to a number of commonly used antibiotics increased (Ricki, 1995). Possibly no other factor is of more importance in the development of antimicrobial resistance than antimicrobial misuse in our environments. Researchers have established that increasing resistance to antimicrobial drug is associated with selective pressure of antibiotics use, poor drug quality, adulteration and inadequate surveillance system (Okeke *et al*., 1999; Okeke *et al*., 2005).

The challenges associated with rising development of drug resistance is mostly observed in developing countries such as Nigeria, Cameroon, Bangladesh, India etc, because there are no adequate surveillance of antimicrobial resistance (Okeke *et al*., 2005). Moreover the conditions worsened due to increase in use and misuse of antibiotics by skilled and unskilled health professionals, sales and unrestricted access to antibiotics over the counter and other commercial centers (Okeke *et al*., 2005). Thus therapeutic failure in such places remains a perpetual challenge because of microorganisms abilities to resist several antimicrobial agents, hence the spread and persistent infectious disease conditions becomes a prominent circumstances.

1.3. Role of *P. aeruginosa* in Nosocomial Infections

Infections caused by *P. aeruginosa* is no longer a new global incidence as reports from around the continents have attested to the disease causing abilities of this non- fermenting Gram negative bacteria. Despite significant clinical changes in the spectrum of organisms causing hospital-acquired infections worldwide, *P. aeruginosa* has persistently remained nearly unchanged in position among the top ranked nosocomial agents during the last four decades (NNIS, 2004, Trautmann *et al*., 2005). Its multiple drug resistance abilities have also been well documented (Juan *et al*., 2005; Poole, 2005; Okonko *et al*., 2009; Hemalatha and Dhasarathan, 2010). In spite of the improvement in healthcare sector in Nigeria and introduction of wide range of antimicrobial drugs against infectious agents, *P. aeruginosa* increasing pathogenicity has continued to cause complications in Nigerian hospitals (Olayinka, 2004; Aibinu *et al*., 2007). This bacterium has been reported to contribute

immensely to wound related morbidity and mortality in Nigeria (Kehinde *et al*., 2004; Oguntibeju and Nwobu 2004). However, in Nigeria little is known about its genetic basis of multidrug resistance (MDR) to available antibiotics.

Pseudomonas aeruginosa is associated with high morbidity and mortality in immunocompromised patients and especially among those admitted in intensive care unit with wound injuries. These infections are often severe and life threatening. They are difficult to treat because of their high rates of resistance to available antimicrobial agents during therapy consequently resulting in longer stay of patients in the hospital and other severe outcomes including cost (Nouér *et al*., 2005). Unfortunately there are no specific measures to prevent nosocomial Pseudomonal infections in Nigeria.

1.4.Molecular Basis of *P. aeruginosa* Resistance

There is paucity of information about the mechanism of transmission and acquisition of resistant genes by *P. aeruginosa* in Nigeria which has led most clinicians to rely on extrapolation of data from other scientific reports from developed nations around the world. Although there has been several reports of phenotypic investigations on the multidrug resistant *P. aeruginosa* isolated from different hospitals in Nigeria [(Oduyebo *et al*., (1997); Oni *et al*. (2002); Brown *et al***.** (2003); Oguntibeju, & Nwobu, (2004); Adeniyi *et al*. (2006); Yah *et al*. (2006); Aibinu *et al*., (2007); Jombo *et al*., (2008); El-Mahmood *et al*., (2009); Okonko *et al*., (2009); Nkang *et al*., (2009); Okesola and Oni (2009); Nwankwo and Shuaibu (2010)]. However, comprehensive information on the genetic basis and identification of resistance mechanisms of *P. aeruginosa* to various antibiotics in Nigeria especially in the southwest is lacking. Given that *P. aeruginosa* possess several intrinsic and acquired mechanisms capable of hydrolyzing all classes of antibiotics available (except very few ones), and is also capable of horizontal gene transfer of newer genes and acquisitions, it is therefore imperative to investigate this pathogen's multidrug resistance to the genetic level. Its increasing rate of resistance to multiple antibiotics in Nigeria necessitates urgent attention and scientific evaluation.

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1.5 Statement of problems

Gram negative bacteria such as *Escherichia coli, Klebsiella Pneumonia, Pseudomonas aeruginosa* and *Acinetobacter. Baumannii* as well as Gram positive bacteria such as *Staphylococcus aureus* are mostly implicated in hospital-acquired infections worldwide. Many of these infections are life threatening, resulting in increased morbidity and mortality rates because of the bacterial resistance nature and ability to quickly acquire newer resistant phenotypes through horizontal gene transfer or by mutation in chromosomally encoded genes (Hocquet *et al*., 2003). Many bacteria harbouring one or more of these novel resistance genes are capable of hydrolyzing several classes of antibiotics and also able to disseminate it among the bacterial population thereby causing increasing rate of multiple drug resistant bacteria. The dissemination of resistance genes among the Gram-negative bacteria in the clinical settings has been previously reported (Peleg *et al.*, 2005). Upsurge and spread of resistance genes among Gram negative bacteria causing infections in Nigeria and other parts of Africa necessitate urgent clinical investigation. In Africa, emergence of resistance genes among Gram-negative bacteria has been documented. Aibinu *et al.* (2003) reported the detection of extended-spectrum *β*-lactamase (ESBL) producing *Enterobacter* spp. from two hospitals in Lagos, Nigeria. Another report documented the detection of CTX-M 15 producing Enterobacteria in Cameroon (Gangoue-Pieboji *et al*., 2005). Similar gene was also reported in uropathogenic *Klebsiella pneumoniae* plasmids in Nigeria (Soge *et al*., 2006). Chouchani (2007) also reported the characterization of TEM-15, extended-spectrum *β*-lactamase-producing *K. pneumoniae* isolate in Tunisia. Recently Aibinu *et al.* (2011) reported the emergence of OXA-10, VEB-1 and CMY *β*-lactamase and mobile genetic elements in *Providencia* spp. in Nigeria. However reports on *P. aeruginosa* genetic acquisition of gene is lacking in these regions.

Frequent detection of ESBL producing bacteria such as *P. aeruginosa* is clinically and epidemiologically important; and data on the current status of ESBL among strains of *P. aeruginosa* will enable effective empirical treatment of infections caused by such strains.

ESBL producing organisms are clinically and epidemiologically significant because they are capable of compromising therapy with cephalosporins and other classes of antibiotics throughout the world. ESBL may confer resistance to ceftazidime, cefotaxime, ceftriaxone, piperacillin and Aztreonam, which are antimicrobial agents widely prescribed for *P. aeruginosa* infections (Nordmann and Guibert, 1998). Routine surveillance of antimicrobial resistance and robust detection of ESBL among strains of *P. aeruginosa* is important to

forestall rapid spread and transfer of resistance and ESBL genes among other nosocomial pathogens. This research study is therefore designed to investigate the level of multiple drug resistance of *P .aeruginosa* in Southwest hospitals in Nigeria and to determine the molecular mechanisms of multidrug resistance of this organism

1.6 Research objectives

1. To determine the antimicrobial susceptibility pattern of *P .aeruginosa* isolates from 5 hospitals in 3 Southwest States of Nigeria.
2. To determine the prevalence of MDR in *P. aeruginosa* from hospitals in three Southwestern states in Nigeria.
3. To phenotypically and genotypically characterize ESBL among the *P. aeruginosa* isolates.
4. To investigate the presence of various mobile genetic elements such as plasmids, integrons and gene cassettes among the *P. aeruginosa* isolates.
5. To detrmine the genetic basis for resistance to aminoglycosides and fluoroquinolones among the *P. aeruginosa* isolates.
6. To determine the mode of transfer of the resistance genes either by transformation or conjugation

CHAPTER TWO

LITERATURE REVIEW

2.1. Nosocomial Infections

Nosocomial or hospital-acquired infections (HAI) have been defined as an infection manifesting in a patient while in an hospital or other health care facility in whom infection was not present or incubating initially (Bolyard *et al*., 1998). Nosocomial infections are classified into thirteen major categories viz. urinary tract infection (UTI), surgical site infections (SSI), blood stream infection (BSI), pneumonia, bone and joint infection (BJ), gastro intestinal infection (GI), central nervous system infection (CNS), reproductive tract infection (RTI), cardio vascular infection (CVI), ear, eye, nose and throat infection (EENT), skin and soft tissue infection (SST), systemic infection (SI), lower respiration tract infection (LRT) and other body sites (Burke, 2003; Pollack, 2010). The four most common types of these infections are urinary infections, surgical site infection, nosocomial pneumonia, and nosocomial bacteremia which are usually associated with Gram-negative bacteria (Weinstein *et al*., 2004).

2.1.1. Classification of Nosocomial Infections

2.1.1.1. Urinary Tract Infection

Urinary tract infection is defined as the symptomatic presence of microbial pathogen within the urinary tract, usually the kidney, urethra, urinary bladder, tissue surrounded the retro-peritoneal and peri-nephric spaces (Garner *et al*., 1996; French, 2006). Urinary tract infection can also be defined as a positive urine culture with at least 10^5 bacteria/ml, with or without clinical symptoms (Girard, 1990). Although it is generally an infection of all age and sex but considered more common in adult women. At least one woman out of five at age 24 is diagnosed of UTI in her lifetime. The reason is probably due to easy accessibility of the bladder by rectal bacteria to the urethra thereby causing infections (Foster, 2008). A higher percent of these infections are associated with the use of an indwelling catheter and also known as catheter-associated UTIs (CAUTI) (Jacobsen *et al*., 2008). There are asymptomatic infected individuals but most people get at least a symptom such as frequent urge to urinate and a painful burning sensation in the bladder or urethra during urination, milky or bloodied

urine may be passed out. In children there may be an irritation, loss of appetite and lose bowel (Health community 2010).

Several risk factors reported includes; hospitalization, diabetes, female gender, pregnancy, renal insufficiency, long duration of catheter usage, insertion of a urinary catheter late in the hospital stay, and several others (Tietjen *et al*., 2003; Falagas and Kompoti, 2006). Other patients with compromised immunological status including patients who have kidney transplant are also at high risk (Dantas *et al*., 2006). Nosocomial urinary tract infections are commonly caused by Gram-negative pathogens (Weinstein *et al*., 2004), such as *E. coli*, *P. mirabilis, Klebsiella* spp., and *P. aeruginosa*, while other causal pathogens include *S. aureus* (Perl and Golub, 1998), *Enterococci*, *Enterobacter* spp (Gaynes and Edwards, 2005) and Candida (Weinstein *et al*., 2004).

2.1.1.2. Surgical Site Infection

A surgical site infection (SSI) is often indicated by the presence of purulent discharge around the wound or by the presence of cellulites which is coming from the wound. A surgical site infection is considered nosocomial if it occurs within 30 days of the operative procedure (Garner *et al*., 1996). Surgical site infections account for approximately 40% of nosocomial infection and are costly in terms of length of stay, morbidity and mortality, and actual costs (Griffin, 2005; Odom, 2006). There are patient-related and surgery-related risk factors implicated in SSI. Patient-related risk factors includes existing infections, low serum albumin concentration, nutritional status, diabetes mellitus, blood transfusion hypothermia, hypoxia, hyperglycemia or trauma while surgery-related risk are anesthesia score, duration of the operation, the use of drains, and inadequate aseptic technique (Griffin, 2005; Odom, 2006, Cheadle, 2006). Surgical site infections are caused by both Gram positive and Gram negative bacteria and they both arise from endogenous and exogenous transmission. Etiological sources of surgical site infections vary and are according to the type of surgery. The most encountered microorganisms are *S. aureus*, *P. aeruginosa*, coagulase-negative Staphylococci, *Enterococcus* spp., *E. coli*, and *Enterobacter* spp. (Gaynes and Edwards, 2005).

2.1.1.3. Nosocomial Pneumonia

Nosocomial pneumonia is another common hospital-acquired infection which is caused by aspiration of bacteria originating in the upper gastrointestinal tract of the patient leading to infection of the lungs called pneumonia. Nosocomial pneumonia is also associated with substantial morbidity and mortality and accounting for 15% to 20% of all nosocomial infections (Burke, 2003; Tietjen *et al*., 2003 Weinstein, 2004). Pneumonia is a general disease that occurs in all age group but are most common among the critically ill patients (Dodek *et al*., 2004) and higher in patients receiving continuous mechanical ventilation (Kollef , 2005; Davis, 2006), because of the aspiration of gastric secretion and other contaminated fluids into the lower airways (Nseir *et al*., 2002). Symptoms include cold, sneezing, coughing with sputum which could be followed by fever. Etiologic agents of pneumonia differ by hospitals but most infections have been reported to be caused by bacteria most of which are Gram negative bacilli (Jimenez *et al*., 1989; Torres *et al.*, 1990; Pugin *et al.*, 1991). The risk factor is high in intubated patients and those having a long duration of stay in the hospital, its costs in terms of morbidity, mortality, and economy are among the highest for hospital-acquired infections (Sehulster and Chinn, 2003; Kollef , 2005).

2.1.1.4. Blood Stream Infection

Bloodstream infections, such as bacteremia and septicemia, can develop as a result of complication from other types of nosocomial infections or infections may occur at the entry site of the intravascular device or along the path of a catheter (tunnel infection). About half of these infections are caused by intravascular devices, primarily central venous catheters (Weinstein, 2004); the risk is even higher when the catheter is inserted in the jugular vein (Tietjen *et al*., 2003). Bloodstream infections stemming from intravascular devices account for approximately 15% of all nosocomial infections, affecting approximately 1% of all hospitalized patients (Hugonnet *et al.*, 2004; Chen *et al.*, 2006). Blood stream infection is associated with both gram positive and gram negative bacteria, once they contaminate the blood through one or more of the medical devices an infection can easily be established (O'Grady *et al*., 2002; Tietjen *et al*., 2003).

2.1.1.5. Other Nosocomial Infections

There are other important hospital-acquired infections that are commonly encountered with potential site of infections such as skin and soft tissue infections i.e. ulcers, bedsores, which often leads to systemic infection. Gastroenteritis is a common nosocomial infection among children (Brady 1989; Singh, 2003) usually caused by rotavirus. In some developed countries gastroenteritis in adult is caused by *Clostridium difficile* also a nosocomial infection as well as sinusitis and other enteric infection (Barbut, 1996; Johnson *et al*., 1998)

2.2. Diagnosis and Treatment of Nosocomial Infections

The diagnosis and identification of nosocomial infection is carried out by clinical assessment of the patient based on clinical signs and symptoms due to an infection and further interpretation of laboratory findings. Fluid or blood specimen can be collected aseptically, directly or indirectly, from specific site of infection and cultured appropriately for the isolation of the pathogen (Garner *et al*., 1996; Cheesbrough, 2001).

Treatments of hospital-acquired infections are usually after the result of thorough investigations and examinations involving the quantitative culture of specimen and microscopic identification of specific samples (Weinstein, 2005). The commonly administered antimicrobial drug includes β-lactam antibiotics, aminoglycosides, fluoroquinolones, Trimethoprim sulfamethoxazole, polymyxin, chloramphenicol and colistin (Oguntibeju and Nwobu, 2004; Weinstein, 2005).

2.3. Prevention of Nosocomial infections

Most of the deaths caused by nosocomial infections encountered in the hospital could be prevented by following evidence-based guidelines and agreed statements on preventive strategies (Burke, 2003). The main goal of prevention of hospital-acquired infection is to reduce as minimal as possible transmission of etiologic agents from patient to staff personnel and to other patients. Several measures and guidelines are being suggested and laid down to prevent nosocomial infection such as hand washing which is the simplest and easiest form of prevention to other techniques such as disinfections, wearing of safety gloves, aseptic cleaning and sterilization of hospital equipment, reduction of patient per ward (Overcrowding) and adherence to recommended safety practice within the hospitals amongst

other preentive measures highlighted, the importance of hand washing in preventing transmission of infection in the hospital has been emphasized (CDC, 1986; Larson, 1995). Proper hand washing with antiseptic lotions and cleaning of hands with alcohol after attending to individual patients must be carried out always.

Personal hygiene of health care workers must be ensured. There must be a proper decontamination of wears, sterilization of materials and equipment, the use and proper disposal of sterile gloves and other disposable materials like syringes must be carried out to avoid cross contamination from exogenous sources i.e. contamination from the environment and health workers (WHO, 2001; Ducel *et al.*, 2004). Hospital environment must always be kept clean and disinfected (CCDR, 1998). Education and orientation of health care workers and caregivers on basic infection control measures and polices must be adequate, thus providing recent information on antimicrobial resistance and prevention of nosocomial infection (Michalopouls and Sparos, 2003).

2.4. Genus *Pseudomonas*

Members of the genus *Pseudomonas* are rod shaped, Gram-negative bacteria with one or more polar flagella, aerobic, non-spore forming and are catalase positive (Krieg, 1984). Members of this genus include *P. aeruginosa, P. fluorescens, P. putida, P. polycolor* and *P. syringae*. The redefinition of the taxonomy of many bacterial species with the 16S rRNA, has made some strains that are formerly classified in the genera *Chryseomonas* and *Flavimonas*, to be included in the genus *Pseudomonas* while other strains that are previously classified in the genus *Pseudomonas* such as *P. cepacia, P. mallei, P. pseudomallei* have been reclassified in the genera *Aminobacter, Brevundimonas, Burkholderia* and *Ralstonia* (Baumann *et al*., 1983)

There are other characteristics that are associated with the *Pseudomonas* species with exceptions of some strains, these includes the production of pyoverdin (fluorescein) and pyocyanin (bluish-green) siderophore, and thioquinolobactin which are commonly associated with *P. aeruginosa* and *P. fluorescens* (Lau *et al*., 2004). The *Pseudomonas* species are typically oxidase positive, indole negative, methyl red negative, Voges-proskauer test negative, citrate negative non-fermenter and beta hemolytic on blood agar.

2.4.1. *Pseudomonas aeruginosa* as a pathogen

Pseudomonas aeruginosa is a specie of the genus Pseudomonas belonging to the class *Schizomycetes*, order *Eubacteriales* and family *Pseudomonadaceae* (N.R.C 1991). This Gram-negative bacillus exists everywhere in nature especially warm, moist environment, and can be frequently isolated from soil, water, plants, sewage and occasionally from the normal human skin (Palleroni, 1984). *P. aeruginosa* is a highly relevant opportunistic bacterium that causes disease in humans and plants. Its minimal nutrient requirement and oxygen usage makes it easier to persist and colonize many artificial and natural habitats (Pier *et al*., 2004).

According to the available surveillance data collected by the CDC National Nosocomial Infections Surveillance System from 1986 to 1998, *Pseudomonas* was named the fifth most frequently isolated nosocomial pathogen, responsible for one tenth of all hospital-acquired infections in the United State. *P. aeruginosa* was also identified as the second leading cause of nosocomial pneumonia, third most common agent of urinary tract infection, fourth most frequently encountered pathogen in surgical site infections and the seventh most implicated leading cause of bloodstream infections (Emori and Gaynes, 1993; NNIS 1998). Pier *et al* (2004) also reported *P. aeruginosa* as responsible for a number of clinical conditions, namely endocarditis, otitis, urinary tract infections, bone and joints infection, central nervous system infection, respiratory infections, gastrointestinal infections, skin and soft tissue infections including wounds, bacteremia, keratitis, neonatal opthalmia, folliculitis and unmanageable forms of acne vulgaris**.**

Due to its ability to survive harsh conditions such as high temperature of 42^{o}C, as well as ubiquitousness in and around the hospitals and the community, *P. aeruginosa* ability in causing hospital and community-acquired infections becomes a constant clinical challenge worldwide. Its intrinsic and acquired nature of antibiotic resistance makes it more difficult to treat (Lee *et al*., 2009). *P. aeruginosa* rarely cause infection in the normal host, but is an efficient opportunistic pathogen in immunologically compromised people such as HIV patients, mechanically ventilated persons, patients with open wounds and people with cystic fibrosis and so on; thereby causing serious infections (Emori and Gaynes 1993). The pathogenicity of *P. aeruginosa* is largely influenced by multiple bacterial virulence factors that contribute to its pathogenicity by aiding its colonization and invasion and its genetic flexibility enabling it to survive in varied environments (Sadikot *et al*., 2005). Its colonization is due to multiple factors including fimbriae or pili, flagella, and surface polysaccharides that enable its adherence to epithelium tissues. While its tissue invasion is by the production of

elastase, alkaline proteases, hemolysins (phospholipase and lecithinase), cytotoxin (leukocidin), siderophores with their uptake systems and diffusible pyocyanin pigment (Mariencheck *et al*., 2003).

Another major determinant of *P. aeruginosa* virulence is the encoding of a secretion system called type III that allows the bacterium to inject toxins into the host cell (Sadikot *et al.*, 2005). This secretion system is associated with acute invasive infections and requires pilin-mediated bacterial–epithelial contact (Hauser *et al*., 1998). Type III system is activated on contact with eukaryotic cell membranes and interferes with signal transduction, consequently terminates the cell or alterations in host immune responses. There are other secreted toxins by *P. aeruginosa* that are via type III system called effector proteins consisting of ExoS, ExoT, ExoU, and ExoY (Epelman *et al.*, 2004). Genetic adaption to the environment by *P. aeruginosa* is enhanced by the development of mechanisms called quorum-sensing systems that controls and coordinates expression of genes important for the adaptation to the environment. Quorum-sensing systems is a complex regulatory circuit involving cell-to-cell signaling that enables *P. aeruginosa* to regulate genes in a density-dependent manner through the production of small diffusible molecules called auto inducers (Fuqua and Greenberg, 2002).

Pseudomonas aeruginosa, is capable of Biofilm formation under favourable nutrient and environmental condition. Biofilms are matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces, forming either single-species or mixed-species micro colonies which are phenotypically distinct from their planktonic counterparts, and which provide primitive homeostasis and metabolic cooperatively within the micro colony (Costerton *et al*., 1999). Biofilm production is essential for *P. aeruginosa* because it is responsible for its resistance to antibiotics (Mah *et al*., 2003), protects it from desiccation and the environment (Drenkard and Ausubel, 2002), enhances cell-to-cell communication (Heydorn *et al*., 2002), increases its plasmid stability and genetic exchange (Koonin 2001), and it also decreases its susceptibility to killing by host defense mechanisms and other important adaptive basis.

2.4.2. Infections caused by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa remains an important cause of both hospital and community infections and have been isolated from patients with infections such as bacteremia, urinary tract infections, cystic fibrosis, septicaemia, wound infection and many more worldwide.

2.4.2.1. Bacteremia

Bloodstream infection otherwise known as bacteremia due to *P. aeruginosa* is a serious infection associated with mortality, and patients with such conditions are often medically complicated (Vincent *et al*., 2010). Most of Pseudomonas bacteremia is hospital-acquired. *P. aeruginosa* ranked seventh in prevalence among the pathogens responsible for bloodstream infections and second only to *Candida* species in bloodstream infection-related mortality (Wisplinghoff *et al*., 2004). Bacteremia is the invasion of bloodstream by bacteria through wound, surgical procedure or an infection. Most of the patients suffering from blood stream infections are immunocompromised people especially those with preexisting severe underlying disease conditions such as diabetes, heart failure, cirrhosis, malignancy, renal failure and patient who have undergone organ transplant (Pier and Ramphal, 2005).

2.4.2.2. Endocarditis

Endocarditis is an inflammation of the inner layer of the heart called the endocardium and it is characterized by a prototypic lesion of microorganisms (Kasper *et al*., 2005). *P. aeruginosa* causes endocarditis by infecting the heart valves of susceptible individual such as intravenous drug users and prosthetic heart valves users by establishing itself on the endocardium by direct invasion from the blood stream (Pier *et al*., 2004).

2.4.2.3. Central nervous System Infections

Central nervous system infection with *P. aeruginosa* is not a common infection but often occur as a result of surgical procedure or head trauma and occasionally bacteremia (Taneja *et al*., 2009). Nosocomial meningitis due to *P. aeruginosa* occurring after a neurosurgery is a serious complication and is associated with high mortality (Huttova *et al*., 2007).

2.4.2.4. Eye Infections

Pseudomonas aeruginosa has been recognized as an important cause of keratitis especially among patient with extended wear of contact lens and corneal trauma (Mah *et al.,* 2005). The corneal is normally resistant to *P. aeruginosa* due to multiple factors attributed to the presence of the human tears in the corneal (Fleiszig *et al.,* 2003), but could be compromised as a result of corneal injury or scratch.

2.4.2.5. Bone and Joint Infections

Bone and joint infection also known as osteomyelitis caused by *P. aeruginosa* occurs as a result of direct inoculation of the pathogen or its hematogenous spread from an infected site in the body (Acc, 2009). Osteomyelitis is a disease condition that occurs primarily in children and the most common site is the rapidly growing and highly vascular metaphysic of growing bones (Kumar *et al*., 2007). The disease presentation in adult is usually as a result of an injury leading to infection, in cases of blood-borne; it is common among IV drug users and in conjunction with urinary tract and pelvic infection (Carek *et al*., 2001). Osteomyelitis caused by *P. aeruginosa* is particularly difficult to treat because of the ease with which the organism may develop drug resistance during therapy (Dan *et al*., 1990).

2.4.2.6. Gastrointestinal Infections

Pseudomonas aeruginosa can produce disease in any part of the gastrointestinal tract from the oropharynx to the rectum. As in other forms of Pseudomonal disease, those involving the GI tract occur primarily in immunocompromised individuals. The organism has been implicated in perirectal infections, pediatric diarrhea, typical gastroenteritis, and necrotizing enterocolitis. The GI tract is also an important portal of entry in Pseudomonas septicemia (Clark, 2008)

2.4.2.7.Wound Infection

Over the several decades, Gram-negative organisms have emerged as the most common pathogens causing invasive infections by virtue of their large arsenal of virulence factors and antimicrobial resistance traits (Clark *et al*., 2003). Multiple drug resistant *P. aeruginosa* is one of the major causes of wound related infections such as skin and tissue infections and other hospital-acquired types with high mortality and morbidity rate among affected individuals (Ludwik *et al.*, 2009). *P. aeruginosa* from the patient's endogenous gastrointestinal flora and/or an environmental source is the most common cause of burn wound infections in many centers (Altoparlak *et al*., 2004). *P. aeruginosa* produces a number of cell-associated and extracellular virulence factors such as adhesins, alginate, pili, flagella, and lipopolysaccharide elastase, exoenzyme S, exotoxin A, hemolysins, iron-binding proteins and so on, that mediate a number of processes, including adhesion, nutrient acquisition, immune system evasion, leukocyte killing, tissue destruction, and bloodstream invasion (Tredge *et al*., 2004). *P. aeruginosa* also carries many intrinsic and acquired antimicrobial

resistance traits that often results to difficulty in treating infected burn wounds (Laupland *et al.*, 2005).

2.4.2.8. Respiratory Infections

Pseudomonas aeruginosa is a major causative pathogen of nosocomial respiratory infections. Significantly, immunocompromised patients are at increased risk for *P. aeruginosa* infection, and it is the predominant cause of morbidity and mortality in patients with cystic fibrosis (CF), a genetically associated infections among the Caucasians once established is difficult to treat (West *et al.*, 2002). Additionally, *P. aeruginosa* is a frequently identified pathogen in patients with ventilator-associated pneumonia (a severe complication of intensive care), and has been reported to have a high mortality rate compared with other pathogens (34–48%). More so, *P. aeruginosa* is associated with exacerbations of chronic obstructive pulmonary disease (Murphy *et al*., 2008).

2.4.2.9. Ear Infections

Otitis externa ear infection also known as "External otitis" and "Swimmer's ear" is an inflammation of the outer ear and ear canal mostly caused by *P. aeruginosa* present in a droplet of water trapped in the ear during shower and are commonly occurring in a contaminated swimming pool water (Roland and Stroman, 2002). Among swimming pools and hot tubs users is also a common infection caused by *P. aeruginosa* called folliculitis, a condition caused by an infection of the ear follicle resulting to its inflammation (James *et al*., 2006).

2.4.2.10. Urinary Tract Infections

Urinary tract infections (UTI) caused by *P. aeruginosa* are usually hospital-acquired and related to urinary tract catheterization, instrumentation or surgery (Alavaren *et al*., 1993). *P. aeruginosa* is the third leading cause of hospital-acquired UTIs, accounting for about 12 percent of all infections of this type (Mahesh *et al*., 2010). Urinary tract infection by *P. aeruginosa* occurring among catheterized patient are mostly of endogenous sources but can also be acquired by cross-contamination from other patients or hospital personnel or by exposure to contaminated solutions or non-sterile equipment

2.4.3 Risk factor for infections caused by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa hardly infect healthy persons on a normal circumstances but chances of being infected is high among hospitalized individuals especially those with compromised state of health. The risk of getting infected is thus high among such individuals. Many studies have reported risk factors for acquiring a multiple drug resistant *P. aeruginosa* infection among the factors documented includes prolong ICU stay, severity of the illness, immunocompromised state, previous treatments with anti-pseudomonal drug and broad-spectrum antibiotics, undergoing an invasive procedure such as surgery and prolonged antibiotics treatment (Aloush *et al.*, 2006; Bou *et al*., 2009).

2.4.4. Clinical Impact of *Pseudomonas aeruginosa*

Even though *P. aeruginosa* is widely distributed in nature and has the potential to cause several community-acquired diseases, serious infections caused by *P. aeruginosa* are predominantly hospital acquired (Mesaros *et al*., 2007). Infections caused by multidrug resistant *P. aeruginosa* results to increase in mortality and morbidity rate among the hospitalized patients (Tacconelli *et al*., 2010). Patients suffering from cystic fibrosis and other systemic infections have increased chances of mortality rate due to inability of antimicrobial agent to efficiently neutralize and eliminate the effect of this pathogen. Long duration of hospital stay and economic cost effect of treating infections caused by this pathogen are part of the menace

Therapeutic failures in infections caused by multiple drug resistant *P. aeruginosa* are as a result of its acquired and intrinsic resistance mechanism. Acquired or imported mechanisms are mostly plasmid mediated and are usually by conjugation or other suitable mode of horizontal gene transfer from other genera such as the *Enterobacteriaceae*. Clinical impact of acquired resistance in *P. aeruginosa* is felt mostly on *β*-lactam drugs such as the penicillins and cephalosporins, and also on aminoglycosides (Livermore and Woodford, 2006). Plasmid mediated resistance to *β*-lactam drugs involves the production of *β*-lactamase, an enzymes which is capable of breaking the *β*-lactam ring and thereby deactivating the drug's antibacterial activities (Bush *et al*., 1995). There are classes and families of *β*-lactamase produced by Gram-negative bacteria which are grouped according to their substrates and range of activities i.e. narrow or extended spectrum. Acquired resistance in *P. aeruginosa* against aminoglycosides also involves an enzymatic inactivation of the drug molecule

through chemical modification (Poole, 2005). These enzymes are also categorized into families based on their chemical modification mediation.

Intrinsic mechanisms of resistance in *P. aeruginosa* are usually mediated by chromosomal adaptations such as mutations in one or two amino acid sequences, membrane structures such as porins channels, efflux pumps and enzymatic activities. The three most commonly observed chromosomal resistance mechanism found in *P. aeruginosa* is the AmpC cephalosporinase, OprD outer membrane porin and the multidrug efflux pumps. The management of these three mechanisms in *P. aeruginosa* makes it dynamic resistant Gram-negative bacteria that will remain relevant in clinical settings.

2.5. Bacterial Resistance

The latest twist in chemotherapy currently is the ability of pathogens that were once susceptible to the killing effect of antibiotics, now being resistant to the same drugs. The term antimicrobial resistance has been defined as the ability of a microorganism to survive at a given concentration of an antimicrobial agent at which its species in the population would be killed. It is also defined as the ability of a microorganism to evade treatment with a clinical concentration of an antimicrobial agent in the body (Kahlmeter *et al*., 2003).

Antibiotics resistance in bacteria may be inherent trait (intrinsic) or acquired by importation of resistance markers from another source also known as horizontal gene transfer or mutation of its own DNA and its transfer to progeny during DNA replication also known as vertical gene transfer.

Inherent or intrinsic resistance by bacteria is a natural ability of bacteria species related to its genetic background which does not require any specific target and often involves the presence of low affinity targets, low cell permeability or efflux mechanisms (Gold and Moellering, 1996). A typical example is *P. aeruginosa* cell wall which is unusually impermeable to antibiotics than the Gram-positive counterpart and also the production of *β*-lactamase enzymes present in many Gram-negative bacteria cell which is capable of hydrolyzing certain *β*-lactam drugs such as penicillin (Bush, 1995). *P. aeruginosa* is intrinsically resistant to a number of structurally unrelated antibiotics due to low permeability of its outer membrane (1/100 of the permeability of *E. coli* outer membrane), it intrinsically expresses efflux pumps and also possess a naturally occurring chromosomal AmpC *β*-lactamase also known as cephalsoporinase which also confer resistance to many *β*-lactam drugs (Nordmann and Guibert, 1998). Knowledge about intrinsic resistance mechanisms is important to predict potential emergence of antibiotic resistance under selective pressure.

Acquired resistance in bacteria is a modification of existing genetic material or acquisition of new gene that makes them to be resistant to drugs that they were previously susceptible. Acquired resistance through chromosomal changes i.e. mutations, which are transferred to progeny during DNA replication, is called vertical gene transfer or vertical evolution. It is a spontaneous event that cannot be predicted. However, acquisition of genetic materials such as plasmid, integrons and other mobile genetic element containing resistant genes from other bacteria in the environment is called lateral or horizontal gene transfer, this is the most common method by which most bacteria acquires resistant genes (Davison, 1999). Acquired resistance genes in bacteria allows for the production and expression of certain enzymes which hydrolyses antibiotics, modification of drug active site and targets or the production of alternative metabolic pathways that are different from the recognize path for drug actions. (Tenover, 2006). There are three possible modes of horizontal gene transfer in bacteria namely; conjugation, transduction and transformation.

2.5.1. Conjugation

Conjugation in bacteria is the process of transferring genetic materials between two bacterial cells having a cell-to-cell contact. Conjugation can be described as bacteria sexual reproduction or mating since there is a physical contact between a donor cell (F^+) and a recipient cell (F^-) (Fig 2.2). Conjugation is not an exchange of genetic material but rather a transfer through a sex pilus which means the ((F^+) strain must possess the gene to be transferred and same gene must be absent in the recipient (F^-) strain. Originally thought to be highly specific occurring between only closely related bacterial specie, but it has now been shown to occur among diverse species including interaction between gram positive and gram negative (Courvalin, 1994) Conjugation in *P aeruginosa* was first described in 1955 between four unrelated strains (Holloway, 1955). Conjugative plasmids such as F-plasmids also known as F-factor, R-plasmids and other genes are transferred during conjugation. The F-plasmid is an episome i.e. a plasmid that is capable of integrating itself into the bacterial chromosomes by homologous combination because it carries its own origin of replication *oriV* and origin of transfer T *ori* (Holmes and Jobling, 1996). Conjugation is the most frequent mode of genetic transfer and the first extensively study method of gene transfer.

2.5.2. Transformation

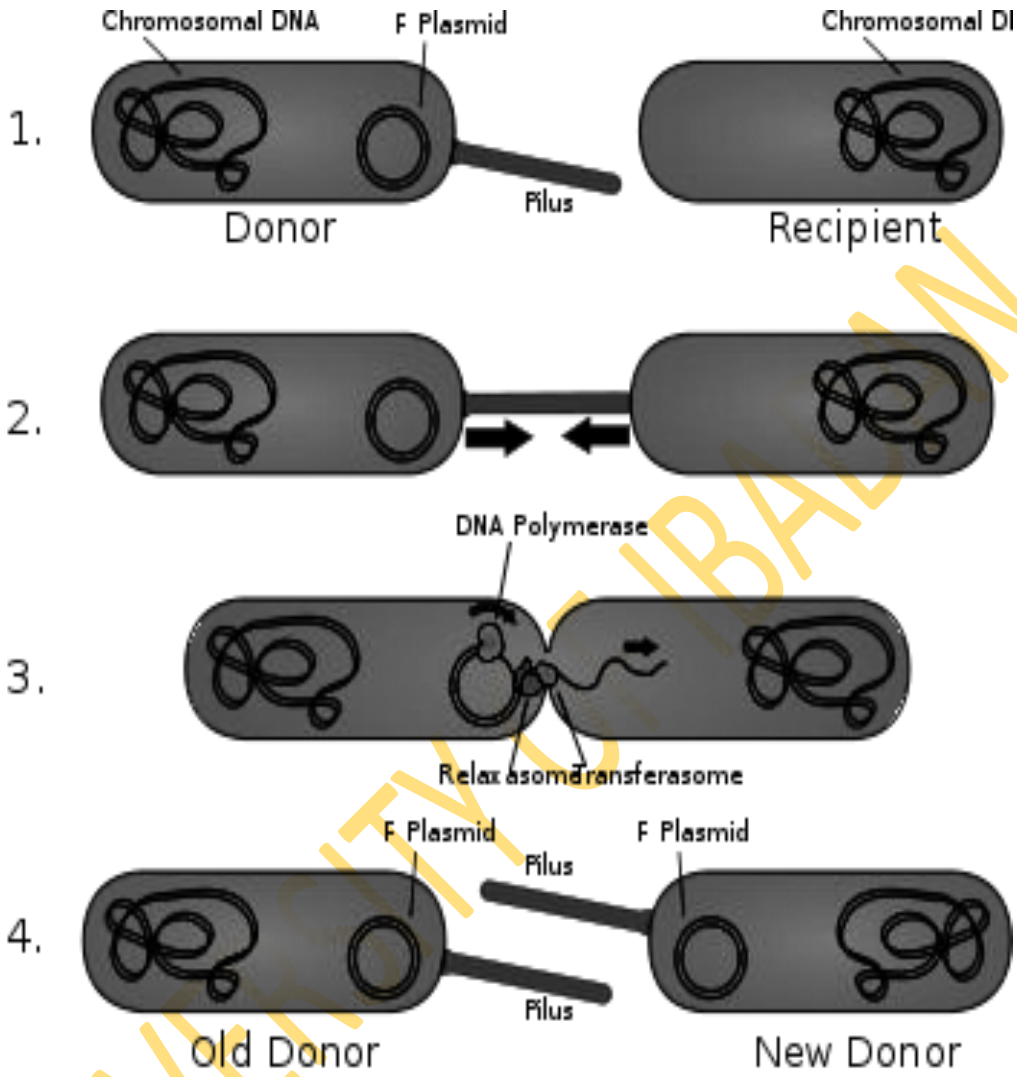
Transformation is the uptake of a naked DNA by a recipient cell. The DNA is usually from a ruptured or lysed bacteria cell found in the environment. Transformation occurs naturally and also leads to increase in resistant population among bacteria. In order to be transformed, a bacterium must be competent i.e. have the ability to take up a DNA from the environment. There are many bacterial species that are either naturally competent or in which competency can be induced by environmental conditions. Bacteria such as *Bacillus, Streptococcus, Neisseria, Pneumococcus* can easily take up DNA from the environment and incorporate it into their own chromosomes (Davison, 1999).

2.5.3. Transduction

Transduction is a mechanism of gene acquisition in bacteria involving the introduction of a foreign DNA into the cell through a bacteriophage (virus that infect bacteria) infection. Transfer of resistance by transduction was first shown with penicillin resistance in staphylococci in 1958 (Garrod and Grady, 1971). Although phages generally have a restricted host range, they are common in many environments and may therefore play an important role in transfer of resistance genes. Genetic transfer in transduction is dependent on the phage ability to have a narrow host range (in this case same bacteria only), or broad host range which can allow for transfer of gene to other species. There are two types of transduction, generalized and specialized. In generalize type any bacterial gene can be transferred while specific bacteria gene is transferred in specialized transduction. Figure 2.3 shows a diagrammatic explanation of phage activity during transduction.

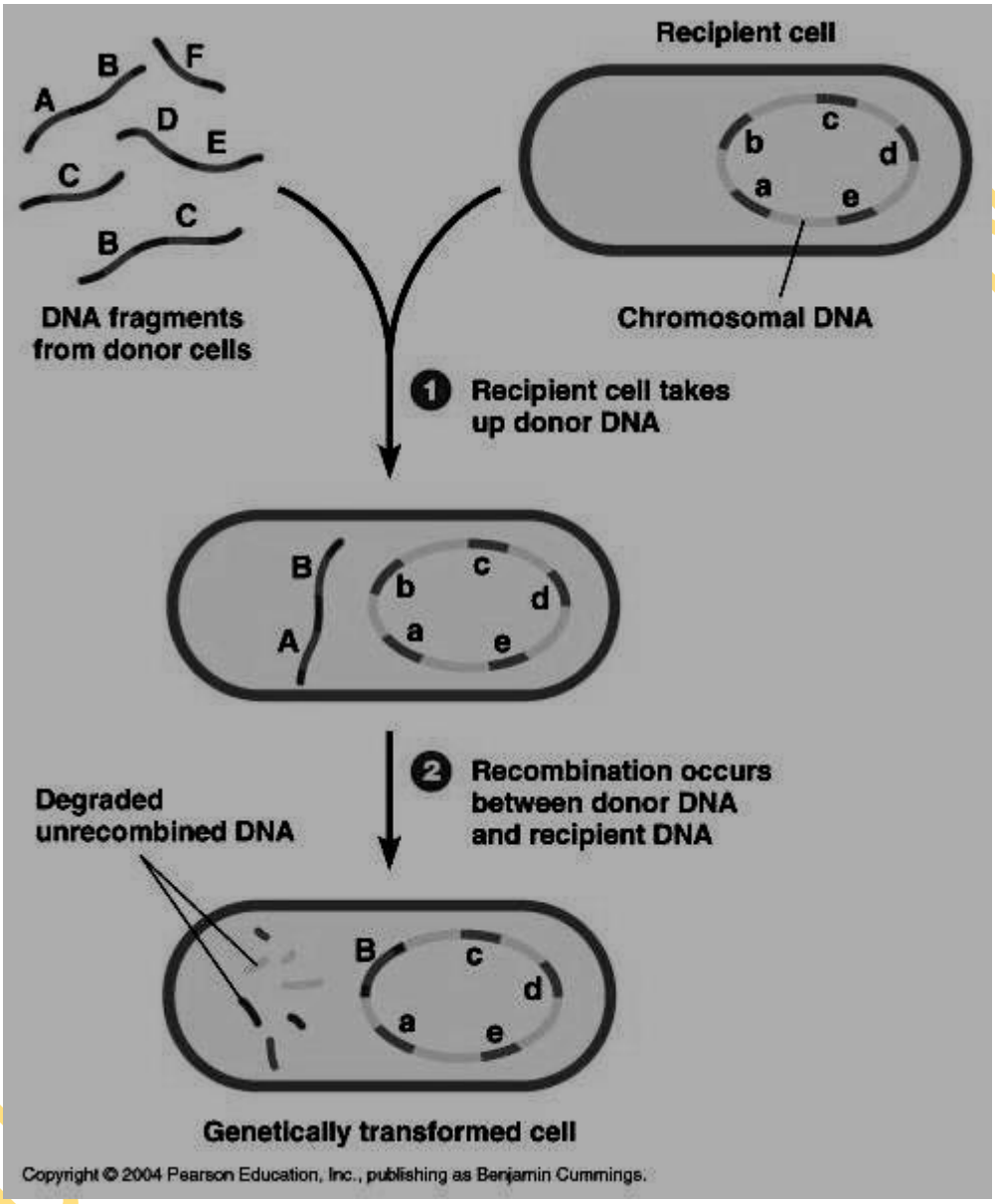
Figures 2.2 to 2.4 schematically show the process of conjugation, transformation and transduction in bacterial cell.

Fig 2.1: Schematic drawing of bacterial conjugation



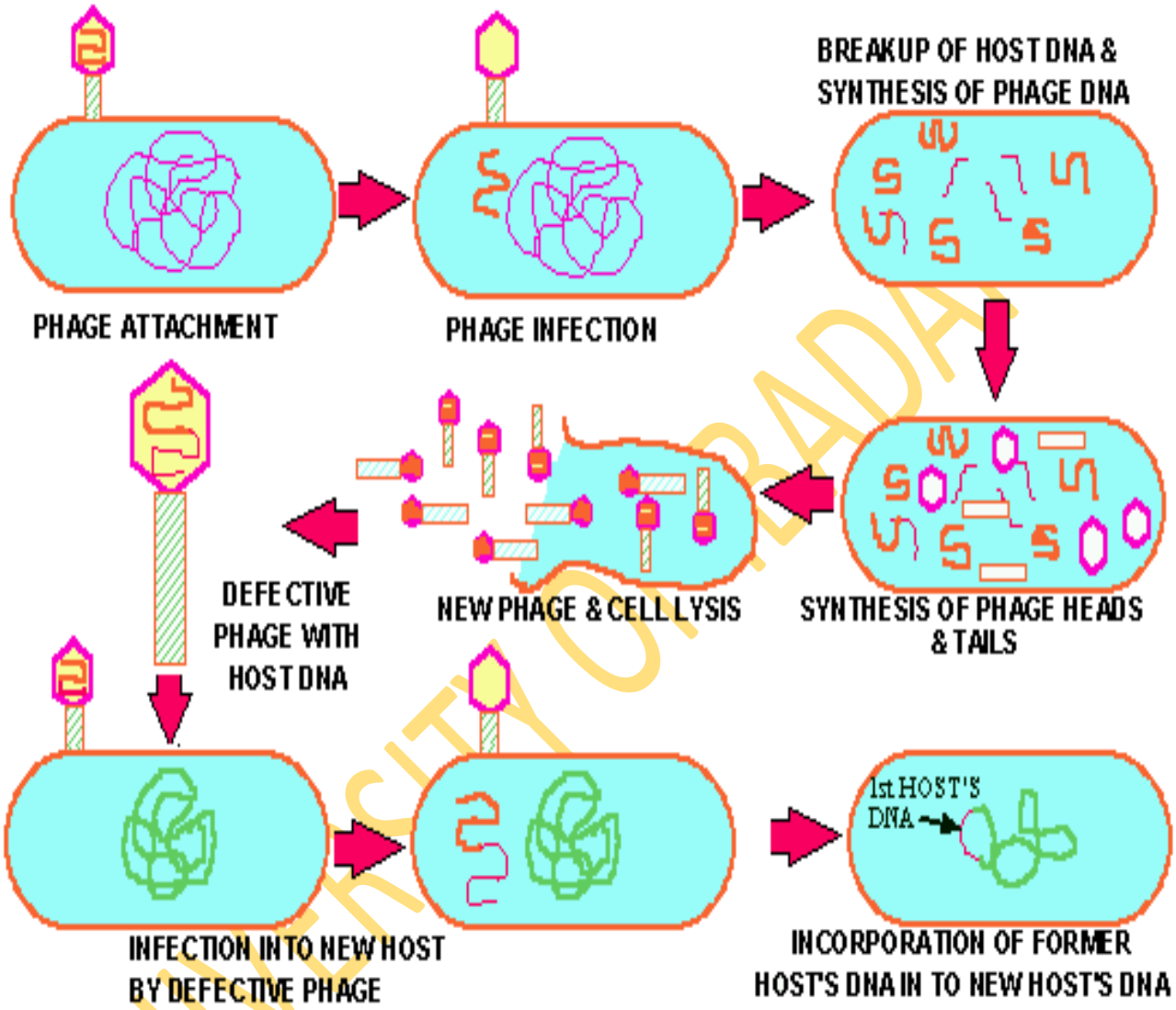
Source: www.en.wikipedia.org

Fig 2.2: Schematic drawing of transformation in bacteria



Source: Pearson Education, Inc USA.

Fig 2.3: Showing transduction between a phage and a bacteria host cell.



Source: www.slic2.wsu.edu

2.5.4. Mobile Genetic Elements

P. aeruginosa has the ability to quickly develop resistance to several unrelated classes of antimicrobial agent because they possess natural resistance mechanisms and great tendency of acquiring new resistance genes from other source. Plasmids, integrons and transposons are among the genetic elements that are easily acquired by *P. aeruginosa* thereby contributing to its menace.

2.5.4.1. Plasmids

Plasmids are double-stranded circular DNA molecules that are capable of independent existence and replication in the host chromosomes and are present in many bacteria and in some yeast and fungi (Fig 2.5). Plasmids play a major role in conjugation in bacteria because they have their own replication origin and are autonomously replicating and stably inherited (Prescort *et al*., 1993). Plasmids that carry the genes necessary for conjugation are called conjugative plasmids, while non-conjugative plasmids can only be spread during conjugation brought on by a conjugative plasmid (Hugh and Datta, 1983). According to the result of a large study carried out by Hugh and Datta (1983), they found out that plasmids are useful in spreading resistance, but their presence does not necessarily mean an organism is resistant. Plasmid genetic information is not essential for the growth and survival of the host but their presence provides bacterium with a competitive advantage over antibiotic-sensitive species (Ricci and Hernandez, 2000). The traits specified by plasmids include antibiotic resistance, toxic heavy metal resistance, degradation of xenobiotic compounds, symbiotic and virulence determinants, bacteriocin production, resistance to radiation and increased mutation frequency (Snyder and Campness, 1997; Thomas, 2000). By transferring genetic material, plasmids play a major role in enhancing the genetic diversity and adaptation of bacteria. Plasmids (with exception of a few small plasmids) contain a large number of inserted and/or extra chromosomal mobile genetic elements such as insertion sequence (IS) elements, transposons, integrons, gene cassettes and conjugative transposons (Osborn *et al*., 2000). These genetic materials are effectively "hitch-hiking" on the plasmid backbone, giving the plasmid (and the bacterial host) a selective advantage in exchange for their maintenance, and possible transfer to other hosts (Osborn *et al*., 2000).

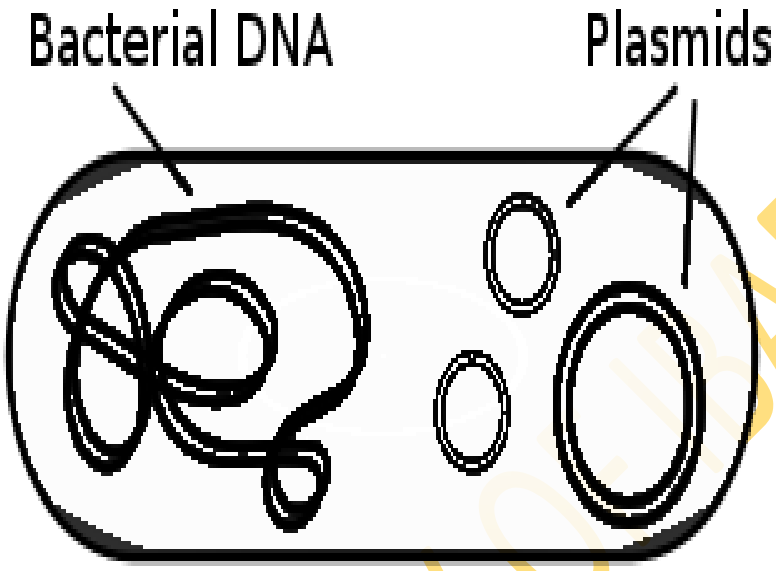
The major types of plasmids available are F factor, R factors, Col factor, metabolic plasmid and virulence plasmids. F factor also known as F plasmid or fertility factor is a type that code for sex pili and plasmid transfer between specific bacteria strains during conjugation. R

factors are plasmids that code for transmissible antibiotics resistance genes and may be transferred by conjugation to closely related species. They are not usually integrated into the host chromosomes and some can have up to 8 resistance genes while some only have a single gene. Col factors are plasmids that contain genes for the synthesis of colicins (bacteriocin) a protein that destroys other bacteria. It gives the host a competitive edge against other closely related strains to which the protein is directed. They are conjugative in nature and harbours resistance genes and the host is unaffected by the bacteriocin it produces. Metabolic plasmids are types that code for the synthesis of enzymes that degrade substances such as aromatic compounds and other carbohydrates. Virulence plasmid makes their host to be more pathogenic through the production of toxins example is enterotoxigenic strains of *E. coli.* (Johnson *et al*., 2009).

2.5.4.2. Transposons

Transposons are DNA elements that have the ability to "jump" or transpose themselves from one place to another within the genome of a single cell. They are capable of creating a significant mutation and alter the cell's genome size by their mode of insertion and deletion or rather "copy and paste" mechanisms, and are aided by transposase enzymes which they encode. The smallest type of transposon is called the insertion sequence (IS) which contains no gene except those that are required for transposition; they are consisting of repeated sequences. Those that contains additional gene are called composite transposons usually begin with a prefix *Tn*, they usually contains toxins and antibiotics resistance genes and are mostly responsible for dissemination of resistance gene among pathogenic bacteria (Tseng *et al*., 2009). Some transposons are also found within the plasmid where they act as source and target for other transposons, this often contributes to the spread of antibiotic resistance genes. Accumulation of transposons in a single plasmid has been described as a basis for its multiple drug resistances (Prescort *et al*., 1993).

Fig 2.4: Illustration of the bacterial cell showing the Plasmids and the genomic DNA



Source: www.fridayharborlistichealth.com

2.5.4.3. Integrons and gene Cassettes

An integron is a genetic element that has the ability to capture and integrate additional mobile DNA known as gene cassettes (that usually carry a resistance gene) by site-specific recombination and which encodes an enzyme, integrase that mediates these site-specific recombination events (Hall and Collis, 1995). All integron characterized to date are composed of three key elements ; an *intI* gene that encodes the *IntI* site-specific recombinase responsible for capturing the small mobile elements known as gene cassettes, an *attI* site into which the cassettes are inserted and a promoter (Pc) that drives expression of cassette-associated genes (Hall and Stokes, 2004). Integrons are mostly located on the bacterial chromosome and sometimes on a plasmid.

Gene cassettes are genetic elements that may exist freely, as circular non-replicating DNA molecules when moving from one genetic site to another, but which are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or bacterial chromosome (Collis and Hall, 1992). These gene cassettes are normally found integrated at a specific site in an integron, where they are often part of multi-cassette arrays (Recchia and Hall, 1995). Gene cassettes consist of an open reading frame (ORF) for the gene encoding the antibiotic resistance, as well as a recombination site, termed a 59-base element (59-be). These 59-be are imperfect inverted repeats (IRs) found at the 3' end of the ORF, which are recognized by the integrase (Mazel, 2004).

At present, five classes of mobile integrons are known to have a role in the dissemination of antibiotic-resistance genes. These classes have been historically defined based on the sequence of the encoded integrases, which show 40–58% identity. All five classes are physically linked to mobile DNA elements, such as insertion sequences (ISs), transposons and conjugative plasmids, all of which can serve as vehicles for the intraspecies and interspecies transmission of genetic material. Three classes of mobile integrons are 'historical' classes that are involved in the multiple-antibiotic-resistance phenotype (Hall, 1997). Class 1integrons were first described by Stoke and Hall in 1989 (Stoke and Hall, 1989) and are the most prevalent and extensively studied class among bacteria. They are associated with functional and non-functional transposons derived from Tn*402* that can be embedded in larger transposons, such as Tn*21* (Brown *et al*., 1996). Class 1 integrons has been described in many Gram-negative genera including *P. aeruginosa* (Severino *et al* 2002**;** Xu *et al*., 2007**)**. The majority of known antibiotic resistance gene cassettes described so far has been found in class 1 integrons.

Class 2 integrons are embedded in the Tn7 family of transposons and consist of an integrase gene followed by gene cassettes (Sundstrom *et al*., 1991). Class 2 integrons have been found

in many bacteria such as *Acinetobacter* (McIver *et al*., 2002), and *Salmonella* (Orman *et al*., 2002) and recently in *P aeruginosa* (Xu *et al*., 2009). The integrase gene of class 2 integrons is 46% similar to the sequence of the class 1 integrase and usually contain *dfrA1-sat1-aadA1* gene cassette array (White *et al*., 2001; Xu *et al*., 2009). Class 3 integrons are thought to be located in a transposon inserted in as-yet-uncharacterized plasmids , they are found mainly among the clinical isolates in Japan and lately in Canada (Arakawa *et al*., 1995; Collis *et al*., 2002; Xu *et al*., 2007). Two gene cassettes were reported to be present in the class 3 gene cassette array; *bla*IMP-1 which encodes a metallo-beta-lactamase, and *aacA4*, which confers resistance to aminoglycosides. The other two classes of mobile integrons, class 4 and class 5, have been identified through their involvement in the development of trimethoprim resistance in *Vibrio* species; one (class 4) is a component of a subset of SXT elements found in *Vibrio cholera* and the other (class 5) is located in a compound transposon carried on a plasmid in *Vibrio salmonicida* (Hochhut *et al*., 2001).

2.5.4.3.1. Superintegrons

A chromosomal superintegron was first described in the genome of *Vibrio cholera* in the late 1990s (Mazel *et al*., 1998). It was so named because of its unique structure that was 126kb long and it contained at least 178 gene cassette. This integron encodes a specific integrase, VchIntIA, which is related to the integrases encoded by mobile integrons but has two main characteristics that distinguish it from known mobile integrons. First, there is a large number of gene cassettes that are associated with the integron, and there is a high degree of identity (>80%) observed between the *attC* sites of these cassettes. Secondly, the integron structure is located on chromosomes and does not seem to be associated with any kind of mobile DNA element. However, unlike the other classes of integrons previously described, the gene cassettes of superintegrons do not appear to encode antibiotic resistance. Instead they encode for adaptive functions such as metabolism and DNA modification. Superintegrons are now known to be integral components of many γ-proteobacterial genomes, and they have been identified in the Vibrionaceae and their close relatives the xanthomonads, and in a branch of the pseudomonads

2.5.5. Chromosomal Resistance in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa chromosomal resistance mechanisms lies predominantly in its ability to extrude antimicrobial agents out of the cell by the help of multidrug efflux pumps, impermeability of its cell membrane, the loss of certain porin channels referred to as OprD and the presence of AmpC cephalosporinase that has the ability to hydrolyze certain antibiotics such as *β*-lactam drugs. These three mechanisms are the most widely studied chromosomal mediated resistance mechanism in *P. aeruginosa* (Livermore, 1995; 2002).

2.5.5.1. AmpC-mediated resistance

AmpC is a type of *β*-lactamase that is found in many *Enterobacteriaceae* and also in other families. It is located at the bacterial periplasm with the exception of the AmpC *β*-lactamase of *Psychrobacter immobilis* (Feller *et al*., 1997). In the Ambler structural classification of β-lactamases (Ambler, 1980), AmpC enzymes belong to class C, while in the functional classification scheme of Bush *et al*. (1995), they were assigned to group 1.

AmpC *β*-lactamase was first identified and named as penicillinase by Abraham and Chain (1940) after observing its activity against penicillin. Its hydrolyzing activity was later discovered to be extended towards cephalosporins and oxyiminocephalosporins such as ceftazidime, cefotaxime, and ceftriaxone; and monobactams such as Aztreonam (Jacoby, 2009). They are generally resistant to *β*-lactamase inhibitors such as clavulanic acid although some are inhibited by sulbactams or tazobactam (Bush *et al*., 1993).

Pseudomonas aeruginosa carries an inducible AmpC cephalosporinase which is similar to the chromosomally encoded AmpC found in several members of the *Enterobacteriaceae (*Hanson and Sanders 1999). Over-expression of AmpC in *P. aeruginosa* through the induction pathway occurs during exposure to specific *β*-lactams and *β*-lactamase inhibitors (e.g., cefoxitin, imipenem and clavulanate). Overproduction of this enzyme in *P. aeruginosa* enables it to develop resistance to all *β*-lactams with the exception of carbapenems (Bagge *et al.*, 2004). The most common cause of AmpC over expression in clinical isolates is a mutation in *ampD* leading to AmpC hyperinducibility or constitutive hyperproduction (Schmidtke and Hanson, 2006). *P. aeruginosa* PAO1 has three *ampD* genes, explaining the stepwise upregulation of AmpC production seen in this organism with the successive inactivation of each *ampD* gene (Juan *et al*., 2006). Mutation of the multiple *ampD* loci contribute to virulence since a *P. aeruginosa* strain partially derepressed by the inactivation

of one *ampD* allele remains fully virulent (Moya *et al* 2008). Although some reports have suggested that the overproduction of this protein may play a role in the intrinsic susceptibility to carbapenems (Livermore, 1992; Mushtaq and Livermore 2004). According to Rodriquez-Martinez *et al.* (2009) overproduction of AmpC alone does not significantly alter *P. aeruginosa* susceptibility to the carbapenems but could certainly contribute to resistance if accompanied by additional resistance mechanisms (e.g., efflux pump overproduction, decreased OprD, and/or production of a class A/class B carbapenemase). Adding even more complexity is the potential for mutational variants of the chromosomally encoded AmpC enzyme (extended-spectrum AmpC) to provide *P. aeruginosa* with carbapenem resistance. Unlike Enterobacteria, *P. aeruginosa* have not yet been found to contain plasmid-mediated cephalosporinases, although some of the plasmid-encoded cephalosporinases demonstrate a remarkably similar structure to that of the pseudomonal AmpC β -lactamase.

2.5.5.1.1. Clinical significance of AmpC overproduction

P. aeruginosa possess arsenal of resistance mechanisms which makes it a bit difficult sometimes to accurately access the impact of its AmpC overproduction. However according to reports, there is a high chances (67.5%) of administering inappropriate antibiotics to patients suffering from infections caused by AmpC overproducing *P. aeruginosa* than with patients with strains that did not over produce AmpC (Tam *et al*., 2009). There are also confirmed reports of resistant *P. aeruginosa* emerging in the course of therapy even with appropriate antibiotics based on initial record of susceptibility. In such cases resistance is often mediated by significant increase in the production of AmpC among the infectious strains (Juan *et al*., 2005). Associated impact of AmpC overproduction in *P. aeruginosa* has resulted to clinical failures in several cases especially among the patients with underlying disease such as cystic fibrosis and neutropenia.

2.5.5.2. OprD-Mediated Resistance

Drug resistant *P. aeruginosa* is a worldwide challenge in the clinical settings because they present difficulties in treatments of infections due to their ability to resist several classes of antibiotics commonly prescribed. Intrinsic resistant abilities of this pathogen has enable it evade treatments with antimicrobial agents. One of its principal intrinsic resistant ability is

reduced outer membrane porosity which often leads to drug impermeability. The outer membrane of *P. aeruginosa* is only 8% as permeable as the outer membrane of *E. coli* (Hancock and Brinkman, 2002). *P. aeruginosa* outer membrane has 163 known proteins with 64 of these outer membrane proteins grouped into three families of porins which play an important physiological roles in the transport of nutrients such as sugars, amino acids, phosphates and divalent cations and siderophores (Hancock and Brinkman, 2002). Porins are proteins capable of forming channels that allows the transport of molecules across lipid bilayer membranes, and shows little permeability for hydrophilic solutes. They provide membranes with multiple functions. Porins can also act as potential targets for adhesion to other cells and binding of bactericidal compounds to the surface of Gram-negative bacteria. Variations in their structure as a mechanism to escape from antibacterial pressure or regulation of porin expression in response to the presence of antibiotics are survival strategies that have been developed by many bacteria (Vila *et al.*, 2007)

Certain hydrophilic antibiotics, such as *β*-lactams, aminoglycosides, tetracyclines, and some fluoroquinolones penetrate the bacteria outer membranes by transversing through porin channels. However the loss of specific porin channels can decrease the susceptibility of *P. aeruginosa* to such antibacterial agents (Yoshimura and Nikaido, 1985; Nikaido, 1989).

The OprD family includes a number of porins in *Pseudomonas* species and other Gram-negative bacteria that appear to exhibit a variety of substrate specificities. Carbapenems such as impenem, meropenem are highly effective against multiple drug resistance *P. aeruginosa*. OprD serve as the preferred route by carbapenem into the cell membrane hence the loss of OprD significantly results to resistance or decreased susceptibility carbapenems in mutant strains (Livermore 2002; Mushtaq and Livermore, 2004). OprD in *P. aeruginosa* has been shown to facilitate the diffusion of basic amino acids and other small peptides that contain these amino acids. It shares a close homology to the non-specific porin OmpF in *E.coli* (Pirnay *et al*., 2002). The expression of OprD is coregulated with another resistant mechanism called the MexEF-OprM efflux thus in this manner resistance to the carbapenems and other substrates of MexEF-OprM can develop in mutants where the expression of OprD and the efflux pump has been altered (Ochs *et al*., 1999).

2.5.5.3. Efflux-mediated resistance

Efflux pumps are mechanism of resistance in *P. aeruginosa* that helps the bacteria in reducing the amount of drug accumulation by extrusion of the drugs out of the cells (Fig 2.5). Bacterial drug efflux pumps have been categorized into five families, based primarily on amino acid sequence identity, the energy source required to drive export, and substrate specificities of the different pumps (Van-Bambeke *et al.*, 2000). The superfamilies includes the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family (a subgroup of the drug/metabolite transporter superfamily, and the resistance-nodulation division (RND) superfamily (Li and Nikaido, 2009). Although all the above superfamily has been revealed in *P aeruginosa* by sequence analysis, the largest number of predicted pumps belongs to the RND family, with a total of 12 RND systems (Stover *et al*., 2000). The resistance nodulation division superfamily (RND) is a major clinically relevant efflux systems mostly found in Gram-negatives bacteria typically composed of a cytoplasmic membrane pumps, an MFP and an OM protein channel, and responsible for drug exporting, a key role in bacteria drug resistance (Li and Nikiado, 2009).

RND pumps typically exist as a tripartite structure made up of a periplasmic membrane fusion protein (MFP), an outer membrane factor (OMF), and a cytoplasmic membrane (RND) transporter. This complex forms a channel spanning the entire membrane, allowing for the transportation of lipophilic and amphiphilic drugs from the periplasmic space and cytoplasm to the extracellular environment. The genes encoding these pumps are organized into operons on the *P. aeruginosa* chromosome (Lister *et al*., 2009). There are more than 10 RND pumps reported in *P. aeruginosa* that is responsible for protection, having different substrate, export various antimicrobial including organic compounds out of the cell. They include MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK, MexGHI-OpmD, MexVW, MexPQ-OpmE, MexMN, and TriABC (Lister *et al*., 2009). Of all the above listed, Mex-AB-OprM is most constitutionally expressed in *P. aeruginosa* responsible for its intrinsic resistance to fluoroquinolones and pathogenicity (Schweizer, 2003). Efflux pumps contribute immensely to multidrug resistance in *P. aeruginosa* because they expel different types of antibiotics and chemicals such as dyes and organic compounds e.t.c. from the cell and are mediated by four genetically different three-component efflux systems that belong to the resistance–nodulation–division (RND) family; MexA–MexB–OprM, MexC–MexD–OprJ, MexE–MexF–OprN and MexX–MexY–OprM. (Livermore, 2001). Summary of their substrates and

components are listed in Table 2.2. MexA–MexB–OprM and MexX–MexY–OprM efflux systems participate simultaneously in natural and acquired antimicrobial-resistance mechanisms of *P. aeruginosa*, while MexC–MexD–OprJ and MexE–MexF–OprN act only in acquired resistance (Poole *et al*., 1996; Kohler *et al*., 1999).

MexA–MexB–OprM overproduction often occurs in clinical isolates of *P. aeruginosa* and usually it is a result of increased transcription of the *mexA–mexB–oprM* operon due to mutations in the chromosomal gene encoding the MexR repressor protein, i.e. mutations at the mexR locus. *nalB* mutants are characterized by increased MICs and corresponding clinical resistance to most of the b-lactams (penicillins, cephalosporins, monobactams, meropenem to some extent, but not imipenem), quinolones, tetracyclines, chloramphenicol and trimethoprim (Livermore, 2001). Masuda and Ohya (1992) were the first to report that MexA–MexB–OprM overexpression in *P. aeruginosa* that determines decreased susceptibility to meropenem, but does not affect the activity of the other carbapenems – imipenem and panipenem (compared to wild-type *P. aeruginosa*). This is due to the different molecular structure of carbapenems, meropenem has a hydrophobic side-chain at the second position, which makes it a substrate for this efflux system, while imipenem and panipenem are not substrates as their side-chains are strongly charged and hydrophilic.

The *mexC–mexD–oprJ* operon cannot be expressed constitutively, but is overexpressed *in P. aeruginosa* mutants possessing mutations in the *nfxB* gene, which encodes a transcriptional repressor (Poole *et al*., 1996). This efflux system predominantly exports extended-spectrum cephems (cefepime and cefpirome) from the bacterial cell, as well as quinolones, macrolides, tetracycline and chloramphenicol (Li *et al*., 2000). The third known efflux operon, *mexE–mexF–oprN*, determines resistance to quinolones, chloramphenicol and trimethoprim, and is overexpressed by the so called *nfxC P. aeruginosa* mutants (having a mutation at the *mexT* locus) (Kohler *et al*., 1999). The *nfxC* mutants also show crossresistance towards carbapenems (predominantly imipenem) as these have decreased expression of OprD outer membrane proteins.

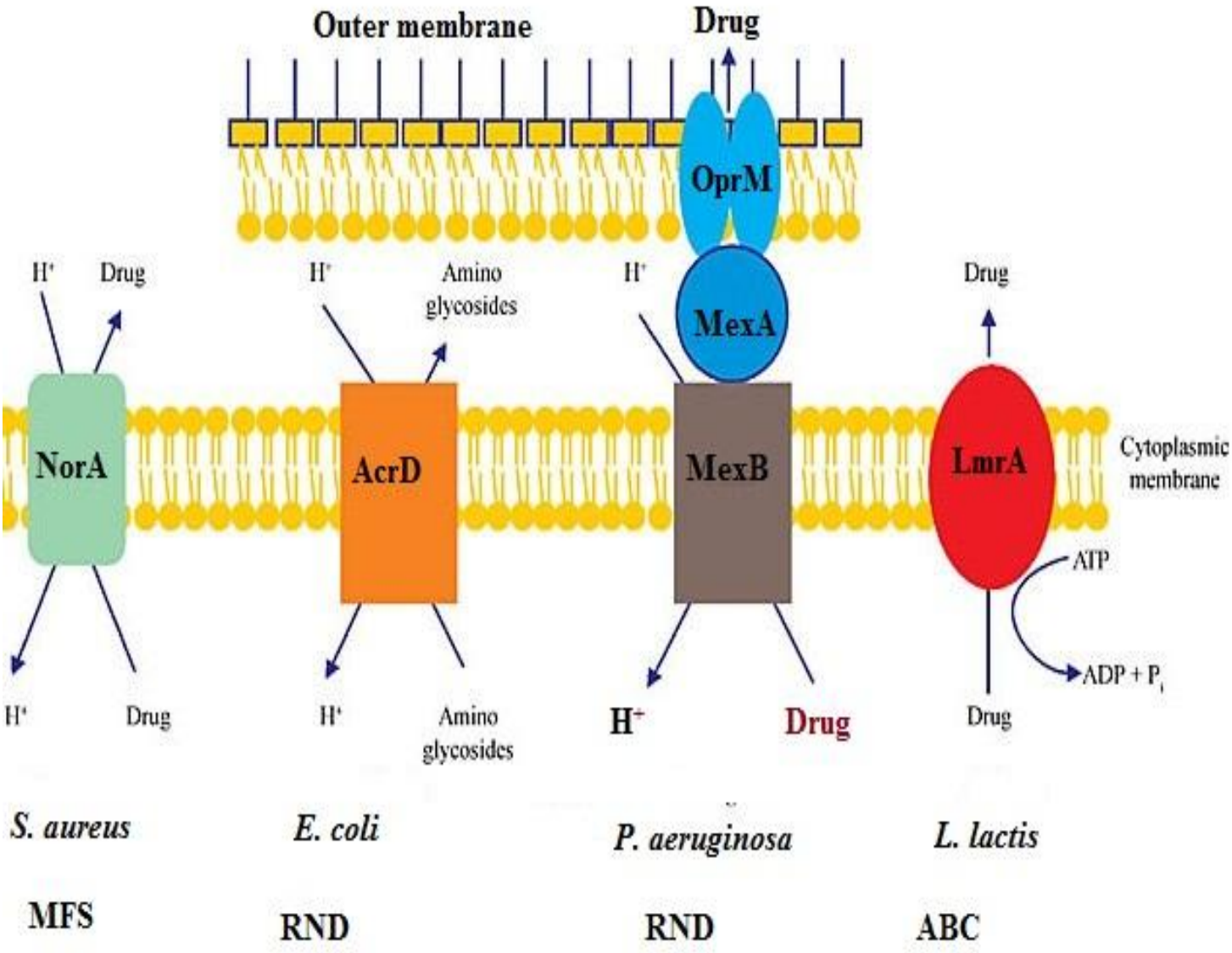


Fig 2.5: Schematic illustration of the main types of bacterial drug efflux pumps shown in *Staphyloccus aureus, Escherichia.coli, Pseudomonas aeruginosa* and *Lactobacillus lactis.* Source: Lister *et al*. (2009).

Table 2.1. Characteristics of RND efflux pumps in *P. aeruginosa* Source: Lister *et al*. (2009)

Operon	Component	Function	Regulator		Substrate(s)	
			Primary	Secondary	Antibiotics	Additional compounds
mexAB-oprM	MexA MexB OprM	MFP RND OMF	MexR NalD	NalC	Fluoroquinolones, β-lactams, β-lactamase inhibitors, tetracyclines, chloramphenicol, macrolides, novobiocin, trimethoprim, sulphonamides	Biocides (e.g., triclosan), detergents, dyes, HSL,f aromatic hydrocarbons
mexCD-oprJ	MexC MexD OprJ	MFP RND OMF	NfxB		Fluoroquinolones, β-lactams, tetracycline, chloramphenicol, macrolides, trimethoprim, novobiocin	Biocides (e.g., triclosan), detergents, dyes, aromatic hydrocarbons
mexEF-oprN	MexE MexF OprN	MFP RND OMF	MexT	MexS MvaT	Fluoroquinolones, chloramphenicol, trimethoprim	Biocides (e.g., triclosan), aromatic hydrocarbons

Table 2.1 Contd.

Operon	Component	Function	Regulator		Substrate(s)	
			Primary	Secondary	Antibiotics	Additional compounds
mexJK	MexJ MexK OprM/OpmH	MFP RND OMF	MexL		Tetracycline, erythromycin	Biocides (e.g., triclosan)
mexGHI-opmD	MexG MexH MexI OpmD	 MFP RND OMF	SoxR		Fluoroquinolones	Vanadium
mexVW	MexV MexW OprM	MFP RND OMF	—		Fluoroquinolones, tetracycline, chloramphenicol, erythromycin	
mexPQ-opmE	MexP MexQ OpmE	MFP RND OMF	—		Fluoroquinolones, tetracycline, chloramphenicol, macrolides	
mexMN	MexM MexN OprM	MFP RND OMF	—		Chloramphenicol, thiamphenicol	
triABC	TriA TriB TriC OpmH	MFP MFP RND OMF	—			Triclosan

However, unlike the rest of the efflux operons, *mexE–mexF–oprN* is subject to positive regulation by MexT protein, which belongs to the LysR family of transcriptional activators (Kohler *et al*., 1999; Li *et al*., 2000). Efflux pumps have also been reported to play crucial role in the antibiotics resistance in Biofilm formation *P. aeruginosa* (Gillis *et al*., 2005). In spite of their broad substrate specificity and, thus, ability to promote resistance to multiple antimicrobial clinical significance of multidrug efflux pumps is seen primarily as determinant of resistance to fluoroquinolones agents.

2.6. Antibiotics

Antibiotics are natural chemical compounds produced by organisms such as actinomycetes or fungus that inhibit or kill other microorganisms in their surroundings. Observation of this phenomenon led to the production of the first antibiotics called penicillin and its variants such as ampicillin, which are still around till today. Antimicrobial agent is a chemically derived substance from a biological source or chemical synthesis that inhibits or kills the growth of microorganisms. Nowadays, more antibiotics and antimicrobial agents are synthetically produced to achieve better activities in combating infections caused by bacteria, fungi and viruses. Antibiotics are also known as antibacterials and are used specifically for the treatments of infections caused by bacteria. Antibiotics mode of actions against bacteria is by targeting and the disrupting the bacterial cell wall, DNA replication and other nucleic activities responsible for promoting the growth and proliferation of the bacteria with selective toxicity i.e. without causing harm to the host cells and flora.

Antibiotics can either be a broad-spectrum or a narrow-spectrum drug based on their activities antibiotics. The latter usually target the Gram-positives or Gram-negatives bacteria, while the former targets both group hence broad-spectrum. Antibiotics that kill bacteria are bactericidal while those that stop their growth are bacteriostatic. Currently more than 8 classes of antibiotics are used in treatments of bacterial infections worldwide examples includes *β*-lactams, aminoglycosides, fluoroquinolones and tetracyclines.

2.6.1. Antibiotics classification

Antibiotics are usually classified based on their structure and/or function. There are five functional groups that cover their mechanisms of actions, which include:

(a) Inhibitors of cell wall synthesis,

(b) Inhibitors of protein synthesis,

(c) Inhibitors of membrane function,

(d) Anti-metabolites,

(e) Inhibitors of nucleic acid synthesis.

Antibiotics that inhibit cell wall synthesis include the *β*-lactams comprising of penicillins, cephalosporins, monobactams and carbapenems, and the glycopeptides such as vancomycin and teicoplanin and the fosfomycin such as Phosphomycin. *β*-lactam antibiotics are bactericidal and operate by inhibiting the final transpeptidation step in the synthesis of the strong peptidoglycan by inactivating the enzymes transpeptidase also known as penicillin-binding proteins (PBPs). The glycopeptides, in addition to their cell wall inhibition, also binds to the amino acids within the cell wall called acyl-D-alanyl-D-alanine and prevent the addition of new units in the peptidoglycan. Fosfomycin inhibits bacterial cell wall biogenesis by inactivating the enzyme UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase also known as MurA, which catalyzes the ligation of phosphoenolpyruvate (PEP) to the 3'-hydroxyl group of UDP-N-acetylglucosamine a committed step in the peptidoglycan biosynthesis. (Brown *et al*., 1996)

Inhibitors of protein synthesis includes aminoglycosides, tetracyclines, phenicols, oxazolidinones, ansamycins, glyclyclines and MLSK (Macrolides, Lincosamides, Streptogramins, Ketolides), (BioMérieux, 2008). Most of the antibiotics in this category are bacteriostatic except for few. Their selectivity is based on the differences in the prokaryotic 70S ribosome and the 80S eukaryotic ribosome. Tetracycline and aminoglycosides irreversibly bind to the 30S subunit of the ribosome, MLSK, phenicol, oxazolidinones, and binds to 50S ribosomal subunits giving rise to abnormal proteins. Ansamycins forms stable complex with RNA polymerase and prevents transcription of DNA into RNA (Bohen, 1998).

Inhibitor of membrane functions includes lipopeptides, polymyxins and cyclic lipopetides, all bactericidal. Polymyxin alters the cell membrane structure by binding and disrupting the cell membrane osmotic balance causing water leakage leading to cell death. Cyclic lipopeptides binds to calcium components of the cell membrane causing depolarization and leading to inhibition of intracellular synthesis of nucleic acid materials (DNA, RNA and protein) (BioMérieux, 2008).

Antibiotics acting as anti-metabolites otherwise known as folate pathway inhibitors include sulphonamides and trimethoprim/sulfamethoxazole. They inhibit the folic acid synthesis in bacteria and since human beings do not synthesis folic acid, their selective toxicity is maintained.

Quinolones and furanes are nucleic acid synthesis inhibitors. Quinolones inhibit DNA synthesis by targeting the DNA gyrase responsible for supercoiling process of the DNA while nitrofurantoin damages the DNA by its reduction to flavoproteins which attacks the ribosomal proteins in the bacterial cell. Both drugs are bactericidal. Tables 2.2. Illustrates how the antibiotics exert their action on different target in the bacterial cell. Figures 2.6 list the mechanisms of actions with respect to the corresponding class of antibiotics and examples of each class.

Table 2.2. Mechanism of action of antibiotics. Source: BioMérieux (2008)

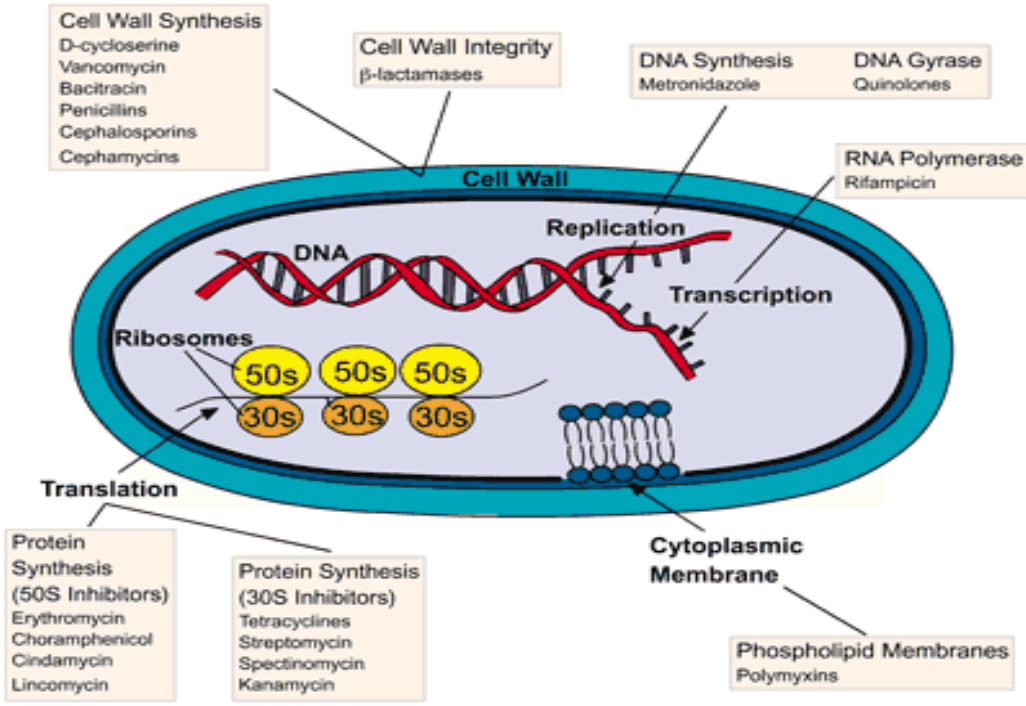
Mechanism of action	Antibiotics	Targeted bacteria
Interference with cell wall synthesis	***β*-lactams**; penicillins, cephalosporins, monobactams and carbapenems,	Gram-negative
	Glycopeptides: vancomycin and teicoplanin	Gram-positive
	Fosfomycin: Phosphomycin	Broad-spectrum
Inhibition of protein synthesis		
Bind to 50S ribosomal unit	*__MLSK__: Macrolides, Lincosamides, Streptogramins, Ketolides	Gram-positive /Gram-negative
	Phenicols:Chloramphenicol	
	Oxazolidinones: Linezolid	broad-spectrum including mycoplasma, rickettsia and Chlamydia
Bind to 30S ribosomal unit	**Aminoglycosides:** Amikacin, gentamicin	Gram-positives
	Tetracyclines: Tetracyclines	Broad-spectrum
	Glycylcyclines: Tigecycline	Broad-spectrum (except for Proteus with natural resistance)
Forms complex with RNA polymerase		Broad-spectrum
	Ansamycins: Rifamycins, Rifamipicin	Gram-negatives

Table 2.2. Contd.

Inhibition of membrane function	Polymyxins: Plymyxin B and Colistin	Gram-negatives
	Cyclic Lipopeptide: Daptomycin	Gram-positives
Anti-metabolites	Sulfonamides Trimethoprim/Sulfamethoxazole	Broad-spectrum of activity. Natural resistance due to low level and poorly expression is seen in *Enterococcus* spp. and *S. pneumoniae* . *P. aeruginosa* resistance is due to impermeability
Inhibitors of nucleic acid synthesis	Fluoroquinolones: ciprofloxacin, norfloxacin and ofloxacin. Furanes: Nitrofurantoins	Broad-spectrum of activity

*****structurally unrelated but similar in spectrum and activity

Fig 2.6: Diagrammatic representation: Mechanism of action of antibiotics



Source: www.wiley.com/college/studentactivities.com

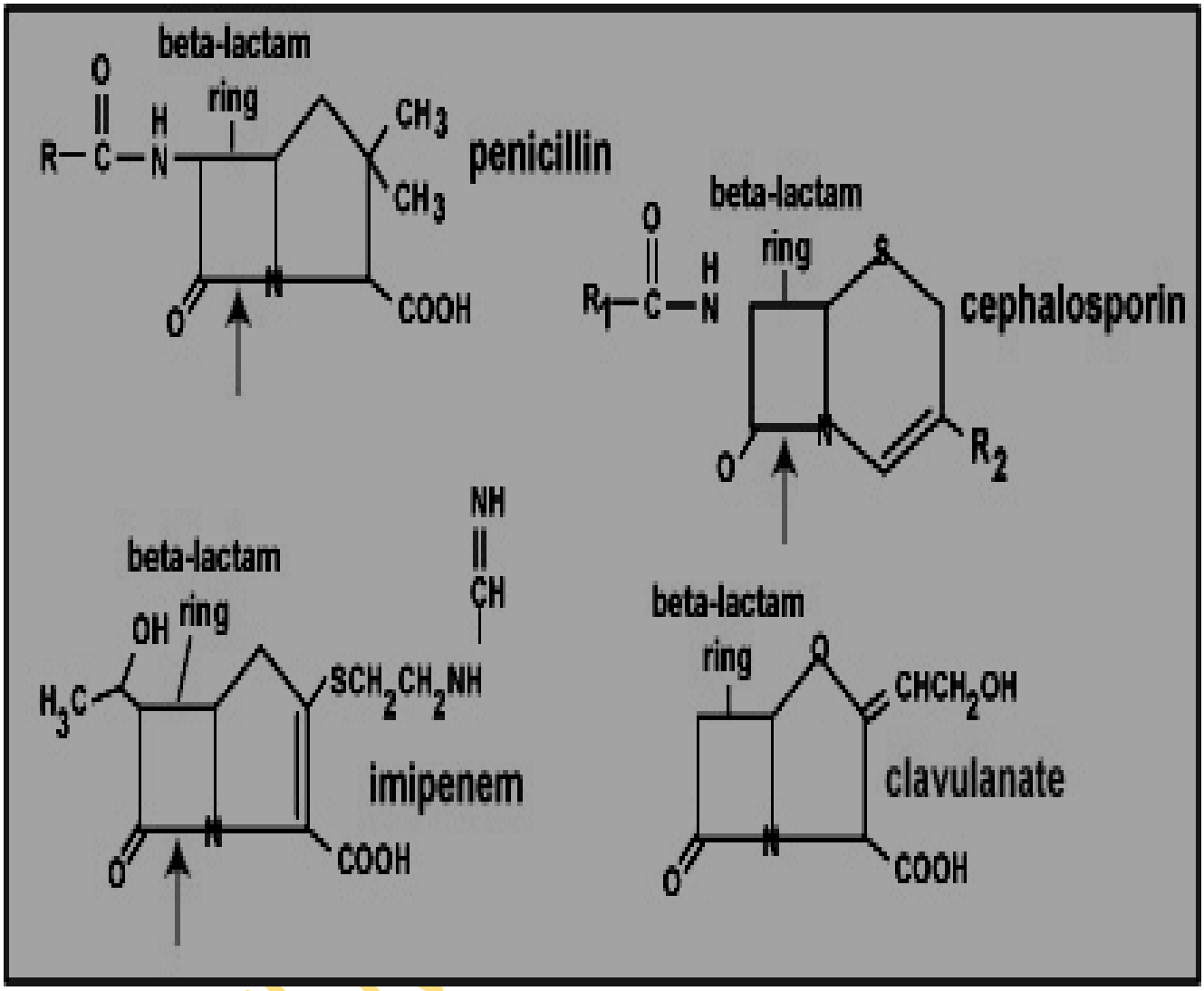
2.7. Beta-Lactam antibiotics

The *β*-Lactam antibiotics are broad class of antibiotics, consisting of all antibiotic agents that contains a *β*-lactam nucleus in its molecular structure (Table 2.2 and Fig 2.7). This includes penicillins and its derivatives, cephalosporins, carbapenems, monobactams, and *β*-lactamase inhibitors (Holten and Onusko, 2000). The *β*-lactam antibiotics are clinically relevant in the treatment of bacterial infections caused by susceptible organisms. *β*-lactam antibiotics were initially narrow spectrum i.e. they were active only against Gram-positive bacteria but became broader in their spectrum and also against Gram-negatives by the introduction of newer members and thus increase their usefulness (Bush 1999). (The *β*-lactam antibiotics got their name as a result of their chemical structure, a four-atom ring known as *β*-Lactam which exerts its activity against bacteria. (Fig 2.7)).

2.7.1. Mode of action of *β*-Lactam antibiotics

The *β*-lactam antibiotics are bacteriocidal, and operate by inhibiting the final transpeptidation step in the synthesis of the strong peptidoglycan by inactivating the enzymes transpeptidase also known as penicillin-binding proteins (PBPs). The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms (McManus, 1997).

Fig 2.7: Beta-Lactam Rings of Penicillins, Cephalosporins, Imipenem, and Clavulanate



Source: www.faculty.ccbcmd.edu

2.7.2. Mechanism of resistance to *β*-Lactam antibiotics

There are four primary mechanisms by which bacteria can overcome β-lactam antibiotics (Babic *et al*., 2006) they include; production of *β*-lactamase enzymes, change in the active site of penicillin binding proteins (PBPs), decreased expression of outer membrane proteins (OMP) and efflux pumps. All these mechanisms are identified with *P. aeruginosa*. However the most common of all these mechanisms against *β*-lactam is the production of *β*-lactamase, although all other methods also play contributory roles (Poole, 2004). β-lactamase acts by breaking the β-lactam ring and binding covalently to the amide bond of the β-lactam ring structure thereby deactivating the molecules antibacterial property (Sykes and Matthew, 1976), but are generally inhibited by clavulanic acid, a β-lactamase inhibitor (Strateva and Yordanov, 2009). According to earlier studies, bacteria cells must have been producing β-lactamases ever before the introduction of penicillins because the genes encoding these enzymes were originally located on the bacterial chromosomes (Livermore, 1995; Bradford, 2001). Additionally, β-lactamases are inducible and constitutively expressed in low quantities. The first report of plasmid-encoded β-lactamases in a Gram-negative bacterium was in 1965 from a Greek patient named Temoniera whose blood culture grew β-lactamase producing *E. coli* hence the designation "TEM" (Datta and Kontomichalou, 1965). This TEM-1 producing *E. coli* hydrolyzed ampicillin, and within a few years after its first isolation, its plasmid-mediated resistance had spread over the world and into many different members of the *Enterobacteriaceae* family, and other bacteria from other genera including *P. aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Jacoby and Munoz-Price, 2005).

Classification of β-lactamase has traditionally been based on either the functional characteristics (Bush-Jacoby-Medieros classification system), or their primary structure (Ambler molecular classification scheme) (Bush and Jacoby 2010). The easiest classification is by protein sequence, whereby the β-lactamase are classified into four molecular classes, A, B, C and D based on conserved and distinguishing amino acid motif (Ambler, 1980; Medeiros, 1997). Classes A, C and D includes enzymes that hydrolyze their substrate by forming acyl enzymes through an active site serine whereas class B β-lactamase are metallic enzymes that utilize at least one active-site zinc ion to facilitate β-lactamase hydrolysis (Bush and Jacoby, 2010). New updated functional subgroups have been added to the scheme as a result of identification. Table (2.4) illustrates an expanded version of the functional classification (Bush, 1989; Bush *et al*., 1995).

Over the years, clinicians keep improving on newer *β*-lactam drugs to circumvent the hydrolytic action of *β*-lactamase. However with each new *β*-lactam antibiotics produced, the pathogen also evolve with a new *β*-lactamase that brings about resistance to the particular drug. Oxyimino-cephalosporins were later introduced and gained an extensive use due to their stability against *β*-lactamase and also effective for treating serious infections caused by Gram-negative bacteria, they were termed Expanded-Spectrum *β*-Lactam antibiotics (Bradford, 2001). Moreover resistance to these newer *β*-lactam antibiotics quickly emerged among the Gram-negatives. All *β*-lactamase capable of hydrolyzing the expanded-spectrum *β*-lactam antibiotics were designated "extended-spectrum *β*-lactamase" (ESBL).

The first recorded of such enzyme (ESBL) was SHV-2, which was found in a German *Klebsiella ozaenae* isolate (Klieb *et al*.,1985). Emergence of closely related TEM-1 and TEM-2 soon began and has been found worldwide in many different genera of *Enterobacteriaceae* and *Pseudomonas*. Today the total number of ESBL now characterized exceeds 200; these are detailed in the authoritative website on the nomenclature of ESBL (http://www.lahey.org/studies/webt.htm). Table 2.3 summaries different *β*-Lactamase and their classes.

2.7.3. Epidemiology of Extended-Spectrum Beta-Lactamase

ESBLs are now a major challenge in the hospitals worldwide especially among infected individuals. After the initial report in Germany and England (Knothe *et al*., 1983) several ESBL-producing organisms have been detected all over the world. In Europe, the first ESBL outbreak caused by *Klebsiella pneumoniae* was reported in France 1986 (Brun-Buisson *et al*., 1987). Ever since then the outbreaks of infections with ESBL-producing organisms and its detection have been reported from virtually every European country (Gunseren *et al*., 1999; Hanberger *et al*., 1999; Babini and Livermore, 2000)

In the United state, first report of ESBL-producing organism occurred in 1988 (Jacoby, 1988). There have been reports of detection of ESBL producing organism in Canada, Chicago and other parts of America (Bedenic *et al*., 2001; Moland *et al*., 2003; Boyd *et al*., 2004). In 1988 and 1989, isolates of *K. pneumoniae* from Chile and Argentina were reported as harbouring SHV-2 and SHV-5 respectively (Casella and Goldberg, 1989). In the same year outbreak of multiresistant *Salmonella enterica* serovar Typhimuruim infections occurred and the pathogen was reported to be harbouring a non-TEM, non-SHV ESBL named CTX-M-2

Table 2.3 Major Families of *β*-lactamase of clinical importance

Enzyme family[a]	Functional group or subgroup	No. of enzymes[bc]	Representative enzymes
CMY	1, 1e	50	CMY-1 to CMY-50
TEM	2b, 2be, 2br, 2ber	172	
	2b	12	TEM-1, TEM-2, TEM-13
	2be	79	TEM-3, TEM-10, TEM-26
	2br	36	TEM-30 (IRT-2), TEM-31 (IRT-1), TEM-163
	2ber	9	TEM-50 (CMT-1), TEM-158 (CMT-9)
SHV	2b, 2be, 2br	127	
	2b	30	SHV-1, SHV-11, SHV-89
	2be	37	SHV-2, SHV-3, SHV-115
	2br	5	SHV-10, SHV-72
CTX-M	2be	90	CTX-M-1, CTX-M-44 (Toho-1) to CTX-M-92
PER	2be	5	PER-1 to PER-5
VEB	2be	7	VEB-1 to VEB-7
GES	2f	15	GES-2 to GES-7 (IBC-1) to GES-15
KPC	2f	9	KPC-2 to KPC-10
SME	2f	3	SME-1, SME-2, SME-3

OXA	2d, 2de, 2df	158	
	2d	5	OXA-1, OXA-2, OXA-10
	2de	9	OXA-11, OXA-14, OXA-15
	2df	48	OXA-23 (ARI-1), OXA-51, OXA-58
IMP	3a	26	IMP-1 to IMP-26
VIM	3a	23	VIM-1 to VIM-23
IND	3a	8	IND-1, IND-2, IND-2a, IND-3 to IND-7

a Enzyme families include those for which numbers have been assigned based on primary amino acid structures (G. Jacoby and K. Bush, http://www.lahey.org/Studies/).

b Compiled through December 2009.

c The sum of the subgroups in each family does not always equal the total number of enzymes in each family, because some enzyme numbers have been withdrawn, and some enzymes have not been assigned a functional designation by the investigators who provided the amino acid sequence. Source: (G. Jacoby and K. Bush, http://www.lahey.org/Studies/).

(Bauernfeind *et al.*, 1996), however organism with various CTX-M genes have since then spread through many part of South America (Radice 2002)

In Africa, ESBL producing *K. pneumoniae* have also been reported in South Africa (Shipton *et al.*, 2001; Bell *et al*., 2002) Israel (Borer *et al*., 2002) and many North African countries (BenRedjeb *et al*., 1990; El-Karsh *et al*., 1995; Mhand *et al*., 1999). In 2006 a novel CTX-M-15 enzyme was identified in Nigeria by Soge *et al* (2006) a similar report was earlier documented in Cameroon (Gangoue-Pieboji *et al*., 2005). Other studies of ESBL producing organisms has been widely reported around Africa such as Mali (Weill *et al*., 2004), Morocco (AitMhand *et al*., 2002) including a nosocomial outbreak with an infection with *P. aeruginosa* expressing GES-2 in South Africa (Poirel *et al*., 2002).

Report of ESBL in Asia was first documented in China in 1988 from *K. pneumoniae* harbouring SHV-2, further reports of ESBL types such as CTX-M and other SHV derivatives have since been described in India (Karim *et al*., 2001), Korea (Pai *et al.*, 2001) Japan (Ma *et al*., 2002). Recently a new *β*-Lactamase called New Delhi metallo-*β*-lactamase (NDM-1) belonging to the class carbapenamase was reported from *K. pneumoniae* isolated from a patients in the United Kingdom who have had treatment from India (Yong *et al*., 2009).

The first detected ESBL producing organisms in Australia were from a collection of gentamicin-resistant *Klebsiella* spp. in a 2-years study (1986-1988) from Perth (Mulgrave, 1990), they were characterized as SHV derivation. However, ESBL-producing organisms have since then been detected in every state of Australia (Bell *et al*., 2002).

2.7.4. Clinical impact of ESBL

Extended-spectrum *β*-lactamase production among Gram-negative bacteria associated with nosocomial and other related infections are known to increase mortality and morbidity cases among the hospitalized due to difficulty in treatment of such infections (Lister *et al*., 2009). Infections due to ESBLs producing organisms also prolong hospitalization and increases hospital expenses (Cosgrove and Carmeli, 2003). ESBL have become increasingly widespread lately and their routine detection is not affordable by all clinical laboratories due to its cost and labor-intensive procedures (Paterson *et al*., 2004).

ESBL producing bacteria often transfer resistant genes encoded in plasmids and other mobile genetic elements through horizontal gene transfer to other bacteria such as *P. aeruginosa, E. coli*, *A. baumannii*, *K. pneumoniae* and *Salonella* spp that are known to be dangerous pathogens capable of causing an outbreak. Third and fouth generation cephalosporin are often used in their treatment but they usually acquire resistance to such drugs. Carbapenem such as Imipenem are effective in such treatments but are expensive, however increasing carbapenem resistant *P. aeruginosa* and *A. baumannii* have also been reported (Strateva and Yordanov 2009; Anuradha *et al*.,2010). Progressive proliferation of ESBL capable of hydrolyzing available antimicrobial drugs among the pathogen may eventually lead to a post antibiotic era if urgent measures are not taken.

TABLE 2.4. Classification schemes for bacterial *β* -lactamases, expanded from Bush *et al*. (1995)

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Molecular class (subclass)	Inhibited by CA or EDTA	TZB*a*	Defining characteristic(s)	Representative Enzyme (s)
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI[b]	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino- β –lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins,	Yes	No	Increased hydrolysis of oxyimino- *β* -lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	

Table 2.4 Contd.

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Molecular class (subclass)	Inhibited by		Defining characteristic(s)	Representative Enzyme (s)
				CA	or EDTA TZB*a*		
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino- β -lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or Oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino- β –lactams	

Table 2.4 Contd.

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Molecular class (subclass)	Inhibited by		Defining characteristic(s)	Representative Enzyme (s)
				CA or TZBa	EDTA		
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum Cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not Aztreonam	CepA
2f	2f	A	Carbapenems	Yes	No	Increased hydrolysis of carbapenems, oxyimino- β -lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B(B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

a CA, clavulanic acid; TZB, tazobactam. *b* NI, not included .
Source: Bush *et al*. 1995

2.8. Aminoglycoside

Aminoglycosides are important group of antimicrobials containing both natural and semi synthetic broad-spectrum antibiotics. The first aminoglycoside, streptomycin was discovered in 1944 from *Streptomyces griseus* and was the first therapeutic for tuberculosis (Schatz and Waksman, 1944). Naturally occurring aminoglycosides are derived from genera Steptomyces and Micromonospora. The aminoglycosides derived from Streptomyces are expressed with suffix "*mycin*" while those produced by Micromonospora are named with the suffix "*micin*" examples are kanamycin and Gentamicin (Paul, 2009). Semi-synthetic aminoglycosides such as amikacin, netlimicin and arbekacin are produced from naturally occurring ones such as kanamycin, gentamicin and sisomicin.

Aminoglycoside are bactericidal and exhibit *in vitro* activity against a wide variety of clinically important Gram-negative bacilli such as *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Citrobacter* spp., *Acinetobacter* spp., *Enterobacter* spp., *Proteus* spp., *Klebsiella* spp., *Serratia* spp., *Morganella* spp and *Pseudomonas* spp. as well as *Staphylococcus aureus* and some *Streptococci* spp (Vakulenko and Mobashery, 2003). They have a weak activity against *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Burkholderia cepacia*, and *Stenotrophomonas maltophilia* except when they are used in synergy with cell-active antibiotics such as *β*-lactams and vancomycin; however they lack predictable *in-vitro* activity against *Bacteroides* (Gilbert, 2000).

2.8.1. Mode of Action of Aminoglycosides

Aminoglycosides are a vital component of antipseudomonal chemotherapy implicated in the treatment of a variety of infections. They kill bacteria by inhibiting protein synthesis via irreversible binding to the 30S smaller subunit of the ribsome, which is responsible for high-fidelity translation of genetic material (Vakulenko and Mobashery, 2003). The action of aminoglycosides usually leads to misinterpretation and premature termination of the mRNA resulting to abnormal proteins which are fatal to the bacterial cell (Nightingale *et al.*, 2007). These agents are bactericidal and exhibit synergy with other antimicrobials, most notably *β* -lactams, with which they are often administered for the treatment of *P. aeruginosa* infections; and problems

with toxicity (aminoglycosides are oto- and nephrotoxic) appear to be ameliorated by increasing the dosing intervals (Turnidge, 2003).

Several groups of aminoglycoside resistance mechanisms are known: enzyme modification (major), low outer membrane permeability, active efflux and, target modification (Vakulenko and Mobashery, 2003; Poole, 2005;). These enzymes are further subdivided into classes based on their site of modification of the drug and the spectrum of resistance. For example AACs can acetylate aminoglycosides at the 1, 3, 2' and 6' amino groups and are correspondingly designated AAC(1), AAC(3), AAC(2') and AAC(6') respectively. Individual variants of these classes are further subdivided using roman numerals such as AAC(3)-I,II and III.

2.8.2. Modifying enzymes

Enzymatic modification of aminoglycosides is the most common type of aminoglycoside resistance among bacteria. These enzymes are called Aminoglycosides-modifying enzymes (AMEs). Their action cause the drug to become inactive hence binds poorly to the ribosome thus permitting bacteria to survive in the presence of the drug (Llano-Sotelo *et al.*, 2002). Three categories of modification enzymes have been described in the bacterial cytoplasm; aminoglycoside acetyltransferase (AAC), aminoglycoside nucleotidyltransferansferase (ANT) and aminoglycoside phosphoryltranserase (APH) which acetylate, adenylate and phosphorylate aminoglycoside antibiotics respectively (Smith and Baker, 2002). These enzymes are further subdivided into classes based on their site of modification of the drug and the spectrum of resistance (Wright, 1999). The genes encoding for AME are usually located on the plasmid or maybe associated with transposable elements and integrons in the chromosomes (Mingeol-Leclercq *et al.*, 1999).

The AACs (N-acetylation) conferring resistance to gentamicin has been known for sometime (Brzezinska *et al*., 1972). Acetylating at the 1, 3, 6′, and 2′ amino groups and it involves virtually all medically useful compounds (e.g. gentamicin, tobramycin, netilmicin and amikacin). AAC family comprises enzymes that modifies 3 position (3-*N*-aminoglycoside acetyltransferases [AAC(3)]) and the 6′ position (6′-*N*-aminoglycoside acetyltransferases [AAC(6′)]). They are discovered early in *P. aeruginosa* and still remain the most common enzymes alongside APH, providing aminoglycosides resistance for this organism. Many types of these enzymes including

their variants have been described in *P. aeruginosa*. AAC (3)-1[a, b, c] are common determinants of gentamicin resistance in *P. aeruginosa* (Phillips *et al*., 1986; Severino and Magalahes, 2002) while the AAC (3)-II and AAC (3)-III are less commonly described enzymes conferring resistance to gentamicin. The AAC (6′) family of enzymes provides resistance to tobramycin, netilmicin, kanamycin, and either amikacin. AAC (6′)-II is more common in *P. aeruginosa* than AAC (6′)-I (Vanhoof *et al.*, 1993).

Aminoglycoside phosphoryltransferase (APH) responsible for the inactivation of aminoglycosides such as kanamycin, neomycin, and streptomycin by resistant strains of *P. aeruginosa* has been known for more than 3 decades (Doi *et al*., 1969). Inactivation is carried out by phosphotransferases [APH (3′)] that modify the 3′-OH of these antimicrobials, and these phosphotransferases are commonly encountered in *P. aeruginosa*. Several APH (3′) enzymes have been described in *P. aeruginosa*, with APH (3′)-I and -II being predominant in clinical isolates resistant to kanamycin (and neomycin) (Miller *et al*., 1994).

Adenylation via aminoglycoside nucleotidyltransferase (ANT) which specifically inactivate streptomycin and gentamicin by resistant strains of *P.aeruginosa* has been previously reported to be in existence more than 20 years ago (Angelatou *et al*., 1982). The most prevalent nucleotidyltransferase is the ANT(2'')-I enzyme, which, with AAC(6') [and, to some extent, AAC(3)], represents the most common determinant of enzyme-dependent aminoglycoside resistance in *P. aeruginosa* (Shaw *et al*., 1991).Other adenyltransferases associated with aminoglycoside resistance in *P. aeruginosa* include ANT(3'') (streptomycin resistance) and ANT(4')-II (amikacin, tobramycin, and isepamicin resistance) (Shaw *et al*., 1991; Sabtcheva *et al*., 2003).

2.8.3. Impermeability

Decrease in uptake and accumulation of aminoglycoside is an intrinsic mechanism of resistance independent of inactivating enzymes commonly associated with microorganisms that are deficient in electron transport system such as Enterococci and other facultative anaerobes, because uptake of aminoglycosides has been shown to require respiration, which generates an electric potential across the cytoplasmic membrane (Mollering, 1991). However in *P. aeruginosa*, impermeability is characterized by resistance to all aminoglycosides due to reduction in accumulation,

i.e. reduced uptake owing to reduced permeability and such was typically referred to as impermeability resistance. In some instances impermeability resistance do occur together with inactivating enzymes in promoting multiple aminoglycoside resistance in *P. aeruginosa* (MacLeod *et al*., 2000). Numerous studies have highlighted the significance of impermeability resistance in aminoglycoside resistant clinical isolates, particularly in cystic fibrosis isolates in which it is often the most common aminoglycoside resistance mechanism (MacLeod *et al*., 2000).

2.8.4. Target Modification

Methylation of 16S rRNA has recently emerged as a new mechanism of resistance against aminoglycosides among Gram-negative pathogens belonging to the family *Enterobacteriaceae* and non-lactose fermenting Gram-negative bacteria, including *P. aeruginosa* and *Acinetobacter* spp (Doi and Arakawa, 2007). This occurrence is mediated by a newly recognized group of 16S rRNA methylases, which share modest similarity to those produced by aminoglycoside producing actinomycetes. The genes responsible are often within transferable plasmids and are mostly encoded on transposons, which provides them with the potential to spread horizontally, and may partially explain the worldwide distribution of this novel resistance mechanism. The first 16S rRNA methylase, called RmtA, was reported in an aminoglycoside-resistant *P. aeruginosa* clinical isolate from Japan, in 2003 (Yokoyama *et al*., 2003). The enzyme was found to confer a high-level resistance to all parenterally administered aminoglycosides, including amikacin, tobramycin, isepamicin, kanamycin, arbekacin and gentamicin.

2.8.5. Active efflux

Active aminoglycoside efflux is a relatively rare resistance mechanism that is due to MexXY proteins operating simultaneously with OprM (Masuda *et al*., 2000; Vogne *et al*., 2004), as well as with some other outer membrane proteins; OpmB, OpmG, OpmI, thus forming three-component active efflux systems. Studies have clarified the involvement of an efflux system of the resistance-nodulation-division (RND) family MexXY in the reduced level of aminoglycoside accumulation that characterizes both

impermeability resistance and adaptive aminoglycoside resistance in *P. aeruginosa*. (Poole, 2004; Vogne *et al*., 2004). MexXY confer resistance to a wide range of antimicrobials, including macrolides, tetracyclines, glycylcyclines, lincomycin, chloramphenicol, novobiocin, fluoroquinolones, and β-lactams aminoglycosides, erythromycin, tetracyclines and glycylcyclines (Okamoto, 2002). Expression of *mexXY* is under the control of MexZ a repressor of the TetR and AcrR family encoded by a gene located immediately upstream of *mexXY* (Aires *et al*., 1999; Westbrock *et al*., 1999). An *in vitro* study demonstrating knockout mutations in *mexZ* have been shown to increase the level of *mexXY* expression but did not provide for aminoglycoside resistance (Westbrock *et al*., 1999). In another study by Sobel and colleagues, MexXY-expressing aminoglycoside resistant clinical isolates lacked mutations in mexZ, suggesting that aminoglycoside resistance attributable to MexXY may require additional components and other means of upregulating mexXY (Sobel *et al*., 2003).

In addition, a recent report highlighting the presence of mexZ mutations in aminoglycoside-resistant clinical isolates expressing mexXY indicates that mexZ mutations may play a role in mexXY expression in clinical strains (Vogne, 2004). It is by no means clear that such mutations were sufficient for aminoglycoside resistance. While the most significant observation regarding the regulation of mexXY is its inducibility by several substrate antimicrobials, it is unclear if this is mediated by the MexZ repressor (e.g., drugs target MexZ directly, obviating repressor activity, thereby permitting mexXY expression, as has been seen for other drug-inducible efflux systems (Poole, 2005).

2.9. Fluoroquinolones

The fluoroquinolone compounds are an important group of antimicrobial agents that have been developed extensively over the past decade (Hooper, 2000a). They have broad acceptance in hospitalized and community patients, and their usage is increasing (Chen, 1999; Hooper 2000b). The quinolones are divided into four groups based on their spectrum of activities (Table 2.5). Recent members have a fluorine substitution which gives them enhanced activity hence are referred to as fluoroquinolones. While older fluoroquinolones (quinolones) are generally effective against aerobic Gram-negative bacteria, newer fluoroquinolones have a broader

spectrum of activity against Gram-negative and Gram-positive bacteria and/or mycobacteria. (Hooper, 2000a). Although some members of the class such as temafloxacin, grepafloxacin and trovafloxacin have been withdrawn because of clinical side effects, new members continued to be developed and approved. Fluoroquinolones are effective against many bacterial infections and well distributed after administration. They are the only available antibiotics for oral treatment of *P. aeruginosa* infections in most countries and are important alternative medicinal product for a veterinarian to have as option for treatment (Jalal *et al.*, 2000; EMEA 2006).

However resistance to this novel class of antibiotics has threatened its use among pathogenic bacteria such as *P. aeruginosa* and *E. coli*. It has been previously stressed that problems associated with resistance to human medicine are correlated to use of antimicrobials in humans (EMEA, 2006) this is also true for fluoroquinolones resistance (Goossen *et al*., 2005). It has been reported that both the overall use of fluoroquinolones and prior patient specific use correlate to the risk of a patient acquiring a nosocomial infection with *P. aeruginosa* (Ray *et al*., 2005).

2.9.1. Mode of Action of Fluoroquinolones

Fluoroquinolones (and earlier quinolones) directly inhibit DNA synthesis by interacting with complexes composed of DNA and either of the two target enzymes, DNA gyrase and topoisomerase IV that are structurally related to each other, both being tetrameric with pairs of two different subunits (Hooper, 2000a). The GyrA and GyrB subunits of DNA gyrase are respectively homologous with the ParC and ParE subunits of topoisomerase IV. DNA gyrase and topoisomerase IV act mutually in bacterial DNA replication, transcription, recombination and repairing of DNA (Nöllmann, 2007). Fluoroquinolones appears to bind to these enzymes and block the DNA synthesis. These complexes apparently act as a kind of cellular poison that eventually kills the cell (Hooper, 2005; Jacoby, 2005).

2.9.2. Mechanism of Fluoroquinolones resistance

Fluoroquinolone mechanism of resistance in *P. aeruginosa* and other Gram-negative bacteria includes one or more of the followings; target mutations, alteration in drug

permeation, plasmid-borne quinolone resistance by the *qnr* gene (Jacoby 2005), efflux mediated resistance and inactivating enzyme (Jacoby *et al*. 2009; Strahilevitz, 2009).

2.9.2. Target mutations

Alteration in target enzymes mode of resistance is one of the extensively studied bacteria resistance mechanism to fluoroquinolones. This mechanism of resistance is usually a consequence of alteration caused by spontaneous mutations occurring in the genes encoding the enzymes subunits and thus can exist in small numbers (1 in 10^6 to 1 in 10^9 cells) in large bacterial populations (Hooper, 1999). A number of mutations in the quinolones-resistance-determining regions (QRDR) of gyrase and topoisomerase IV may result to resistance to fluoroquinolones. Notably is a specific mutation that occurs at position 83 of GyrA that corresponds to residue 80 in ParC which causes reduced binding of fluoroquinolones to the gyrase-DNA complex (Cabral *et al*., 1997). However for the GyrB and ParE subunits of resistant bacteria, amino acid changes, when present (mutations in these subunits are much less common than those in GyrA or ParC), are usually localized to the mid-portion of the subunit in a domain involved in interactions with their complementary subunits (GyrA and ParC, respectively) (Hooper, 2000).

2.9.3. Alteration in drug permeation

Resistance to fluoroquinolones in Gram-negative bacteria is also associated with reductions in porins and reduced bacterial accumulation of drug. Fluoroquinolones are sufficiently small and have charge characteristics that allow them to cross the outer membrane through porin proteins, which form general diffusion channels; they also appear to cross the cytoplasmic membrane by diffusion (Hooper, 1999). However, measurements of diffusion rates suggest that porin reductions alone are generally not sufficient to account for resistance (Nikaido and Thanassi, 1993). Studies have shown that resistance caused by reduced accumulation in MDR *P. aeruginosa* requires the presence and enhanced expression of active efflux pump systems that actively pumps the drug from the cytoplasm (Sugawara *et al*., 2006). Four multidrug efflux systems have been described to date in *P. aeruginosa*, two of which, MexAB-OprM and MexXY-OprM contribute to intrinsic resistance to fluoroquinolones (Masuda *et al*., 2000).

Table 2.5. Classification of different groups of fluoroquinolones based on their antimicrobial spectrum

Groups	Antimicrobial spectrum	Antimicrobial agents
1st group	Enterobacteriaceae	Nalidixic acid
		Cinoxacin
2nd group In addition	*Pseudomonas aeruginosa*, many Gram-positive cocci, *Neisseria* spp.	Ciprofloxacin
		Norfloxacin
		Ofloxacin
		Lomefloxacin
		Levofloxacin
3rd group In addition:	*Streptococcus pneumonia*, some other Gram-positive cocci	Gatifloxacin
		Grepafloxacin
		Sparfloxacin
4th group In addition:	enhanced activity against anaerobes	Moxifloxacin
		Gemifloxacin
		Sitafloxacin

Source: Andriole, 2005

2.9.4. Efflux mediated resistance

Resistance to fluoroquinolones can also result from the decreased accumulation of the drug inside the bacterial cell due to increase efflux. The efflux determinants of fluoroquinolones resistance are multidrug transporters encoded by endogenous chromosomal genes. However it is mostly members of a single resistance/nodulation/division super family (RND) found in Gram-negative species that are implicated in clinically relevant resistance (Poole, 2000). Efflux mediated fluoroquinolones resistance was found to play a significant role in *P. aeruginosa* and many other clinically relevant bacteria (Kohler *et al.*, 1997). In *P. aeruginosa* at least 4 RND type multidrug efflux systems namely; MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN are involved in quinolone resistance (Masuda *et al*., 2000). MexAB-OprM and MexXY-OprM are constitutively expressed providing baseline or instrinsic resistance to fluoroquinolone antibiotics while MexCD-OprJ and MexEF-OprN efflux systems are involved in acquired quinolone resistance in *P. aeruginosa* (Kohler *et al*., 1997).

2.9.5. Plasmid-borne quinolone resistance

Quinolone resistant genes (*qnr*) are plasmid-mediated group of genes, which produces proteins that reduce susceptibility to the quinolones by protecting the complex of DNA with either DNA gyrase or topoisomerase IV enzymes from the inhibitory effect of the quinolones (Jacoby *et al*. 2009). They were first discovered in a *K. pneumoniae* strain that was isolated in July 1994 from the urine of a patient at the University of Alabama at Birmingham Medical Center (UAB) (Martínez-Martínez *et al*., 1998). Plasmid pMG252 on which *qnrA1* was originally identified also encodes the AmpC-type ß-lactamase FOX-5, which has been found at other sites in the United States (Queenan *et al*., 2001). Two additional PMQR mechanisms that could be more prevalent than Qnr proteins have also been reported: *aac(6')-Ib-cr* a variant of aminoglycoside acetyltransferase which inactivate ciprofloxacin, and *oqxAB* and *qepA* which encode efflux pumps which can extrude the drug out (Strahilevitz *et al*., 2009). Several reports have documented the dissemination of plasmid-mediated fluoroquinolones resistance in Gram-negatives bacteria but to date plasmid-mediated

quinolone-resistance determinants, such as described above has not be found in *P. aeruginosa.*

2.10. Macrolide, Lincosamide and Streptogramin (MLS)

Macrolides are class of natural and semi synthetic antimicrobial agent with a unique macrocyclic lactone ring to which various amino sugars are attached. This unique structure form basis from which their name was derived. They are composed of 14 to 16 member lactones to which are attached amino and/or neutral sugars via glycosidic bonds. Members of macrolide antibiotics includes erythromycin, clarithromycin, azithromycin, spiramycin and josamycin. Erythromycin is a natural product of *Saccharopolyspora erythraea* while the newer macrolides are semi-synthetic molecules with substitution on the lactone. Clarithromycin and azithromycin are modified newer macrolide with improved intracellular and tissue penetration , greater stability, better absorption, lower incidence of gastrointestinal side effects, and reduced likelihood of interaction with other drugs (Bryskier, 1999).

Lincosamide members includes clindamycin and lincomycin with good activity against a variety of Gram-positive and Gram-negative anaerobes as well as the parasite *Trichomonas gondii* (Camp *et al*., 2002). Streptogramins consist of two chemically distinct components, streptogramin A and streptogramin B. Streptogramins are a distinct group of antibiotics, but the streptogramin B shares an overlapping binding site with the macrolides and lincosamides on the ribosomes (Giguère, 2006).

2.10.1. Mode of action of the MLS

Antimicrobial agents within macrolide-lincosamide-streptogramin (MLS) classes of antibiotics target the ribosome to inhibit protein translation by binding to the 23S rRNA bacterial ribosomal subunit (Alekshun and Levy, 2007). The macrolides inhibition of protein synthesis proceeds by stimulating the dissociating of the peptidyl-tRNA molecule from the ribsomes during elongation which results in chain termination and a reversible stoppage of protein synthesis causing premature detachment of incomplete peptide chains and subsequent cell death (Vannuffel and Cocito, 1996).

2.10.2. Mechanism of MLS resistance

Mechanism of resistance to macrolide, lincosamide and streptogramin (MLS) has been observed in three ways namely intrinsic, acquired and mutational resistance mechanism. Naturally or intrinsically MLS resistance is a common feature that are observed among the macrolide-producing Streptomycetes that are harbouring genes that provide a self-protective mechanism, as well as the naturally macrolide resistant *Mycobacterium tuberculosis* complex (Andini and Nash, 2006) and several rapidly growing Mycobacteria (Nash *et al*., 2006) that carry unique chromosomal *erm* genes (erythromycin ribosomal methylase). Some of these mycobacterial innate methylase genes confer ML resistance, but not resistance to streptogramins (Roberts, 2008). Equally, innate resistance genes (like *mrs*(C)) for macrolide streptogramin resistance) coding efflux proteins have been described in Enterococci (Roberts, 2008). Enterobacteriaceae such as *E. coli*, *Salmonella* spp. and other Gram-negative bacilli such as *P. aeruginosa* and *A. baumannii* have generally a low susceptibility to macrolides, because of the poor permeability of these hydrophobic substances across their bacterial wall however azithromycin shows activity against *Salmonella* spp. (Vaara, 1993).

The most common acquired resistance mechanism against MLS is a target site modification mediated by at least 34 different rRNA methylases (*erm* genes) described in 34 bacterial genera (Diner and Hayes, 2009). The *erm* genes have been identified so far in 32 bacterial genera, including Gram-negative and Gram-positive as well as aerobic and anaerobic bacteria (Roberts, 2008). Transfer of this gene has been associated with mobile elements like transposon (Tn1545, Tn917, 5384, Tn2009, or Tn53982010) which also confer resistance to other antimicrobials such as tetracyclines and other heavy metals (Martel *et al*., 2003; Schmitt-Van de Leemput and Zadoks, 2007). The *erm* genes can be expressed constitutively or inducibly (Giguère, 2006). When the gene is constitutively expressed, the bacterial strain harbouring the gene will be phenotypically resistant to all or most MLS antimicrobials. However, some of the genes are inducibly regulated by different mechanisms and, in absence of inducers, the enzyme is not produced and the corresponding strain shows a phenotype resistant to the inducing group of molecules only. Induction is generally triggered by exposure of the microorganism to 14-member or 15-member ring macrolides (due to a cladinose sugar moiety), but not by

the 16-member ring macrolides. Inducibly expressed genes can convert to constitutively expressed resistance by deletions or mutations in the regulatory gene. Mutational events in the ribosomal RNA and ribosomal proteins confer reduced susceptibility to MLS by bacteria. Mutations in ribosomal RNA and ribosomal proteins were first identified for proteins L4 and L22 in the 50S subunit of the ribosome (Lovmar *et al*., 2009). From the MLS resistance perspective, the most important are mutations in genes coding for 23S rRNA (domain V), whereas the role of mutations affecting the genes coding for ribosomal proteins L4 and L22 have been less studied (EMA, 2011).

2.11. Tetracycline

The tetracyclines antibiotics was one of the most widely used antibiotics in the 1950s and 1960s in the United States. It had a broad spectrum of activity against a variety of different bacteria and was effective against intracellular and extracellular pathogens (O'Brien and Members, 1997). Tetracycline was first isolated from *Streptomyces aureofaciens*, *Streptomyces rimosus* and *Streptomyces viridofaciens*. Other members of the tetracycline class include 6-deoxy-5-hydroxytetracycline (doxycycline) and minocycline (Chopra and Roberts, 2001). Tetracycline has been particularly useful for outpatient therapy because it is relatively cheap, can be taken orally, and has a relatively few side effects (Standiford, 1990). It does, however, have some important limitations. It is bacteriostatic rather than bactericidal, and it cannot be used for treatment of pregnant women or small children because it causes depression of skeletal growth in premature infants and discoloration of teeth in children (Standiford, 1990). There is also a problem with patient compliance because treatment generally involves multiple doses. Nonetheless, the combination of low toxicity and broad spectrum of activity has far outweighed any drawbacks tetracycline might have (Speer and Sayers, 1992). Although tetracycline is used clinically as an antibacterial agent, it also has activity against some protozoal parasites. Tetracycline derivatives inhibit the growth of *Giardia lamblia*, *Trichomonas vaginalis*, *Entamoeba histolytica*, *Plasmodium falciparum*, and *Leishmania* (Katiya and Elend, 1991).

2.11.1. Mode of Action

The tetracyclines are a family of antibiotics that inhibit the growth of bacteria by entering the bacterial cell, binding to bacterial ribosomes thereby weakening the ribosome-tRNA interaction, and stopping protein synthesis (Schnappinger and Hillen, 1996). A highly conserved region of 16S rRNA may also be part of the binding site (Rasmussen *et al*., 1991), a feature that would explain the broad spectrum of tetracycline. The direct effect of tetracycline binding to ribosomes is that aminoacyl-tRNAs do not bind productively to the A site on the ribosome (Epe *et al*., 1987). The semi-synthetic tetracycline derivatives, colloquially termed the glycylglycines, act at the bacterial ribosome to arrest translation. The glycylglycines bind the ribosome more tightly than previous tetracyclines, so that the *TetM* resistance factor is unable to displace them from this site, hence *TetM* is unable to protect the ribosomes from the action of these new drugs. The *TetA*-mediated efflux system is ineffective against the glycylglycines, as they are not substrates for the transporter.

Tetracycline binds to the 70S ribosomes found in mitochondria and can aslo inhibit protein sysnthsis in mitochondria (Chopra *et al*., 1981). Tetracycline binding activity on 70S and 80S is selective on bacterial cell because of its preference for the 70S ribosomes, this is as a result of the existence of a tetracycline affinity site on this ribosome and its reduced penetration into the mammalian cells (Butaye *et al*., 2003).

2.11.2. Mechanisms of Tetracycline Resistance

Tetracycline resistance in bacteria could occur by the use of three strategies: limiting the access of tetracycline to the ribosomes, altering the ribosome to prevent effective binding of tetracycline, and producing tetracycline-inactivating enzymes.

2.11.3. Limiting Tetracycline Access to Ribosomes

This mechanism is also known as reduced uptake or efflux-mediated tetracycline resistance of the drug, fueled by an energy-dependent mechanism that removes the tetracycline from the bacterial cell which is mediated by membrane-associated proteins (Tet), which exchange a proton for a tetracycline-cation complex (Paulsen *et al*., 1996). This process reduces the intracellular concentrations of tetracycline

because it pumps the antibiotic out of the cell at a rate equal to or greater than its uptake. This resistance mechanism is the best-studied and most familiar mechanism of tetracycline resistance (McMurry *et al*., 1980).

All Tet efflux protein belongs to the 'Major Facilitator' family. To date, eight classes of tetracycline efflux genes have been identified. Classes A to E are found among members of Enterobacteriaceae, *Pseudomonas*, *Aeromonas* and *Vibro* (Buu-Hoi *et al*., 1989; Butaye *et al.,* 2003), Class P are found *Clostridium* spp while class K and L are found mainly in Gram-positive bacteria such as *Staphylococcus*, *Bacillus* and *Streptococcus* (Abraham *et al*., 1988; Butaye *et al.,* 2003). The efflux *tet* genes of classes A, B, D and H are associated with non-conjugative transposons or transposon-like elements; those of classes C, E, and G are often found on plasmids (Butaye *et al.*, 2003).

2.11.4. Tetracycline Resistance by Ribosomal Protection

The ribosomal protection is a less familiar resistance mechanism than tetracycline efflux among bacteria. The cytoplasmic protein interacts or associates with the base of h34 protein, within the ribosome. This interaction leads to a disruption of the tetracycline binding sites on the ribosomes and the tetracycline molecules are displaced. Ribosomal protection resistance genes; tet(M), tet(O), and tet(Q) and others totalling up to 11 classes have been characterized and sequenced (LeBlanc *et al*., 1988; Salyers *et al*., 1990).

2.11.5. Tetracycline Resistance by Enzymatic Inactivation

The third type of tetracycline resistance in bacteria involves enzymatic inactivation by *tet*(X) gene coding for NADPH-requiring oxidoreductase, which inactivates tetracycline in the presence of oxygen and NADPH. This gene was found on two closely related Bacteriodes transposons that also carry a gene for erythromycin resistance However, the gene worked only in aerobically grown *E. coli* cells and did not confer resistance on anaerobically grown *E. coli* or on *Bacteroides* spp. (Speer *et al.*, 1992). The clinical significance of *tet*(X) is unclear. Not only does it not confer resistance on the Bacteroides strains in which it was originally found, but also requires such high levels of aeration to function as a resistance factor in *E. coli* that it probably could not confer meaningful levels of resistance in the microaerophilic

environment found in most sites on the human body. At this point, the possibility cannot be ruled out that some interaction with hemoglobin or other oxygenbearing molecules allows it to function in the human body (Speer *et al*., 1992).

CHAPTER THREE
MATERIAL AND METHODS

3.1. Materials

3.1.1. Equipments, Media, Buffers and other materials

The culture media, enzymes, buffers, chemicals, antibiotic disks and other equipment employed for this study are listed in Appendix 1

3.1.2. Bacterial isolates

Eighty-five bacterial isolates were obtained from various clinical specimens submitted at Medical Microbiology Units of hospitals from 3 Southwestern States of Nigeria; Oyo, Ogun and Ondo (Table 3.1). The isolates were obtained from blood, urine, pus, wound swab, ear swab, and high vagina swab of infected patients during a seven-month period (March-September 2010). All the organism were initially identified in the various laboratories of isolation as *P. aeruginosa* but 54 of these strains were finally verified as *P. aeruginosa* by standard biochemical criteria (Barrow and Feltham, 1993) in the department of Pharmaceutical Microbiology, Univerisity of Ibadan. Further confirmation of the identity of the isolates was carried out at the Department of Environmental Microbiology, CSIR-Indian Institute of Toxicology Research Lucknow, India, using standard biochemical criteria.

3.1.3. Standard bacteria strains

E. coli V 517 and *E. coli* strain DH5α (Microbial Type Culture Collection (MTCC) Chandigarh India) were used for plasmid quantification and conjugation respectively in this study (Table 3.2). American Type Culture Collection (ATCC) strain *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used for positive and negative control strains respectively in the susceptibility studies.

3.1.4. Plasmid markers and DNA Ladders

Plasmid markers and DNA ladders used in this study for the estimation of the size of the plasmids and DNA are listed in Table 3.2.

3.1.5. Polymerase Chain Reaction (PCR) and Sequencing Primers

The PCR primers used for the detection of the various resistance genes investigated in this study and the primers used for the sequencing of genes are listed in Table 3.3

Table 3.1. Sources of Bacterial isolates

STATE	LOCATION	NUMBER OF STRAIN
OYO	University College Hospital, Ibadan	20
	St Mary Catholic Hospital Eleta Ibadan.	7
	Catholic Hospital Oluyoro Ibadan	12
OGUN	Federal Medical Center Abeokuta	10
ONDO	Federal Medical Center Akure	5

Table 3.2 Plasmid and DNA markers used in this study

Standard circular plasmid marker/ Linearized DNA ladders	Description
E. coli V517 Plasmid marker (MTCC Chandigarh India)	Contains 8 plasmids with sizes 58, 7.3, 5.6, 5.2, 4.0, 3.0, 2.7 and 2.1 kb.
1kb ladder (Genei, Banglore, India)	It has 11 fragments consisting of 500bp repeats from 0.5 – 3kb. 1kb repeats from 3 – 6kb and 2kb repeats from 6 – 10kb.
500bp ladder	17 fragments consisting of 50 – 500bp by 50; 600 – 900 bp by 100 and 1000 – 3000 bp by 1000.
*Hind*III Lambda DNA ladder (FINN, Finland)	Contains 8 fragments, 125 – 23, 130bp
Supermix DNA ladder (Genei, Banglore, India)	Contains 13 bands of double stranded DNA fragments ranging from 500bp to 33.5kb
100bp ladder (Genei, Banglore, India)	It has 10 fragments consisting of 100bp repeats from 100 – 1000bp

Table 3.3 Primers used for the PCR and sequencing of genes in this study

Primer	Sequence	Gene	Reference
aac(3)-I- F	AGCCCGCATGGATTTGA	*aac(3)*	Kim *et al.*, 2008
aac(3)-I-R	GGCATACGGGAAGAAGT	*aac(3)*	
aac(6′)-I-F	CGCGCGGATCCCACACTGCGCCTCATGA	*aac(6)*	Kim *et al.*, 2008
aac(6′)-II-F	AGCCCGCATGGATTTGA	*aac(6)*	
aac(6′)-II-R	GGCATACGGGAAGAAGT	*aac(6)*	Kim *et al.*, 2008
aac(6′)-I-R	GACGGGTCGTTTGAATTCTGGTG	*aac(6)*	
aac3-II.F	CGTATGAGATGCCGATGC	*aac(3)*	Kim *et al.*, 2008
aac3-II-R	AAGATAGGTGACGCCGAAC	*aac(3)*	
AmpC-F	TTA CTA CAA GGT CGG CGA CAT GAC C	*AmpC*	Pitout *et al.*, 1998
AmpC-F	GGC ATT GGG ATA GTT GCG GTT G	*AmpC*	
ant(2′′)-I-F	GACACAACGCAGGTCACATT	*ant*	Kim *et al.*, 2008
ant(2′′)-I-R	CGCATATCGCGACCTGAA AGC	*ant*	
aph(3′)-VI-F	GACGACGACAAGGATATGGAATTGCCCAATATTATT	*aph*	Kim *et al.*, 2008
aph(3′)-VI-R	GGAACAAGACCCGTTCAATTCAATTCATCAAGTTT	*aph*	
CTXM1-F	AAAAATCACTGCGCCAGTTC	bla_{CTX}	Woodford *et al.*, 2006
CTXM1-R	AGCTTATTCATCGCCACGTT	bla_{CTX}	
CTX-M1GF	CGC TTT GCG ATG TGC AG	bla_{CTX}	Woodford *et al.*, 2006
CTX-M1GR	ACC GCG ATA TCG TTG GT	bla_{CTX}	
CTX-MU1	ATG TGC AGY ACC AGT AAR GT	bla_{CTX}	Pagani *et al.*,2003
CTX-MU2	TGGGTRAARTARGTSACCAGA	bla_{CTX}	

Table 3.3 Contd.

Primer	Sequence	Gene	Reference
Hep58-F	TCATGGCTTGTTATGACTGT	*attI*	White *et al*., 2001
Hep71-R	CGGGATCCCGGACGGCATGCACGATTTG TA	*attI2*	
Hep 83-F	CACTCAAGGATGTATTGTG	bla_{CTX}	Pitout *et al.*, 1998
Hep 84-R	TTAGCGTTGCCAGTGCTCG	bla_{SHV}	
Hep35-F	TGCGGGTYAARGATBTKGATTT	*Int-1,2,3*	White *et al*., 2001
hep36-R	CARCACATGCGTRTARAT	*Int-1,2,3*	
IntI3-F	GTGGCGCAGGGTGTGGAC	*IntI3*	Falbo *et al*., 1999
IntI3-R	ACAGACCGAGAAGGCTTATG	*IntI3*	
MexR-F	TCGGCCAAACCAATGAACTAC	*mexR*	Jalal *et al*., 2000
MexR_R	GGGTGAGCGGGGCAAACAACT	*mexR*	
NfxB-F	CGCCCCGATCCTTCCTATTGC	*nfxB*	Jalal *et al*., 2000
NfxB-F	ACGAGCGTCACGGTCCTTTGC	*nfxB*	
OXA-10	ATT TTC TTA GCG GCA ACT TAC	bla_{OXA}	Alipour *et al*., 2010
OXA-10	GT CTT TCG AGTACG GCA TTA	bla_{OXA}	
OXA-DEG	CAICCIGTIARCCAICCIACYTG	bla_{OXA}	Brown *et al*., 2004
OXA-DEG	CIYTIISIMGIGCIAAYAMIGARTAYG	bla_{OXA}	
PER- 1	ATGAATGTCATTATAAAAGC	bla_{PER}	Celenza *et al*., 2006
PER- 1	AATTTGGGCTTAGGGCAGAA	bla_{PER}	

3.2. Methods

3.2.1. Identification of isolates

Slant cultures of *P aeruginosa* collected from 5 different hospitals stated earlier were brought to the laboratory for further identification and confirmation on various media selective for isolation and cultivation of *P. aeruginosa* such as cetrimide agar, Pseudomonas agar base, Hifluoro Pseudomonas agar and MacConkey agar.

3.2.1.1. Gram Staining

Gram staining is a differential staining method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls.

Procedure: A small portion of fresh culture of the bacteria was picked with a sterile inoculating loop on a glass slide to form a smear by first air-drying and gentle heat fixing on a Bunsen burner. The smear was flooded with crystal violet for 60 sec and the dye was washed in a stream of tap water for a few seconds. The smear was again covered with mordant (Gram iodine) for 60 sec before washing slide in a gentle and indirect stream of tap water. This was followed by decolourization with 2-3 drops of acetone for 5-10sec. The smear was counter-stained with safranin for 60sec and slide was washed in a gentle stream of water until no colour appears in the effluent. The slide was bloth-dried with absorbent paper and was observed under the oil immersion x100 object lens of microscope (Figure 4.1).

3.2.1.2. Oxidase Test

Oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidized colored product.

Procedure: A flamed platinum wire loop or sterile wooden tooth pick was used to pick a colony from the bacterial plate (18-to-24-hour culture) and was rubbed on a filter paper that was previously damped with N, N, N,'N'-Tetramethyl-p-phenylene diamine dihydrochlroide also called oxidase reagent. The colour of the rubbed portion changes to purple within 5 to 10 seconds indicating positive oxidase reaction. *P. aeruginosa* is positive while *E. coli* the control strain is negative.

3.2.1.3. Catalase Production Test

Catalase test detects the presence of catalase enzymes by the decomposition of hydrogen peroxide to release oxygen and water

Procedure: A 3-4 drops of hydrogen peroxide was added to an overnight growth of the test strain on an agar slant. Vigorous bubbling within 5-10 seconds is a positive indication of catalase. *Pseudomonas aeruginosa* is positive while *E. coli* the control strain is negative.

3.2.1.4. Urease Production Test

Urease test detects the ability of microorganisms to degrade urea by means of the enzyme urease.

Procedure: Overnight culture of the test organisms were heavily inoculated on urea slants and incubated for 18-24hours. A rose-pink colour change in the medium indicates a positive urease reaction. *Pseudomonas aeruginosa* is negative while *Proteus mirabilis* the conrol strain is positive.

3.2.1.5. Citrate Utilization

The citrate test detects the ability of an organism to use citrate as the sole source of carbon and energy. The medium contains inorganic ammonium salts, which are utilized as sole source of nitrogen by the bacteria. The utilization of citrate for bacteria growth results in the production of alkaline-by-products which turns the green media colour to bright blue.

Procedure: The Koser's citrate medium was inoculated with a straight inoculating wire from an overnight peptone water culture. The tubes were incubated at 37^{o}C for 24 –72hours. A positive result was indicated by the change in the medium's colour from green to bright blue. *Pseudomonas aeruginosa* is positive while *E. coli* is negative.

3.2.1.6. Methyl Red Test

The methyl red test is used to identify enteric bacteria based on their pattern of glucose metabolism. All enteric initially produce pyruvic acid from glucose metabolism.

Procedure: An isolate was inoculated into a glucose-broth medium with a sterile transfer loop. The tube containing the medium was incubated at 35°C for 2-5 days. After incubation, 2.5ml of the medium was transferred to another tube. Five drops of the pH indicator methyl red was added to this tube. The tube was gently rolled between the palms of the hands to disperse the methyl red. A bright colour is a positive test while yellow or orange colour is a negative test. *Pseudomonas aeruginosa* is negative whereas *E .coli* is positive.

3.3. Antimicrobial susceptibility Tests

3.3.1. Disk Diffusion

Antimicrobial susceptibilities were determined for all the 54 isolates by the disk diffusion method on Mueller Hinton Agar (Bauer *et al*., 1966) (MHA) [OXOID, England] according to the Clinical and Laboratory Standards Institute (CLSI, 2010). Briefly, a 0.5 McFarland bacterial suspension of 6-8 hours broth culture was made by comparing the turbidity against a freshly prepared 0.5 McFarland standard at 620nm absorbance using the spectrophotometer. A sterile swab was dipped into the adjusted bacterial suspension and firmly rotated against the wall of the tubes to remove excess fluid. The swab was used to inoculate the entire surface of the MHA plates by streaking at separate 60^{o} rotations to obtain uniform inoculation. The antibiotic disk was aseptically applied onto the surface of the inoculated agar plates at the centre. The plates were incubated at 37^{o}C for 18-24 hours. The zones of inhibition were measured and resistance was determined using the CLSI disk breakpoints (CLSI, 2010). American Type Culture Collection (ATCC) strain *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as positive and negative control strains respectively

3.3.2. Determination of MIC by ETest Strip

Etest is a method combining the principles of the disc diffusion method and the dilution method. An E-test is a plastic strip with specific concentrations of an antimicrobial agent on one side and a MIC scale printed on the other side. After spreading a standard amount of bacteria in dilution on the agar, the strip is placed with the MIC scale heading up. The antimicrobial agent will then diffuse into the

agar, and establish a consistent and stable concentration gradient under the strip. After incubation, the antibiotic gradient gives rise to an elliptical-shaped inhibitory area around the strip. The MIC value is read where the ellipse intersects the strip.

The bacterial suspension of an overnight broth culture was adjusted to 0.5 McFarland standards. A sterile swab was dipped into the bacterial suspension and firmly rotated against the wall of the tubes to remove excess fluid. The swab was used to inoculate the entire surface of the MHA plates by streaking at separate 60^{o} rotations to obtain uniform inoculation and allowed to dry for 10-15minutes before the antibiotics strips were applied to the agar surface with the aid of a sterile forceps and press firmly but gently ensuring that the side containing the drug is facing the agar, in accordance to manufacturer's instructions (HiMedia Pvt., India) The plates were inoculated at 37^{o}C for 24hours. Quality control was performed using *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922.

3.4. Double Disc Synergy Test method for phenotypic detection of ESBL

Double disc synergy test (DDST) for all the cephalosporin-resistant and susceptible strains was performed as a standard disk diffusion asay on Mueller Hinton agar as described previously by Jarlier *et al*. (1988) with modifications. An overnight broth culture of test strains was adjusted to 0.5 McFarland bacterial suspensions and was immediately inoculated on the entire surface of Mueller Hinton agar with the aid of a sterile swab. Excess fluid was removed by pressing the tip of the swab against the wall of the tube. Antibiotic disc containing amoxicillin/clavulanic was firmly placed at the centre of the agar with the aid of a sterile forceps. Disks containing 30 µg of aztreonam, ceftazidime, ceftriaxone, and cefotaxime, were placed 20 mm and 15 mm apart (centre to centre) consecutively from a disk containing amoxicillin (20 µg) plus clavulanic acid (10 µg) and incubated for 18 - 24 h at 37^{o}C. Enhancement of the inhibition zone towards the amoxicillin-clavulanate disc, indicating synergy between clavulanic acid and any one of test antibiotics, was regarded as presumptive ESBL production (Jarlier *et al*., 1988; Pagani *et al*.,2004).

3.5. Molecular Methods used in this study

3.5.1. Plasmid Extraction by Alkaline Lysis

The *P. aeruginosa* isolates were grown overnight at 37^{o}C on Luria Bertani (LB) Agar plates supplemented with ampicillin (100mg/L). A discrete colony from the overnight culture was inoculated into a 100ml volume of LB broth supplemented with ampicillin (100µg/ml) and incubated in a shaker incubator for 8 hours at 37^{o}C with vigorous shaking at 200 rpm. A 2ml of the 8 hour culture was centrifuge in a microfuge at a speed of 13,000 rpm for 2 mins at 4^{o}C. The supernatant was discarded and the bacterial pellet was wash with double distilled water in order to remove any extra media still present and left to dry. The pellet was re-suspended in 150µl cold alkaline solution I (50mM Tris pH 8.0 with HCL, 10mM EDTA, 100µg/mL RNase A stored at 4°C). This was followed by lysis step by adding 300 µl of alkaline solution II (200mM NaOH, 1%SDS) and was mix properly by flicking and incubates on ice for 5 minutes. Complete lysis was indicated by a clear solution. 150 µl of alkaline solution III (3.0M Potassium Acetate, pH 5.5) was added to the clear solution and inverted several times then was incubated on ice for 5 minutes. The bacterial lysate was centrifuged at 13,000 rpm for 10minutes at 4^{o}C and supernatant was carefully transferred to a new 1.5ml Eppendorf tube. Equal volume of phenol: chloroform was added to the supernatant and vortex to form emulsion and was centrifuged at 13,000 rpm for 5minutes at 4^{o}C. The upper layer of the supernatant was carefully removed using

a 100µl Pasteur pipette into a fresh 1.5ml Eppendorf tube. Plasmid was precipitated by adding double volume of ice cold ethanol and vortex briefly to increase the plasmid yield and was allowed to stand for 30 minutes at 4^{o}C before centrifuging at 13,000 rpm for 15 minutes. Supernatant was removed by gentle aspiration and fluids adhering to the walls were also removed by Kimwipe leaving the pellet as dry as possible. One thousand microlitre (1ml) of 70% ethanol was added to the pellet and centrifuge at 5000 rpm for 2minutes, supernatant was discarded and pellet was dried. Plasmid was dissolved in 50 µl of TE buffer (10mM Tris pH 8.0 with HCl, 1mM EDTA) and was stored in −20^{o}C until ready for electrophoresis on agarose gel.

3.5.2. Chromosomal DNA Extraction

Chromosomal DNA extraction was carried out as described previously (Sambrook *et al*., 1989) with modifications. Overnight broth culture of the bacterial cell was transfered into a 1.5ml Eppendorf tube and centrifuge at 5000 rpm for 5minutes at 4^{o}C. The supernatant was discarded and pellet re-suspended in 400 µl of TE pH 8.1 (Appendix I). This was followed immediately by the addition of 500 µl of freshly prepared lyzozyme (4mg/ml) and the mixture was incubated for 20 minutes at 37^{o}C. The reaction mixture was centrifuge at 13,000rpm for 15 minutes at 4^{o}C. The supernatant was discarded and the pellet re-suspended in 200 µl TE. The lysis stage followed immediately with the addition of 70 µl 20% SDS, with swirling and the mixture incubated at 55^{o}C for approximately 20 minutes. After the incubation step, 13 µl of proteinase K was added and the mixture subjected to another incubation stage for 1hour at 37^{o}C. One thousand microlitres (1ml) of Phenol-chloroform was added. The suspension was thoroughly mixed and centrifuged at 13,000 rpm for 15minutes at 4^{o}C. The supernatant containing the DNA was removed into a new 1.5ml microfuge tube and 30 µl of 3M sodium acetate and ice cold 100% ethanol was added to fill the tube. This was mixed gently but thoroughly and store at -20^{o}C overnight. On the next day, the mixture was centrifuge at 13,000 rpm for 15 minutes at 4^{o}C. The ethanol was drained off with Pasteur pipette, and DNA pellet was air dried. The DNA was re-suspended in 100 µl TE plus 10 µl RNAse in TE. The DNA was stored at -20^{o}C until required for use as templates for amplification of the resistance genes.

3.5.2.1. Genomic DNA extraction by Boiling Method

Genomic DNA extraction was carried out as described previously (Sambrook *et al*., 1989) with modifications. Briefly, fully grown culture of the *P. aeruginosa* isolates were suspended in 500µl of Tris EDTA (TE) buffer and vortex gently to homogenize the cells. The cells were lysed

by placing the tube in boiling water bath for exactly 10 mins; afterwards it was immediately cooled on ice, and centrifuged at 14,000 x g for 5min to remove any cell debris. The DNA was store at -20^{o}C until required for use as templates for amplification of the resistance genes.

3.5.2.2. DNA quantification and Analysis

Procedure: The spectrophotometer was initially calibrated to 260nm and 280nm using 1ml double distilled water (ddH_2O) in a cuvette. After this, a 10 µl of each DNA sample were added to 900µl TE (Tris-EDTA buffer) and were mixed together to homogenize the mixture. Values of each samples at both optical densities (OD_{260} and OD_{280}) on the spectrophotometer were noted and the OD_{260}/OD_{280} ratio were calculated.

A ratio between 1.8 – 2.0 indicates the range due to pure nucleic acids, a lower value such as 1.8 indicates the presence of protein or other UV absorbers while a higher ratio >2.0 indicates the presence of other contaminants such as phenol or chloroform used for the extraction in either case re-precipitation of the DNA will be required.

The amount of DNA was quantified using the formula:

$$\text{DNA concentration (µg/ml)} = \frac{OD_{260} \times 100 \text{ (dilution factor)} \times 50 \text{ µg/ml}}{1000}$$

3.5.3. Agarose Gel Electrophoresis

The concentration of agarose gels used for electrophoresis in this study ranged from 0.7-2.0% (w/v). The agarose was dissolved in the same running buffer used for electrophoresis (TAE). A loading buffer e.g. bromophenol blue was added to DNA or plasmid to make the sample denser than water so that DNA sinks evenly into the well. It also add colour to the sample, thereby simplifying the loading process and also contained dye, that in electric field migrate towards the anode at predictable rate.

Agarose gels were cast by melting 0.7% (w/v) in the presence of TAE buffer until a clear transparent solution was achieved. The melted solution was slowly poured into a casting tray

containing a comb at the left side unit and allowed to harden. Comb was gently removed by pulling at an angle after 15-20mins of gel solidifying and thus adding sufficient volume of buffer to cover the gel. The electrophoresis unit was connected to the power supply ensuring the wells are at the cathode because DNA is negatively charge hence will migrate to the anode.

Ten microlitres (0.01ml) of the purified plasmid DNA was added to 3 µl of the blue loading buffer and loaded into the agarose gel wells. Same method of loading is carried out for PCR products and other molecular analysis such as Restriction Fragments Length Polymorphism (RFLP) carried out in this study. Electrophoretic conditions for each analysis on agarose gel varies according to type and size of the fragments expected to be visualized. Generally 100V for 120minutes was sufficient for most analysis carried out in this study.

3.5.3.1. Staining and photographing of agarose gels

Ethidium bromide was used in staining each gel after every electrophoresis by adding 20µl (10mg/ml) of its concentration in 100ml of sterile distil water for 15-20mins and destaining in distill water before photographing under UV transillumination at 120nm using SynGene Documentation system (G:BOX Chemix T16) which was connected to a computer. The picture was retrieved from the computer and the resolution was adjusted as appropriate.

3.5.4. Plasmid curing analysis

Ethidium bromide (EtBr) (Sibisco Research Laboratory, India), Acridine Orange and Sodium dodecyl sulphate (SDS) (Hi-Media India) were used as the curing agents. The minimal inhibitory concentration of ethidium bromide and Acridine Orange was determined for the bacterial isolates in Luria Bertani broth and the highest concentration permitting growth was used for plasmid curing while curing using SDS was carried out using the method described by Tolmasky *et al.,* (1993). Curing was performed by overnight growth at elevated temperature (42^{o}C) in LB broth for both acridine orange and ethidium bromide. Subcultures were initially done on Luria Bertani agar (LB agar) and the colonies were tested for antibiotic susceptibility on Mueller Hinton Agar.

3.5.4.1. Acridine Orange

Luria Bertani broth tubes containing different concentrations of acridine orange to a final concentration of 10, 15, 20, 25, 30, 35, 40 and 45µg ml were inoculated with a fresh active overnight culture of *P. aeruginosa*. The tubes were incubated at 37°C for 24 h. The highest concentration of acridine orange that showed turbidity (0.7-0.8 at OD 660 nm) with a viable isolate was selected for the final curing procedure. The selected tubes containing the appropriate concentrations were inoculated on LB agar medium and incubated at 42°C for 24 h. The cured isolates were tested for their antibiotic sensitivity and presence of plasmids on agarose gel.

3.5.4.2. Ethidium Bromide

LB broth tubes containing different concentrations of ethidium bromide to a final concentration of 10, 15, 20, 25, 30, 35, 40 and 45µg/ml were inoculated with fresh active overnight cultures of *P. aeruginosa*. The tubes were incubated at 42^{o}C and other procedures as described previously for the method used in acridine orange.

3.5.4.3. Sodium dodecyl sulphate (SDS)

The curing was carried out according to Tolmasky *et al.* (1993) with modifications. Ten ml of LB broth (pH 7.6) containing 100µl of 10% SDS was inoculated with 100µl of 10 fold diluted overnight culture of test strains and was incubated at 37°C for 24hrs. Two fold dilutions were made, after that, 5µl was streaked over LB agar medium (Difco Laboratories, Detroit, Mich) plates and incubated at 37°C for 24 h. The separate colonies (mutants) were resubcultured on Mueller Hinton (Difco Laboratories, Detroit, Mich) agar plates to ensure their purity. The isolates were tested again for their antibiotic sensitivity and presence of plasmids.

3.5.5. Endonuclease Restriction Analysis

Endonuclease restriction involves the cutting of double stranded circular DNA into linearized fragments which produces specific banding pattern after agarose gel electrophoresis. A restriction enzyme recognizes, and cuts DNA only at a particular sequence of nucleotides.

The bacterial endonuclease enzymes usually recognize specific nucleotide sequences and split the phosphodiester bond of the polynucleotide chain at the recognition site or very close to it. The commercially available endonuclease enzymes are always supplied with appropriate buffer and the optimum incubation temperature that would ensure good enzyme activity. Restriction digest of plasmid DNA was carried out by using a total reaction volume of 15.0 µl comprising of

sterile distill water, enzyme buffer, bovine serum albumin, plasmid DNA and restriction enzyme as below:

Sterile distill water	7.5 µl
Appropriate enzyme buffer	1.5 µl
Bovine Serum Albumin	1.0 µl
Plasmid DNA	4.0 µl
Enzyme	1.0 µl.

Digestion of integron PCR product was as follows

Restriction buffer	3.0 µl
Integron cassette PCR product	20 µl
Restriction enzyme	1.0 µl.

Incubation of the reaction was done at 37^{o}C as recommended by the manufacturer for 1-2hrs and the enzyme activity was terminated by the addition of 3 µl of blue loading buffer. The products of the restriction digests were then electrophoresed in 0.8% agarose at 100V for 1hr; stained in ethidium bromide and photographed under UV transillumination.

3.5.6. Polymerase Chain Reaction (PCR)

The PCR master mix provided to each reaction:

10.0mM Tris-Cl (pH 8.3 at room temperature)
50.0mM KCl
1.5mM $MgCl_2$
0.2mM each dNTPs
2.5U Taq polymerase (0.5µl)

The PCR tube of a 25 µl total volume of reaction contained:

10.5 µl sterile ddH_2O for PCR
0.5 µl primer 1
0,5 µl primer 2

12.5 µl master mix

1.0 µl template DNA

A PCR condition for amplification in a molecular study depends on the quality of the DNA template, primer specific melting temperature (Tm) used and specificity of the PCR, hence varying PCR conditions were employed in this study. Examples of PCR conditions used for the amplification of *bla*$_{OXA-10}$ an ESBL gene and AmpC beta-lactamase detected in this study is as follows:

For OXA-10 ESBL detection.

Initial denaturation	5mins at 94^{o}C
Denaturation	30 sec at 94^{o}C (for 35 cycles)
Annealing Temp.	60 sec at 60^{o}C (Primer specific T $_{m}$)
Extension	45 sec. at 72^{o}C
Final Extension	10mins. at 72^{o}C
Hold at	4^{o}C.

For AmpC beta-lactamase detection

Initial denaturation	5mins at 94^{o}C
Denaturation	30 sec at 94^{o}C (For 25 cycles)
Annealing Temp.	64 sec at 60^{o}C (Primer specific T $_{m}$)
Extension	60 sec. at 72^{o}C
Final Extension	7mins. at 72^{o}C
Hold at	4^{o}C.

3.5.7. Purification of PCR products for sequencing

The PCR products (amplicons) of the desired DNA-fragments were used for sequencing. Purification was carried out with QIAGEN ® QIAquick PCR purification kit or the in-vitrogen PCR purification kit. The amplicons were extracted from the agarose gel with a clean sharp scapel on a UV illuminated slab. The extracted piece of gel was placed in an Eppendorf tube of known weight and net weight of the gel calculated. To one volume of the derived excised gel weight was added three volumes of buffer QG (solubilisation buffer). The mixture was thoroughly mixed and then incubated at 50^{o}C for 10 minutes. At an interval of 3mins of incubation, the mixture was vortexed to ensure complete melting of the gel matrix. The

solubilisation was followed by the addition of 1 gel volume of isopropanol and mixed before dispensing it into a QIAquick chromotagraphy column. Binding of the DNA to the silica gel membrane was achieved by centrifugation at 14,000 rpm for 1min. The column was then washed with 750µl PE buffer followed by centrifuging at 14,000 rpm for 1 min and the flow-through discarded. The centrifugation was repeated in order to expel all residual PE buffer in the column. The purified DNA was eluted from the column by adding 30 µl sterile distilled water, allowed to sit at room temperature for 1 min and then centrifuge at 14,000 rpm for 1min. All the buffers used were as supplied by the manufacturer.

3.5.8. Molecular detection of genes encoding resistance to extended-spectrum *β*-lactam, fluoroquinolones and aminoglycoside modifying enzymes

Twenty isolates showing drug resistance to 2 or more antipseudomonas drugs were randomly selected for further molecular studies. Genes conferring resistance to beta-lactams, expanded-spectrum beta-lactam drugs, fluoroquinolones and aminoglycosides in this study, were detected using molecular methods i.e. PCR amplification. The PCR amplifications were carried out using published degenerate primers and gene specific oligonucleotides in most cases. Details and sequences of the primers were stated in Table 3.2. General PCR condition used are as stated in previous sections. Other primer specific PCR conditions for DNA amplification of other genes were different for each genes investigated in this study. All resulting PCR products in this study were analysed on 1.2 – 2.0% range of agarose gel depending on the product sizes expected.

3.5.9. Detection and characterization of class 1, 2 and 3 integrons

This was done using the method of White *et al*. (2000). Integrons belonging to classes 1 2 and 3 were detected by PCR using two sets of primers for each integron class and typed by restriction fragment length polymorphism (RFLP) analysis as described previously by (White *et al*., 2000). In brief, degenerate primers hep35 and hep36 (Table 3.2) which hybridized to conserve regions of integron-encoded integrase genes *intI1*, *intI2*, and *intI3* were initially used to detect the presence of integron among the tested samples for PCR. The class of integron was determined by analyzing integrase PCR products by enzyme digestion using *Hin*fI as described in previous section and confirmed by PCR amplification using Class 2 and class 3 integron specific primers (table 1). The amplified fragments were electrophoresed on 1.8% agarose gel and view as described in previous section.

3.5.9.1. Characterization of cassette arrays

Class 1 integrons cassette regions were amplified using hep58 and hep59 primers (Table 3.2) while class 2 cassette regions were amplified using hep74 and hep51 (Table 3.2) which binds to *attI*2 and *orf*X situated downstream of the cassette region respectively. Cassette PCR product was digested with H*inf*I as described previously (White *et al*., 2001). A single amplicons each representing fragments of the same amplification sizes were further sequenced for the determination of the gene cassette array present.

3.5.10. Conjugation/Mating Experiment

The principle behind conjugal mating is that antibiotic resistance genes borne on either plasmid or other mobile genetic elements are transferred to a recipient cell during mating of both donor and the recipient. Standard recipient cell are used for the demonstration of this experiment. The final selection for transconjugants acquiring the resistance genes of interest is usually done on agar plates containing two antibiotics to which either the donor or the recipient would be separately susceptible. In this study *E. coli* DH5α was used a recipient strains while the donor were the parental *P. aeruginosa* OXA positive and *IntI1* positive strains.

The conjugation experiment was carried out as previously described by Shohayeb and Sonbol (1994). In brief, 1ml of LB broth of both donor *P. aeruginosa* and recipient nalidixic acid-resistant *E. coli* DH5α cells were each grown separately to logarithmic phase. Equal volume (500µl) of each strain were thoroughly mixed together in a reacting tube and centrifuged at 5000 rpm for 3 min. The pellets were inoculated on Mueller Hinton agar plates containing no antibiotics and incubated at 37^{o}C for 24 hr. The next day, the mating culture was suspended in 3ml 0.85% saline and tenfold serial dilution was made. Antibiotics used for selection of transconjugant were Tet (25 µg/ml), Rif (25 µg/ml), Str (50 µg/ml) and the transconjugant were picked onto series of plates containing nalixidic acid plus one of the antibiotics mentioned above to check the co-transfer of resistance determinant. The mating cells were subcultured onto nutrient agar plates containing 30 µg nalidixic acid plus one of the antibiotics to which the donor strain was resistant as described previously (Shohayeb and Sonbol, 1994).

CHAPTER FOUR

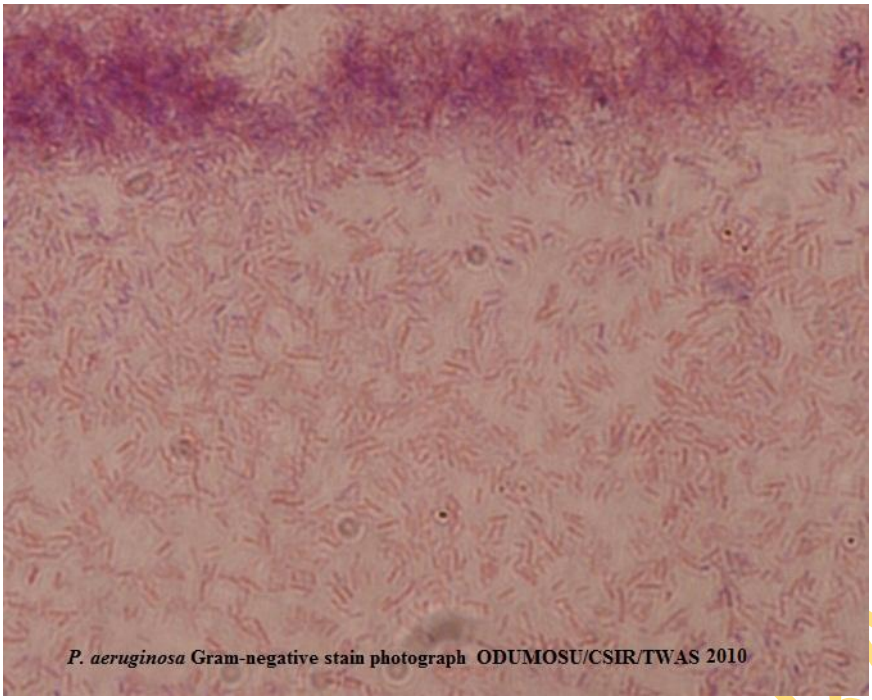
RESULTS

4.1. Identification of Isolates

Eighty five isolates were tentatively identified as *P aeruginosa* at the Medical Microbiology department of the various hospitals where they were stored on slants. A total of 54 isolates were identified by further biochemical characterization at the Department of Pharmaceutical Microbiology University of Ibadan. The isolates were further confirmed at the Environmental Microbiology Section, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow UP India by Gram-staining (Plate 4.1) and growth on selective media specific for the identification of *P. aeruginosa* from clinical source such as *Pseudomonas aeruginosa* agar base (HiMedia, India), Hi-Fluoro Pseudomonas agar (HIMedia, India), excretion of pyocyanin by growth on Pseudosel agar (Centrimide agar Sigma Alrdrich (USA), Kings *et al.* (1954)) and other standard biochemical tests. The isolates were obtained from 5 different hospitals in 3 different states of the south west Nigeria (Catholic Hospital Eleta Oyo State, Catholic Hospital Oluyoro Oyo State, University College Hospital Oyo State, Federal Medical Center Ogun State, Federal Medical Center Ondo State).

4.2. Age and gender distribution of patients from which the 54 *P. aeruginosa* isolates were isolated

The *P. aeruginosa* isolates employed in this study were isolated from patients with various kind of infections commonly associated with *P. aeruginosa* and other diagnosed infections comprises of 29 females and 25 males including 2 elderly patients and 2 young patients. The gender and age distribution of the patients are graphically represented in Figs 4.1 – 4.3



B

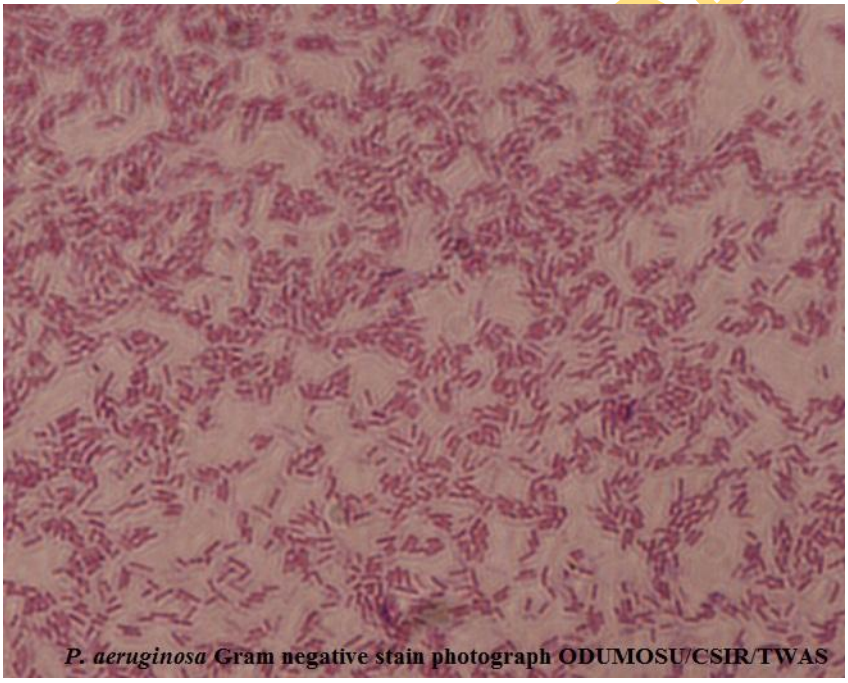


Plate 4.1: Gram Stain of *P. aeruginosa* strain

4.3. Antimicrobial susceptibilities by disk diffusion and resistance patterns

The susceptibilities of the 54 strains of *P. aeruginosa* determined by disk diffusion to a total of 21 antibiotics are given in Table 4.1 Resistance patterns displayed by these isolates and their percentages are shown in Table 4.1. Representative classes of antibiotics are listed in Table 4.3. (Plate 4.2 also shows photograph excerpt of 2 Petri dishes used for the E-test). Cefotaxime, ceftazidime, ceftriaxone, piperacillin, ciprofloxacin, levofloxacin, amikacin and gentamicin were tested by the E-test against selected strains. The overall MICs for all the tested antibiotics ranged from 0.001 – ≥256 µg/ml. Among the *β*-lactam drugs, cefotaxime was the most active agent with all (100%) of isolates having MIC ≤ 15µg/ml (range 1.0 – 15 µg). Ceftriaxone was the least active agent with 29 (50%) of the total isolate tested having MIC ≤ 240 µg/ml (MIC range 3.0 – 240 µg/ml) followed by piperacillin with 21 (25%) (MIC range 1.0 – 240µg/ml) and ceftazidime with 10% (MIC range 1.0 – 240µg/ml).

Ciprofloxacin and levofloxacin representing fluoroquinolones in this assay had similar inhibitory concentration with 13-40 (65-70%) of isolates inhibited by a MIC ≥ 0.001 (MIC range 0.001–2µg/ml) respectively. The remaining 6-7 (30-35%) have high level of MIC >2 µg- 240 µg/ml for levofloxacin and ciprofloxacin respectively. Both drugs were highly resisted by the same set of isolates with high MIC concentration. Among the two aminoglycosides investigated in this study, gentamicin was the least active with 8 (40%) of the isolates having MICs ≤ 4µg/ml and 12 (60%) having MICs ≥ 256µg/ml while 12 (60%) had MICs ≤ 4µg/ml and 8 (40%) had MIC ≥ 256 for amikacin.

4.4. Phenotypic detection of extended-spectrum β-lactamase (ESBL) by double disk method

ESBL was detected in only 5 (9.3%) of 54 isolates investigated while 24 (44.4%) cephalosporin-resistant isolates suspected to be ESBL-producers showed no synergy with clavulanic acid. Among the strains that were ESBL positive by DDST, 2 (40%) of the isolates were from ELETA (ODM5, 17), while 3 (60%) were from UCH (ODM8, 46) and FMC Abeokuta (ODM42) (Table 4.1). Twenty-five (46.3%) strains were cephalosporin-susceptible and were also ESBL-negative by DDST. ESBL was detected at the 15 mm distance in 4 cephalosporin-resistant isolates and at the 20 mm distance from the *β*-lactamase inhibitor disk for *P. aeruginosa* strain ODM 46. Synergy was common at the ceftazidime and cefotaxime disks towards the amoxicillin/clavulanate among the 5 positive strains. All the 5 (9.3%) ESBL positive strains were susceptible to imipenem.

Table 4.1. DDST diameter of the zones of inhibition of ESBL marker antibiotics and cephalosporin resistance patterns

Strain	Clinical source	ESBL status	Zones of inhibition of ESBL marker antibiotics (mm) (CAZ,	CTX,	ATM)	Cephalosporin resistance patterns
ODM 5	Pus	Positive	16	15	18	CAZ,CRO, CTX
ODM 8	Wound	Positive	25	20	25	CRO, CTX
ODM 17	Urine	Positive	20	15	22	CRO, CTX
ODM 42	Pus	Positive	15	6	17	CAZ, CRO, CTX
*ODM 46	Urine	Positive	18	23	20	CAZ, CRO, CTX, FEP

ATM= aztreonam, CAZ= ceftazidime, CRO= ceftriaxone, CTX= cefotaxime, FEP= cefepime
* ODM 46 was detected at the 20mm distance from the amoxicillin/clavualante disk

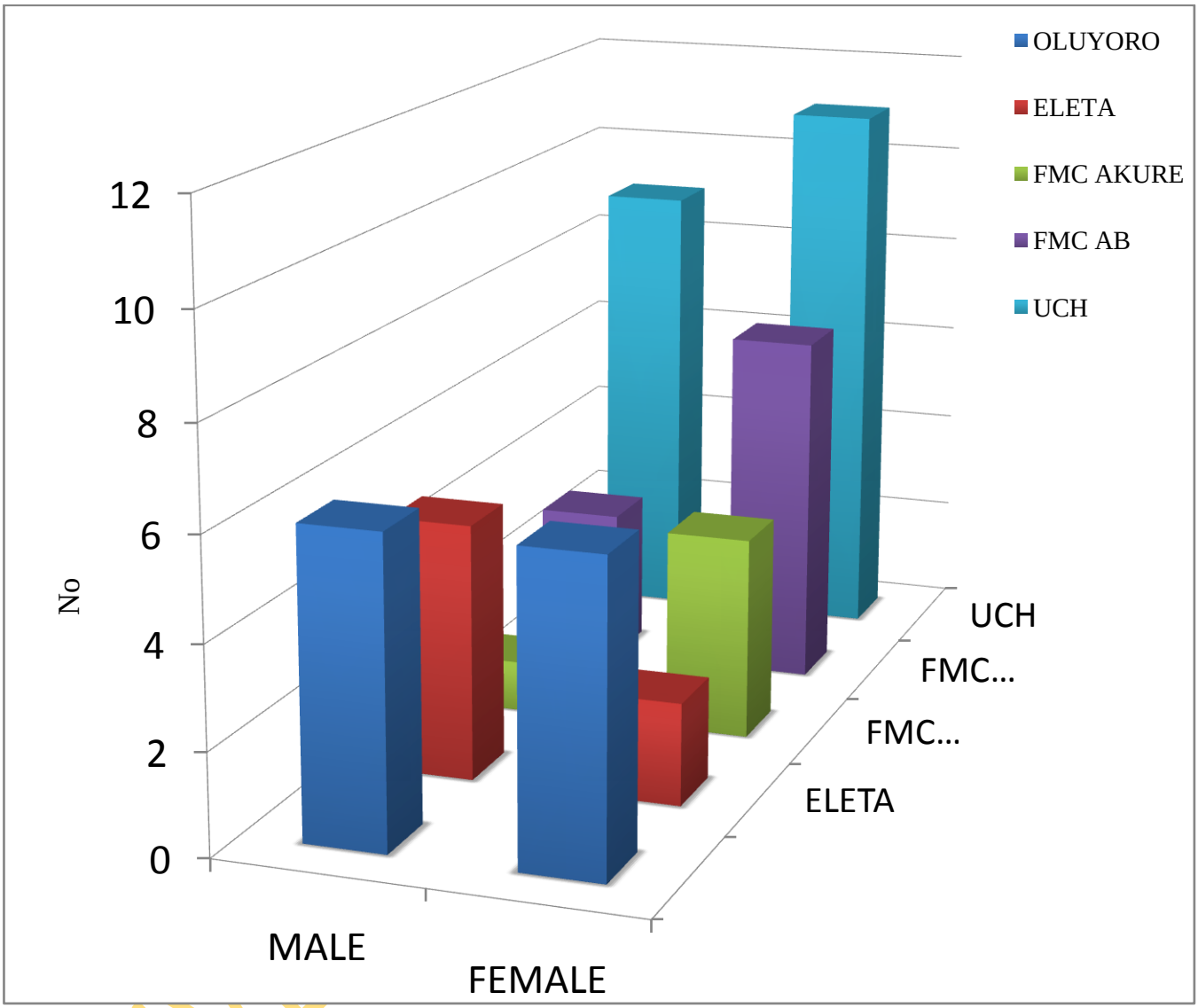


Fig 4.1: Gender distrubution of patients infected with *P. aeruginosa* from 5 hospitals in Southwest Nigeria

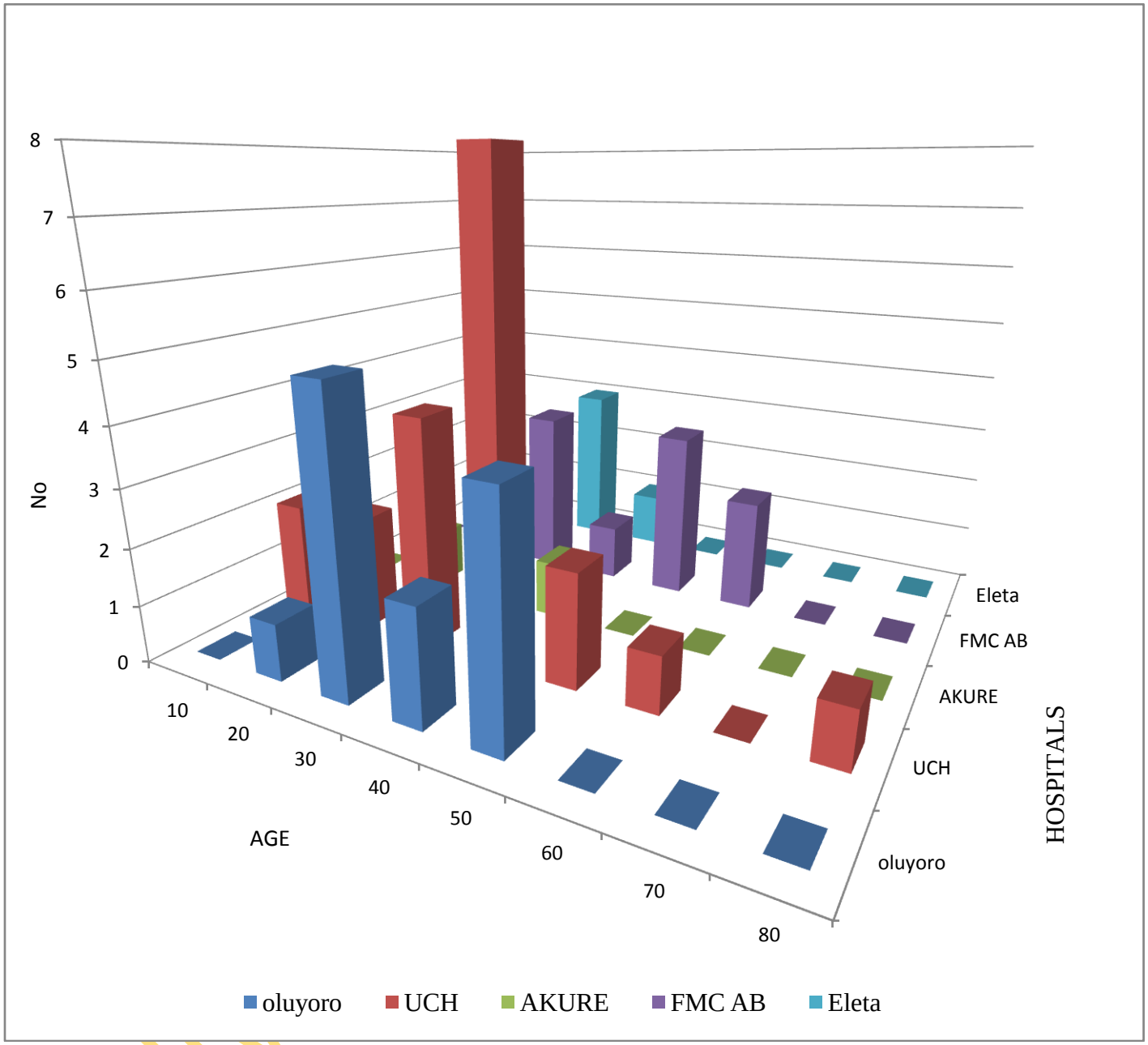


Fig 4.2: Age distribution of patient infected with *P. aeruginosa* isolate from 5 hospitals in Southwest Nigeria

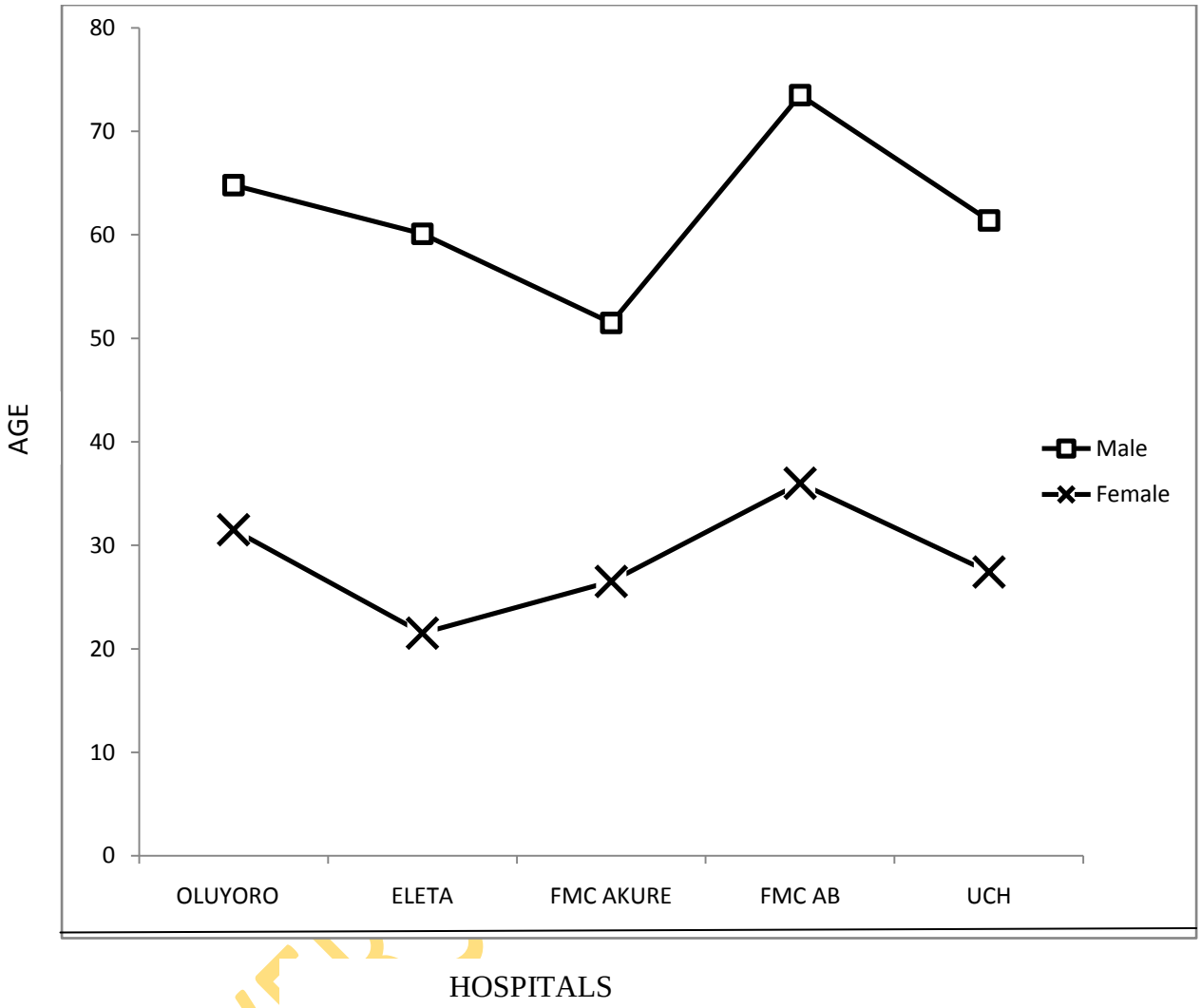


Fig 4.3: Mean age distrubution of patients infected with *P. aeruginosa*

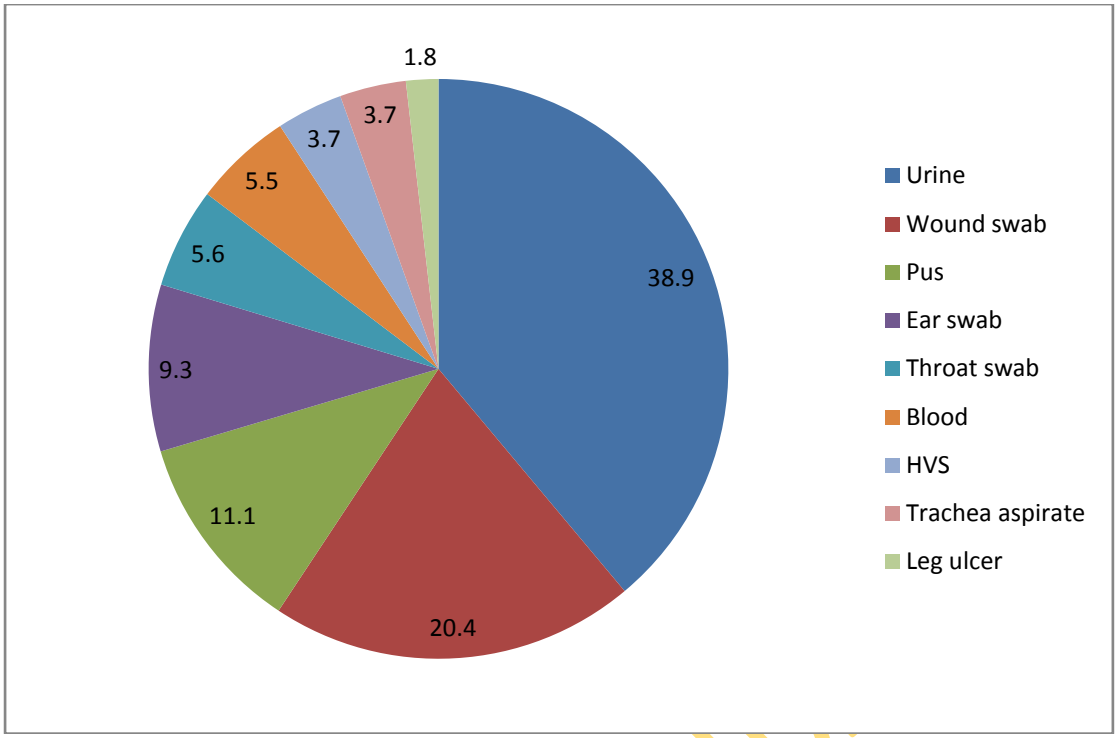


Fig 4.4: Distribution of *P. aeruginosa* obtained from various cilia specimens

Table 4.2 Antimicrobial Resistance Phenotypes of 54 *P. aeruginosa* isolates from 5 hospital in Southwestern Nigeria

Isolates ID	HOSPITAL	RESISTANCE PHENOTYPE
P. aeruginosa ODM1 *ms1*	OLUYORO	AMC, AMP, CAR, CRO, CTX, STR, TET, TIM,
P. aeruginosa ODM2	UCH	AMC, AMP, TET, TIM
P. aeruginosa ODM3	UCH	AMC, AMP, ATM, CAR, CRO, K, LEV, NOR, TET, TIM, TZP
P. aeruginosa ODM4	UCH	AMC, AMP, AK, CAR, CIP, LEV, NOR, TET, TIM, TZP
P. aeruginosa ODM5 *ms2*	ELETA	AMC, AMP, CAZ, CRO, CTX, GEN, K, LEV, NOR, PIP, TET, TIM
P. aeruginosa ODM6	OLUYORO	AMC, AMP, CAR, K, LEV, NOR, STR, TET,TIM, TZP
P. aeruginosa ODM7	FMC ONDO	AMC, AMP, CAR, CIP, STR, TET, TIM, TZP
P. aeruginosa ODM8 *ms3*	UCH	AMC, AMP, ATM, CAR, CIP, CRO, CTX, GEN, STR, TET, TIM, TZP
P. aeruginosa ODM9	FMC ABEOKUTA	AMC, AMP, K, STR, TET, TIM
P. aeruginosa ODM10	OLUYORO	AMC, AMP, TET, TIM
P. aeruginosa ODM11	FMC ABEOKUTA	AMC, AMP, K, TET, TIM

Isolates numbered *ms1-20* were randomly selected for further genotypic studies

AK:Amikacin (30µg), **AMC**: Amoxycillin/clavulanic (20/10µg), **AMP**: Ampicillin (25µg), **ATM**: Azetronam (30µg), **CAR:** Carbenicillin (100µg), **FEP**; Cefepime (30µg), **CTX**: Cefotaxime (30µg), **CAZ**: Ceftazidime (30µg), **CRO**: Ceftriaxone (30µg), **CIP**: Ciprofloxacin (5µg), **COL**: Colistin (10µg), **GEN**: Gentamicn (10µg), **IPM**: Imipenem (10µg), **K**:Kanamycin (30µg), **LEV:** Levofloxacin (5µg), **NOR**; Norfloxacin,(10µg), **PIP**: Piperacillin (100µg), **TZP**: Piperacillin/Tazobactam (110µg), **STR**: Streptomycin (10µg), **TET**:Tetracyclin (30µg), **TIM**: Ticarcillin clavulanic: (75/10µg),

Table 4.2 Contd.

Isolates ID	HOSPITAL	RESISTANCE PHENOTYPE
P. aeruginosa ODM12 *ms4*	UCH	AMC, AMP, AK, ATM, CAR, CAZ, CIP, CRO, CTX, FEP, GEN, IPM, K, LEV, NOR, PRL, STR, TET,TIM, TZP,
P. aeruginosa ODM13	FMC ABEOKUTA	AMC, AMP, ATM, CAR, CRO, CTX, K, STR, TET ,TIM, TZP
P. aeruginosa ODM14	UCH	AMC, AMP, K, LEV, NOR, STR, TET, TIM, TZP
P. aeruginosa ODM15	FMC ONDO	AMC, AMP, K, TET, TIM, TZP
P. aeruginosa ODM16 *ms5*	OLUYORO	AMC, AMP, K, TET, TIM, TZP
P. aeruginosa ODM17 *ms6*	ELETA	AMC, AMP, ATM, CAR, CRO, CTX, K, NOR, PRL, TET, TIM, TZP
P. aeruginosa ODM18	FMC ABEOKUTA	AMC, AMP, K, TET, TIM, TZP
P. aeruginosa ODM19	ELETA	AMC, AMP, GEN, K, STR, TET, TZP
P. aeruginosa ODM20	ELETA	AMC, AMP, CAR, CIP, CRO, CTX, K, LEV, NOR , PRL, STR, TET,TIM, TZP
P. aeruginosa ODM21	FMC ABEOKUTA	AMC, AMP, CAR, CIP, K, STR, TET
P. aeruginosa ODM22	ELETA	AMC, AMP, ATM, K , LEV, NOR, STR, TET,
P. aeruginosa ODM23	FMC ONDO	AMC, AMP, ATM, CAR, CIP, CRO, CTX, K, NOR, STR, TET, TIM, TZP
P. aeruginosa ODM24 *ms7*	OLUYORO	AMC, AMP, CAR, CIP, CRO, CTX, GEN, IPM, K, LEV, NOR, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM25 *ms8*	UCH	AMC, AMP, ATM, CAR, CAZ, CIP, CRO, CTX, FEP, GEN, K, LEV, NOR, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM26	UCH	AMC, AMP, CAR, K, LEV, NOR, TET, TIM, TZP
P. aeruginosa ODM27 *ms9*	ELETA	AMC, AMP, ATM, CAR, CIP, CRO, CTX, K, LEV, NOR, PRL, STR, TET, TIM, TZP .
P. aeruginosa ODM28 *s10*	OLUYORO	AMC, AMP, AK, ATM, CAR, CAZ, CRO, CTX, FEP, GEN, K, STR, TET, TIM, TZP
P. aeruginosa ODM29	ELETA	AMC, AMP, K, STR, TET, TIM, TZP
P. aeruginosa ODM30	UCH	AMC, AMP, ATM, CAR, CAZ, CRO, CTX, K, PRL, TET, TIM, TZP

Table 4.2 Contd.

Isolates ID **HOSPITAL** **RESISTANCE PHENOTYPE**

P. aeruginosa ODM31	OLUYORO	AMC, AMP, CAR, K, LEV, NOR, TET, TZP
P. aeruginosa ODM32 ms*11*	UCH	AMC, AMP, AK, ATM, CAZ, CRO, CTX, FEP, GEN, K, STR, TET, TIM, TZP
P. aeruginosa ODM33	UCH	AMC, AMP, CAR, CRO, CTX, K, LEV, NOR, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM34 ms*12*	FMC ABEOKUTA	AMC, AMP, AK, ATM, CAR, CIP, CRO, IPM, K, LEV, NOR, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM35	UCH	AMC, AMP, ATM, CAR, CAZ, CRO, CTX, FEP, K, LEV, NOR, PRL, TET, TIM, TZP
P. aeruginosa ODM36	FMC ABEOKUTA	AMC, AMP, CAR, CRO, CTX, K, LEV, NOR, TET, TIM, TZP
P. aeruginosa ODM37	UCH	AMC, AMP, K, LEV, NOR, STR, TET, TZP
P. aeruginosa ODM38 *ms13*	OLUYORO	AMC, AMP, AK, ATM, CAR, CIP, CRO, CTX, GEN, K, LEV, NOR, PRL STR, TET, TIM, TZP
P. aeruginosa ODM39	FMC ABEOKUTA	AMC, AMP, K, STR, TET, TIM, TZP
P. aeruginosa ODM40 *ms14*	UCH	AMC AMP, AK, ATM, CAR, CIP, CRO, CTX, GEN, K, LEV, NOR, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM41	UCH	AMC, AMP, CRO, CTX, GEN, IPM, K, NOR, STR, TET, TIM, TZP
P. aeruginosa ODM42*ms15*	FMC ABEOKUTA	AMC, AMP, ATM, CAR, CAZ, CRO, CTX, K, LEV, NOR, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM43	FMC ABEOKUTA	AMC, AMP, ATM, CAR, CRO, CTX, GEN, K, PRL, TET, TIM
P. aeruginosa ODM44	OLUYORO	AMC, AMP, CAR, CIP, K, TET, TIM, TZP
P. aeruginosa ODM45 *ms16*	OLUYORO	AMC, AMP, AK, ATM, CAR, CRO, CTX, FEP, GEN, K, PRL, STR, TET
P. aeruginosa ODM46 *ms17*	UCH	AMC, AMP, CAR, CAZ, CIP, CRO, CTX, FEP, K, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM47	UCH	AMC, AMP, K, LEV, NOR, TET,TIM, TZP
P. aeruginosa ODM48 *ms18*	OLUYORO	AMC, AMP, ATM, CAR, CIP, CRO, CTX, GEN, K ,LEV, NOR, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM49 *ms19*	UCH	AMC, AMP, ATM, CAR, CIP, COL, CRO, CTX, FEP, GEN, K, PRL, STR, TET, TIM, TZP
P. aeruginosa ODM50	UCH	AMC, AMP, K, LEV, NOR ,TET, TIM, TZP
P. aeruginosa ODM51	OLUYORO	AMC, AMP, STR, TET,TIM, TZP
P. aeruginosa ODM52 *ms20*	FMC ONDO	AMC, AMP, ATM, CAR, CAZ, CIP, CRO, CTX, FEP, PRL, TET, TIM, TZP
P. aeruginosa ODM53	FMC ONDO	AMC, AMP, TET, TIM, TZP
P. aeruginosa ODM54	UCH	AMC, AMP, ATM, GEN, K, STR, TET, TIM

Table 4.3. Percentages resistance of isolates of *P. aeruginosa* strains

Antibiotics	No. of isolates showing resistance	Resistant Isolates (%)
Amikacin (AMK)	12	22.2
Amoxicillin/clavulanic acid (AMC)	54	100.0
Ampicillin (AMP)	54	100.0
Aztreonam (ATM)	24	44.4
Carbenicillin (CAR)	34	63.0
Cefepime (FEP)	10	18.6
Cefotaxime (CTX)	28	51.9
Ceftazidime (CAZ)	9	16.7
Ceftriaxone (CRO)	29	53.7
Ciprofloxacin (CIP)	19	35.2
Colistin (COL)	1	1.9
Gentamicin (GEN)	22	40.7
Imipenem (IPM)	4	7.5
Kanamycin (K)	43	79.6
Levofloxacin (LEV)	21	38.9
Norfloxacin (NOR)	21	38.9
Piperacillin (PIP)	21	38.9
Piperacillin-tazobactam (TZP)	21	38.9
Streptomycin (STR)	22	40.7
Tetracycline (TET)	54	100.0
Ticarcillin/Clavulanate (TIM)	47	87.0

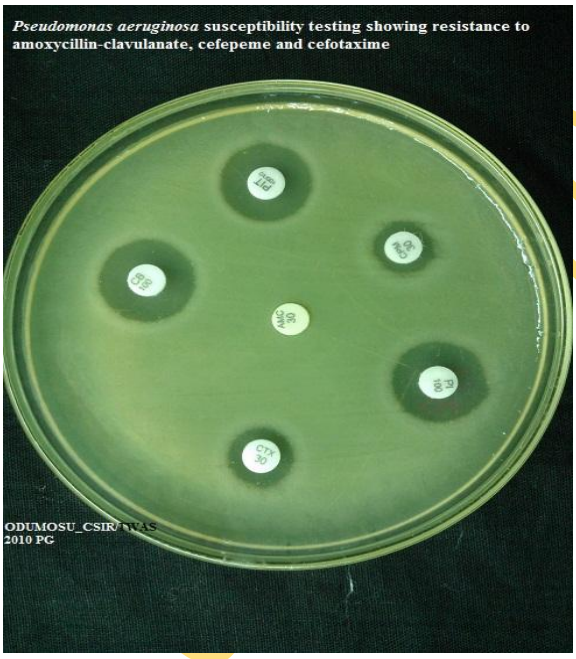
Table 4.4 MICs of different antibiotics for selected *P. aeruginosa* isolates

	Concentrations are in µg/mL							
Strain No	**PIP** b.p = ≥128	**CAZ** b.p = ≥ 32	**CTX** b.p = ≥ 64	**CRO** b.p = ≥ 64	**CIP** b.p = ≥ 4	**LEV** b.p = ≥8	**AMK** b.p = ≥ 64	**GEN** b.p = ≥ 16
P. aerug. ODM1	240.0	240.0	15.0	240.0	0.01	0.01	2.0	256.0
P. aerug. ODM5	240.0	240.0	15.0	240.0	0.25	1.0	4.0	256.0
P. aerug. ODM8	60.0	15.0	7.5	240.0	1.0	2.0	0.5	256.0
P. aerug. ODM12	5.0	7.5	3.0	3.0	0.001	0.01	0.1	0.5
P. aerug. ODM16	5.0	15.0	3.0	7.5	0.01	0.25	0.1	0.1
P. aerug. ODM17	30.0	30.0	15.0	240.0	0.004	0.25	0.5	256.0
P. aerug. ODM24	120.0	120.0	15.0	240.0	0.5	0.5	256.0	256.0
P. aerug. ODM25	120.0	5.0	15.0	240.0	1.0	1.0	256.0	256.0
P. aerug. ODM27	1.0	3.0	3.0	7.5	30.0	0.005	0.05	0.1
P. aerug. ODM28	1.0	1.0	3.0	15.0	0.008	0.005	0.05	0.1
P. aerug. ODM32	5.0	15.0	15.0	240.0	240.0	240.0	256.0	256.0
P. aerug. ODM34	240.0	240.0	15.0	240.0	240.0	240.0	256.0	256.0
P. aerug. ODM38	5.0	1.0	3.0	240.0	240.0	240.0	256.0	256.0

Note
b.p = breakpoint
MIC breakpoints interpretations is according to CLSI 2001

Table 4.4 Contd.

	Concentrations are in µg/mL							
Strain No	**PIP** b.p = ≥128	**CAZ** b.p = ≥ 32	**CTX** b.p = ≥ 64	**CRO** b.p = ≥ 64	**CIP** b.p = ≥ 4	**LEV** b.p = ≥ 8	**AMK** b.p = ≥64	**GEN** b.p = ≥ 16
P. aerug. ODM40	240.0	120.0	15.0	240.0	240.0	240.0	256.0	256.0.
P. aerug. ODM42	240.0	15.0	3.0	240.0	0.01	0.25	0.05	0.5
P. aerug. ODM45	240.0	120.0	7.5	240.0	240.0	240.0	256.0	256.0
P. aerug. ODM46	1.0	7.5	1.0	15.0	0.01	0.05	2.0	4.0
P. aerug. ODM48	120.0	30.0	15.0	240.0	240.0	240.0	256	256.0
P. aerug. ODM49	240.0	15.0	15.0	240.0	2.0	2.0	0.1	0.1
P. aerug. ODM52	1.0	7.5	3.0	7.5	0.008	0.05	0.05	0.05



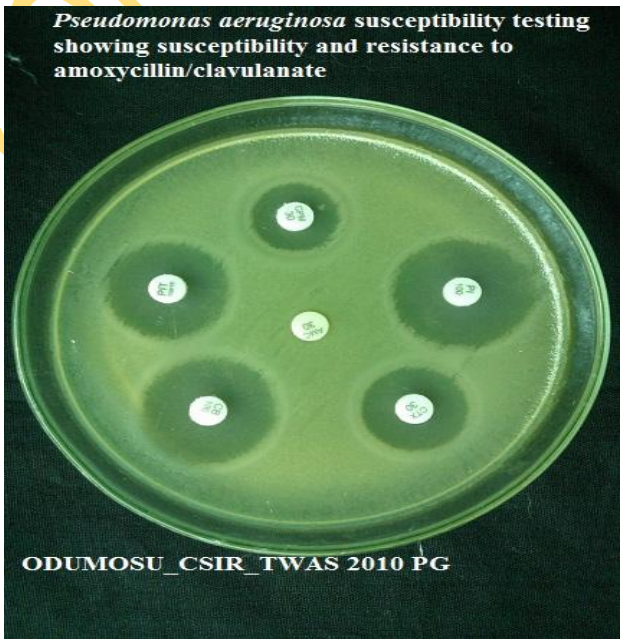


Plate 4.2: MIC by Etest method (above) and susceptibility testing of *P. aeruginosa*

4.5. Analysis and size determination of the *P. aeruginosa* plasmids

The plasmid profiles and quantification of isolates is depicted on Table 4.5 and Plate 4.3. Plasmid size and estimation was done using the reference plasmid markers of the *E. coli* V517 (Pedraza and Diaz, 2002), and by endonuclease restriction digest of the plasmids (RFLP) Plate 4.4. All the *P. aeruginosa* strains investigated were harbouring 1 – 4 plasmids with sizes ranging from 2.2kb – >58kb as revealed by the corresponding *E. coli* V517 standard marker. Plasmid sizes up to 58kb were determined by comparison with the corresponding *E. coli* V517 plasmids, while sizes above this were determined by using the SynGene Gel Documentation in-built software system. The RFLP of the plasmids using H*indIII* for the purpose of typing the strains with respect to the presence of plasmids gave similar band patterns of 7. The cohesive ends of the fragments 1 and 4 previously noted by the manufacturer could not be separated at the temperature (37^{o}C) used in this study resulting in 7 bands instead of 8. The 6th band of the plasmid digest corresponds with the highest band size 23kb of the λ H*indIII* digest that was used as a standard marker in this study (Plate 4.4). Restriction with *Eco*R1 and *Bam*HI showed no cleavage for all the plasmids investigated.

4.5.1. Plasmid curing analysis

All the 54 isolates were subjected to curing using the three different curing agents; acridine orange, ethidium bromide (EtBr) and SDS. The three curing agents were each able to cure the *P. aeruginosa* plasmids at varying concentration and extents. EtBr and acridine orange were able to cure the highest strains in this study than SDS. EtBr at concentration of 40µg/ml cure 44 (81%) out of the 54 isolates while acridine orange at 35 µg/ml concentration cured 40 (74%). SDS curing effect was only successful for 25 (46%) (Plate 4.5). Increase in susceptibilities of the cured isolates was observed against antibiotics they had previously been tested with. The cured strains were also investigated for the presence of plasmids but were found to have been lost (Plate 4.5). Even though some strains retained their plasmids but its resistance factors were suspected to have been lost since they failed to show any resistance.

Table 4.5 Plasmid profiles and sizes of 54 *P. aeruginosa* isolates

Isolates ID	Source	PA*	HOSPITAL	Number of Plasmid (s)	Approximate sizes (kb)
ODM1	Urine	1	OLUYORO	3	2.2, 17.0, > 58
ODM2	HVS		UCH	1	58
ODM3	Urine		UCH	2	58, 63
ODM4	Wound sawb		UCH	1	58
ODM5	Pus	2	ELETA	2	3.2, > 58
ODM6	Urine		OLUYORO	1	58
ODM7	Urine		FMC AKURE	1	58
ODM8	Wound swab	3	UCH	2	58, 210
ODM9	Wound swab		FMC ABEOKUTA	1	58
ODM10	Chronic leg ulcer		OLUYORO	1	58
ODM11	Urine		FMC ABEOKUTA	1	58
ODM12	Wound swab	4	UCH	1	58
ODM13	Urine		FMC ABEOKUTA	1	58
ODM14	HVS		UCH	1	58
ODM15	Urine		FMC AKURE	1	58
ODM16	Urine	5	OLUYORO	1	58
ODM17	Urine	6	ELETA	2	58,> 210
ODM18	Urine		FMC ABEOKUTA	1	58

Table 4.5 Contd.

Isolates ID	Source	PA*	HOSPITAL	Number of Plasmid (s)	Approximate sizes (kb)
ODM19	Wound swab		ELETA	1	58
ODM20	Pus		ELETA	1	58
ODM21	Urine		FMC ABEOKUTA	1	58
ODM22	Urine		ELETA	1	58
ODM23	Wound swab		FMC AKURE	1	58
ODM24	Urine	7	OLUYORO	3	17, 58, >210
ODM25	Urine	8	UCH	2	58, > 63
ODM26	Ear swab		UCH	1	58
ODM27	Throat swab	9	ELETA	1	58
ODM28	Ear swab	10	OLUYORO	1	> 58
ODM29	Ear swab		ELETA	1	58
ODM30	Ear swab		UCH	1	58
ODM31	Ear swab		OLUYORO	1	58
ODM32	Wound biopsy	11	UCH	2	2.7, > 58
ODM33	Wound swab		UCH	1	> 58
ODM34	HVS	12	FMC ABEOKUTA	4	2.4, 3.2, 58, > 65
ODM35	Urine		UCH	4	2.2, 3.2, 58, > 70
ODM36	Pus		FMC ABEOKUTA	1	> 58
ODM37	Pus		UCH	1	> 58

Table 4.5 Contd.

Isolates ID	Source	PA*	HOSPITAL	Number of Plasmid (s)	Approximate sizes (kb)
ODM38	Urine	13	OLUYORO	3	2.7, 3.2, > 58
ODM39	Urine		FMC ABEOKUTA	1	58
ODM40	HVS	14	UCH	2	2.2, >58
ODM41	Umbilica swab		UCH	1	58
ODM42	Pus	15	FMC ABEOKUTA	2	58, >210
ODM43	Pus		FMC ABEOKUTA	2	58, >210
ODM44	Pus		OLUYORO	2	58, >180
ODM45	Wound swab	16	OLUYORO	1	> 58
ODM46	Urine	17	UCH	2	58,>210
ODM47	Wound biospy		UCH	1	58
ODM48	Urine	18	OLUYORO	1	58
ODM49	Urine	19	UCH	1	> 58
ODM50	Blood		UCH	2	58, >63
ODM51	Urine		OLUYORO	1	58
ODM52	Urine	20	FMC AKURE	1	58
ODM53	Urine		FMC AKURE	1	58
ODM54	Wound swab		UCH	1	58

***PA represents those strains that were further selected for molecular studies**

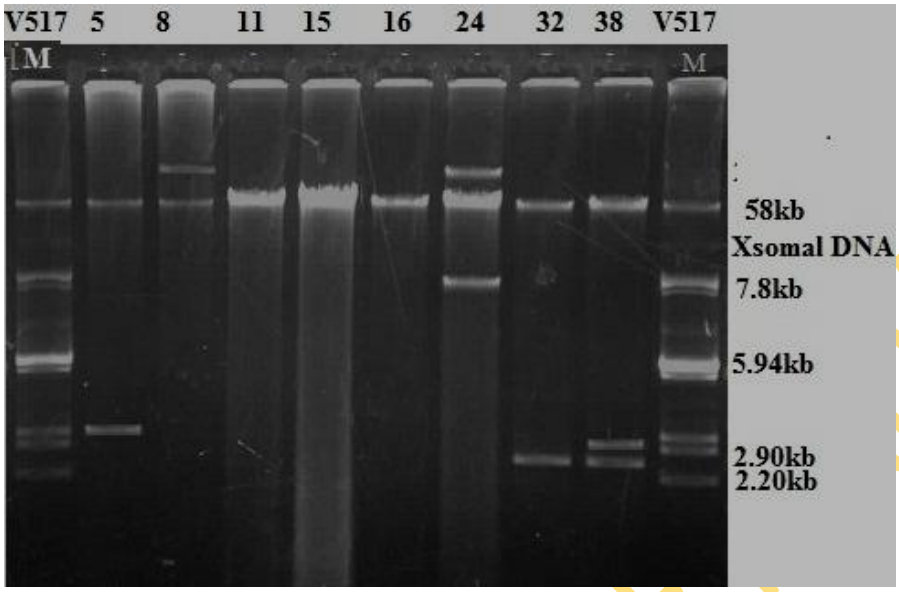
Table 4.6 Association of plasmid counts and antimicrobial resistance among *P. aeruginosa* strains

Number of plasmids present	*Multidrug Resistant	Non-Multidrug Resistant
4	2	0
3	3	0
2	12	0
1	35	2
Total isolates	52	2

* Multidrug resistance for this statistical analyses was defined as isolates resistance to >4 antibiotics

Statistical analyses by Fisher's Exact Test indicate a significant trend in the association of plasmid counts and antimicrobial resistance among *P. aeruginosa*. The MDR *P. aeruginosa* strains harbouring plasmids (≥3) shows significant resistance compared to the non-MDR counterparts, which carried (< 2) plasmids ($p<0.01$) (Table 4.6)

A



B

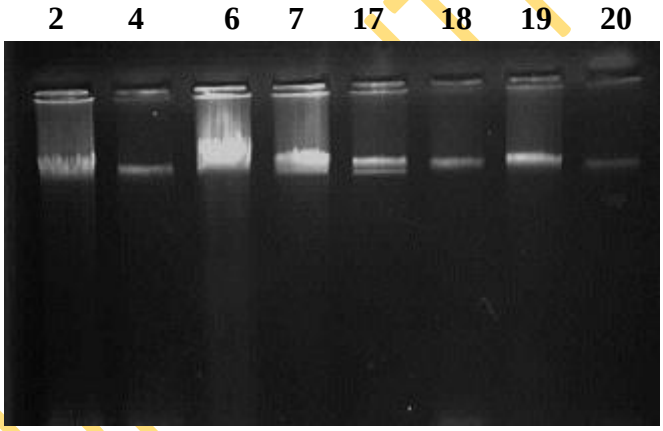
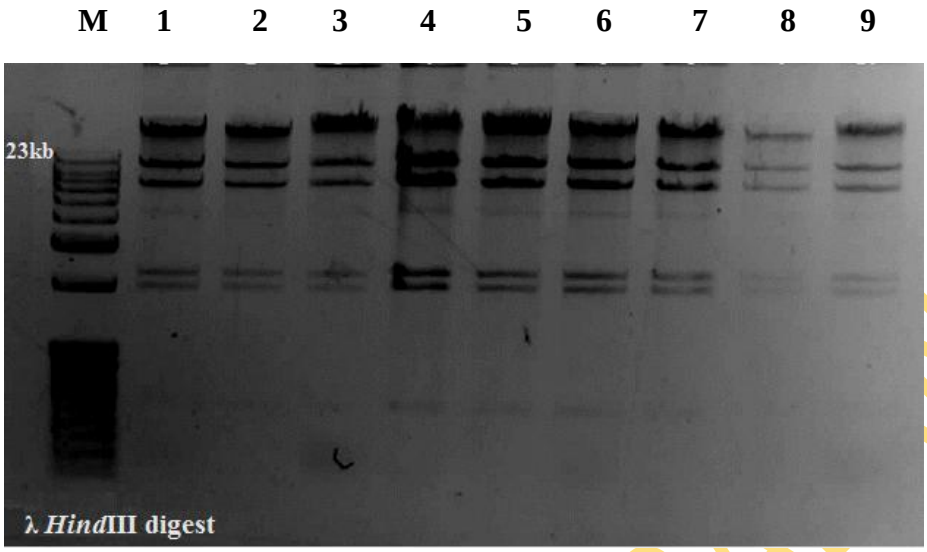


Plate 4.3: Plasmids picture for *P. aeruginosa* isolates showing varying patterns.

Note Plate 4.3 A shows agarose gel electrophoresis of *P. aeruginosa* plasmids with *E.coli* V517 standard marker [0.7% agarose in 5x TAE]. Plate 4.3B shows plasmid pictures for 8 isolates.



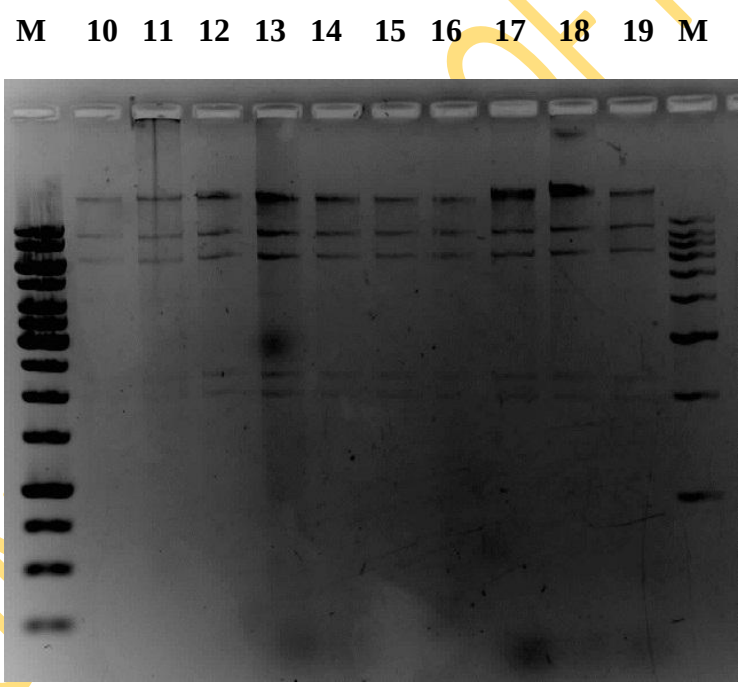


Plate 4.4: Restriction Fragment Length Polymorphism of selected plasmids with λ DNA-*Hind*III

Note M=supermix DNA ladder [1% agarose in 5x TAE]

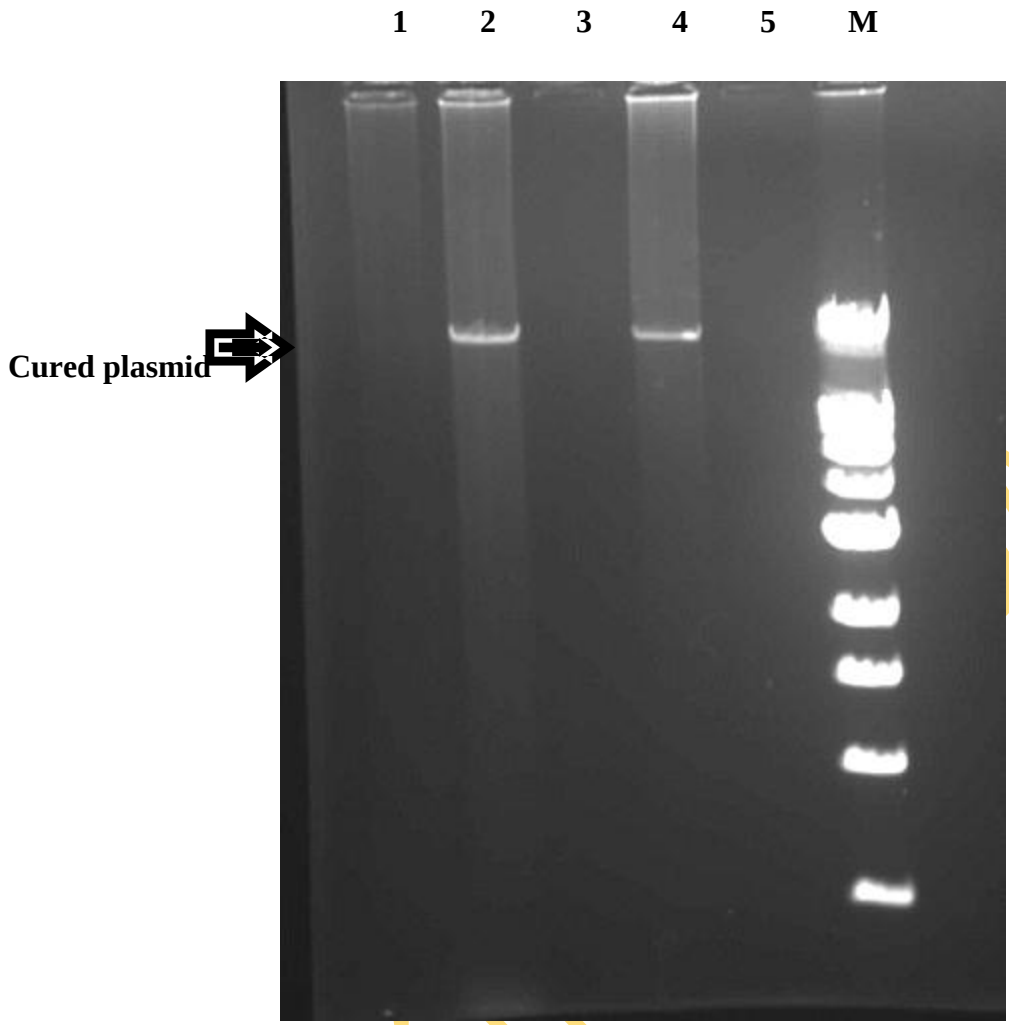


Plate 4.5: Agarose gel electrophoresis of cured isolates showing cured plasmids without resistance genes and those without the presence of plasmids bands.

Note Organisms in Lanes 1, 3 and 5 have lost the plasmids to curing while lanes 2 and 4 shows the presence of plasmids without any antibiotic resistance.

4.6. Detection of *β*-lactamase, ESBL and respective location of the enzymes

The *bla* genes *bla*$_{OXA}$, *bla*$_{CTX}$ are usually plasmid encoded in *P. aeruginosa*. The 20 multidrug resistant *P. aeruginosa* strains selected for molecular studies in this investigation all harboured plasmids of varying sizes and numbers (Table 4.5). Sixteen expanded-spectrum cephalosporin resistant *P. aeruginosa* strains ODM5, ODM8, ODM12, ODM17, ODM24, ODM25, ODM28, ODM32, ODM34, ODM38, ODM42, ODM45, ODM46, ODM48, ODM49 and ODM52 harboured large plasmids (≥58kb) that carried the *bla*$_{\text{OXA}}$ genes. A single isolate each was positive for *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$ (ODM42 and ODM46 respectively). All β-lactamase and ESBL enzymes were detected on plasmid by PCR assay. Fourteen (70%) of the 20 strains (ODM 1, ODM3, ODM12, ODM 17, ODM20, ODM23, ODM24, ODM25, ODM27, ODM28, ODM32, ODM38, ODM40, ODM48) were positive for AmpC *β*-lactamase, which was chromosomally encoded in all the strains investigated in this study.

4.6.1. Characterization of the ESBL enzymes

For both *bla*$_{\text{OXA}}$ and *bla*$_{\text{CTX-M}}$ PCR assay, two different degenerate primer pairs which amplify the entire OXA and CTX-M groups respectively were initially used, suggesting the presence of *bla* genes among the isolates. All the OXA gene positive were then confirmed with specific primers corresponding to the amplified PCR product sizes which was *bla*$_{\text{OXA-10}}$. Sixteen out of twenty (80%) isolates were positive for *bla*$_{\text{OXA-10}}$ with amplification sizes of 720bp (plate 4.6). A representative of the strains positive for the production of *bla*$_{\text{OXA-10}}$ was sequenced. The sequence result revealed the presence of *bla*$_{\text{OXA-10.}}$ A BLAST result for the alignment is available at the appendix II. Specific primers were used for the amplification *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{SHV.}}$ Single isolate ODM46 (Plate 4.7) and ODM42 (Plate 4.8) respectively were amplified for both enzymes but were not sequenced further. AmpC *β*-lactamase also could not be quantified due to the presence of multiple mechanisms of resistance present among the isolates investigated in this study (Plate 4.9).

4.7. Detection of Aminoglycosides Modifying Enzymes (AMEs) and Genetic Locations

Twelve strains (60%) among the 20 that was selected for molecular studies showed a high level (≥ 256µg) resistance to aminoglycosides. All the 12 strains were investigated for the presence of three aminoglycoside modifying enzymes (*aac*, *aph*, and *ant)* responsible for aminoglycosides resistance in *P. aeruginosa* and other Gram-negative bacteria. Ten (83%) were positive for *aac (6')* – I, (Plate 4.10) while 9 (75%) were positive for *ant (2'')* – I (Plate 4.11), 7 (58%) strains harboured both *aac (6')* – I and *ant (2'')* – I enzymes. None of the isolates investigated in this study were positive for *aph, aac (3)* genes and *aac (6'')* – II enzymes. Both enzymes that were detected in this study were located on the chromosomes of the investigated strains, none of the enzymes was plasmid encoded. One representative each for the positive strains was sequenced and aligned at the NCBI website (Appendix VII). Result from the Basic Local Allignment Search Tool (BLAST) showed the respective enzymes are 100% similar to aminoglycosides modifying enzymes (AME) *aac (6′)* – I and *ant* (2′′) – I

4.8. Fluoroquinolone resistance among the *P. aeruginosa*

The presence of *mexR* and *nfxB* genes responsible for the regulation of efflux pumps MexAB-OprM and MexCD-OprJ respectively were detected in 9(45%) (*P. aeruginosa* ODM 24, 25, 32, 34, 38, 40, 45, 48 and 49) among the 20 that was selected for molecular studies (Plate 4.12 and 4.13). Six (30%) of these strains (*P. aeruginosa* ODM 32, 34, 38, 40, 45 and 48) showed a high level resistance (≥ 240µg/ml) to the fluoroquinolones (FQ) (ciprofloxacin and levofloxacin) in this study (Table 4.4). The remaining 3, (*P. aeruginosa* ODM 24 25 and 49) had MIC (>120 µg/ml) against piperacillin and ceftriaxone but were all susceptible to both FQ. One *mexR* and *nfxB* positive amplicons were further confirmed by sequencing of PCR products and both revealed the presence of *mexR* and *nfxB* previously described as responsible for the FQ resistance efflux pump regulatory genes in *P. aeruginosa* (Appendix VII).

4.9. Detection of Class 1 and 2 integrons among the multidrug *P. aeruginosa* isolates

Thirty-one (57%) of the 54 *P. aeruginosa* isolates investigated were positive for integrase gene showing amplification size of 491bp. Analysis of the PCR product by RFLP with *Hin*fI

revealed 31 (57%) 491bp confirming the presence of *intI1* genes in all the 31 integron positive strains (Plate 4.14). PCR amplification using *intI2* and *intI3* specific primers showed no amplification suggesting the absence of class 2 and 3 integrons in this study.

4.9.1 Characterization of gene cassette presents in the class 1 integron

Using the hep58 and hep59 primers for gene cassette characterization, two different fragments sizes of approximately 1.6 kb and 1.2 kb were obtained for all the 31 *intI1*-positive isolates (Plate 4.15, 4.16). Twenty-three (74%) isolates yielded a single fragment of ~ 1.6 kb while the remaining 8 isolates gave a single fragment of ~ 1.2 kb. Sequence data obtained from sequening of the 1.6 kb gene cassette fragment from strain ODM24 gave 100% homology to *aadA6* conferring resistance to streptomycin and spectinomycin and *orfD* of unknown function [accession number JX195555] whereas the1.2kb fragment of strain *P. aeruginosa* ODM8 was 100% identical to *aadA13* which also confer resistance to streptomycin and spectinomycin [accession number JX195556].

4.10. Conjugation assay for demonstration of plasmids transferability and genetic localization of class 1integrons among positive strains

The 31 *P. aeruginosa* isolates that harboured the *intI1*gene, 16 of which are $bla_{\text{OXA-10}}$ positive were selected for conjugation assay using standard recipient strain *E.coli* DHα rec [-], F [-] nalixidic acid resistance (nal [r] F [-] recA gyrA. Of the total strain, 10 (32.0%) *P. aeruginosa* strains were capable of transferring their genetic resistance determinants by conjugation. All the transconjugants obtained from the conjugation experiments harboured the same plasmid profiles as the parental strains. However, PCR amplification with specific primers revealed the absence of integrons and gene cassettes among the transconjugants; indicating the non-transference of the integrons and associated gene cassettes along with the conjugative plasmids, suggesting a chromosomal location of the integron and gene cassette.

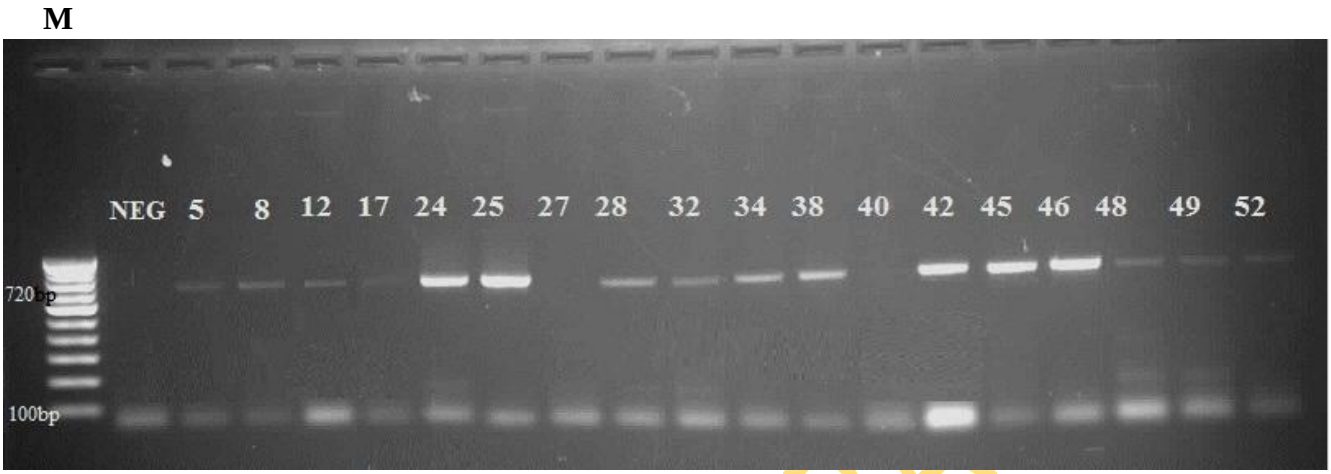


Plate 4.6: Agarose gel electrophoresis of OXA-10 PCR product

Note Lane M= 100bp DNA ladder (1.5% agarose in 5x TAE).

* 100bp ladder has 10 fragments consisting of 100bp repeats from 100-1000bp

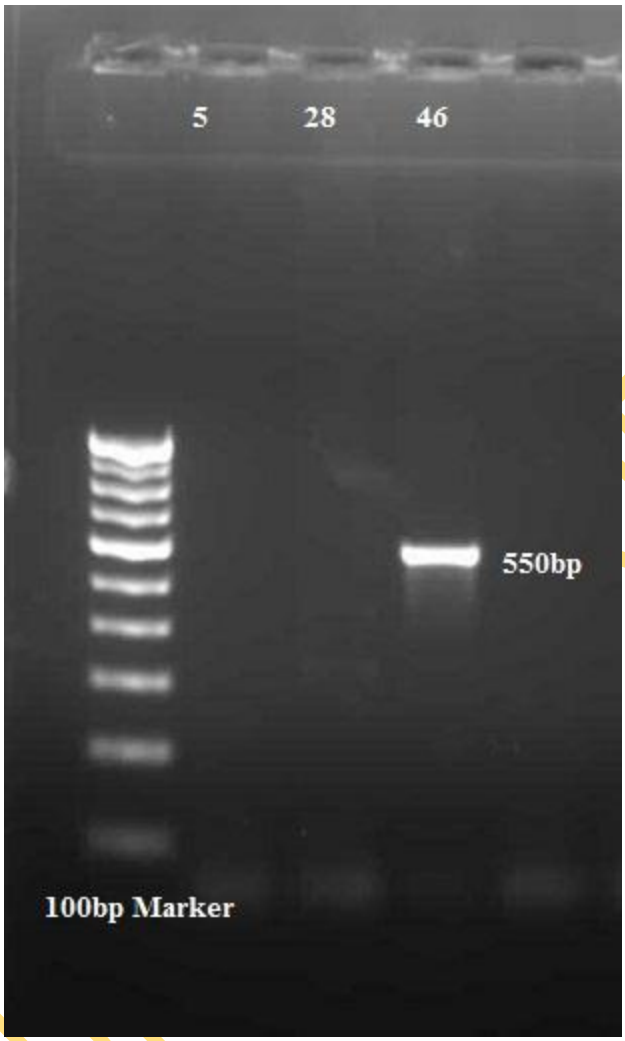


Plate 4.7: Agarose gel electrophoresis of CTX-M-1 PCR product
Note. Lane M, 100bp ladder, Lane 4,ODM46(1.8% agarose in 5X TAE).

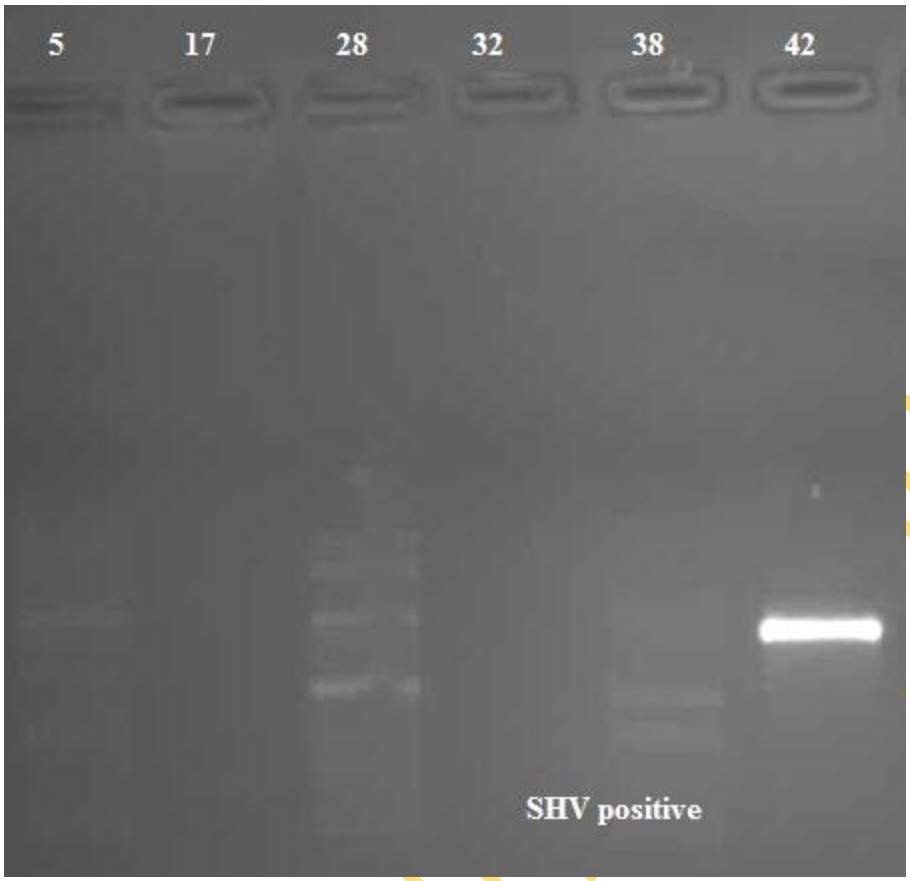


Plate 4.8: Agarose gel electrophoresis of SHV PCR product.

Note. Lane 6, ODM42 Showing positive gene (1.8% agarose in 5X TAE).

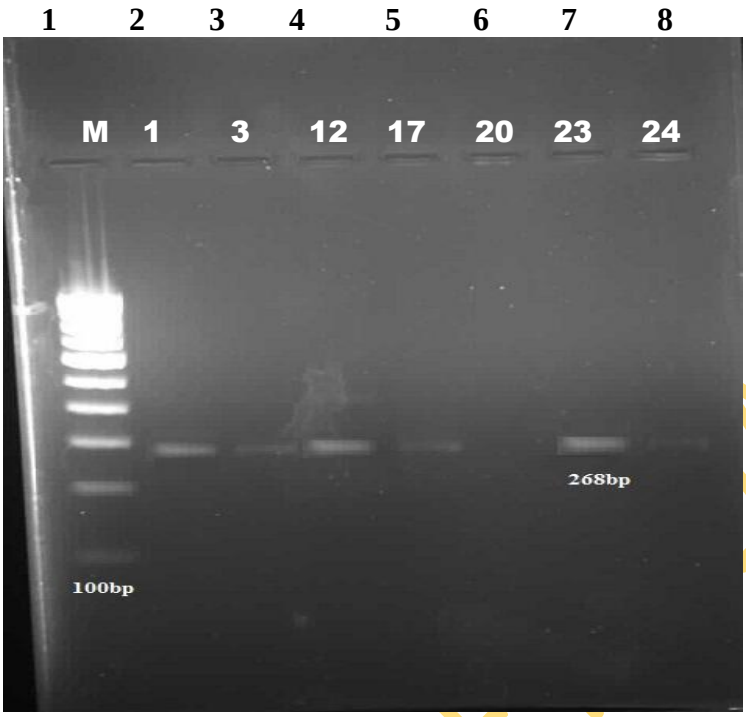


Plate 4.9: Agarose gel electrophoresis of AmpC *β*-lactamase.

Note. Lane 1, 100bp ladder, Lane 2 – 8 AmpC positive isolates, Lane 6, AmpC negative strain. (1.8% agarose gel 5X TAE)

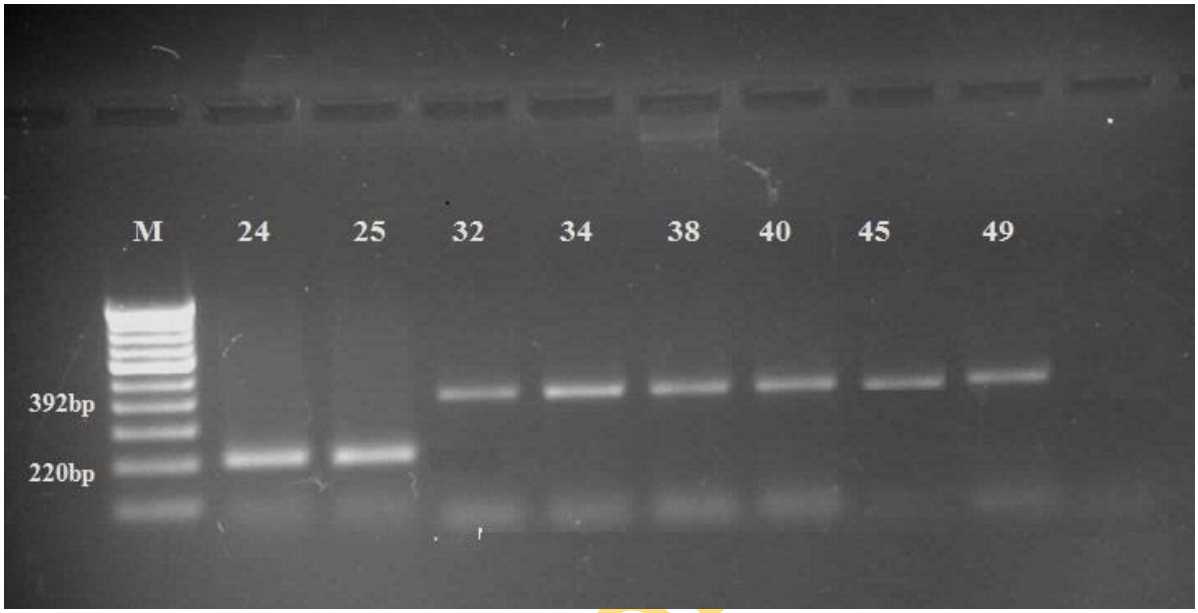


Plate 4.10: Agarose gel electrophoresis of *aac*(6′) – I PCR assay of ***P. aeruginosa*** . **Note.** Lane M 100bp ladder, ODM 24, ODM 25,ODM32,ODM34,ODM38, ODM40,ODM45 and ODM49 are positive isolates for the enzyme *aac(6′)* – I.

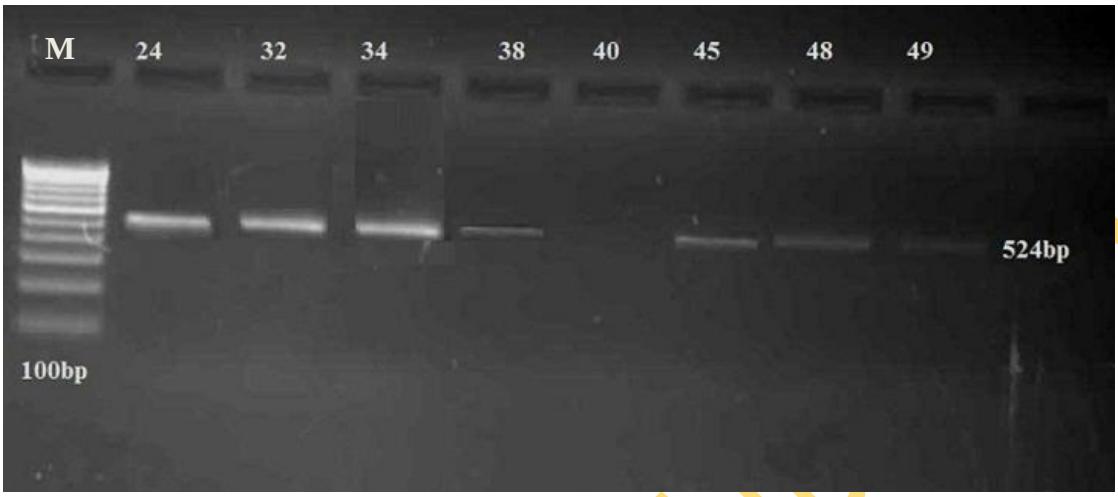


Plate 4.11: Agarose gel electrophoresis of *ant* (2″) – IV PCR assay of ***P. aeruginosa.*** **Note.** Lane M 100bp ladder, ODM24, ODM25, ODM32, ODM34, ODM38, ODM45 and ODM49 are positive isolates for the enzyme *ant* (2″) – IV.

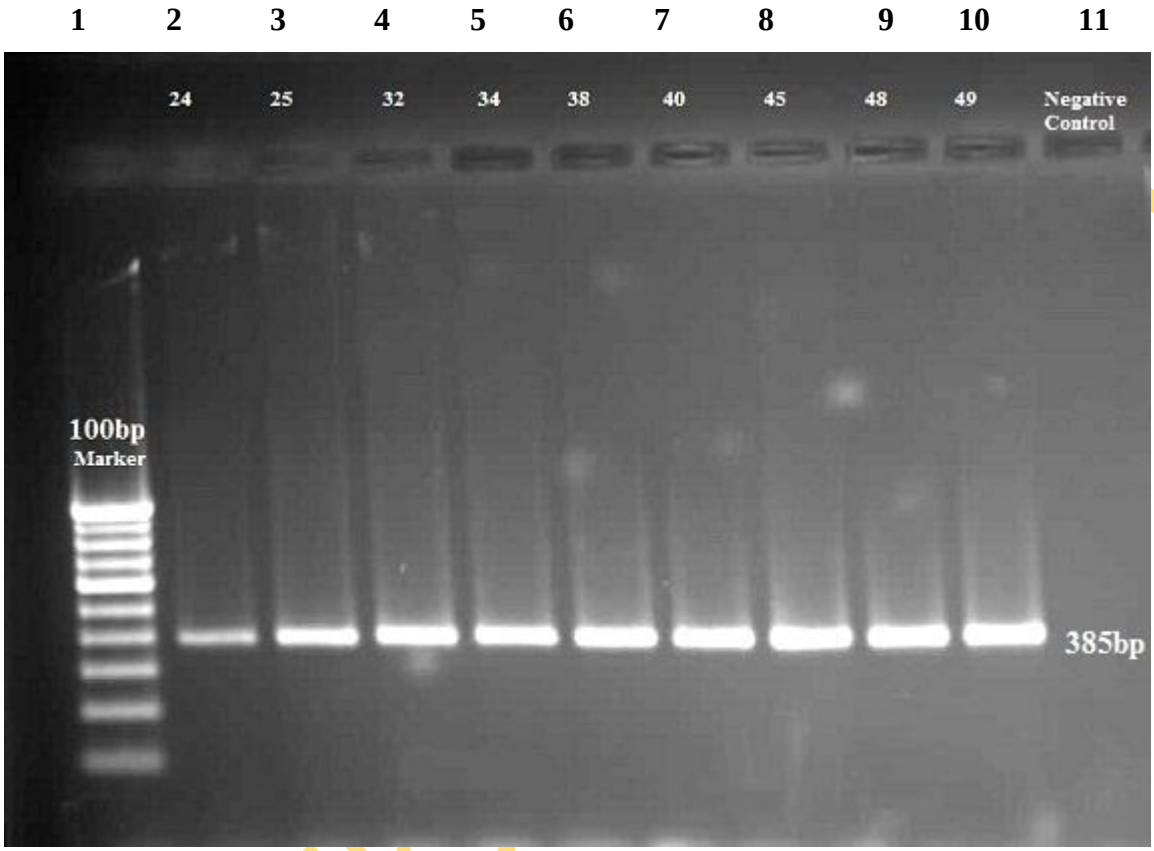


Plate 4.12: Agarose gel electrophoresis of *mexR* PCR assay of fluoroquinolones resistant *P. aeruginosa* strains.

Note. Lane 1, 100bp ladder, Lane 2 – 10 *mexR* positive *P. aeruginosa* isolates, Lane 11, Negative control.

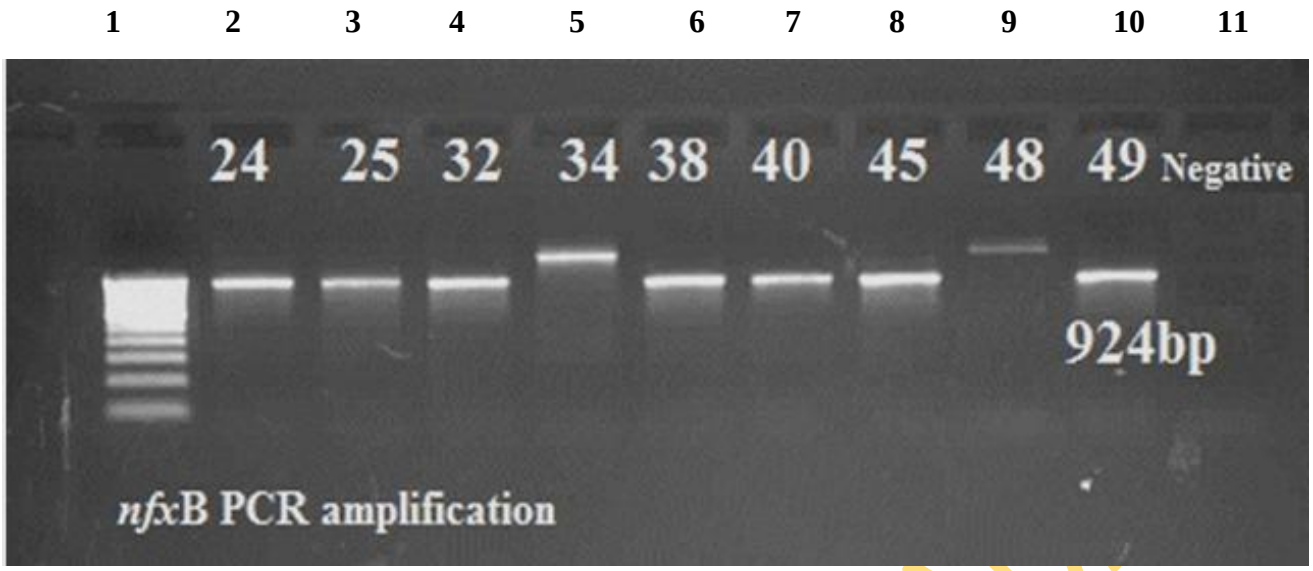
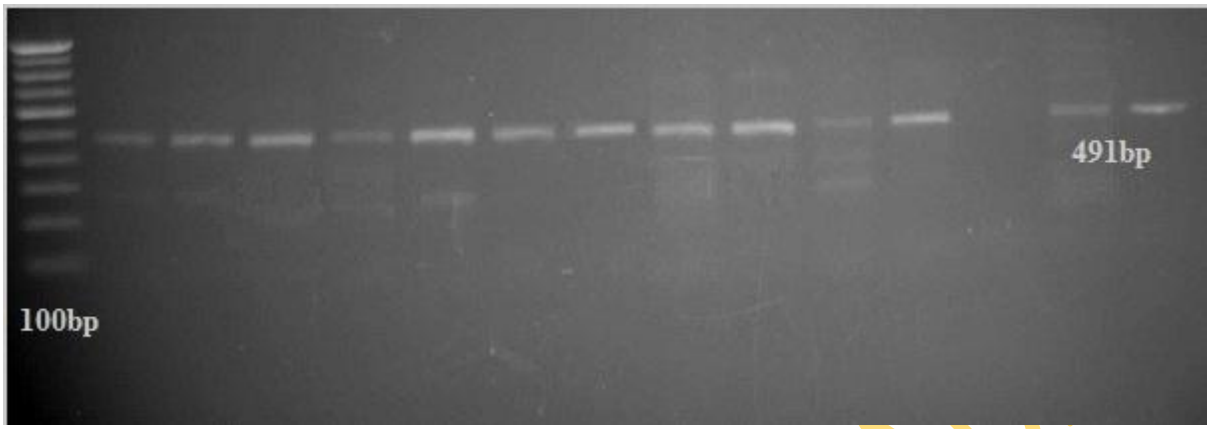


Plate 4.13:. Agarose gel electrophoresis of *nfx*B PCR assay of fluoroquinolones resistant *P. aeruginosa* strains.

Note. Lane 1, 100bp ladder, Lane 2 – 10 *nfx*B positive *P. aeruginosa* isolates, Lane 11, Negative control.

(a)

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14



(b)

M 1 2 3 4 5 6 7

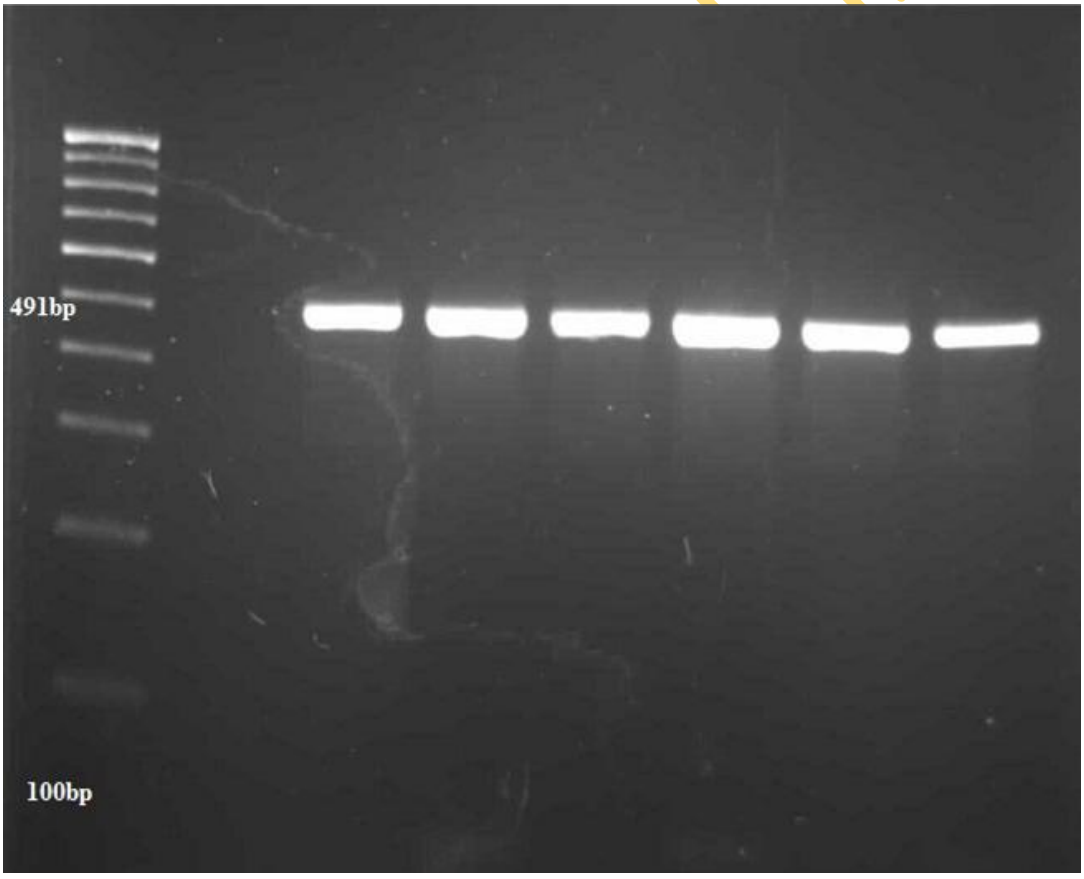


Plate 4.14: Class 1 integron with 491bp amplification. **Note.** Figure shows (a) 1.2% agarose gel electrophoresis of amplified *IntI1* gene fragments. (b) Amplification *IntI1* gene fragment on 1.8% agarose gel electrophoresis.

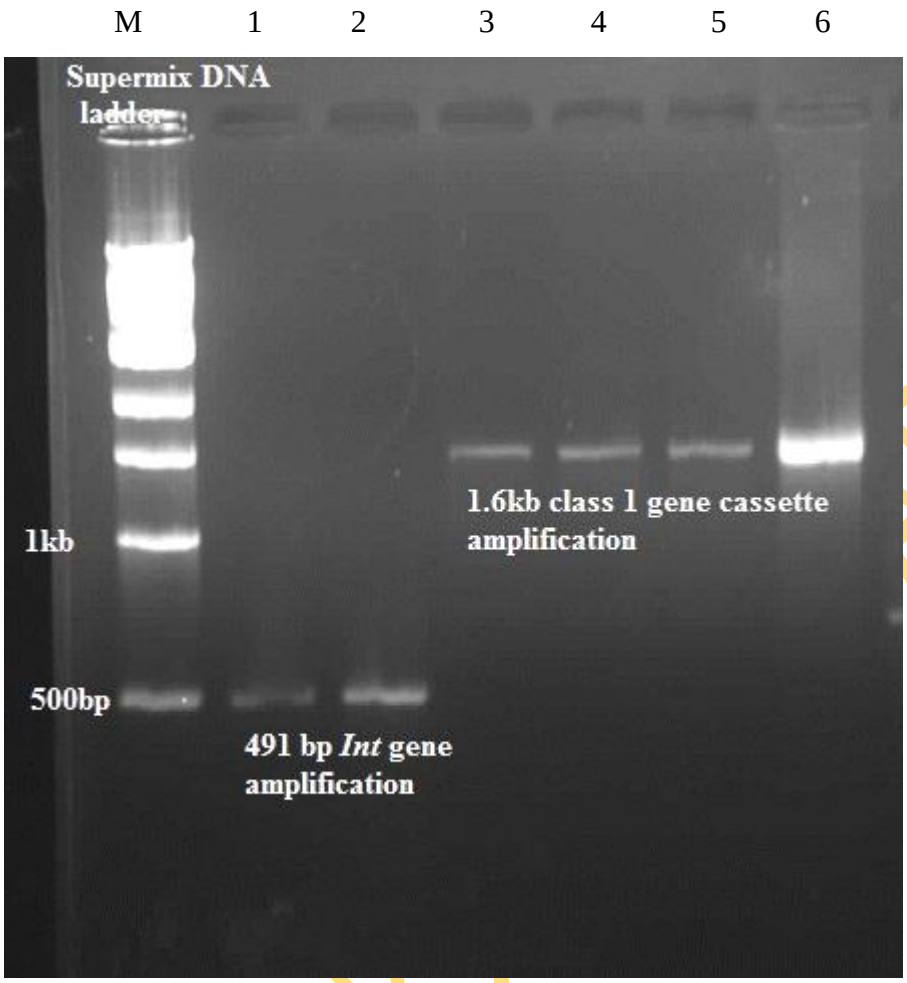


Plate 4.15: Agarose gel electrophoresis of PCR amplification of class 1 integron and class 1 gene cassette amplication of *P. aeruginosa* strains on 1.2% agarose gel.

Note. Lane M; 500bp, lane 1-2; *IntI1*gene positive amplicons of 491bp, lane 3-6 *IntI1* gene cassette of 1.6kb amplicons size.

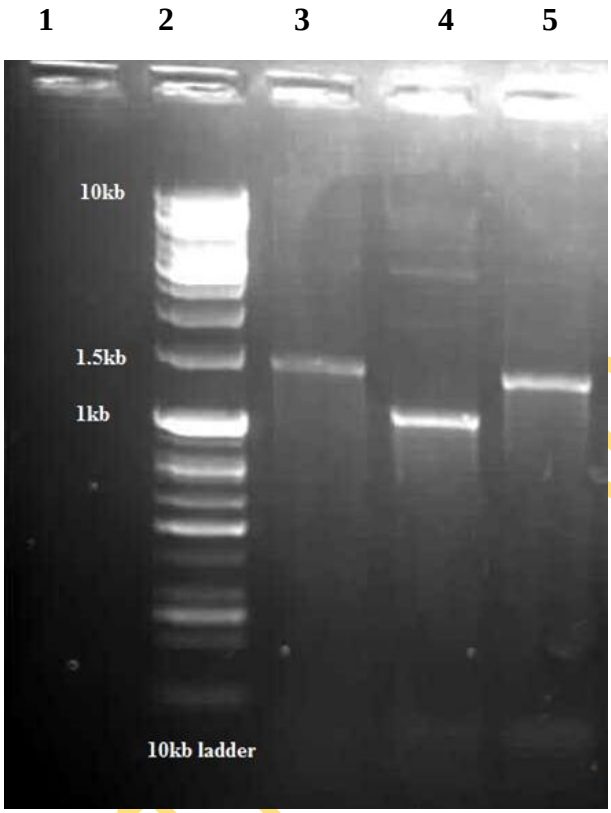


Plate 4.16: Agarose gel electrophoresis of PCR assay of gene Cassette amplification.

Note. Lane 1, 10kb ladder, Lane 2, *aadA6-orfD* gene cassette, Lane 3, *aadA13*, Lane 4, *aadA6-orfD*.

CHAPTER FIVE

DISCUSSION

5.1. Identification and distrubition of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa are usually identified by their unique growth and biochemical reactions to several reagents and media. All the *P. aeruginosa* investigated in this study grew on cetrimide agar which differentially allows *P. aeruginosa* to grow with the production of pyocyanin (Kings *et al*., 1954; Cassanovas *et al*., 2010). HiFluoro Pseudomonas agar Base specific for *P. aeruginosa* was also used, this medium allows *P. aeruginosa* to fluoresce under UV light because *P. aeruginosa* breaks the fluorogenic compound to release the fluorogen which produces a visible fluorescence under long wave UV light (http://www.krackeler.com/products/sigma/FLUKA/78996). Although it has been reported that some strains of Gram-negative bacilli (especially *Klebsiella* spp. and *Providencia* spp) may also grow on cetrimide agar however no other bacteria can produce pigmentation such as pyocyanin on cetrimide and on other agar at 42^{o}C (Reyes *et al*., 1981). Among the 85 presumptive *P. aeruginosa* isolated, 54 were identified as *P. aeruginosa* by the above methods and other standard biochemical tests such as oxidase test, catalase test, citrate test e.t.c.

P. aeruginosa is an opportunistic pathogen that is generally considered a ubiquitous bacterium (Feinbaum *et al*., 2012) among the Gram-negative bacteria commonly encountered in the hospital. *P. aeruginosa* isolates obtained from the urinary tract 21 (38.9%) dominated the population of *P. aeruginosa* isolates in this study (Fig 4.4). This finding is consistent with previous studies in Nigeria where high rate of isolation of *P. aeruginosa* was obtain from urine among the infected patients (Olayinka *et al*., 2004; Okonko *et al*., 2009; Okesola and Oni, 2012). Similar high rate of isolation from urine has been reported from other countries, indicating urinary tract infection (UTI) as the most common nosocomial infection (Tambekar *et al.,* 2006; Tavajjohi and Moniri, 2011). Among many other reasons, *P. aeruginosa* is associated with wound and surgical related infections, because of its opportunistic and pathogenic tendies, and ability to quickly colonize surfaces including inanimate objects such as sinks, surgical instruments, catheters e.t.c in and around the hospitals. Eleven (20.4%) of the isolates in this study were from wound related infections followed by 6 (11.1%) from pus (Fig 4.4). More women were found to be associated with Pseudomonal infections than men in this study (Fig

4.1), while highest frequencies of samples were recovered from both sexes of age range 20-30 (Fig 4.2). Since *P. aeruginosa* infections are not sex related, hence the data from this study cannot be used as an establishing fact.

5.2. Antibiotics resistance among the *P. aeruginosa* isolates

All the 54 *P. aeruginosa* strains in this study were resistant to at least 4 antimicrobial agents to which they were tested. All the strains of *P. aeruginosa* were found to be resistant to ampicillin, tetracycline and amoxicillin-clavulanic acid, while 28 (51.9%), 29 (53.7%), 34 (63.0%), 43 (79.6%) and 47 (87.0%) were resistant to cefotaxime, ceftriaxone, carbenicillin, kanamycin, and ticarcillin-clavulanic acid respectively. These high rates of resistance are consistent with previous studies on *P. aeruginosa* by different workers in Nigeria (Brown *et al*., 2003; Chah *et al*., 2003; Aibinu *et al*., 2007; Ogbolu *et al*., 2008; Okesola *et al*., 2009; Ohieku *et al*., 2010;) and those from other developing countries (Sader *et al*.,2002; Oliveira *et al*., 2007; Waigh *et al*., 2009; Ravichandra *et al*., 2012). High resistance by *P. aeruginosa* is not only prevalent in developing countries as studies from other developed nations have also reported these high rate among *P. aeruginosa* isolates (Oie *et al*., 1999; Kim *et al*., 2008), indicating a global incidence of high rates of drug resistance among these species.

High rate of resistance to carbenicillin observed in this study is worrisome because carbenicillin is a carboxypenicillin that serve as a good anti-pseudomonas drug due to its high resistance to efflux pumps (Pechere and Kohler, 1999; Poole, 2001) in *P. aeruginosa* and is currently prescribed infrequently in Nigeria. The observed resistance for this drug might hinder its future prescription as an antimicrobial agent. The resistance 34 (63%) observed for carbenicillin in this study is lower than 93.1% and 78% reported from Bulgaria and India respectively (Strateva *et al.*, 2007; Bashir *et al.* (2011) but comparably higher than 25% and 36.2% resistance rates reported from two studies from Iran (Nikbin *et al.*, 2007; Pal *et al*.,2010).

There was an observed decrease in fluoroquinolones (FQ) susceptibilities among the *P. aeruginosa* isolates in this study as compared to earlier studies in Nigeria. Fluoroquinolones susceptibilities results in this study are Ciprofloxacin 35 (64.8%), levofloxacin 33 (61.1%) and norfloxacin 33 (61.1%). Various studies in Nigeria had previously documented higher susceptibilities to FQ by clinical *P. aeruginosa*. Jombo *et al*. (2008) reported 92% susceptibilities to ciprofloxacin while Okonko *et al*. (2009) reported 100%. In another study by Olayinka *et al*. (2009), 90% susceptibility to ciprofloxacin was reported while El-Mahmood *et*

al. (2009) reported 71.4% susceptibilities as well as Ogbolu *et al*. (2008) and Nwankwo and Shuaibu (2010) who both reported 72.0% and 82.5% susceptibilities respectively. Compared to earlier reports, the result of this study indicates a lower susceptibility to ciprofloxacin among clinical *P. aeruginosa* indicating an increasing trend of resistance among *P. aeruginosa* against fluoroquinolones especially ciprofloxacin which is the most frequently prescribed. High resistance to ciprofloxacin (59.8%) and levofloxacin (69.1%) reported by Aibinu *et al*. (2007) is similar to result of susceptibility for both FQ in this study while higher resistance (80%) and (98%) to ciprofloxacin were obtained in Pakistan and India respectively (Saghir *et al*., 2009; Ravichandra *et al*., 2012), thus suggesting a global rise in the resistance to ciprofloxacin among clinical strains of *P. aeruginosa.*

Fluoroquinolones frequent empiric use in Nigeria raises concern for a rise in resistance. The findings from this study also confirm the previous report on emerging fluoroquinolones resistance *P. aeruginosa* in Nigeria by Lamikanra *et al*. (2011). The increase in resistance to FQ is alarming and the implication might be as a loss of confidence in the use of FQ alone in the management of *Pseudomonas* infection considering the fact that FQ are potent antimicrobial agent with broad-spectrum activities that has gained a wide use in management of many infections in Nigeria. Fluoroquinolones still remains the only available antibiotics for oral treatments of *P. aeruginosa* infection in most countries (Jalal *et al.,* 2000) hence its resistance might yield an undesirable clinical effect.

This study also revealed an increasing trend of resistance to the cephalosporins among the third-generation investigated. Ceftriaxone was found to be the least active as 25 (46.3%) isolates were susceptible, followed closely by cefotaxime 26 (48.1%). Previous workers in Nigeria (Brown *et al*., 2003; Nwachukwu *et al*., 2009; Okesola and Oni, 2009) have also obtained low susceptibilities in their studies with ceftriaxone and cefotaxime against *P. aeruginosa* while ceftazidime inhibited 45 (83.30%) of the isolates investigated in this study. Aibinu *et al* (2007) also reported a close value of 79.4% susceptibility to this drug against *P. aeruginosa* in their study. Although a decrease in activity was observed for ceftazidime as compare to previous reports (Oni *et al*., 2002; Nwankwo and Shuaibu 2010) where over 90% susceptibilities to *P. aeruginosa* were reported for ceftazidime. A high level of resistance (MIC $\geq$ 240µg) was observed in 3 (15%) and 14 (70%) of the resistant strains tested in this study against ceftazidime and ceftriaxone respectively. An inference from the above results is that these

strains are speedily acquiring resistance against third-generation cephalosporins and high reduction in the susceptibility rate of *P. aeruginosa* is gradually emerging.

A notable finding in this study is the emerging resistance to cefepime. Cefepime is a fourth-generation cephalosporin, one of the few remaining antimicrobial agents that has a reliable activity against *P. aeruginosa* (Akhabue *et al*., 2011) due to its broad-spectrum activities against ESBL. However resistance to cefepime observed in 10 (18.4%) of the isolates investigated this study is worrisome considering the fact that cefepime use is rarely reported Nigeria. Prompt attention is thereby needed to check this emergence in order to prevent complications in the treatments of MDR *P. aeruginosa* in Nigeria.

There was a high prevalence of multiple drug resistance among the 54 *P. aeruginosa* isolates investigated in this study as all the isolates showed resistance to more than three antibiotics. Multidrug resistance has been defined as resistance to 3 or more antimicrobial agent (Flamm et *al*., 2004). However according to recent MDR *P. aeruginosa* definition by Lee *et al*. (2012), which classifies a MDR *P. aeruginosa* strain as one that is resistant to 3 or more antipseudomonas drug, the prevalence of MDR *P. aeruginosa* in this study is 68.5%

The high prevalence of MDR *P. aeruginosa* observed in this study is comparable to previous studies from Nigeria which reported the prevalence rates ranging from 19.6% to 79.0%. For instance, Olayinka *et al*. (2004) reported 19.6% of MDR resistant *P. aeruginosa* obtained from clinical specimens from Zaria Northern Nigeria. In another related study, 22.6% incidence of MDR *P. aeruginosa* obtained from clinical specimens in a tertiary hospital in Maiduguri Northeastern Nigeria (Okon *et al.*, 2010), while Amadi *et al* (2009) reported high prevalence of MDR of 79% MDR *P. aeruginosa* from pus and wound from 2 tertiary hospitals in Enugu Eastern Nigeria. Although these authors had not classify their MDR based on resistance to 3 or more antipseudomonas drugs, their classifications has either been on resistance to more than 3 classes of antimicrobial agents or more than 3 antibiotics. Irrespective of this, the rate obtained in this study is comparably higher. Difference in geographical locations could suggest the varying susceptibility results obtained in these studies. High prevalence in MDR *P. aeruginosa* from other developing countries have also been reported. For example Tavajjohi and Moniri, (2011) reported a 30% *P. aeruginosa* resistance to at least 3 antimicrobial agents tested in Iran. In another two studies from India, Amutha *et al*. (2009) and Pal *et al*. (2010) reported a high prevalence of 45.2% and 62.3% respectively among clinical strains of *P. aeruginosa* while 48% MDR prevalence was reported from China (Jiang *et al*.,2006).

Similar findings of MDR *P. aeruginosa* as documented in this study have also been reported from developed countries in the world. According to Pagani *et al*. (2005) an outbreak involving 15 patients in a tertiary care and clinical research institute in Italy was reportedly caused by MDR *P. aeruginosa* isolates derived mostly from the lower respiratory tract. Studies in Bulgaria by Strateva *et al*.(2001) reported 49.8% MDR *P. aeruginosa* isolates from a tertiary hospital while in another study from France, the prevalence of multiple antibiotics resistant *P. aeruginosa* isolates was 72% among the clinical isolates investigated in 5 tertiary hospitals (Cholley *et al*., 2011). In another report Tam *et al*. (2010), found 14.0% prevalence of MDR *P. aeruginosa* isolated from bloodstream infection among 20 patients in a single hospital in Texas (USA). The findings from this study and its consistency with other reports worldwide affirms the global prevalence of MDR *P. aeruginosa*.

P. aeruginosa resistance to multiple antibiotics especially to the third-generation cephalosporin, aminoglycosides and fluoroquinolones is a formidable problem associated with adverse clinical outcomes such as high mortality and morbidity rate among the compromised individuals, long hospital stay and increased cost of therapy for such infections. Due to limited available therapeutic option, drugs such as carbapenems (imipenem and meropenem) shown to be very effective against multidrug resistant isolates including *P. aeruginosa*, are expensive and unaffordable by everybody. Although emergence of carbapenem resistance *P. aeruginosa* has been reported in other countries (Pagani *et al*., 2005), 50 (92.6%) of the isolates in this study were susceptible to imipenem confirming its effective inhibitory activity against MDR *P. aeruginosa.* In situations where carbapenem resistance is prevalent as reported in some developed countries, colistin and polymyxin B have been proven to be effective substitutes to this *β*-lactam drug (Gupta *et al*., 2009). Colistin resistance in *P. aeruginosa* has not been widely reported, 1 (98.1%) of the isolates in this study also showed susceptibility to colistin indicating its high inhibitory action against *P. aeruginosa*.

5.3 Mechanisms of resistance to antbiotics

Multiple drug resistance in bacteria is due to several intrinsic and extrinsic factors like the presence of resistant plasmids, efflux pumps, hydrolyzing enzymes e.t.c. *P. aeruginosa* is well known for harbouring multiple copies of plasmids, some of which are conjugative with resistance genes that are responsible for multiple drug resistance. All the *P. aeruginosa* strains investigated in this study harboured 1 to 4 plasmids with sizes ranging from 2.2kb to≥ 210 kb. All the strains also show resistance to 4 – 21 antibiotics. Highest number of plasmids was

extracted from isolates obtained from urine followed by vaginal swab and wound swab. The result of this study contradicts previous studies (Olayinka *et al*., 2004; Yah *et al*., 2006) on *P. aeruginosa* that reported low molecular weight plasmids (<2kb) in clinical isolates of *P. aeruginosa* from Nigeria but is in agreement with result from other countries (Nikbin *et al*., 2007; Raja and Selvam, 2009; Ranjbar *et al*., 2011) where high molecular weight plasmids were detected. The relationship between drug resistance and the presence of plasmids has been established (Laporta *et al*., 1986; Hu *et al*., 2009). The relationship between plasmid profiles and the multiple drug resistance patterns observed in this study suggests that plasmids may have played a significant role in the multidrug resistance of *P. aeruginosa* because resistance genes and virulence genes have often been found clustered together on a plasmid (Villa and Carattoli, 2005). *P. aeruginosa* isolates investigated in this study showed both plasmid and chromosomal mediated resistance to antibiotics tested. Elimination of plasmids from antibiotic resistant *P. aeruginosa* and increase in antibiotic susceptibility of cured *P. aeruginosa* isolates to third generation cephalosporins observed in this study confirmed plasmid borne nature of certain antibiotic resistance markers. Plasmid mediated resistance to the third generation cephalosporins by *P. aeruginosa* has widely been reported (Naas *et al*., 1998; Villegas *et al*., 2007). Resistance to cefotaxime, ceftazidime, ceftriaxone third generation cephalosporins and aztreonam monobactam are usually as a result of extended-spectrum *β*-lactamase production which are mostly plasmid mediated.

The simplest explanation for the presence of an integron within a clinical isolate of bacteria is due to the multitude of resistances conferred by the gene cassettes that are located in the same place. This study detected a high prevalence of integron among the clinical *P. aeruginos*a investigated. Thirty-one (57.4%) out of 54 isolates were found harbouring class 1 integrons in this study. The 57.4% incidence rate of class 1 integrons observed in this study is higher than previously reported rates of 41.5% from Brazil (Fonseca *et al*., 2005), 45.8% from China (Xu *et al*., 2009), and 56.3% from Iran (Yousefi *et al*., 2010). The prevalence of class 1 integrons in clinical isolates of *P. aeruginosa* from this study is of great concern because these genetic elements are highly stable in resistant pathogens; and also capable of easy spread and capture of other multidrug resistance gene cassettes leading to increase in resistance to broad-spectrum antibiotics (Tenover, 2006). Consistent with other previous studies that documented no detection of class 2 and class 3 integrons in clinical *P. aeruginosa* (Naas *et al*., 1998; Naas *et*

al., 1999; Gu *et al*., 2007; Xu *et al*., 2009; Martinez *et al*., 2012), none of the isolates in this study harboured class 2 or class 3 integron.

In Africa, class 1 integrons have been reported previously in clinical isolates of *P. aeruginosa.* Labuschagne *et al.* (2008) reported bla_{GES-5} and bla_{GES-5}-like genes as part of the variable region of class 1 integrons, occurring in three clinical *P. aeruginosa* isolates from South Africa. Another study reported class 1 integron containing bla_{VIM-2}, *aacA7* and *aacA4*, as well as *aadB* and *arr6*, a novel rifampin resistance gene among 35 clonally related *P. aeruginosa* isolated from a hospital in Tunisia (Hammami *et al*., 2010). This current study along with others from Africa and other parts of the world confirms that class 1 integrons are strongly associated with multiple drug resistance and are frequently detected among clinical isolates of *P. aeruginosa* (Martinez *et al*., 2012).

Clinically significant antimicrobial resistance was found among isolates that were positive for class 1 integrons (31/54), with unacceptably high resistance rates to cefotaxime 42 (77.4%), carbenicillin 44 (80.6%), ceftriaxone 47 (87.1%), streptomycin 49 (90.3%), kanamycin 45 (83.8%), tetracycline 54 (100%) and amoxicillin/clavulanate 54 (100%). This is comparable with previously reported high rates of resistance to ceftriaxone (88.7%) and cefotaxime (90.1%) (Chen *et al*., 2005), tetracycline (100%) and gentamicin (78.6%) (Gu *et al*., 2007) among integron positive clinical isolates of *P. aeruginosa* from China.

The gene cassettes mostly detected in all the class 1 integrons belong to *aadA* family of genes encoding aminoglycoside-3″-adenylytransferases (AAD) which confer resistance to streptomycin and spectinomycin by adenylylation (Naas *et al*., 1999, White and Rawlinson, 2001). The *aadA6-ofrD* gene cassette array derived from sequencing of the 1.6 kb gene cassette fragment from isolate *P. aeruginosa* ODM-24 showed complete homology with the *aadA6-ofrD* of class 1 integron reported from previous studies (Naas *et al*., 1999; Sekiguchi *et al*., 2007). Naas *et al.* (1999) first reported gene cassette *aadA6* as novel gene cassette in *P. aeruginosa*; and it has been reported to be highly conserved among the class 1 integrons gene cassettes of the *Enterobacteriaceae* most especially *E. coli* (Zhao *et al*., 2001; Roe *et al*., 2003). The complete sequence of *aadA13* obtained from sequencing of the 1.2kb gene cassette fragment derived from isolate *P. aeruginosa* ODM-08 was identical to previously reported *aadA13* sequences *[accession numbers* DQ779002, DQ779001] (Heuer and Smalla, 2007). To date, there are few reports of *aadA13* in clinical strains of *P. aeruginosa*. Yuan *et al* (2008) reported *aadA13* cassette, which was present in a new array of *aac(6')-II-aadA13-cmlA8-oxa-*

10 gene cassette from *P. aeruginosa*. To the best of our knowledge, this is the first study to report carriage of class 1 integrons and associated gene cassettes in *P. aeruginosa* isolates from Nigeria.

All the transconjugants obtained from the conjugation experiments harboured the same plasmid profiles as the parental strains. However, PCR amplification with specific primers revealed the absence of integrons and gene cassettes among the transconjugants; indicating the non-transference of the integrons and associated gene cassettes along with the conjugative plasmids. This is suggestive of chromosomal location of the integrons and gene cassettes as previously observed elsewhere that non-plasmid lateral exchange of resistance regions may be common in *P. aeruginosa* (Martinez *et al*., 2012). In addition, the result of this study also agrees with a recent publication on the dispersal of resistance regions from chromosomally located class 1 integrons possibly serving as the major genetic element of global dissemination in *P. aeruginosa* (Stokes *et al*., 2012). With the emergence of chromosomally located integrons capable of capturing more resistance gene cassette among *P. aeruginosa* on the steady rise, the chromosome may become an important platform in the dispersal of complex resistance regions in *P. aeruginosa* in the future through lateral gene transfer.

Clinical implication of this is the dissemination and spread of highly resistant *P. aeruginosa* with limited option for treatment once established in an infected host due to the presence of multidrug resistant integrons present in its genome. *P. aeruginosa* is a versatile organsim with arsenal of intrinsic resistance mechanisms and capable of acquisition of many more resistance gene through mobile genetic elements such as plasmids and integrons. Transfer of gene cassettes from other Gram-negative bacteria with multiple gene cassettes in their integrons such as *E. coli*, *K. pneumoniae* and *A. baumannii* that are found also among clinical isolates to *P. aeruginosa* would gravely complicate treatments of Pseudomonal infections in our hospitals if allowed to persist.

The conjugation assay with *E. coli* DH5α that possess no plasmids was successful among 10 (32.0%) of the 31 isolates investigated. All transconjugant plasmids mirrored the parental donor in this study indicating a successful transfer of the plasmids. The transconjugants were also resistant to amoxicillin/clavulanic acid, cefotaxime, carbenicillin and piperacillin in the susceptibility testing, suggesting the transfer of resistance markers. Five among the parental donor strains successful for conjugation also harboured $bla_{\text{OXA-10.}}$ However, efforts to demonstrate the $bla_{\text{OXA-10}}$ gene transfer to the recipient *E. coli* strains were unsuccessful.

Consistent with other previous reports (Naas and Nordmann. 1999; Bradford, 2001; Poirel *et al*., 2001), *bla*$_{\text{OXA-10}}$ are plasmid mediated as found in several other ESBL, however this study was unable to demonstrate the transfer of the *bla*$_{\text{OXA}}$ gene by conjugation. The reason for the failure could partly be due to the antibiotics used for the selection of the transconjugants. Other possible reasons for the unsuccessful transfer of the *bla*$_{\text{OXA}}$ genes via conjugation to the recipient cells in this study might be due to instability of the gene in the standard recipient *E. coli* DH5α strains. It has been previously shown that OXA-type enzymes provide weak resistance and becomes inactive when cloned into *E. coli* transconjugant as contrary to when *P. aeruginosa* transconjugant is used (Hall *et al*., 1993). Another possible reason might be due to the fact that the enzyme might be produced at a very low inducible rate which is not sufficient for an expression in the *E. coli* transconjugant.

5.4. Prevalence of ESBL among *P. aeruginosa* isolates

Low prevalence of ESBL by DDST among *P. aeruginosa* was observed in this study. Only 5 (9.3%) isolates showed synergy in the presence of the *β*-lactamase inhibitor (clavulanic acid) while the remaining strains, which showed resistance to the ESBL marker antibiotics remained negative even at a reduced distance of 15 mm of cephalosporin to the amoxicillin/clavulanate disk. Similar distance of 15 mm for DDST has been previously shown to be most reliable for detecting ESBL (Sanders *et al*., 1996). Previous studies in Nigeria have documented higher prevalence of ESBL in *P. aeruginosa* by DDST method. Aibinu *et al*. (2007) reported 45% detection of ESBL among clinical strains of *P. aeruginosa* investigated in 2 hospitals in Lagos while Akinjogunola *et al*. (2010) reported 30% detection among isolates obtained from UTI in the South-South Nigeria. In another study, Osazuwa *et al* (2011) reported 14% detection among *P. aeruginosa* investigated along with other Gram-negative bacteria isolated from HIV infected patients in Benin Metropolis while in a recent study by Okesola and Oni (2012), 22.2% rate of detection was reported from clinical isolates from University College Hospital in Ibadan. However, a lower rate of detection of ESBL with DDST observed in this study compared to the previous reports might be due to the presence of other resistance genes conferring multiple resistance to the investigated strains. Similar low rates of 4.0% and 8.1% of ESBL detection with DDST have been reported in Turkey and Iran respectively (Gençer *et al*., 2002; Tavajjohi *et al*., 2011)

The antimicrobial susceptibility results showed that the *P. aeruginosa* from this study were resistant to 3rd generation cephalosporins especially ceftriaxone (53.7%) and cefotaxime (51.9%), and were also resistant to aztreonam (44.4%) (Table 4.3) suggesting the presence of ESBL among these strains. Difficulty in the phenotypic detection of ESBL by DDST in *P. aeruginosa* has been previously reported to be problematic due to the frequent chromosomal *β*-lactam resistance mechanisms such as the over-expression of AmpC *β*-lactamase and/or one of the several efflux pumps encoded in its genome (Vahaboglu *et al*., 1998; Aubert *et al*., 2004; Juan *et al*., 2009). AmpC *β*-lactamase resist clavulanic acid hence prevents synergy between *β*-lactam and clavulanic acid. Resistance of the isolates in this study against ticarcillin/clavulanate 47 (87.0%) and piperacillin/tazobactam 21 (38.9%) indicates the presence of AmpC *β*-lactamase among these strains. Additionally, *P. aeruginosa* strains in this study have also been shown to be resistant to other classes of antimicrobial agents including fluoroquinolones and aminoglycosides (Odumosu *et al*., 2012). This further suggests the presence of other resistance mechanisms such as outer membrane impermeability, efflux pumps and integrons that are associated with multidrug resistance and capable of masking ESBL detection among the strains.

Tzelepi *et al*. (2000) suggested the use of cefepime to inhibit the activities of the AmpC enzymes and efflux pumps in *P. aeruginosa* thereby increasing the chance of the detection of ESBL by DDST. However, based on the resistance data obtained in this study, inclusion of cefepime may have shown little or no effect especially among the isolates (18.6%) that were resistant to cefepime, suggesting interplay of multiple resistance mechanisms among the *P. aeruginosa* strains. The low sensitivity of DDST for detecting ESBL among the cephalosporin-resistant isolates from this study could also be indicative of the presence of different *β*-lactamases, which are not easily detected by conventional phenotypic ESBL detection methods such as DDST. Therefore, data on the presence of ESBL among clinical *P. aeruginosa* obtained by DDST could be insufficient to assess prevalence of ESBLs. Previous studies have documented tazobactam inhibitory activity against ESBL and AmpC beta-lactamase to be almost 10 fold greater than clavulanic acid (Bush *et al*., 1993; Phillippon *et al.,* 2002). Tazobactam could therefore be used as a beta-lactamase inhibitor for DDST method along with cefepime or cloxacillin, especially for bacteria that co-produce ESBL and AmpC beta-lactamases.

Currently in Nigeria, there have not been any documented reports on molecular characterization of ESBL enzymes in *P. aeruginosa*, although as stated earlier ESBL and MBL have detected phenotypically in some strains of *P. aeruginosa* isolated in Nigerian hospitals (Aibinu *et al*.,2007; Akinjogunola *et al*.,2010; Osazuwa *et al*.,2011; Okesola and Oni 2012). In another recent report by Ogbolu *et al*. (2011), OXA variants and CTX-M variants were reportedly found among *P. aeruginosa* and other Gram-negative bacteria isolated from four teaching hospitals in the southwest Nigeria however the study did not indicate the specific variant detected nor was any documentation regarding a sequence report of the aforementioned.

Several workers from other parts of Africa have reported multiple *β*-lactamases in *P. aeruginosa* recovered from several sources in hospitals. These includes reports from Tunisia (Kalai *et al*., 2007; Mansour *et al*., 2009; Hammami *et al*., 2011; Ktari *et al*., 2011), Ghana (Samuelsen *et al*., 2009) Algeria (Drissi *et al*., 2008;) Egypt (Gad *et al*., 2007), South Africa (Poirel *et al*., 2002; Jacobson *et al*., 2012). This current study along with reports from Nigeria and other African countries showed the prevalence of *β* -lactamases and ESBL in African isolates of *P. aeruginosa.*

This study report multiple *β*-lactamases and ESBL detected by PCR and other molecular methods among the *P. aeruginosa* isolates from hospitals in Southwest Nigeria. The result of this study is consistent with previous studies where OXA-types and AmpC *β*-lactamases were detected at very high frequencies among *P. aeruginosa* strains. (Strateva *et al*., 2007; Du *et al*., 2010; Cabot *et al*., 2011). Similarly, low detection of SHV and CTX-M beta-lactamase enzymes in *P. aeruginosa* as observed in this present study have also been reported in France (De Champs *et al*., 2004) and China (Cai *et al*., 2012) indicating the low incidence and proliferation of these two enzymes in strains of *P. aeruginosa*. Expectedly, PER-1 (Pseudomonas extended resistance) was not found among any of the isolate investigated probably because PER-1 *β*-lactamase is an ESBL predominantly found in *P. aeruginosa* from Turkey (Vahaboglu *et al*., 1997) although other PER-1 detections from Italy, France and Belgium have been noted (Claeys *et al*., 2000; De Champs *et al*., 2002; Pagani *et al*., 2002), there have no report of the detection of this enzyme in studies carried out in Africa.

Contrary to the phenotypic detection of ESBL among the isolates in this study, which gave 5 (9.3%) prevalence, the PCR method of ESBL detection using specific primers revealed a high prevalence among the isolates. This further confirm that phenotypic detection of ESBL by DDST is not a reliable method for *P. aeruginosa* as was previously reported (Odumosu et al.,

2012a). Hence a call for a more reliable method especially by the CLSI can never be overempahsized.

The agarose gel electrophoresis of the PCR assay products showed that 16 out of 20 randomly selected *P. aeruginosa* for molecular studies in this work gave a 720bp amplification using OXA-degenerate primers OXA-MU1 and OXA-MU2 (Table 3.3 Plate 4.6). Further assay with OXA-10 specific primers (Table 3.3) and sequencing of an amplicon confirmed that the amplified oxacillinase gene is $bla_{\text{OXA-10}}$ belonging to class D *β*-lactamase previously described (Bert *et al.*, 2002). The remaining 15 positive isolates whose amplicons were not sequenced were also resistant to Amoxicillin/clavulanic, carbenicillin and ticarcillin and 15 (75%) of the isolates demonstrated a high level resistance to piperacillin which is characteristic of OXA-10 *β*-lactamase (Bert *et al*., 2002), suggesting that they also harbour the same $bla_{\text{OXA-10}}$ *β*-lactamase. OXA-10 is a classical OXA type enzyme (oxacillinase) belonging to molecular class D and functional group 2d *β*-lactamase and the second largest family of *β*-lactamase (Bush and Jacoby 2010). They determine resistance to carboxypenicillins (carbenicillin and ticarcillin) and ureidopenicillins (azlocillin and piperacillin) but not to ceftazidime (Bert *et al*., 2002) and are insensitive to clavulanic acid. They are mostly plasmid mediated and predominantly occur in *P. aeruginosa* and have been found in isolates originating in Turkey and France (Naas and Nordmann. 1999; Bradford, 2001).

This study also identified chromosomal AmpC cephalosporinase at a high frequency. Reports across the globe including Africa have also described the carriage of AmpC genes in *P. aeruginosa* (Drissi *et al*., 2008; Bhattacharjee *et al*., 2008; Tomas *et al*., 2010; Cabot *et al*., 2011; Ogbolu *et al*., 2011, VinodKumar *et al*., 2011). *P. aeruginosa* strains are known to frequently harbour AmpC cephalosporinase as a part of their chromosomal genes which additionally confer their resistance against multiple *β*-lactam drugs (Sahid *et al*., 2003).

Worryingly, most of the AmpC *β*-lactamase positive isolates in this study are positive for ESBL by PCR, harbouring $bla_{\text{OXA-10}}$ genes. The coexistence of these two enzymes may render an organism resistant to virtually all the *β*-lactam drugs. This suggests the high-level penicillin resistance (including all the ureidopenicillins and carboxypenicillin) and the cephalosporins (3^{rd} generation and cefepime) and other beta-lactam drugs in this study is as a result of the presence of or coexistence of both OXA-10 and AmpC enzymes identified in the same strains of *P. aeruginosa*. The coexistence of these enzymes do not only result in high level resistance to the above drugs but could also give false-negative test results for the detection of ESBLs.

Interestingly, all the *P. aeruginosa* employed in this study had diverse plasmids profile of varying sizes and restriction patterns (Plate 4.3 and 4.4) they also showed different patterns of susceptibility to the various antimicrobial agents tested. This further confirms non-relatedness of the isolates and diversity of their origins.

Clinical implications of these identified enzymes in *P. aeruginosa* strains is the frequent encounter of multidrug resistance in infections, which become difficult to manage due to a limited therapeutic options (Jacoby and Munoz-Price, 2005). The failure to detect the presence of ESBL phenotypically in such isolates harbouring OXA-10 and AmpC may also lead to clinical failure because of inappropriate antimicrobial treatment due to false negative ESBL detection routinely. This situation is however further worsened in Nigeria because most clinical laboratories do not perform tests to monitor ESBL production among clinical isolates due to its financial constraints. The detection of AmpC beta-lactamase producing strains is difficult and the phenotypic tests for AmpC detection are not well defined (Jacoby and Munoz-Price, 2005), hence clinical management of such infections is difficult. AmpC beta-lactamase is usually responsible for the development of resistance in the course of therapy when it is over-expressed (Jacoby and Munoz-Price, 2005).

Observers from around the world have attributed the multidrug resistance/ESBL production among bacteria pathogens to the use of third-generation cephalosporins and other broad-spectrum *β*-lactam drugs such as carbapenems (Urbánek *et al*., 2007; Ahmad *et al*.**,** 2010)**.** In this current study, the high rate of multidrug resistance and beta-lactamase production observed in *P. aeruginosa* in Southwest Nigeria was very high. This could also be attributed to the indiscriminate use or misuse of antibiotics and prescription of broad-spectrum drugs by medical personnel in the hospitals and by individual over-the-counter purchase without doctor's prescription. Studies have shown that 40 to over 90% of antibiotic prescription are unnecessary; likewise the use of antibiotics as growth promoters in animal husbandry has contributed largely to this spread (Okeke *et al*., 1999)

5.5. Aminoglycosides modifying enzymes (AMEs)

Aminoglycosides as a class of antimicrobial agent have enjoyed wide use due to their stability against many bacterial isolates which are becoming resistant to the newer antibacterial agents (Falagas *et al*., 2008). This study describes the carriage of aminoglycosides modifying enzymes (AMEs) among the clinical *P. aeruginosa* isolates investigated. Three classes of AMES; APH,

AAC and ANT were investigated in this study. Among the AMEs examined, *aac(6')-I* and *ant(2'')-I* are the two most frequently detected. None of the isolates produced *aac(3)-I,II, aac(6)-II, aph(3')-IV*. This finding correlated with studies from Europe and USA (Miller*et a.,l* 1997; Poole, 2005; Dubois *et al*., 2008) where the *aac (6')-I* gene and *ant(2'')-I* were the most frequently detected, but is in contrast to studies from Korea and Iran where the most common gene was *aph (3')-IV* (Kim *et al*., 2008; Vaziri *et al*., 2011). It has been previously reported that the occurrence of these combination of enzymes varied by geographic regions and among hospitals (Miller *et al*., 1997) hence the inconsistencies. Seven (35%) of isolate studied harboured at least one of the enzymes and were distributed among 4 out of the 5 hospitals; this is also consistent with previously reported frequencies (Dubois *et al*., 2008; Kim *et al*., 2008; Vaziri *et al*., 2011) but at variance with studies from USA and Europe where most of the genes were present as a single AG modifying enzymes (Miller., *et al* 1997; Poole, 2005; Dubois *et al*., 2008).

The aminoglycoside mechanisms of resistance have been rarely studied in *P. aeruginosa* here in Nigeria. To our knowledge; this is the first report of AMEs in clinical *P. aeruginosa* isolates from Nigeria. Unfortunately there are no sufficient data from other African countries for comparison because Pubmed and other literature search revealed scanty AMEs documented report on *P. aeruginosa* isolates among African reports. Poirel and his colleagues had earlier reported a class 1 integron carrying a gene cassette encoding *β*-lactamases and an aminoglycoside modifying AAC(3)1-like enzymes from clonally related *P. aeruginosa* from a nosocomial outbreak in South Africa (Poirel *et al*., 2000).

The *aac(6')-I* is significant for tobramycin, amikacin and kanamycin resistance while *ant(2'')-I* inactivates gentamicin, tobramycin and kanamycin but not amikacin (Vakulenko and Mobashery, 2003; Poole, 2005). An unexpected resistance phenotype leading to some inconsistency between the AST result and the PCR amplification of AMEs was observed in this study regarding the above fact. Out of the 10 (50%) isolates positive for AMEs in this study, only 3 (15%) isolates were in consonant between the PCR and AST result. For instance 5 isolates harboring *aac (6')-I* which has amikacin as a substrate showed resistance (>256µg/ml) to gentamicin with amikacin and some without amikacin, while 2 isolates showing resistance (>256µg/ml) to amikacin and gentamicin do not harbor the enzymes at all and an isolate that showed susceptibility to both drugs in spite of the presence of both genes (Table 4.2, 4.4 and Plate 4.10, 4.11). Similar result and observation have been reported previously from Korea

(Kim *et al*., 2008) and Iran (Vaziri *et al*., 2011) after investigating the presence of AMEs by PCR and its correlation with the AST results. The result of this study agrees with previous presumption (Kim *et al*., 2008) that the reason for these inconsistencies in resistance phenotype might be due to the presence of other resistance mechanisms such as efflux pumps or some form of rare AMEs present in integrons and transposons among the isolates as observed in our study too. In addition, it is also believed that non-enzymatic resistance (NER) as previously reported (Dubois *et al*., 2008) could also be at play among the phenotypes. Furthermore, in *P. aeruginosa*, due to the combination of several mechanisms and variable levels of their expression, the involved mechanisms cannot be easily deducted from the resistance profile.

The incidence of *aac(6')-I* and *ant(2'')-I* detected among the clinical isolates of *P. aeruginosa* isolates reflects the high aminoglycoside (AG) usage in Nigerian hospitals. A positive correlation between the use of an AG and the occurrence of enzyme-mediated resistance in geographic regions and among hospitals has been described (Miller *et al*., 1997; Schmitz *et al*., 1999). Further increase in use of these drugs may result in the increase in the spread of genes coding for this enzymes which are not only transferable as they are commonly found in gene cassette and transposons but have also been seen to co-exist very frequently with other ESBL from previous reports and as observed in this current study (Poirel *et al*., 1999; Poole, 2005; Kim *et al*., 2008).

Co-existence of aminoglycoside modifying enzymes and extended-spectrum *β*-lactamase was prevalent among the *P. aeruginosa* isolates in this study. Among the *β* –lactamase gene, $bla_{\text{OXA-10}}$ was the most prevalent. All the isolates harbouring *aac(6')-I* or *ant(2'')-I* genes also co-harboured $bla_{\text{OXA-10}}$ and class 1 integron. Highest frequency was observed for isolates harbouring *aac(6')-I* being the most prevalent AME detected in this study. This finding is consistent with previous reports (Poirel *et al*., 1999; Kim *et al*., 2008; Dubois *et al*., 2008). The finding of this study also supports the fact that $bla_{\text{OXA-10}}$ is the most prevalent *β* -lactamase gene that is frequently associated with *ant(2'')-I* and as a part of an integron (Poirel *et al*., 1999). Although both genes were not identified as a part of an integron in this study, however gene cassette responsible for streptomycins and spectinomycins as a part of class 1 integron was detected among the isolates in this study (Plate 4.16). Occurrence of class 1 integron, AMEs and *β* –lactamase gene co-existence in *P. aeruginosa* investigated in this study is of a serious concern. There is the possibility of co-selection, dissemination of multidrug resistance and acquisition of more resistance genes, considering the fact that *β*–lactam drugs and

aminoglycosides/fluoroquinolones are both employed synergistically as antipseudomonas drugs against multidrug resistant *P. aeruginosa*. The presence of these resistance genes in already intrinsically resistant *P. aeruginosa* may increase the chances of total clinical failure and management of infections caused by *P. aeruginosa* in southwest Nigerian hospitals if urgent measures are overlooked.

5.6. Fluoroquinolones resistance mechanism

Transferable fluoroquinolones (FQ) resistance genes are frequently detected among the *Enterobacteriaceae* and have already been reported in all continents. The first plasmid mediated *qnrB1* gene from Nigeria uropathogenic *Klebsiella pneumoniae* was reported by Soge *et al*. (2006). Shortly afterwards, disturbing transferable FQ resistance reports among members of *Enterobacteriaceae* in Nigeria were been documented (Lamikanra *et al*., 2011; Ogbolu *et al*., 2012). None of these FQ resistance genes has been found in clinical *P. aeruginosa* to date (Poole, 2001; Coban *et al*., 2011), although Martı´nez-Martı´nez *et al*. (1998) demonstrated that *qnrA* gene does confer resistance in this species if introduced via conjugation.

Mutations in the quinolone-resistance-determining regions (QRDR) of the gyrase and topoisomerase IV resulting to resistance in FQ in *P. aeruginosa* have been extensively studied in most pathogens including *P. aeruginosa* (Hooper, 2000; Wong and Kassen, 2011; Ogunleye, 2012). However in *P. aeruginosa*, increased efflux of antibiotics is typically achieved by the upregulation of chromosomally encoded efflux pumps which also occurs independent of mutations (Ziha-Zarifi *et al*., 1999; Srikumar *et al*., 2000). This mechanism has been reported to play a major role in its resistance to FQ (Kohler *et al* 1997; Masuda *et al*., 2000). To our knowledge, no Nigerian studies have investigated the efflux mediated resistance of *P. aeruginosa* to FQ.

Genes *mexR* and *nfxB* responsible for the regulatory protein that controls the expression of the efflux operon *mex*AB-*opr*M and *mex*CD-*opr*J commonly found in *P. aeruginosa* (Poole, 2005) was detected in this study at a high prevalence. Both genes were found among 9 (45%) of the 20 strains that were selected for molecular studies. Six (30%) of these strains (*P. aeruginosa* ODM 32, 34, 38, 40, 45 and 48) were found with a high level resistance (≥ 240µg/ml) to FQ (ciprofloxacin and levofloxacin) (Table 4.4). *nfxB* gene was originally identified as a determinant of fluoroquinolones resistance (Hirai *et al*., 1987), however, both genes (*mexR* and *nfxB)* are responsible for extrusion of a number of antimicrobial agents, their other substrate

apart from fluoroquinolones includes; *β*-lactam, *β*-lactamase inhibitors, tetracycline, macrolide, trimethoprim, chloramphenicol, novobiocin and other compounds (Poole, 2005). Three strains (*P. aeruginosa* ODM 24, 25 and 49) remained susceptible to FQ but showed high level resistance to ceftriaxone (>240µg/ml) and to piperacillin suggesting a possibility of the extrusion of these drugs by the efflux pumps or synergistically with other mechanisms. Interestingly, all the above strains were found harbouring class 1 integron and $bla_{\text{OXA-10}}$ with majority of them also producing AmpC *β*-lactamase. It has been previously shown that MexCD-OprJ exports both 3rd and the 4th generation of cephalosporin (Srikumar *et al*., 1997; Gotoh *et al*., 1998), while MexAB-OprM in some instances plays a more important role than the chromosomally-encoded *β*-lactamase of *P. aeruginosa* (Nakae *et al*., 1999; Masuda *et al*., 1999).

As stated in the previous section of this study, the observed resistance phenotypes cannot be conclusively ascribed to a particular resistance mechanism in this study because of the level and expression of various multidrug resistance mechanisms found and the possibility of synergy in such mechanisms among the *P. aeruginosa* strains. However, understanding the significance of efflux mechanisms in fluoroquinolones resistance in *P. aeruginosa* will give more room for therapeutic intervention against MDR efflux pumps present in these strains.

5.7. Conclusion and recommendations

In summary, this study focused on the prevalence of multidrug resistant *P. aeruginosa* isolated from five hospitals in southwest Nigeria, characterisation of the isolates on the genetic basis of their antibiotic resistance and demonstrate the transferability of their resistance genes. This study found a high prevalence of multidrug resistance with all the 54 isolates being resistance to more than 3 antibiotics and 68.5% of these isolates resistant to 3 or more antipseudomonas class of antimicrobial agents. This study also involved detection and characterization of plasmids and integrons among the mobile gentic elements, which led to the identification of novels gene cassettes not previously reported in *P. aeruginosa* in Nigeria. The integrons which were all chromosomally encoded harbours *aadA6-ofrD* and *aadA13* which determines resistance to aminoglycosides in Gram negative bacteria. Transferable plasmids of sizes greater than 58kb and copies 1 – 4 in range were detected among all the isolates investigated in this study. Plasmid encoded ESBL OXA-10, CTX-M and SHV were detected in this study of which OXA-10 was highly prevalent among the isolates investigated, while AmpC that was also detected at a high prevalence was chromosomally encoded.

This study represent the first report of OXA-10 among clinical isolates of *P. aeruginosa* in West Africa.

Aminoglycosides resistance observed among the isolates is credited to the presence of AMEs *aac(6) – I* and *ant(2'')–I* detected at high prevalence and their association with $bla_{\text{OXA-10}}$ genes among the isolates in this study. This study represent the first report of AMEs and it association with extended-spectrum *β*-lactamase in *P. aeruginosa* isolates in Nigeria. Furthermore, for the first time the two efflux pumps *mexR* and *nfxB* determining MDR resistance in strains of *P. aeruginosa* and conferring resistance to fluoroquinolones among *P. aeruginosa* were described in this study. This work detected class 1 integron and gene cassette array *aadA6-ofrD* and *aadA13* at high prevalence making it the first report of class 1 integrons and associated gene cassettes in *P. aeruginosa* isolates from West Africa. The class 1 integron and gene cassette detected in this study are also associated with aminoglycoside resistance. The *aadA13* gene cassette and *aadA6-ofrD* detected in this study have not been previously reported in Nigeria.

The data presented in this thesis has provided a significant contribution to the knowledge of the prevalence and type of ESBL, AMES and integron in *P. aeruginosa* in Southwest Nigeria, encompassing a comprehensive investigation into the resistance mechanisms to anti-pseudomonas drugs. In line with other previous studies where multiple *β*-lactamases were

detected among strains of *P. aeruginosa,* PER-1, CTX-M and SHV *β*-lactamases is not widespread among *P. aeruginosa* strains in Southwest hospitals in Nigeria. High rates of antibiotic resistance recorded in this study is an indicative of widespread dissemination of resistance genes which could gravely compromise treatments of infection if prompt adequate measures are not put in place.

Resistance to expanded-spectrum cephalosporins among clinically and epidemiologically important Gram-negative bacteria including *P. aeruginosa* should serve as a warning signal to the presence of ESBL; and the detection of such ESBL-producing strains among the patients should necessitates urgent implementation to prevent outbreaks arising from cross-transmission to other patients. Importantly, early and accurate detection of ESBL-producing *P. aeruginosa* and other Gram-negative bacteria is crucial for effective treatment and control of the rapid spread of plasmid-encoded ESBL genes among these pathogens.

As a recommendation for the control of the spread of antibiotic resistant *P. aeruginosa,* It is imperative to ensure adequate measures to contain the spread and dissemination of resistance genes among *P. aeruginosa* and other relevant pathogens commonly encountered in hospitals are in place. Hence, the call for an extensive surveillance and monitoring of MDR pathogens especially the notorious ones such as *P. aeruginosa, K. Pneumoniae, S. aureus* and *E. coli* in Nigeria can never be overemphasized.

The following recommendation will help to circumvent the emergence, spread and dissemination of antibiotic resistant bacteria implicated in several fatal infections in Nigeria and other parts of the world:

1) Proper monitoring of the use, sales and prescription of antibiotics in the community and the hospital. The use of broad-spectrum antibiotics should be restricted to chronic infections and not just any type of infections.
2) Laboratory scientist and clinicians should be well informed and updated in the current resistance/susceptibility trends and spectrum of clinically important bacteria. Accurate method of identification of resistance mechanisms will help to determine the epidemiology, risk factors, and appropriate option for treatments.
3) Routine detection of different antibiotics resistance mechanisms, especially the ones with epidemiological consequences, such as ESBL production, is vital for the clinical laboratories. The double disk synergy test (DDST) method used worldwide in the detection of ESBL production has been proved unsuitable for *P. aeruginosa* in this study, due to the

effects of AmpC β-lactamase and the production of $bla_{\text{OXA-10}}$ among the strains. Hence, cheap, fast and reliable method is required for *P. aeruginosa* and relations. Although, a combination of phenotypic and molecular detection methods remains the best reliable, robust surveillance system for detecting the diverse group members of the ESBLs. This will improve the empirical treatment and management of infections caused by ESBL producing bacteria.).

4) Adequate funding of molecular research in Nigeria should be encourage. This will increase the knowledge base and pave way for more novel discoveries.

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LIST OF AWARD, PUBLISHED PAPERS, ABSTRACTS AND PAPERS ACCEPTED FOR PUBLICATION FROM THIS RESEARCH THESIS

Awards.

The World Academy of Science (TWAS) Postgraduate Fellowship 2010.

Recipient of University of Ibadan postgraduate school Teaching and Research Assistanship.

Publications

1). Odumosu, Bamidele .T, Adeniyi, B. A, Dada-Adegbola H and Ram Chandra**.** Multidrug resistant *Pseudomonas aeruginosa* from Southwest Nigeria hospitals. **(***Int. J. Pharm. Sci. Rev. Res*. 15: 11-15 2012.)

2). Odumosu Bamidele .T, Adeniyi B.A., Soge Olusegun O., Dada-Adegbola Hannah O**.** Phenotypic detection of extended-spectrum beta-lactamase producing *Pseudomonas aeruginosa* from Hospitals in Southwest Nigeria. (*The Global Journal of Pharmaceutical Research Vol. 1(4), 708-714, 15 Sep, 2012;*).

Abstracts.

- Multidrug resistant *Pseudomonas aeruginosa* from Southwest Nigeria Hospitals 52nd Annual conference of Association of Microbiologist of India (AMI) Panjab University, India, November 2011

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Odumosu, Bamidele .T , Adeniyi, B. A, Soge, O. O and Ram Chandra**.** Analysis of integrons and associated gene cassettes in clinical isolates of multidrug resistant *Pseudomonas aeruginosa* from Southwest Nigeria (In Press).