MOLECULAR CHARACTERISATION OF METALLO-BETA LACTAMASE AND OTHER RESISTANCE GENES IN *PSEUDOMONAS AERUGINOSA* FROM SEVEN TERTIARY HOSPITALS IN SOUTHWESTERN NIGERIA

BY

OLUWATOYIN BOLA, OLANIRAN

MATRIC No.: 160617

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ABSTRACT

The emergence of resistance to carbapenems, a last resort antibiotic, among *Pseudomonas aeruginosa* is of great health concern. Detailed studies on the molecular basis of carbapenem resistance in clinical *P. aeruginosa* isolates are scanty in Nigeria. Therefore, this study was aimed at determining the incidence of Metallo-Beta Lactamase (MBL) and other mechanisms mediating carbapenem resistance, and evaluating clonal spread among carbapenem-resistant *P. aeruginosa* isolates.

Four hundred and forty-seven presumptive *P. aeruginosa* isolates collected from seven tertiary hospitals laboratories in southwestern Nigeria were identified using biochemical tests and amplification of *oprI* and *oprL* genes. Antibiogram of the isolates and Minimum Inhibitory Concentrations (MIC) were determined by Kirby-Bauer disk diffusion and broth microdilution, respectively. Phenotypic detection of carbapenemases was carried out using Modified-Hodge and combined disc tests. Carbapenem-resistant *P. aeruginosa* isolates were screened for class A, B and D carbapenemases, integrons and type III secretion effectors by Polymerase Chain Reaction (PCR) followed by sequencing of amplified carbapenemase genes. Transferability of MBL genes was determined by transformation experiments. Quantitative reverse transcription PCR (RT-qPCR) was used to quantify expression levels of eight efflux pump genes, *ampC* cephalosporinase and outer membrane porin *oprD*. The isolates were further genotyped using three PCR-based fingerprinting techniques. Fisher's exact test was used to determine the association between MBL and integrons at $p \leq 0.05$.

Four hundred and thirty isolates were identified as *P. aeruginosa* of which 185(43.0%) were multidrug resistant and 50(11.6%) were extensively drug resistant. All the isolates were resistant to ampicillin, cephalothin and cefuroxime, while sensitivity to polymyxin B was most common (96.3%). The MICs ranged from 0.125 to >64 µg/mL and 0.0625 to >64 µg/mL against imipenem and meropenem, respectively. All the isolates were negative for Modified-Hodge test, while combined disc test revealed the presence of MBL. Two class B carbapenemases were detected in 86.3% of the carbapenem resistant isolates: bla_{VIM} and bla_{NDM} in 35.6% and 38.4% isolates, respectively, co-existing in 12.3% isolates. Fifty-one (57.5%) carbapenem-resistant *P. aeruginosa* strains carried class 1 integrons while class 1 and 2 integrons were present concomitantly in 12.3%. Type III effector genes, *exoY* and *exoT* were found in all

isolates, while *exoU* and *exoS* were present in 49.3% and 53.4%, respectively. Two isolates possessed both *exoU* and *exoS*. Sequence analysis of bla_{VIM} and bla_{NDM} revealed maximum identity with bla_{VIM-5} and bla_{NDM-1}, respectively. MBL genes were successfully transferred into *Escherichia coli* DH5α. MexXY-OprM was the most overexpressed pump (5.0 - 996.3 fold increase) occurring in 58.3% of the isolates. The *ampC* was overexpressed in 27.1% isolates, while *oprD* porin down-regulation was observed in 77.1% of the isolates. Nine disseminated clones were identified across southwestern states. There was positive association between integrons and MBL (p = 0.0064).

There is a high incidence of transmissible metallo-beta lactamase genes in *Pseudomonas aeruginosa* from tertiary hospitals in southwestern Nigeria with different mechanisms mediating carbapenem resistance. bla_{VIM-5} and bla_{NDM-1} were found co-occurring for the first time. There is a need for surveillance of resistance to carbapenems and associated resistance genes.

Keywords: Carbapenem resistance, *Pseudomonas aeruginosa*, Efflux pumps, Integrons, Metallo-Beta Lactamases

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All glory the Most-High God: the source of my strength.

CERTIFICATION

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

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Supervisor

O. E. Adeleke (Ph.D)

Professor of Pharmaceutical Microbiology,

University of Ibadan, Ibadan, Nigeria.

DEDICATION

This research work is dedicated to Most-High God for His endless faithfulness towards me.

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LIST OF MAIN ABBREVIATIONS

Abbreviations	Full meaning
ABC	Adenosine triphosphate-binding cassette superfamily
AIM	Adelaide Imipenemase
ampC	Chromosomal cephalosporinase
ATCC	American Typed Culture Collection
BLAST	Basic local alignment search tool
BIC-1	Bicêtre carbapenemase
CDC	Centre for disease prevention and control
CI	Chromosomal integrons
CLSI	Clinical laboratory standard institute
CRPA	Carbapenem-resistant *Pseudomonas aeruginosa*
DHP-I	Dehydropeptidase I
DIM	Dutch imipenemase
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ERIC-PCR	Enterobacterial repetitive intragenic consensus sequence PCR
ESBL	Extended spectrum beta-lactamase
FIM	Florence Imipenemase
FMCA	Federal Medical Centre, Abeokuta
FMCI	Federal Medical Centre, Ido-Ekiti
FMCO	Federal Medical Centre, Owo
GES	Guiana extended spectrum
GIM	Germany Imipenemase
GIs	Genomic islands
HAI	Healthcare associated infections
ICU	Intensive care unit
IMI-1	Imipenem-hydrolysing beta-lactamase
IMP	Imipenem type metallo-beta lactamase
Intl	Integrase gene
IS	Insertion sequence
IS*CR19*	Insertion sequence common region 19
kb	kilobase
kDa	kilodalton

KHM	Kyorin Hospital Imipenemase
KPC	*Klebsiella pneumoniae* carbapenemase
LTHO	Ladoke Akintola University Teaching Hospital, Osogbo
MATE	Multidrug and toxic compound extrusion family
MBL	Metallo beta-lactamase
MDR	Multidrug resistance
MFS	Major facilitator superfamily
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MITEs	Miniature Inverted-repeat Transposable Elements
MLEE	Multilocus enzyme electrophoresis
MLST	Multi Locus Sequence Typing
NDM	New Delhi metallo-beta lactamase
NmcA	not metalloenzyme carbapenemase A
OmpF	Outer membrane protein F
OprD	Outer membrane porin D
OTHI	Obafemi Awolowo University Teaching Hospital Complex. Ile-Ife
OTHS	Olabisi Onabanjo University Teaching Hospital, Sagamu
OXA	Oxacillin-hydrolyzing beta-lactamases
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
REP-PCR	Repetitive extragenic palindromic PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RND	Resistance nodulation division family
RT-PCR	Reverse transcriptase polymerase chain reaction
s	seconds
SDS	Sodium dodecyl sulphate
SFC-1	*Serratia fonticola* carbapenemase-1
SIM	Seoul imipenemase
SME	*Serratia marcescens* enzyme

SMR	Small multidrug resistance family
SPM	Sao Paulo Imipenemase
SSI	Surgical site infection
T3SS	Type III secretion system
TAE	Tris acetate EDTA
Tns	Transposons
UCHI	University College Hospital, Ibadan
UTI	Urinary tract infection
UV	Ultraviolet
VAP	Ventilator-Associated Pneumonia
VIM	Veronese Imipenemase
WHO	World Health Organisation
XDR	Extensively drug resistance

Detection of Bacterial Pathogens in Cerebrospinal Fluid using Restriction Fragment Length Polymorphism

AT Kalghatgi,[*] AK Praharaj,[+] AK Sahni,[#] D Pradhan,[**] S Kumaravelu,[++] PL Prasad,[##] and A Nagendra, (Retd)[***]

CHAPTER ONE
INTRODUCTION

1.1 *Pseudomonas aeruginosa* as a nosocomial pathogen

Pseudomonas aeruginosa is a Gram-negative opportunist pathogen which takes advantage of the breach in the host immune system to cause infection. This is the reason *P. aeruginosa* is commonly implicated in deadly bacterial infections of immunodepressed people but rarely causes disease in healthy individuals (Dean *et al*., 2008). *P. aeruginosa* produces a high mortality rate of over 70% in immunocompromised individuals (Mackie and MacCartney, 1996). *P. aeruginosa* is the main nosocomial pathogen which causes about 10% of infections in most hospitals followed by *Staphylococcus aureus* (Fazeli *et al*., 2012). The US Centre for Disease Control and Prevention (2014) estimated that *P. aeruginosa* accounted for 51,000 healthcare-associated infections and over 6,000 (13%) are caused by multidrug resistant *P. aeruginosa* with about 400 deaths occurring every year in the USA. *P. aeruginosa* is the second and third most frequently isolated nosocomial pathogen in respiratory tract infection (17%), urinary tract and surgical establish infections (11%), respectively (Richards *et al*., 1999).

1.2 Healthcare-Associated Infections (HAIs)

Healthcare associated infections otherwise known as nosocomial infections are infections that were not present prior to patients' admission but acquired within 72 hours of hospitalisation (World Health Organisation, 2002). Patients that are admitted in the sickbay, specifically those in the critical care units are liable to contract infections from hospital staff or infected medical devices (Khan *et al*., 2017). Nosocomial infections are caused by bacteria, fungi and viruses. However, bacteria especially Gram-negative bacteria, are more often than not, connected with nosocomial infections (Ige *et al*., 2011; Khan *et al*., 2017). HAIs contribute to death in infected individuals because majority of these infections are produced by microbes that are not sensitive to many antibiotics (Ige *et al.*, 2011). Nosocomial infections majorly accompany the use of invasive devices and mostly affect sick individuals in critical

care divisions (Dia *et al*., 2008). Surgical-site infection was the principal infection in hospitals in both advanced and unindustrialised countries (Atif *et al*., 2006; Ige *et al*., 2011; Nejad *et al*., 2011). Frequently reported nosocomial infections include bloodstream, catheter-related urinary tract, surgical site and ventilator-linked pneumonia (Khan *et al*., 2017).

HAI is a global challenge but the burden of HAIs is considered highest in developing countries (Nejad *et al*., 2011). Most hospitals in developing countries are overcrowded and understaffed leading to poor infection control measures. Moreover, absence of infection control strategies and skillful personnel also worsen the issue (Nejad *et al.,* 2011). There is little statistics on the epidemiology of nosocomial infections in African countries (Vincent *et al*., 1995; Nejad *et al*., 2011). The pooled predominance of nosocomial infection from some countries in Africa was 15·5% which was more than rate reported from the USA and Europe (Nejad *et al*., 2011). Occurrence rate of 2.5%, 10.3%, 10.9%, 14.8% were observed from Algeria, Ethiopia, Senegal and Tanzania, respectively (Gosling *et al.*, 2003; Atif *et al.*, 2006; Dia *et al*., 2008; Mulu *et al*., 2012). Prevalence of HAIs in a tertiary hospital in Ibadan increased slowly from 2.4% in 2005 to 3.1% in 2008 (Ige *et al*., 2011). Other studies from Tanzania and Nigeria showed a reduction in the prevalence of HAI from 9.5 in 2001 to 4% in 2005, and 5.8% in 2003 to 2.8% in 2006, respectively when an infection control practice was implemented (Atif *et al*., 2006; Abubakar, 2007).

1.3 *Pseudomonas aeruginosa* Type III Secretion System (T3SS)

Type III secretion systems (T3SS) are virulence factors found in Gram-negative bacteria which enable them to bypass the extracellular milieu by injecting bacterial effector proteins straight into the cytoplasm of the infected individual cell (Coburn *et al*., 2007). *Many P. aeruginosa strains produce an extremely harmful T3SS upon contact with human. In P. aeruginosa,* there are four known type III effector toxins that function as a virulence factor. Over 80% of *P. aeruginosa isolates from serious infections* express these toxins (Hauser *et al*., 2002). The complement of effectors differs among strains of *P. aeruginosa*. Nevertheless, most strains have *exoT* and *exoY*. *exoU* and *exoS* are not usually present together in one strain; that is the presence of one may mean the absence of the other and vice versa (Roy-Burman *et al.,* 2001). Exotoxin U is the most destructive of the type III effector proteins with phospholipase A_2 activity which is only expressed by a few hospital isolates (Sato *et al*., 2003).

Strains expressing *exoU* are highly cytotoxic. Exotoxin Y is an adenylate cyclase and has a moderate impact on virulence. Exotoxin S and exotoxin T are double-functional enzymes, though effects of *exoT* in overall virulence were not as impressive as *exoS*. Exotoxin S adds to the capacity of *P. aeruginosa* to pass over the epithelial barricade (Soong *et al*., 2008). T3SS has been linked with additional severe proven illness in mortal patients (Sato *et al*., 2003). For instance, the existence of efficient T3SS has been linked with poor aftermath of infection in ventilator-related pneumonia patients (Hauser *et al*., 2002). It has been established that type III toxins are virulence determinants that aid the spread of *P. aeruginosa from burn wounds* (Nicas *et al*., 1985). A study that examined the contribution of a lively T3SS in *P. aeruginosa* infection has established that great relationship exists between T3SS manifestation and mortality in patients infected with *P. aeruginosa* (Roy-Burman *et al*., 2001).

1.4 Carbapenem resistance

Resistance of bacteria to antibiotics is no longer a new burden worldwide. As new antibiotics come into clinical use, bacteria also devise means of surviving the antibacterial action of antibiotics. *P. aeruginosa* is notorious having resistance to a lot of antibiotics intrinsically with ability to obtain and disseminate resistance genes among themselves and other bacterial genera. Their ability to form biofilm and survive in low nutrient environments such as in catheters also aids the spread of this organism in the hospital settings (Mayhall, 1996). Multidrug resistant *P. aeruginosa* are becoming difficult to treat (Bassetti *et al*., 2018). Therefore, *P. aeruginosa* infection in critically ill patients is a great concern.

Carbapenems are used in the treatment of infections caused by Gram-negative bacteria that produce expanded spectrum beta-lactamase as well as *P. aeruginosa*. However, resistance to carbapenems is being witnessed in these isolates. Carbapenem resistance in bacteria is a serious threat to the healthcare system because carbapenem resistant bacteria are well-known to be resistant to several beta lactam drugs and other antibiotics, however only susceptible to polymyxins, leaving physicians with few or no treatment options (Varaiya *et al*., 2008). Carbapenem resistance among *P. aeruginosa* has been shown to be mediated by acquired carbapenemases most especially the metallo-beta lactamases (MBLs) (Villegas *et al*., 2007). However, the presence of intrinsic resistance devices such as upregulation of efflux pump systems, exceeding manifestation of chromosomal cephalosporinase and decreased outer membrane

permeability has also been observed. There is paucity of information on the genetic basis of carbapenem resistance in *P. aeruginosa* from Nigeria. Therefore, the present study will be conducted to molecularly characterise carbapenem resistance genes in clinical isolates of *P. aeruginosa* from Southwest Nigeria.

1.5 Justification for this study

Pseudomonas aeruginosa is an emerging pathogen and the frequent source of urinary tract and surgical site infections in patients in critical care units. It also plays a serious part in producing persistent respiratory infections in cystic fibrosis patients (Mackie and MacCartney, 1996; Gaynes and Edwards, 2005). *P. aeruginosa* has mortality rate in immunosuppressed individuals particularly patients with severe burns or cancer (Mackie and MacCartney, 1996). Carbapenems are potent beta-lactam antibiotics that are used in serious nosocomial infections particularly those that are triggered off by Gram-negative bacteria that produce extended spectrum beta-lactamases owing to their broad spectrum of action and steadiness to hydrolysis by majority of beta-lactamases (Gupta, 2008; Armand-Lefèvre *et al.*, 2013). However, increased prevalence of resistance to carbapenems is being observed amongst Gram-negative organisms more frequently in non-fermenters comprising *P. aeruginosa* and *Acinetobacter* species (Lolans *et al*., 2005; Mohammed and Raafat, 2011).

The genes for carbapenem resistance are typically carried on mobile genomic elements such as plasmids and transferred among bacterial genera and species (Nicasio *et al*., 2008). The ability to acquire resistance genes has made *P. aeruginosa* resistant to most beta-lactam antibiotics and to develop resistance to many other antibiotics, bringing about very limited therapeutic possibilities (Poulakou *et al*., 2014). There have been reports on outbreak of pandrug-resistant *P. aeruginosa*. For instance, in Belgium, an outbreak of pan-resistant *P. aeruginosa* (resistant to all antibiotics except the polymyxins) having upregulation of efflux pump systems, chromosomal *ampC* cephalosporinase and deficient in outer membrane porins was reported (Deplano *et al*., 2005). An epidemic of pan-drug resistant *P. aeruginosa*, which produced $bla_{\text{VIM-2}}$ metallo-beta lactamase, chromosomal *ampC* beta-lactamase and aminoglycoside modifying enzymes (*aacA7* and *aaC-A5*) in a critical care unit in Chicago has also been documented (Lolans *et al.*, 2005). Also, in Lithuania, increased prevalence of MBL-producing *P. aeruginosa* from 15.8% in 2003 to 61.9% in 2008 has also been

documented (Vitkauskiene *et al*., 2011). It is crucial to monitor dissemination of *P. aeruginosa* and identify carbapenem resistance encoding genes to aid infection control. An increase in knowledge about the predominance and tools of resistance to carbapenems in *P. aeruginosa* may be a valuable guide in the selection of antibiotics and possibly help to prevent associated morbidity and mortality (Meradji *et al*., 2015). There are few antimicrobial options for multi-drug resistant organisms in clinical trials; hence, regular monitoring of this pathogen is highly essential.

The genomic basis for carbapenem resistance in *P. aeruginosa* has been intensively studied in developed countries, but there are limited data from sub-Saharan Africa (Cholley *et al*., 2014), including Nigeria. Some studies from Nigeria have reported the occurrence of MBL in Gram-negative bacteria by phenotypic methods, while the presence of bla_{KPC}, bla_{NDM} and bla_{VIM} type carbapenemase in carbapenem resistance Enterobacteriaceae from Northern, Nigeria have also been documented (Mohammed *et al*., 2015; Abdullahi *et al*., 2017). There is scarcity of information on the prevalence and tool of resistance to carbapenems in clinical isolates of *P. aeruginosa* from Nigeria. Understanding of the prevalence and tools of carbapenem resistance in *P. aeruginosa* is of paramount importance in order to aid infection control and monitor nosocomial spread.

1.6 Hypotheses

Resistance to carbapenems in *P. aeruginosa* has been known to result from outer membrane protein loss, efflux systems, enzyme production (MBL) and mutations among which MBL production is of great clinical concern because of its broad hydrolysis profile and rapid rate of dissemination. The research hypotheses are:

1. MBL genes have high prevalence among carbapenem-resistant *P. aeruginosa* from Southwest Nigeria hospitals
2. Occurrence of MBL resistance genes is closely related to the occurrence of efflux pump genes in carbapenem-resistant clinical isolates of *P. aeruginosa*
3. Occurrence of metallo-beta lactamases and integrons in carbapenem-resistant *P. aeruginosa* from Southwest Nigeria are not mutually exclusive
4. Carbapenem-resistant clinical isolates of *P. aeruginosa* from Southwestern Nigeria hospitals are clonally related

1.7 General objective

To determine the prevalence of carbapenem resistance and characterise carbapenem-resistance encoding genes in clinical isolates of *P. aeruginosa* from Southwestern Nigeria.

Specific objectives

1. To determine the antibiotic susceptibility profile of clinical isolates of *P. aeruginosa* to antibiotics and relate the antibiotic susceptibility pattern of MBL-positive to MBL-negative *P. aeruginosa* clinical isolates
2. To characterise carbapenemase genes and determine expression level of efflux pump systems, AmpC chromosomal cephalosporinase and outer membrane porin (OprD) in carbapenem-resistant *P. aeruginosa*
3. To detect the presence of resistance-plasmids among MBL-producing isolates and determine the transferability of carbapenem-resistance encoding genes by transformation experiment
4. To determine the molecular epidemiology of carbapenem-resistant *P. aeruginosa* and establish the mechanisms for carbapenem resistance in *P. aeruginosa* from Southwest Nigeria.

CHAPTER TWO
LITERATURE REVIEW

2.1 The test organism: *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a rod-formed Gram-negative bacterium which has its place in Pseudomonads family. *P. aeruginosa* is an opportunistic nosocomial pathogen and the major cause of depression and death in immunosuppressed host or those with comorbid illness such as prolonged renal failure or cystic fibrosis (Chastre and Fagon, 2002). *P. aeruginosa* causes infection in various parts of the body, including ear (otitis externa), eye (corneal ulcers and keratitis), heart (endocarditis), central nervous system (meningitis) and infections of the urinary tract, burns, bone, joints, wound as well as, infection of the respiratory tract (Bielecki *et al*., 2008; Galle *et al*., 2012). *P. aeruginosa* possess a large collection of virulence features that permit them to flourish in an animate domicile and escape host response (Reinhart and Oglesby-Sherrouse, 2016). Virulence factors play a critical role in establishment of infections by *P. aeruginosa*. Virulence factors include endotoxin (lipopolysaccharides), pili, capsule and several toxins including type III secretion system (T3SS) toxins which makes it easy for *P. aeruginosa* to escape host defence (Davies and Bilton, 2009). The existence of biofilm also allows *P. aeruginosa* to stick to diverse medical tools such as indwelling catheter and the air route of cystic fibrosis patients and heavily contributes to wound related morbidity and mortality globally (Mayhall, 1996). Figure 2.1 shows virulence factors produced by *P. aeruginosa*.

2.2 The *Pseudomonas aeruginosa* type III secretion system (T3SS)

Type III secretion system is found in majority of Gram-negative bacteria. It is a main virulence factors that maneuver eukaryotic host cell response (Galle *et al*., 2012). T3SS introduces effector toxins straight into the mammalian host cell in a very well-coordinated manner (Galle *et al*., 2012). T3SS and its effectors were first detected in *P. aeruginosa* in 1996 and are the foremost virulence determinants of *P. aeruginosa* (Galle *et al*., 2012). The T3SS of *P. aeruginosa* comprises of many proteins that form compound which is structurally and functionally conserved (Moraes *et al*., 2008). Four

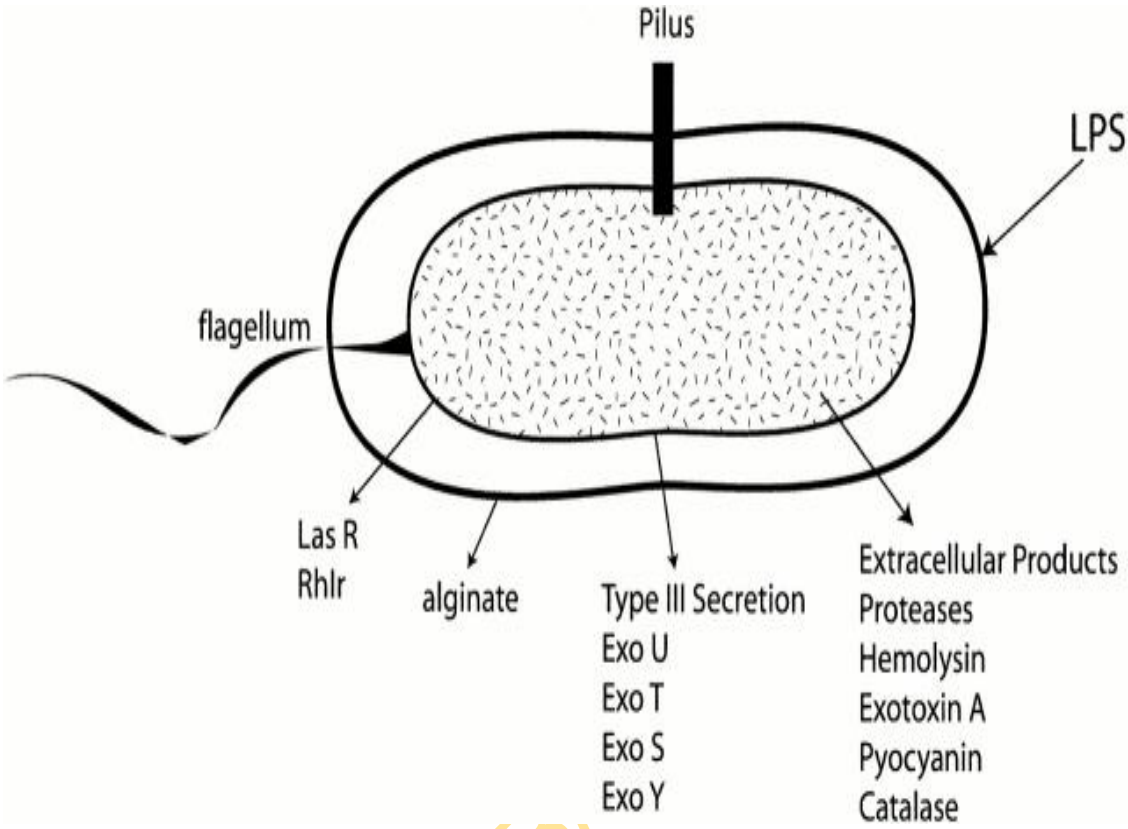

Figure 2.1. A typical image of *P. aeruginosa* with its virulence factors

Source: Sadikot *et al*. (2005).

effector toxins identified in *P. aeruginosa* thus far include: *exoY*, *exoT*, *exoU* and *exoS*. Not all the four effector toxins can be found in one strain, though *exoT* and *exoY* may be existing in all strains but dissimilar strains have one or the other of the *exoS* and *exoU* gene that is, *exoS* and *exoU* are mutually exclusive in *P. aeruginosa* so that the populace is at present set apart into a main *exoS*-containing strain, a negligible *exoU*-containing strain, while both *exoS* and *exoU* T3SS may be lacking in some strains of *P. aeruginosa* (Freschi *et al*., 2015). Exotoxin U is extremely cytotoxic and more destructive than *exoS*. The expression of T3SS effector proteins correspond with poor outcome of a disease (Hauser, 2009).

Apart from the four recognised effector toxins, additional two effector toxins have been established in *P. aeruginosa; pemA* and *pemB* but these genes are not indispensable machineries of T3SS. The effectors are typically found in all strains of *P. aerginosa* as with *exoY* and *exoT* (Burstein *et al*., 2015). The two effector cells fail to stimulate a toxic reaction in *Saccharomyces cerevisiae*. Therefore, Burstein and his fellow worker concluded that lack of toxicity observed in their study may stipulate that the two genes may be toxic on lesser collection of hosts or could be activated by yet undiscovered signals. The effectors may also be host particular, in other words, the two effector genes may be toxic in another host. On the other hand, these effectors may interrelate with the host inherent protection or normalise definite passage-ways for the advantage of the bacteria. This has opened way for additional investigation into translating the roles and devices of these novel effectors (Burstein *et al*., 2015).

2.3 *Pseudomonas aeruginosa* as a nosocomial pathogen

Hospitalised patients (especially prolonged ICU stay) stand a great danger of being inhabited and infected with *P. aeruginosa* and causes between 10 to 20% of nosocomial infections (Fazeli *et al*., 2012). Amongst the family of Pseudomonads, *P. aeruginosa* is the greatest regularly isolated nosocomial pathogen in urinary tract infection, ventilator associated pneumonia and bacteraemia (Elkhatib *et al*., 2008). It is the chief cause of the long-lasting debilitating lung infection, which is the primary basis of death in those suffering from cystic fibrosis (Govan and Nelson, 1992), and the second best regular pathogen of the lower respiratory tract infections (Jones, 2001).

2.4 Common Hospital-Acquire Infections (HAIs) caused by *P. aeruginosa*

2.4.1 Ventilator-Associated Pneumonia (VAP)

An infection which progresses in the lung of patient who is on a ventilator is referred to as ventilator-associated pneumonia (CDC, 2012). *P. aeruginosa* has a high mortality in causing ventilator-associated pneumonia (Chastre and Fragon, 2002). *P. aeruginosa* is the most important bacterial source of serious ventilator-related pneumonia and protracted lung contaminations in patients with cystic fibrosis (Goldberg, 2010). The properties of the *P. aeruginosa* strains connected to VAP and critical respiratory letdown are very unique from those related to *P. aeruginosa* strains that reside in the air routes of patients with cystic fibrosis (Sadikot *et al*., 2005). Pulmonary infections caused by *exoU* secreting *P. aeruginosa* are usually more severe. Exotoxin U toxin secretion in isolates obtained from patients with VAP is a symbol for exceedingly contagious *P. aeruginosa* and is linked with reduced clinical outcome in patients with VAP (Hauser *et al*., 2002).

2.4.2 Catheter-Associated Urinary Tract Infections (CAUTIs)

About 15 to 25% of patients admitted to hospitals receive urinary catheters during hospitalisation (CDC, 2017). Relatively, 75% of nosocomial urinary tract infections (UTIs) are allied with a urinary catheter (CDC, 2017). Lengthy use of urinary catheter is the greatest essential reason which predisposes patients to catheter-linked urinary tract infections. Catheter bruises the natural barrier and damages the mucosal layer thereby allowing bacterial establishment. CAUTI is the major root of nosocomial infection and *P. aeruginosa* is the third chief pathogen of CAUTIs accounting for 40% of all nosocomial infections. UTIs due to *P. aeruginosa infections are* connected with high death in hospitalised patients, especially those with underlying diseases (Lamas-Ferreiro *et al*., 2017).

2.4.3 Central line-Associated Bloodstream Infections (CLABSIs)

Bloodstream infections (BSI) are infections that result when there is presence of viable bacteria or fungi in the bloodstream (Viscol, 2016). When a central-line catheter was in place at the period of or within 48 hours earlier, the commencement of an infection is referred to as CLABSIs. CLABSI is also known as primary bloodstream infection because infection does not result from other parts of the body. BSIs are a foremost cause of death universally, particularly those connected with patients on admission in intensive care units has fatality rate of 35-50% (Timsit and Laupland, 2012). *P.*

aeruginosa is the third best common Gram-negative organism identified in CLABSI. *P. aeruginosa* bloodstream infection is connected with great hospital death and is topmost among patients getting improper preliminary antimicrobial therapy (Micek *et al*., 2005).

2.4.4 Surgical Site /Wound Infections

Surgical site infections can occur on the surface of skin or encompass muscles beneath the membrane, structures or inserted material (CDC, 2012). Skin infections such as boils or abscesses which develop at sites other than the operation site is a sign that the infection was acquired in the ward. Primary surgical site infections are infections caused by patients' normal flora and some other environmental sources in the operating theater. It becomes evident within 5–7 days after surgery and is usually more serious. The deep-rooted sepsis that occurs within 30 days after surgery and before the wound is dressed is also an indication of theatre infection. Surgical site or wound infections specifically caused by multidrug-resistant *P. aeruginosa* isolates are connected with amplified illness and death. The ability of *P. aeruginosa* to form biofilms makes them persist in hospital water systems and serve as potential reservoir for *Pseudomonas* surgical site infections (Falkinham *et al*., 2015).

2.5 Other infections caused by *Pseudomonas aeruginosa*

2.51 Gastrointestinal infections

The gastrointestinal tract (GI) is thought-out to be the best essential source of *P. aeruginosa*. GI tract is also an essential route of entry in *Pseudomonas* septicaemia and bacteraemia. The rate of mortality of patients travailing from bowel colonisation by *P. aeruginosa* is undoubtedly advanced than that of patients lacking establishment by *P. aeruginosa* in critical care unit (Marshall *et al*., 1993).

2.5.2 Central nervous system (CNS) infections

Pseudomonas aeruginosa rarely cause infection in the CNS except in conditions such as postneurosurgical procedures or head trauma to cause meningitis or brain abscesses (Juhi *et al*., 2009). Previous studies showed that *Pseudomonas* meningitis which results after surgical operations is always accompanied with pronounced mortality (Huang *et al*., 2007; Juhi *et al*., 2009). *P. aeruginosa* was accountable for 8.3% to 10.7% of meningitis in postneurosurgical patients (Erdem *et al*., 2008). Postoperative *Pseudomonas* meningitis is linked with increased mortality (Huang *et al*., 2007; Juhi *et al*., 2009).

2.5.3 Ocular infections

Pseudomonas aeruginosa is a chief cause of bacterial keratitis. Infections of the eye have been reported infrequently in individuals wearing contact lenses for extended period. The most common form of infection is corneal ulcer, which spreads with frightening rapidity to panophthalmitis. The infection often follows penetration of the cornea by a foreign body which becomes embedded there. In a lot of cases, loss of vision in the affected eye ensues or evisceration may be necessary. Most infections of the eye with *P. aeruginosa* occur as a result of chemical, mechanical or thermal injury to the eye (Spencer, 1953).

2.5.4 Endocarditis

Pseudomonas aeruginosa infect the endocardial surface of the heart through invasion from the blood stream and establish itself. If left untreated, *P. aeruginosa* invasion may result to incurable heart failure and myocardial abscesses (Brusch, 2017). Infective endocarditis (IE) caused by *P. aeruginosa* is also rare accounting for roughly 3% of all patients with infective endocarditis. Almost 90% of patients with *P. aeruginosa* endocarditis use intravenous drugs. Most of these reports reveal that infective endocarditis caused by *P. aeruginosa* may be life threatening and has also been linked with high mortality rate (Lin *et al*., 2016).

2.5.5 Ear infections

Pseudomonas aeruginosa is the most principal bacterial pathogen of external otitis and the major cause of ear infections in children. Otitis media is an indigenous infection of the exterior ear passage often associated with warm humid conditions after exposure to inadequately chlorinated hot tubs or swimming pools (Havelaar *et al*., 1983).

2.5.6 Skin infections

Pseudomonas aeruginosa can cause both localised and diffuse skin infections. Skin and soft tissue infections with *P. aeruginosa* result when the normal skin is bridged as in burns wound, surgical wound, trauma or dermatitis. *P. aeruginosa* is among the most frequent causes of burn wound infection and a main cause of death in burn patients (Lari *et al.*, 1998).

2.5.7 Bone and joint infections

Pseudomonas aeruginosa accounts for up to 20% of Gram-negative bacteria osteomyelitis infection and is linked with the use of orthopaedic devices and complicates the surgical management of this infection (Rodríguez-Pardo *et al*., 2014).

2.6 Antibiotics

The word antibiotic was devised from the name "antibiosis" which exactly means "against life". In the previous years, antibiotics were deliberated to be organic compounds produced by one microorganism which are lethal to other microorganisms (Russell, 2004). As a result of this conception, an antibiotic was initially, largely defined as a substance, produced by one microorganism, or of biological source which at low concentrations can inhibit the growth of, or are lethal to other microorganisms (Russell, 2004). On the other hand, this description has been modified in contemporary times, to comprise antimicrobials that are also manufactured partially or entirely through synthetic means (Etebu and Arikekpar, 2016). In September 1928, late Sir Alexander Fleming, an English Bacteriologist by chance discovered penicillin, the first antibiotic produced by *Penicillium notatum*, a soil residing mould (Russell, 2004). The discovery of penicillin has continued to transform the management and battle against bacterial infections (Etebu and Arikekpar, 2016). Antibiotic may be expansive-spectrum or constricted-spectrum. Expansive-spectrum antibiotics are active against both Gram-positive and Gram-negative bacteria while constricted-spectrum antibiotics are active on either Gram-positive or Gram-negative bacteria. Antibiotic could be bactericidal or bacteriostatic in action. Bactericidal antibiotics act by killing the bacteria and their action is irreversible while bacteriostatic antibiotics work by hindering the growth of bacteria and the action is reversible. Antibacterial agents implicated in the treatment of bacterial infections are considered in accordance with their tool of exploit (Figure 2.2).

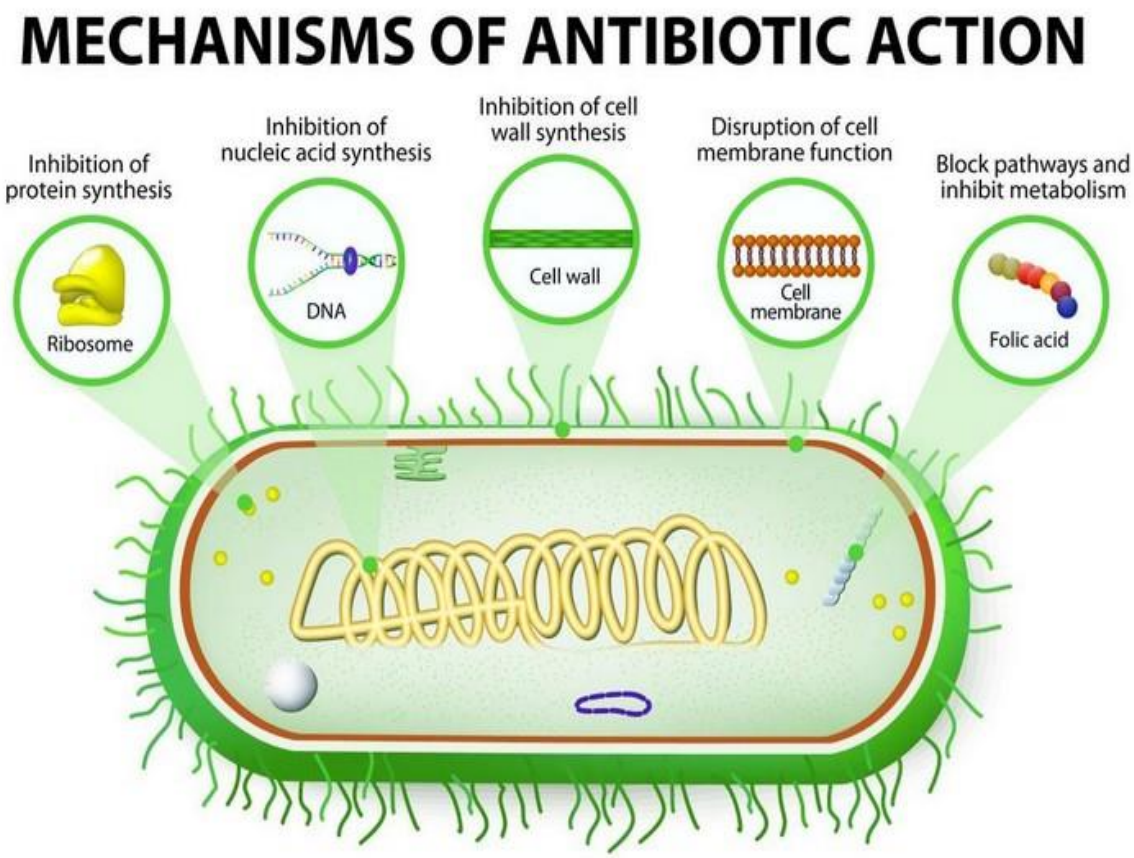

Figure 2.2. Mechanisms of action of antibacterial drugs

Source: healthlove.in/antibiotics/how-do-antibiotics-work-mode-mechanism-of-action-of-antibiotics

2.7 Beta lactam antibiotics

The manner of exploit of beta-lactam agents is closely connected to the arrangement and biosynthesis of the bacterial cell wall (Williamson *et al.,* 1986). Beta-lactam antibiotics are composed of four-membered beta-lactam ring at the centre of their organisation, which is indispensable to their manner of exploit. Beta-lactam antibiotics could be bacteriostatic or bactericidal in action. The bacteriostatic effect of beta-lactam antibiotics ensue as a consequence of inhibition of transpeptidase and carboxypeptidase enzymes implicated in the coming together of the bacterial cell wall and consequent inhibition of cell splitting up and development (Lee *et al.*, 2001). Beta-lactam antibiotics include:

- Penicillins: ampicillin, carbenicillin and amoxicillin
- Cephalosporins: ceftazidime, cefotaxime and cefepime
- Cephamycins: cefoxitin, cefmetazole and cefotetan
- Monobactams: aztreonam
- Carbapenems: imipenem, meropenem, doripenem, ertapenem, panipenem and biapenem

2.7.1 Carbapenems

Carbapenems are potent member of beta-lactam antibiotics which are structurally related to penicillins but have extended activity profile against most beta-lactamases for instance the extended spectrum beta lactamases (Walsh *et al.*, 2005). Carbapenems utilise their exploit by hindering transpeptidase enzyme situated on the exterior of the cytoplasm (Huang *et al.*, 1995). Carbapenems which are in clinical use include doripenem, imipenem, ertapenem, panipenem, meropenem and biapenem (Codjoe and Donkor, 2018). Broad spectrum of activity is found among meropenem, doripenem, panipenem and ertapenem but the effectiveness of panipenem against Gram-negative bacterial strains is negligible (Codjoe and Donkor, 2018).

2.7.1.1 Imipenem

Imipenem was the principal antibiotic in carbapenem family to be accepted for the management of infections initiated by multiple drug resistant bacteria. Imipenem exhibited great attraction for penicillin binding proteins (PBPs) and is stable towards most beta-lactamases. However, both imipenem and panipenem can be easily degraded by dehydropeptidase I (DHP-I) enzyme, present in human renal tubules (Hikida *et al.*, 1992).

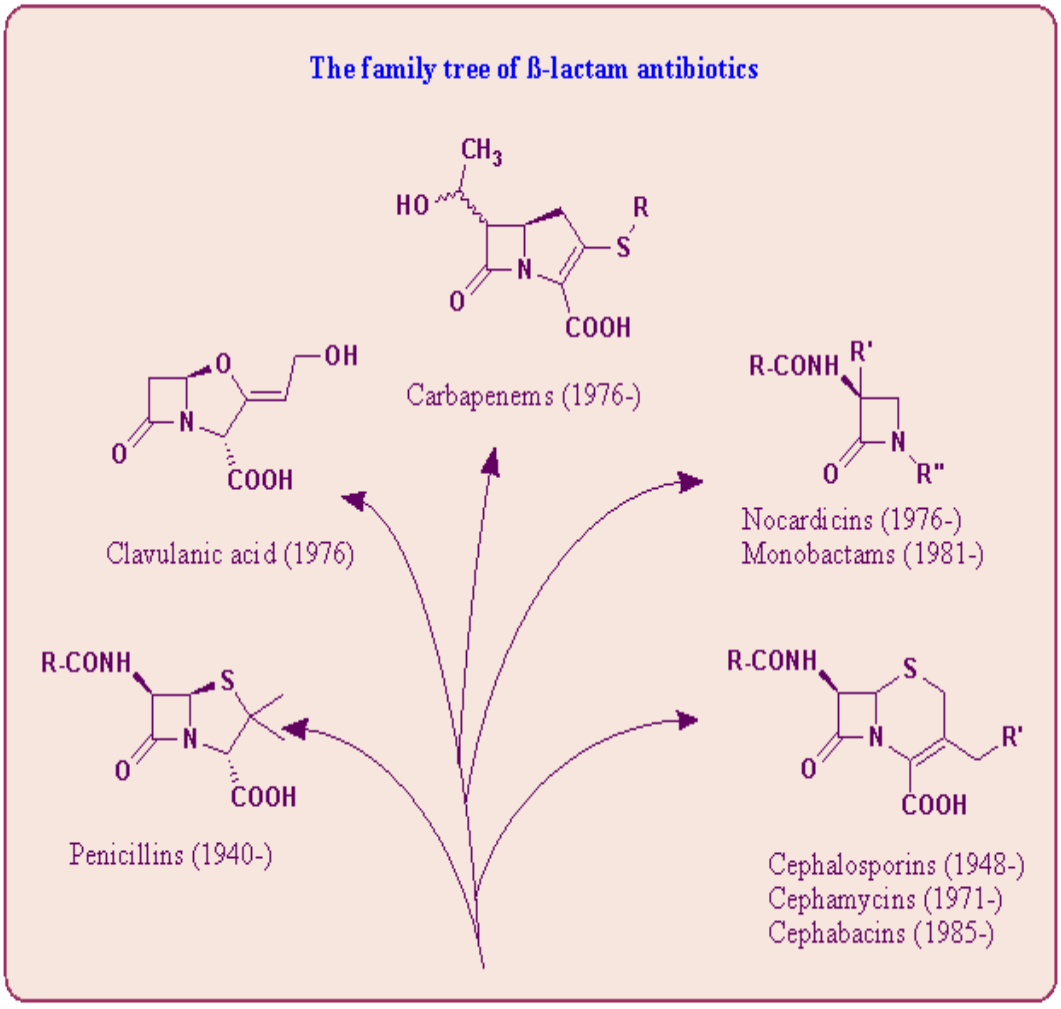

Figure 2.3. Structures of beta-lactam antibiotics

Source: http://dragon.klte.hu/~gundat/betalaca.htm

Therefore, it requires coadministration with an inhibitor, cilastatin or betamipron (Hikida *et al*., 1992).

2.7.1.2 Meropenem

Meropenem demonstrated increased stability to enzyme dehydropeptidase-1 (DHP-1) and does not require to be administered with dehydropeptidase-1 inhibitor (cilastatin) hence, its being administered without a DHP-1 inhibitor (Zhanel *et al*., 2007).

2.7.1.3 Doripenem

Doripenem does not require beta-lactamase inhibitor and has potent activity towards a varied collection of Gram-positive and Gram-negative clinical isolates (**Mushtaq *et al*., 2004**). Relatively, amongst the carbapenems, doripenem is steady towards degradation by majority of beta-lactamases and is not inactivated by human renal dehydropeptidase I as against imipenem (**Mushtaq *et al*., 2004**).

2.7.1.4 Ertapenem

The efficacy of ertapenem against Gram-negative non-fermenters is low in comparison with other carbapenems (Papp-Wallace *et al*., 2011).

2.8 Carbapenem resistance

Normally, Gram-negative bacteria are impervious towards many antibiotics than Gram-positive bacteria in line with the dissimilarities in their cell wall physiology (Armand-Lefèvre *et al*., 2013). Bacteria elude the antibacterial action of beta-lactam antibiotics through four mechanisms including:

1. reduced heights of drug buildup due to excessive manifestation of efflux-pumps
2. decreased or loss of the outermembrane porin
3. alteration in the active site of penicillin-binding proteins (PBPs)
4. production of beta-lactamases such as *ampC* hyperproduction, metallo beta-lactamase (Taneja *et al*., 2010).

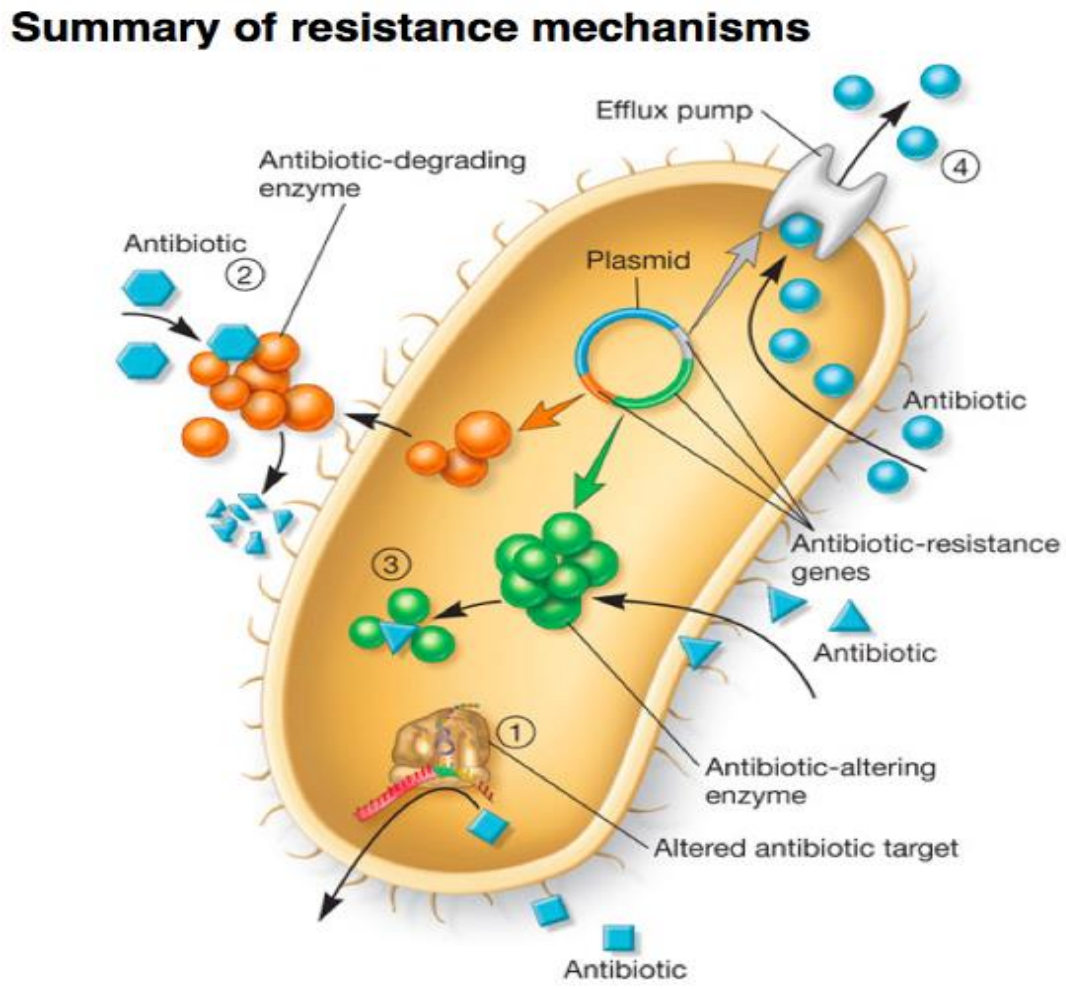

Figure 2.4. Mechanisms of antibiotic resistance in bacteria

https://www.easynotecards.com/print_list/59058

2.8.1 Impermeability of antibiotics mediated by overexpression of efflux pump systems

Efflux pumps are carriage proteins that are implicated in the ejection of noxious substances (together with almost entire groups of clinically important antibiotics) from inside cells into the exterior milieu (Bambeke *et al*., 2000). These proteins are found in very nearly all bacterial species including Gram-positive and Gram-negative bacteria. The genetic material encoding efflux pump proteins can be sited on plasmids or chromosomes. Bacterial efflux pumps are categorised into five major families

1. Small multidrug resistance family (SMR)
2. Major facilitator superfamily (MFS)
3. Resistance nodulation division family (RND)
4. Multidrug and toxic compound extrusion family (MATE)
5. Adenosine triphosphate-binding cassette superfamily (ABC)

With exception of the ABC family, which hydolyse ATP to export substrates, other efflux systems use proton motive force as their basis of drive. The RND family is connected widely with clinically important bacteria and is present only in Gram-negative bacteria while other families were established in both Gram-negative and Gram-positive bacteria (Handzlik *et al*., 2013).

Four efflux pumps have been seen as the most clinically relevant out of the twelve efflux pumps of RND family identified so far in the genome of *P. aeruginosa*. These include: MexEF-OprN, MexAB-OprM, MexCD-OprJ and MexXY-OprM, and are well distinguished as antibiotic carriers (Mesaros *et al*., 2007). Inherent resistance of *P. aeruginosa* to most antibiotics was first believed to occur due to impermeability of the cell membrane but is at the present established to consist of interaction of impenetrability with multiple drug resistance efflux, controlled primarily by MexA-MexB-OprM. Inherent expressions of efflux pumps in 'wild-type' strains perform a meaningful function in the comparatively reduced susceptibility of *P. aeruginosa* to antibiotics (Lister, 2002). However, overproduction of these genes in mutant strains leads to high-level resistance to multiple antibiotics (Lister, 2002). Although efflux typically confers a modest degree of resistance, its role in the chemotherapy of infectious diseases could be relevant, because clinical concentration of antibiotic at the target site of action could not be achieved leading to cross-resistance to unrelated

antibiotic classes (Lister, 2002). Upregulation of efflux system in *P. aeruginosa* affects the activity of meropenem more than imipenem (Walsh, 2000).

2.8.2 Down-regulation of outer membrane porin (*oprD*)

The *oprD* is a specific porin with a unique function in the take-up of positively charged amino acids like arginine, glutamine, histidine (Huang *et al*., 1995). The *oprD* function as the main porin for the entrance of carbapenems in *P. aeruginosa* because it allows the entry of carbapenems in the bacterial cells and its diminished manifestation is usually observed in carbapenem-resistant isolates (Livermore, 1992). Deficiency in the regulation of outer membrane porins (*oprD*) as a result of mutations has been recognised to convene a minor level of resistance to meropenem and doripenem but majorly to imipenem (Quale *et al*., 2006). The lack of a 45-49 kilodalton (kDa) *oprD* was described as the peculiarity of imipenem-resistant *P. aeruginosa* (Huang *et al*., 1995).

The *oprD* protein shared between 41% and 58% similarity with members of the porin family and have the overall topography of other porin. Normal porin comprise a 16-strand transmembrane β-barrel arrangement which composed of 7 slight-turn sequences on the periplasmic side, which acts as a link which connect the outer surface of the 8 loop structures to form a thin channel, causing reduced penetrability through the outward membrane in *P. aeruginosa* as compared with *Escherichia coli ompF* porin (Huang *et al*., 1995).

2.8.3 Overexpression of *ampC* cephalosporinase

The *ampC* beta-lactamases have its place in class C group of beta lactamases. Chromosomal *ampC* cephalosporinase production poses a grave danger to the effectiveness of beta-lactam antibiotics (Lister, 2002). The *ampC* beta-lactamase was detected in 1940 in *Escherichia coli* as the major beta-lactamase which hydrolyse penicillin. The expression of *ampC* is low in majority of Gram-negative bacilli, but inducible in reaction to contact by beta-lactam and is typically associated with multidrug resistance (Jacoby, 2009). Antibiotics like aztreonam, oxacillin and cloxacillin are able to repress *ampC* beta-lactamases but beta-lactamase inhibitors and ethylenediamine tetraacetic acid (EDTA) do not have activity against them (Bush *et al*., 1995).

The tool by which these organisms induce *ampC* expression is complex (Jacobs *et al*., 1997). The mutation in *ampD* gene resulting in *ampC* constitutive hyperproduction is most frequent cause of *ampC* overexpression in clinical isolates (Schmidtke and Hanson, 2006). When *ampC* expression increases through mutational events, overexpression of this cephalosporinase provides high-level resistance to all beta-lactams except the carbapenems (Lister, 2002). The *ampR* modifications are unusual but can also lead to highly-constitutive or hyperinducible phenotypes (Hanson and Sanders, 1999).

P. aeruginosa PAO1 contains three *ampD* genes, describing the gradual overexpression of *ampC* observed by means of subsequent triggering of every single *ampD* gene (Juan *et al*., 2006). The presence of numerous loci in *ampD* adds to the virulence ability of *P. aeruginosa*, which subsequently results in a derepressed *P. aeruginosa* strain following inactivation of one *ampD* allele while twofold or threefold mutation in *ampD* gene results in the inability of *ampD* mutant to strive in a typical infection with mouse. The regulation of *ampC* in *P. aeruginosa* is more multifaceted than the regulation observed in Enterobacteriaceae (Moya *et al*., 2008). Beta-lactam antibiotics differ in their inducing potentials and the prompting effect of clavulanic acid is markedly essential for *P. aeruginosa*. For instance, when *ampC* expression is induced, clinically reached concentrations of clavulanic acid have been documented to upset antibacterial activity of ticarcillin (Lister *et al*., 1999).

2.8.4 Mechanism of action and classification of Beta lactamases

The chief tool for resistance to beta-lactam drugs in Gram-negative bacteria is the generation of beta-lactamases. Beta-lactamases are degradative enzymes that dislocate the amide connection of the four-membered beta-lactam ring characteristic of beta-lactam antibiotics making the efficacy of beta-lactams useless. Beta-lactamases have been described numerous times in many bacterial species and may be present on the chromosome or plasmid and repeatedly connected with moveable genomic features such as transposons and integrons (Nijssen *et al*., 2005). Beta-lactamase produced by different bacteria holds a spectrum of physical, biochemical and practical assets that are unique for particular beta lactam antibiotics just as penicillinases for penicillins, cephalosporinases for cephalosporins, and carbapenemases for carbapenems while some of this beta-lactamases may have affinity for one or more additional groups (Livermore and Brown, 2001). Grouping of beta-lactamases has conventionally

remained grounded on either the primary structure or operative features of the enzymes (Bush *et al*., 1995). The straightforward classification is by protein sequence which is grounded on the amino acid motifs and then grouped into four classes. Class A, C, and D enzymes employ serine for beta-lactam hydrolysis and class B enzymes are those that have need of divalent zinc ions for hydrolysis (Bush and Jacoby, 2010). Carbapenemases have their place in the molecular classes A, B, and D. Production of carbapenemase especially the class B (acquired MBLs) is a key tool through which Gram-negative bacteria confer resistance to carbapenems.

2.8.4.1.1 Class A carbapenemases

Class A enzymes have comprehensive spectrum of hydrolysis towards most beta-lactams like the penicillins, aztreonam, first group cephalosporins and carbapenems. Examples of class A enzymes that are existing in the genome of bacteria are NmcA (not metalloenzyme carbapenemase A), SME (*Serratia marcescens* enzyme), IMI-1 (Imipenem-hydrolysing beta-lactamase), SFC-1 (*Serratia fonticola* carbapenemase-1) (Codjoe and Donkor, 2018). SME-1 was originally detected in two *Serratia marcescens* from England in 1982. The IMI and NMC-A were identified in *Enterobacter cloacae* from hospital, with amino acid similarity to 97% and 70% to SME (Nordmann *et al*., 2011). DNA segment coding IMI-2 beta-lactamases were detected on plasmids in *Enterobacter* species. They are resistant to carbapenem, susceptible to third group of cephalosporins and are partially hindered by clavulanic acid (Garcia *et al*., 2013).

Plasmid encoded class A carbapenemases include KPC (*Klebsiella pneumoniae* carbapenemase) (KPC-2 to KPC-13) and GES (Guiana extended spectrum). These members of class A carbapenemases are also partly hindered by clavulanic acid and hydrolyse carbapenems (Nordmann *et al*., 2011). KPC is the most dominant class A carbapenemase present mainly on plasmids (Nordmann *et al*., 2011). There has been report of KPC in other regions after its rapid spread in the United States (Queenan and Bush, 2007). Concomitant with the increasing reports of KPC-2, KPC-3 which is a sole amino acid substitute of KPC-2 was described in New York in a 2000 to 2001 epidemic of *K. pneumoniae*. The GES and IBC (integron-borne cephalosporinase) groups are not frequently come across and were principally described in 2000. IBC-1 was identified in Greece from *E. cloacae* while GES-1 was discovered from France in a *K. pneumoniae* isolate (Poirel *et al*., 2001). The arrangement of their amino acid

confirmed that they are remotely linked to KPC-2, SME-1 and NMC-A with identities of 36%, 35% and 31%, respectively (Poirel *et al*., 2001). These enzymes were at the start grouped as ESBLs but further categorised to include imipenem hydrolysis when a clinical isolate of *P. aeruginosa* was identified to contain GES-2 which inactivates imipenem (Poirel *et al*., 2001). Although GES enzymes are not common, they had been identified worldwide in developed countries and have not been connected with outbreaks. Nevertheless, *P. aeruginosa* strains carrying GES-2 have been linked to minor nosocomial epidemic in eight patients (Poirel *et al*., 2002). In Africa, KPCs were famous to be widespread among Enterobacteriaceae but later discovered in a *P. aeruginosa* isolate from a teaching hospital in northwestern, Tanzania (Mushi *et al*., 2014).

2.8.4.2 Class D carbapenemase

The OXA beta-lactamase with carbapenemase function was first found in *Acinetobacter baumannii* in Scotland. These carbapenemases are of the OXA (oxacillin-hydrolyzing beta-lactamases) enzyme type. They are poorly repressed by EDTA and clavulanic acid and have weak activity against carbapenems (Codjoe and Donkor, 2018). In the later 1970s and initial 1980s, OXA was among the utmost dominant plasmid-encoded beta-lactamase. OXA carbapenemases have ability to rapidly mutate which results in large variability in their amino acid sequences with extended spectrum of activity (Codjoe and Donkor, 2018). Recurrent discovery of class D carbapenemases among the clique of Enterobacteriaceae has been reported. OXA-48 was also reported in *P. aeruginosa* from Tanzania (Mushi *et al*., 2014).

Endemicity of OXA-48 containing Enterobacteriaceae in northern part of Africa has been reported (Manenzhe *et al*., 2015). OXA-48, OXA-58, OXA-51-like, OXA-181, OXA-163 producing Enterobacteriaceae were reported from Tunisia, Egypt and Morocco. Non-fermenters are also found to contain class D beta-lactamases in Africa (Manenzhe *et al*., 2015). Outburst of OXA-23 containing *A. baumannii* was reported from Tunisia while *A. baumannii* isolates from Egypt and Algeria have been documented to carry OXA-24-like enzymes. Most remarkable is the report of OXA-48 gene in *P. aeruginosa*. This enzyme is known to be widespread in Enterobacteriaceae but now described in non-fermenters suggesting horizontal transfer of this gene (Mushi *et al*., 2014).

2.8.4.3 Molecular Class B Carbapenemases (Metallo beta-lactamases)

Metallo beta-lactamases (MBLs) have its place in class B of Ambler grouping system. MBLs are group of beta-lactamase that requires divalent cation usually Zn^{2+} as a cofactor for the enzyme activity (Queenan and Bush, 2007). They are categorised by the capability to inactivate carbapenems and are inhibited by EDTA chelators of Zn^{2+} (Queenan and Bush, 2007). MBLs are of great clinical importance because they are capable of hydrolyzing virtually all beta-lactam antibiotics with the exclusion of aztreonam and most clinically existing inhibitors such as clavulanate, sulbactam and tazobactam do not hinder their activity (Bonomo and Szabo, 2006). On the grounds of amino acid relatedness, ten MBL types and different variants of these families have been recognised (Pollini *et al*., 2013).

MBL genes could exist on the chromosome or plasmid mediated. Acquired MBL genetic factors are situated on the integron configurations that exist on moveable genomic features like plasmids or transposons, and can rapidly spread to other bacterial species (Walsh *et al.,* 2005). MBLs have been identified in countless Gram-negative bacteria and are linked with clonal spread (Bush *et al*., 1995). The bla_{IMP} and bla_{VIM} types are the most widely reported except in Brazil where bla_{SPM} was the most prevalent enzyme (Lucena *et al*., 2014). Both bla_{IMP} and bla_{VIM} possess the broadest hydrolysis profile (Queenan and Bush, 2007). *P. aeruginosa* isolates that produce MBLs are more prevalent in Brazil and Italy and accounted for 43.9% and 39.1%, respectively (Toleman *et al.,* 2005). The bla_{VIM-2} has also been described in West and Central Africa (Cholley *et al.,* 2014). Comprehensive evidence on the predominance of carbapenem resistance and molecular characterisation of carbapenem-resistant clinical isolates of *P. aeruginosa* from Nigeria are lacking.

2.8.5 TYPES OF MBL

2.8.5.1 Imipenem (IMP) type MBL

IMP (active on imipenem) was the first MBL known in imipenem-resistant *P. aeruginosa* isolate from Japan in 1988 (Watanabe *et al.,* 1991). IMP was present on a transferable conjugative plasmid. After three years, the existence of IMP gene was also established in *Serratia marcescens* from Japan. MBLs were primarily restricted to South-east Asia, but have extended quickly to Europe, North America, Oceania, including Africa especially after 2000 (Walsh *et al*., 2005; Manenzhe *et al*., 2015).

2.8.5.2 Veronese Imipenemase (VIM) type MBL

Veronese imipenemase (VIM-1) was first recognised in *P. aeruginosa* in a hospital in Verona, Italy (Lauretti *et al.,* 1999). Later a *bla*$_{VIM-1}$ genetic material was found in *Achromobacter xyloxidans* in similar hospital. The *bla*$_{VIM-1}$ gene was also found on gene cassette integrated into class 1 integron (Lauretti *et al.,* 1999). The *bla*$_{VIM-1}$ are predominant in *Pseudomonas aeruginosa* strains but have been noticed in additional Gram-negative bacteria. In 1996, *bla*$_{VIM-2}$ closely interconnected to *bla*$_{VIM-1}$ was first identified in *P. aeruginosa* from France (Poirel *et al.,* 2000). The existence of *bla*$_{VIM-2}$ has been acknowledged in many countries presenting its circulation universally (Mohammed *et al*., 2015; Zubair and Iregbu, 2018). A novel *bla*$_{VIM}$ type identified in clinical isolates of *P. aeruginosa* in Japan was recently reported by Hishinuma *et al.* (2019). This variant was designated *bla*$_{VIM-60}$ with two amino acid substitutions at positions 228 and 252 compared with *bla*$_{VIM-2}$

2.8.5.3 Sao Paulo Imipenemase (SPM-1) type MBL

A novel gene *bla*$_{SPM}$ was first identified in *P. aeruginosa* in Sao Paulo, Brazil in 1997. The *bla*$_{SPM-1}$ showed 35.5% identity to IMP-1 (Toleman *et al.,* 2005). The *bla*$_{SPM-1}$ is linked with common region elements but not integrons or transposons. The *bla*$_{SPM-1}$ can inactivate the entire classes of beta-lactams but does not hydrolyse clavulanic acid and aztreonam like *bla*$_{IMP-1}$ and *bla*$_{VIM-1}$ (Murphy *et al.,* 2003).

2.8.5.4 Germany Imipenemase (GIM-1) type MBL

The *bla*$_{GIM-1}$ is the fourth MBL enzyme to be identified. It was first detected in 2004 in five identical *P. aeruginosa* isolates from a German hospital. The isolates were susceptible to polymyxin only (Walsh *et al.,* 2005). The *bla*$_{GIM-1}$ showed highest similarity with IMP-types. Reports have shown that *bla*$_{GIM}$ do not inactivate azlocillin and aztreonam but are able to inactivate beta-lactamase inhibitors as several MBLs do (Gupta, 2008).

2.8.5.5 Seoul *Imipenemase (*SIM-1) type MBL

The *bla*$_{SIM-1}$ was principally discovered in multidrug resistant *Acinetobacter baumannii* and exhibits 64–69% similarity with the bla$_{IMP}$ and can hydrolyse majorly all beta-lactam antibiotics (Poirel *et al.,* 2000). The *bla*$_{SIM}$ have not been detected in *P. aeruginosa*.

2.8.5.6 Adelaide Imipenemase (AIM-1) type

The *bla*$_{\text{AIM-1}}$ was discovered in *P. aeruginosa* from Australia (Yong *et al.*, 2012). The *bla*$_{\text{AIM-1}}$ is found on two ISCR elements not on integrons as most MBL-genes. The presence of ISCR 10 adjacent to *bla*$_{\text{AIM-1}}$ showed that ISCR elements are implicated in its mobility (Yong *et al.*, 2012).

2.8.5.7 Kyorin Hospital Imipenemase (KHM)

In 1997, bla$_{\text{KHM-1}}$ *was identified in clinical isolate of Citrobacter freundii* from a patient with catheter-linked urinary tract infection in Japan (Sekiguchi *et al.*, 2008).

2.8.5.8 New Delhi metallo-beta lactamase (NDM)

The *bla*$_{\text{NDM-1}}$ was majorly recognised in *Klebsiella pneumoniae* in 2008 but later detected in *E. coli* from the same patient (Yong *et al.*, 2009). The *bla*$_{\text{NDM-1}}$ encoding gene is found on diverse big plasmids which also convey genes that confer resistance to practically all antibiotics; hence generating quick distribution in clinically related bacteria poses a severe danger for therapy. The *bla*$_{\text{NDM-1}}$ parts very slight uniqueness with other MBLs. The *bla*$_{\text{VIM-1}}$/*bla*$_{\text{VIM-2}}$ is the most identical with 32.4% similarity (Pitout *et al.*, 2005). After the first discovery of *bla*$_{\text{NDM-1}}$, infections with *bla*$_{\text{NDM-1}}$ containing *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae* and *Acinebacter baumannii* were also identified in New Delhi.

2.8.5.9 Dutch Imipenemase (DIM)

DIM-1 (Dutch imipenemase) was principally recognised in *Pseudomonas stutzeri* in the Netherlands. DIM-1 was remotely interconnected to other class B beta -lactamases, having maximum amino acid resemblance of 52% with *bla*$_{\text{GIM-1}}$, 48% with *bla*$_{\text{KHM-1}}$, 45% with *bla*$_{\text{IMP}}$ and merely 30% uniqueness with *bla*$_{\text{VIM}}$. The *bla*$_{\text{DIM-1}}$, like other MBLs did not hydrolyse aztreonam (Walsh *et al.*, 2005; Sekiguchi *et al*., 2008).

2.8.5.10 Florence Imipenemase (FIM)

The *bla*$_{\text{FIM-1}}$ was recognised in a *Pseudomonas aeruginosa* strain from hospital in Italy (Pollini *et al.*, 2013). The *bla*$_{\text{FIM-1}}$ enzyme exhibited the uppermost amino acid connection of 40% with *bla*$_{\text{NDM}}$-type. The *bla*$_{\text{FIM-1}}$ genetic factor was supposedly present on the chromosome and connected with IS*CR19*-like features which may be involved in its incorporation and mobilisation (Pollini *et al.*, 2013). Analysis of the kinetic factors displayed that *bla*$_{\text{FIM-1}}$ has expanded substrate specificity and do not hydrolyse aztreonam like other MBLs (Pollini *et al.*, 2013).

2.9 Clinical significance of carbapenem-resistant Gram-negative bacteria

The risk of acquiring carbapenem resistant Gram-negative bacteria is greater in the unindustrialised world especially in sub-Saharan Africa where irrational use of antibiotics predominates (Donkor *et al*., 2012). Carbapenem resistant Gram-negative bacilli are mainly involved in infections in patients with prolonged length of hospital stay or critical care units, extensive antibiotic use, lengthy usage of indwelling therapeutic procedures such as catheter, or immunocompromising conditions. They are mostly associated with healthcare-acquired infections. For instance, *bla*$_{NDM}$-producing Enterobacteriaceae were identified most commonly among hospitalised patients in critical care units and is linked with high mortality (Barguigua *et al*., 2012; Gniadek *et al*., 2016). High death rate in patients carrying isolates that are resistant to carbapenem has been documented (Meradji *et al*., 2015). The potential for spread of carbapenem resistant genes to other bacterial species is worrisome. For instance, *bla*$_{IMP-4}$ was discovered firstly in *P. aeruginosa* from Australia and later described in *Aeromonas junii* from this country (Peleg *et al*., 2006). Nosocomial outbreaks of carbapenem resistant Gram-negative bacilli have persistently produced evidence that significant outbreaks are occurring (Gniadek *et al*., 2016). For instance, a study from Tunisian university hospital described an outburst of *bla*$_{VIM-4}$-producing *K. pneumoniae* isolates (Ktari *et al*., 2006). In addition, a clonal outbreak of carbapenem-resistant *P. aeruginosa* strains in hospitalised patients in Algeria have also been reported (Meradji *et al* 2015). Likewise, a report from the Netherlands described a nosocomial explosion of *bla*$_{VIM-2}$-containing *P. aeruginosa* isolates. Multifocal outbreaks of MBL-containing *P. aeruginosa* from Japan have also been described (Senda *et al*., 1996). All these reports have shown that MBLs are disseminated through horizontal transfer of these genes.

2.10 Mobile genetic elements

2.10.1 Integrons

Integrons were first described in the 1980s as mobile genetic element that can capture and incorporate small mobile elements known as gene cassettes, especially gene responsible for antibiotic resistance through site-specific recombination event (Hall and Collis, 1995). The antibiotic resistance genes are located on gene cassettes and can exist as free circular DNA. More than one gene cassette can be inserted into an integron (Hall and Collis, 1995). Integrons consist of an *intI* gene that is required

within the integron for site specific recombination, an *attI* site that is identified by the integrase and a P_c promoter necessary for gene cassette expression (Boucher *et al*., 2007). Integrons were majorly clustered into few classes centered on genetic relatedness of *intI* gene but class 1, 2 and 3 were the most recognised. Later, over 90 different variants of *intI* gene were identified (Boucher *et al*., 2007). Integrons may also be present on the chromosome, called chromosomal integrons (CI). Chromosomal integrons are thought to be motionless but movement of gene cassettes has been reported (Cambray *et al*., 2010). They can carry varying number (from zero to hundreds) of gene cassettes that are not particularly involved in antibiotic resistance (Cambray *et al*., 2010).

Approximately 10% of bacterial chromosomes consist of integrons that are often connected to MGEs specifically the class 1 integrons (Domingues *et al*., 2012). Class 1 integrons are the most widespread, basically in medical settings and contribute majorly to widespread antibiotic resistance (Cambray *et al*., 2010). The genesis of Class 1 integrons is not clear, that they were believed to be in existence in bacteria prior to the advent of antibiotics and are implicated in global transmission of antibiotic resistance genetic material (Cambray *et al*., 2010). Class 1 integrons have been reported in both Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, class 1 integrons are identified mainly from the clinical isolates while class 1 integrons have been isolated from diverse sources like soil, water, hospitals, animals, food and so on in Gram-negative bacteria (Domingues *et al*., 2012).

Typical class 1 integrons basically consist of a crucial adjustable area where the gene cassettes are placed and two highly conserved regions; the 5′-preserved section (5′-CS) and the 3′-preserved section (3′-CS). The 5′-CS consists of the integrase gene (*intI1*), the recombination site *attI1* and the promoter P_c. A growing number of class 1 integrons with structure different from the typical class 1 integrons have also been documented (Toleman *et al*., 2005). They are associated with insertion sequence common region 1 (IS*CR1*) and are often referred to as composite class 1 integrons.

Majority of class 1 integrons are naturally connected or inserted in mobile genetic elements like Miniature Inverted-repeat Transposable Elements (MITEs), insertion sequences (ISs), transposons (Tns), genomic islands (GIs) and plasmids, thus promoting the transfer of integrons within and between bacterial genome (Domingues

et al., 2012). The same class 1 integron identified in different bacterial host has been reported from different geographical locations suggesting horizontal dissemination.

2.10.2 Plasmids

Plasmids are extrachromosomal spherical DNA particles that reproduce freely of the bacterial genetic material. Plasmids perform a major role in horizontal spread of genetic material especially the antimicrobial resistance genes and occur in many bacterial strains. One strain can harbor multiple plasmids (Bennett, 2008). A resistance plasmid (R-plasmid) is every plasmid that conveys one or additional antibiotic resistance DNA segment. A R-plasmid may perhaps be a metabolic plasmid if it encrypts a degradative role or a virulence plasmid, if it owns virulence genetic material (Bennett, 2008). Plasmids may be conjugative or mobilisable. It is conjugative if it codes for task required for its movement or mobilisable if it relies on conjugative ones to become moveable. Plasmids are the key route for antibiotic resistance spread, including resistance genes encoded by class 1 integrons (Hu and Zhao, 2009). For instance, the plasmid-mediated transfer of class 1 integron from clinical isolates of *Serratia marcescens* to *Escherichia coli* through conjugation has been reported (Hu and Zhao, 2009).

2.10.3 Miniature Inverted-repeat Transposable Elements (MITEs)

MITEs are dependent moveable genetic elements that consist of small repeat sequences that do not code for any protein but are found at different locations in the chromosome of many bacteria. For instance, two copies of a 288 bp MITEs have been detected in a class 1 integron present in the plasmid of a clinical isolate of *Enterobacter cloacae* (Poirel *et al*., 2009).

2.10.4 Genomic islands (GIs)

Genomic islands are large chromosomal regions which contain genes that encode different functions. They are referred to as resistance islands when they encode resistance genes. Genomic islands are usually incorporated on the chromosome of many bacteria at specific positions. Genomic islands harbouring class 1 integrons in Gram-negative bacteria have been acknowledged (Douard *et al*., 2010).

2.10.5 Insertion sequences (ISs)

ISs are smallest mobile elements surrounded by terminal inverted repeats which are composed of a middle region that code for a transposase, which accounts for its

mobility and are essential for transfer of resistance genes (Domingues *et al*., 2012). When a DNA fragment is flanked on both sides by the one and the same IS, it is called complex transposon. There have been reports of class 1 integron whose sides are flanked by IS as found in IS*6* family that are found in transposons (Doublet *et al*., 2009).

2.10.6 Transposons

Transposons are referred to as jumping genes. Resistance transposons incorporate one or more resistance gene inside the element (Bennett, 2004). They have the ability to jump from one plasmid to another or from bacterial chromosome to a plasmid or *vice versa (Bennett, 2008).* These mechanisms do not necessarily need corresponding DNA between the sequence and the positions of attachment but on rare occasion, a specific transposon may have solid preference for a specific nucleotide sequence at an attachment position. Some transposons are mobilisable while others are conjugative. There is also information on other mobile transposons containing class 1 integrons *(*Petrova *et al*., 2011).

2.12 Molecular typing

*The development of multiple drug resistance in Pseudomonas aeruginosa has strengthened the requirement for epidemiological studies, unfolding their predominance and resistance profile. Numerous numbers of techniques including phenotypic and genotypic approaches have been established for typing P. aeruginosa, the selection of each technique depend on the capacity to type large number of strains. Such a method should also h*ave good discernment with capability to identify a sensible number of types, a method that will not be costly and be reproducible over an extended period (Olive and Bean, 1999). Genotypic methods that have been successfully used to type *P. aeruginosa* include: Pulsed-field gel electrophoresis, multilocus sequence typing and PCR-based typing among others.

2.12.1 Pulsed-field gel electrophoresis (PFGE)

Of all the molecular typing methods, PFGE is frequently regarded as the 'gold standard'. In this procedure, overnight culture of bacterial cells implanted into melted agarose are lysed in situ and treated with restriction enzymes to obtain an agarose plug comprising whole DNA digested with varying fragment sizes. Electrophoresis is done after the bacterial plug is implanted into electrophoretic tool whose current

polarity is changed at programmed period of time. Subsequently, gels are visualised for pattern of arrangement of DNA which is separated according to size after staining with fluorescent dye (Olive and Bean, 1999). This technique permits perfect split-up of bulky DNA fragments of between 10 and 800 kb. Snapped gels can then be analysed with accessible software bundles. PFGE is acknowledged to be better than other methods for molecular typing. It has displayed worthy discriminatory supremacy over repetitive extragenic palindromic polymerase chain reaction (REP-PCR) in distinguishing strains of P. aeruginosa (Grundmann et al., 1995). Distinguishing capability of PFGE can be improved through the use of two or more restriction endonucleases (Olive and Bean, 1999).

The most important rewards of this method are great degree of replication and discernment while its greatest disadvantages lie in the use of costly apparatus and timewasting procedure that requires 2-3 days subject to specific protocols (Olive and Bean, 1999).

2.12.2 Multi Locus Sequence Typing (MLST)

The procedure of MLST is grounded on the explicit categorisation of bacterial isolates and other organisms in a consistent reproducible and convenient way using nucleotide sequence (Urwin and Maiden, 2003). MLST adopted the recognised ideas of multilocus enzyme electrophoresis (MLEE) but contrary to relying on the electrophoretic movement of the loci products, MLST is established on direct sequencing of the loci products to define the alleles at each locus (Urwin and Maiden, 2003). MLST classically denotes logical sequencing of five to six greatly-preserved housekeeping genes within the bacterial genomic DNA. Alteration in the nucleotide sequence of every locus is categorised and documented and a sequence type or ancestry is assigned by comparing the sequence obtained with sequence of other isolates in the databank (Maiden, 2006).

Many typing systems have been suggested for typing P. aeruginosa with PFGE being the standard against which other techniques are justified. The inordinate need for clear-cut and reproducible worldwide procedure for typing P. aeruginosa led to the development of MLST system for P. aeruginosa typing and has provided improved information on epidemiology of P. aeruginosa (Curran et al., 2004). A lot of studies have proven that MLST satisfies these standards (Curran et al., 2004).

MLST has lot of benefits above other techniques of typing in the sense that it can identify variations in nucleotide sequence, that is, at the level of DNA that are not obvious by phenotypic techniques. Moreover, the method is reproducible and did not necessitate the use of living bacterial cells or standard-quality genomic DNA, thereby preventing problems accompanying transportation and handling of pathogens. Records obtained through MLST can be accessed in research laboratories all over the world via internet (Maiden, 2006).

2.12.3 Repetitive element sequence-based polymerase chain reaction (rep-PCR)

Repetitive element sequence-based polymerase chain reaction (rep-PCR) is a typing technique that permits the creation of DNA fingerprinting. It involves the use of definite primers for PCR magnification of scattered repetitive DNA elements present at different positions in prokaryotic genomic constituent (Hiett and Seal, 2009). These elements seem to be well preserved amongst various bacterial species (Versalovic et al., 1991). The rep-PCR method involves the use of isolated DNA from bacteria or the use of whole cells in PCR amplification of either a single primer or combination of primers succeeded by gel electrophoresis after staining with ethidium bromide. When the amplified DNA fragments are parted by electrophoresis, a genomic pattern that can be employed for strain representation and discernment is obtained (Hiett and Seal, 2009).

Repetitive extragenic palindromic PCR (REP-PCR) and enterobacterial repetitive intragenic consensus sequence (ERIC-PCR) are the frequently used repetitive element. REP comprises of six degenerate locations with 38 bp arrangements and flanked by a 5 bp variable loop on each side of a preserved palindromic structure (Versalovic et al., 1991). REP is best described among the repetitive sequences in bacterial genome and around 500-1000 replicas of these elements are found in the genome of *E. coli* and *Salmonella typhimurium* (*Hiett and Seal, 2009). ERIC is the second PCR-based typing method that has been used effectively for differentiating bacterial strains. ERIC sequences are situated in the extragenic areas of the bacterial genome and composed of 126 bp elements of exceedingly preserved indispensable reversed replication (Sharples and Lloyd, 1990). Several studies have successfully typed P. aeruginosa with REP- and ERIC-PCR; while ERIC-PCR has been reported to show equal discriminatory potential with PFGE and MLST (Kidd et al., 2011; Faridi and Javidpour, 2015). Both REP and ERIC sequences have been described in many*

bacteria. REP patterns are extra challenging than ERIC patterns; nonetheless the combination of both REP-PCR and ERIC-PCR has proven to augment the discriminatory control as compared to when the methods are used independently (Olive and Bean, 1999).

While REP- and ERIC-PCR are the best recurrently used targets for DNA typing, BOX elements was primarily believed to be exclusive to Streptococcus pneumoniae but is at present established in many bacterial species as with REP and ERIC elements (Kwon et al., 1998). Sequences of BOX elements are not in any way related to the two rep-PCR methods above. Wolska and coworkers (2012) demonstrated that BOX-PCR is a pronounced investigational strategy useful for categorising P. aeruginosa because it is quick, extremely replicated and with good ability to distinguish bacterial strains. Rep-PCR is extremely relevant and largely more appropriate than genomic fingerprinting and plasmid analysis and has shown worthy connection with PFGE with less differentiating ability (Liu and Wu, 1997).

2.12.4 Restriction Fragment Length Polymorphism (RFLP)

This method is centered on the belief that various restriction enzyme recognition sites in a certain genetic region of interest may possibly vary and be located at diverse sites in the chromosome of different bacteria which invariably give different banding arrangement. In this method, chromosomal DNA is broken down with restriction enzyme and then analysed on agarose gel through electrophoresis, the gel is observed for discrepancy in restriction pattern which signifies strain disparity. RFLP may involve the transfer of electrophoretic products to any of nylon or nitrocellulose membrane using a probe that is homologous to the target site in a practice called Southern blotting or by amplifying the chromosome with a primer specific for the gene of interest and subjected to gel electrophoresis to obtain a banding arrangement particular for a certain strain in the process named PCR-based RFLP (Olive and Bean, 1999).

2.12.5 Randomly amplified polymorphic DNA (RAPD)

This method depends on the use of short primer of between 6-10 base pair of random sequence that hybridise to DNA at low annealing temperature. The quantity and position of these arbitrary primer sites differ for diverse strains of a bacterial species. A PCR product with size equivalent to the distance amidst the two primers will form if

the two primers anneal within limited kilobases of one another. Therefore, subsequent segregation of PCR products on agarose gel produces a band arrangement which is perculiar to specific bacterial strain (Olive and Bean, 1999). RAPD is able to differentiate strains than RFLP but with not as much of discrimination as rep-PCR. In RFLP, since primer is not directed towards a precise locus, many of the annealing procedures result from defective alignment between the target position and the primer. Also, amplification procedure is exceedingly sensitive to minor variation in the annealing temperature which brings about inconsistency in banding arrangements.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment and glassware

All equipment and glassware used in this study are listed below:

NanoDrop® spectrophotometer (Thermo Scientific), Hot air oven (Gallenkamp England), Autoclave (Healthquip England), Incubator (Gallenkamp England), Centrifuge (Merck), Microscope (Olympus England), Q125 Sonicator (QSONICA), Weighing balance (Ohaus London), Vortex mixer (Griffin & George Britain), Refrigerator (-20^{o}C and -80^{o}C), Digital water bath (Thomas Scientific), Water bath shaker (Bionics Scientific), Ice machine (fisher Scientific), Thermal cycler (AB Applied Biosystem), Step One® real time polymerase chain reaction (RT-PCR) system (AB Applied Biosystem), Gel Documentation and Analysis System (GenoSens 1560), Gel casting trays and combs, Electrophoresis tank and Electrophoresis Power supply.

Glassware

Beakers**,** Conical flasks**,** Measuring cylinder**,** Test tube, Petri dish**,** Inoculating loop**,** Bunsen burner and Micropipettes

3.1.2 Media, Buffers, Chemicals

All media, antibiotics, buffers and chemicals used in this study are listed in Appendix I

3.1.3 DNA isolation and purification kits, enzymes, molecular weight markers and Master mix

DNA isolation and purifica tion kits, enzymes, molecular weight markers and Master mix are listed in Appendix II.

3.1.4 List of primers

Primer sequences used for polymerase chain reaction (PCR) and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and sequencing are listed in Table 3.1

3.1.5 Study design and place of study

Study design: This work is a cross-sectional study

Place of study: University Teaching Hospitals and Federal Medical Centers in five states, southwest Nigeria (Ekiti, Ogun, Ondo, Osun and Oyo states). These included Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife, Osun state (OTHI), University College Hospital, Ibadan, Oyo state (UCH), Ladoke Akintola University Teaching Hospital, Osogbo, Osun state (LTHO), Federal Medical Centre, Abeokuta, Ogun state (FMCA), Olabisi Onabanjo University Teaching Hospital (OTHS) Sagamu, Ogun state, Federal Medical Centre, Owo, Ondo state (FMCO), Federal Medical Centre, Ido-Ekiti, Ekiti state (FMCI)..

3.1.6 Bacterial isolates

Four hundred and forty-seven (447) *P. aeruginosa* isolates recovered were collected from Microbiology units of seven tertiary hospitals from September 2014 to June 2017. The isolates were obtained from urine, pus, catheter tips, wound swabs, wound biopsy, sputum, eye swab, cerebrospinal fluid (CSF), tracheal aspirate, genital discharge, blood and sputum.

Table 3.1. Primers used for molecular screening in this study

Primer	Sequence (5′ to 3′)	Gene	Amplicon size, bp	Reference
OprI-F	ATGAACAACGTTCTGAAATTCTCTGCT	*oprI*	249	De Vos *et al.*, 1992
OprI-R	CTTGCGGCTGGCTTTTTCCAG			
OprL-F	ATGGAAATGCTGAAATTCGGC	*oprL*	504	Lim *et al.*, 1997
OprL-R	CTTCTTCAGCTCGACGCGACG			
SME-F	ACTTTGATGGGAGGATTGGC	bla_{SME}	551	Hong *et al.*, 2012
SME-R	ACGAATTCGAGCATCACCAG			
GES-F	GCTTCATTCACGCACTATT	bla_{GES}	323	Hong *et al.*, 2012
GES-R	CGATGCTAGAAACCGCTC			
NMC-A F	TGCGGTCGATTGGAGATAAA	bla_{NMC-A}	399	Hong *et al.*, 2012
NMC-A R	CGATTCTTGAAGCTTCTGCG			
BIC-1 F	TATGCAGCTCCTTTAAGGGC	bla_{BIC-1}	537	Hong *et al.*, 2012
BIC-1 R	TCATTGGCGGTGCCGTACAC			
IMP-F	GGAATAGAGTGGCTTAAYTCTC	bla_{IMP}	188	Ellington *et al.*, 2007
IMP-R	CCAAACYACTASGTTATCT			
VIM-F	GATGGTGTTTGGTCGCATA	bla_{VIM}	390	Ellington *et al.*, 2007
VIM-R	CGAATGCGCAGCACCAG			
SPM-F	AAAATCTGGGTACGCAAACG	bla_{SPM}	271	Ellington *et al.*, 2007
SPM-R	ACATTATCCGCTGGAACAGG			
GIM-F	TCGACACACCTTGGTCTGAA	bla_{GIM}	477	Ellington *et al.*, 2007

Table 3.1. Primers used for molecular screening in this study (cont'd)

Primer	Sequence (5′ to 3′)	Gene	Amplicon size, bp	Reference
GIM-R	AACTTCCAACTTTGCCATGC			
SIM-F	TACAAGGGATTCGGCATCG	*bla*$_{SIM}$	570	Ellington *et al*., 2007
SIM-R	TAATGGCCTGTTCCCATGTG			
AIM-F	TGAGAAATGGCTACGCACTG	*bla*$_{AIM}$	1241	Yong *et al*., 2007
AIM-R	GTACGGAAAACTCAGCACCC			
DIM-F	GCTTGTCTTCGCTTGCTAACG	*bla*$_{DIM}$	699	Poirel *et al*., 2011
DIM-R	CGTTCGGCTGGATTGATTTG			
NDM-F	GGGCAGTCGCTTCCAAGGT	*bla*$_{NDM}$	475	Deshpande *et al*., 2010
NDM-R	GTAGTGCTCAGTGTCGGCAT			
OXA-48A	TTGGTGGCATCGATTATCGG	*bla*$_{OXA}$	744	Poirel *et al*., 2012
OXA-48B	GAGCACTTCTTTTGTGATGGC			
OXA-58A	CGATCAGAATGTTCAAGCGC	*bla*$_{OXA}$	529	Poirel and Nordmann, 2006
OXA-58B	ACGATTCTCCCCTCTGCGC			
ampC-PA-A	CTTCCACACTGCTGTTCGCC	*ampC*	1063	Rodriguez-Martinez *et al*., 2009
ampC-PA-B	TTGGCCAGGATCACCAGTCC			
OprD –F	GCTCGACCTCGAGGCAGGCCA	*oprD*	242	Gutiérrez *et al*., 2007
OprD-R	CCAGCGATTGGTCGGATGCCA			
MexB-F	CAAGGGCGTCGGTGACTTCCAG	*mexB*	272	El Amin *et al*., 2005
MexB-R	ACCTGGGAACCGTCGGGATTGA			

Table 3.1. Primers used for molecular screening in this study (cont'd)

Primer	Sequence (5′ to 3′)	Gene	Amplicon size, bp	Reference
MexY-F	GGACCACGCCGAAACCGAACG	*mexY*	522	El Amin *et al*., 2005
MexY-R	CGCCGCAACTGACCCGCTACA			
MexD-F	GGACGGCTCGCTGGTCCGGCT	*mexD*	236	Rodriguez-Martinez *et al*., 2009
MexD-R	CGACGAAGCGCGAGGTGTCGT			
MexF-F	CGCCTGGTCACCGAGGAAGAGT	*mexF*	255	El Amin *et al*., 2005
MexF-R	CGCCTGGTCACCGAGGAAGAGT			
rpsL-F	GCAAGCGCATGGTCGACAAGA	*rpsL*	201	Dumas *et al*., 2006
rpsL-R	CGCTGTGCTCTTGCAGGTTGTGA			
MexA-F	GGCGACAACGCGGCGAAGG	*mexA*	203	Tomás *et al*., 2010
MexA-R	CCTTCTGCTTGACGCCTTCCTGC			
MexC-F	GCAATAGGAAGGATCGGGGCGTTGG	*mexC*	102	Tomás *et al*., 2010
MexC-R	CCTCCACCGGCAACACCATTTCG			
MexE-F	TCATCCCACTTCTCCTGGCGCTACC	*mexE*	150	Tomás *et al*., 2010
MexE-R	CGTCCCACTCGTTCAGCGGTTGTTCGATG			
MexX-F	AATCGAGGGACACCCATGCACATCC	*mexX*	82	Tomás *et al*., 2010
MexX-R	CCCAGCAGGAATAGGGCGACCAG			

Table 3.1. Primers used for molecular screening in this study (cont'd)

Primer	Sequence (5′ to 3′)	Gene	Amplicon size, bp	Reference
hep35	TGCGGGTYAARGATBTKGATTT	*int 1, 2 3*	491	White *et al*., 2000
hep36	CARCACATGCGTRTARAT			
hep58	TCATGGCTTGTTATGACTGT	*intI* cassette	Variable	White *et al*., 2001
hep59	GTAGGGCTTATTATGCACGC			
ExoS	MP5, GCGAGGTCAGCAGAGTATCG	*exoS*	118	Ajayi *et al*., 2003
	MP3, TTCGGCGTCACTGTGGATGC			
ExoT	MP5, AATCGCCGTCCAACTGCATGCG	*exoT*	152	Ajayi *et al*., 2003
	MP3, TGTTCGCCGAGGTACTGCTC			
ExoU	MP5, CCGTTGTGGTGCCGTTGAAG	*exoU*	134	Ajayi *et al*., 2003
	MP3, CCAGATGTTCACCGACTCGC			
ExoY	MP5, CGGATTCTATGGCAGGGAGG	*exoY*	289	Ajayi *et al*., 2003
	MP3, GCCCTTGATGCACTCGACCA			

where B = C or G or T; K = G or T; R = A or G; Y = C or T; D = A or G or T

Table 3.2. Primers used for typing in this study

Primer	Sequence (5′ to 3′)	Reference
REP1R-I	IIIICGICGICATCIGGC	Versalovic *et al*., 1991
REP2-I	ICGICTTATCIGGCCTAC	
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	Versalovic *et al*., 1991
ERIC2	AAGTAAGTGACTGGGTGAGCG	
BOX-A1R	CTACGGCAAGGCGACGCTGAC	Versalovic *et al*., 1994

3.2 Methods

3.2.1 Phenotypic identification of the study isolates

Pseudomonas aeruginosa was identified phenotypically by Gram staining, growth on cetrimide agar, motility, pigment production, oxidase reaction, gelatin liquefaction, catalase production, citrate utilisation, urea hydrolysis, oxidative utilisation of sugars.

3.2.1.1. Cultural identification of isolates

Clinical isolates collected on agar slants were inoculated on *Pseudomonas* cetrimide agar (Oxoid) and incubated at 37^{o}C for 24 - 48 hours to observe for growth and pigment production.

3.2.1.2 Gram's staining

A smear of the cell culture was made on a clean grease-free microscope slide with a bit of colony from 18 - 24 hour old culture grown on *Pseudomonas* cetrimide agar (Oxoid). The smear was air dried and passed through the Bunsen burner flame thrice to heat fix the smear. Crystal violet (primary stain) was applied on the smear and left for 60 seconds. The smear was washed under the tap for 3 seconds to remove the remaining unbound crystal violet. Lugol's iodine used as a mordant, was added to the smear to fix the crystal violet, for 60 seconds. The slides were gently washed under the tap, rinsed with alcohol for 3 seconds and then rinsed again with water. Carbol fuschin (secondary stain) was added to the slide for 30 seconds and washed with water for 5 seconds, allowed to dry and then observed under oil immersion x100 objective lens of light microscope. The Gram reaction that showed red rod-shaped cells under the microscope was indicative of *P. aeruginosa*. *P. aeruginosa* ATCC 27853 served as control strain.

3.2.1.3 Oxidase test

Oxidase test strip (Oxoid™) was put on a sterile Petri dish with the use of sterile pair of forceps, 3 - 4 well isolated colonies of bacteria from 18 hour culture on *Pseudomonas* cetrimide agar were smeared onto the strip with sterile cotton swab. Presence of a blue or purple colour within 30 seconds indicates a positive test characteristic of *P. aeruginosa*. *P. aeruginosa* ATCC 27853 served as control strain.

3.2.1.4 Gelatin liquefaction test

A heavy inoculum of 18-24 hour old culture of each isolate was stab inoculated on the tube containing nutrient gelatin medium. The tubes were inoculated at 37°C for 24 - 48 hours along with uninoculated control tube. Thereafter, the tubes were placed in refrigerator for 1 hr and then examined. Liquefaction of the test while the control remains solidified showed a positive test, characteristic of *P. aeruginosa*. *P. aeruginosa* ATCC 27853 served as control strain.

3.2.1.5 Catalase test

Well isolated colony of the test organism from overnight culture is placed onto a clean microscope slide. Two drops of 3% hydrogen peroxide (H_2O_2) solution was placed separately on a slide, a colony of the test isolate was placed on one of the H_2O_2 smear. Immediate bubbles observed by rapid evolution of oxygen (O_2) indicate a positive result. *P. aeruginosa* ATCC 27853 served as control strain.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

3.2.1.6 Urease test

Overnight cultures of the isolates were obtained in 5mL nutrient broth and used to inoculate Motility Indole Urea (MIU) medium. With an inoculating wire, the centre of the tube was stabbed to about one-half its length. The tubes were incubated aerobically with caps loosened at 37^{o}C for 24 hours. Intense pink-red colour indicates a positive result while colour change to pale yellow indicates a negative result. *P. aeruginosa* ATCC 27853 served as control strain.

3.2.1.7 Hydrogen sulphide production

Sulphide indole motility medium (SIM) tubes were stab inoculated with overnight culture of the test isolates. Tubes were incubated at 37^{o}C for 24 hours. Hydrogen sulphide will react with sodium thiosulphate and ferric ammonium citrate in the medium if hydrogen sulphide is present, to give ferrous sulphide which turns the medium into a black colour. The presence of hydrogen sulphide means that the bacteria produce the enzyme cysteine in the medium to give hydrogen sulphide. *P. aeruginosa* does not produce hydrogen sulphide. *P. aeruginosa* ATCC 27853 served as control strain.

3.2.1.8 Citrate test

Overnight broth culture of the test organism was obtained in 5ml nutrient broth. The overnight culture was used to inoculate sterile 5mL Koser's citrate broth. The inoculated tubes and an uninoculated control tube were incubated at 37^{o}C for 72 hours. The medium was later examined for colour change. Colour change from green to blue specifies a positive test whereas no colour change specifies a negative result. *P. aeruginosa* ATCC 27853 served as control strain.

3.2.1.9 Oxidative utilisation of sugars

Nutrient broths containing each of glucose, sucrose or mannitol (Appendix II) were inoculated with overnight culture of the test isolates, followed by incubation at 37^{o}C for 24-72 hours. If acid is produced, colour changes from red to yellow, while bubbles in the inverted Durham tube indicate production of gas. *P. aeruginosa* ATCC 27853 served as control strain.

3.2.2 Antimicrobial susceptibility testing (AST)

3.2.2.1 Disc diffusion

Antimicrobial susceptibility testing was done to determine the antibiotic susceptibility profile of the isolates to various antipseudomonad antibiotics using Kirby-Bauer disc diffusion procedure. Overnight nutrient broth culture of the test bacterium was calibrated to achieve McFarland turbidity standard of 0.5, equivalent to 1.5 x 10^{8} cells/mL. Then, 0.1 mL of the suspension was added to 20 mL molten Mueller Hinton agar and cooled to about 45^{o}C. The mixture was swirled gently and then poured into sterile Petri-dishes. The plates were left on the bench for 15 minutes and dried for 20 minutes at 37^{o}C in an oven. Antibiotic discs were positioned on the surface of the plates. The plates were left on the bench for 1 hour preincubation period and then incubated at 37^{o}C for 24 hours. Thereafter, zones of growth inhibition were then determined and reported in millimeter (mm). The result was interpreted as resistant, sensitive or intermediate according to CLSI (2015). *P. aeruginosa* ATCC 27853 served as control strain.

The following antibiotics were tested against the isolates: amoxicillin clavunate (30 µg), piperacillin (100 µg), ticarcillin (75 µg, piperacillin tazobactam (100/10 µg), cephalothin (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefoperazone (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg),

doripenem (10 µg), aztreonam (30 µg), amikacin (30 µg), gentamicin (10 µg), tobramycin (30 µg), ciprofloxacin (5 µg), levofloxacin (10 µg), ofloxacin (5 µg), colistin sulphate (10 µg), polymyxin B (300 unit).

MDR was defined as resistance to at least one antimicrobial agent in three or more antimicrobial classes listed while XDR was defined as non-susceptible to at least one agent in all but two antimicrobial classes listed (Magiorakos *et al.*, 2012). Antimicrobial classes include:

1. Aminoglycosides: Gentamicin, Tobramycin, Amikacin
2. Antipseudomonal cephalosporins: Ceftazidime, Cefepime
3. Antipseudomonal fluoroquinolones: Levofloxacin, Ciprofloxacin
4. Antipseudomonal carbapenems: Meropenem, Imipenem and Doripenem
5. Antipseudomonal penicillins plus beta-lactamase inhibitors: Ticarcillin clavulanic acid, Piperacillin-tazobactam
6. Monobactams: Aztreonam
7. Polymyxins: Colistin, Polymyxin B

3.2.2.2 Determination of minimum inhibitory concentrations (MICs)

This was carried out to look at the bacteriostatic activity of selected antibiotics on the test isolates. Minimum inhibitory concentrations of selected antibiotics against the *P. aeruginosa* were determined by broth microdilution (CLSI, 2015).

In a 96 well plate, 100 µL of double-strength Mueller-Hinton broth was introduced into each well with the aid of multi-channel pipette followed by 50 µL of x4 strength antibiotic dilutions (Appendix I) and 50 µL of the test organism suspension at a concentration of $2x10^6$/mL. The contents of each well was mixed and incubated at 37°C for 24 hours. Two control wells one without antibiotic and the other without test organism were set up. Standard strain (*P. aeruginosa* ATCC 27853) was run with the test to check the reagents and conditions. The lowest concentration showing inhibition of growth assessed by lack of turbidity in the well was considered the MIC of the organism. *P. aeruginosa* ATCC 27853 served as control strain.

3.2.3.1 Detection of beta-lactamase production

Beta-lactamase detection in clinical strains of *P. aeruginosa* was performed with Iodometric cell suspension method as used by Adeleke and Odelola (2000). Bacterial

colonies were emulsified into a 0.5 mL of freshly prepared Penicillin G phosphate buffer to form suspension of McFarland standard no 6 and the suspension was homogenised. The tubes were incubated for 1 hour at 28^{o}C (room temperature). Then, 2 drops of newly processed starch suspension (1%) were introduced into the mixture and shaken smoothly. One drop of iodine solution was introduced gently into the mix and allowed to stay at 28^{o}C for 10 minutes to observe for colour conversion from blue-black to white if beta-lactamase is produced. *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 36318 served as controls.

3.2.3.2 Screening of pathogen for carbapenemase production

Well isolated colonies of *E. coli* ATCC 25922 from overnight culture emulsified in normal saline (0.5 McFarland standard) was swabbed on the surface of Mueller Hinton agar plate. Meropenem (10 µg) disc was placed at the centre of the Mueller Hinton agar plate aseptically. Well isolated colonies of the test organism from overnight culture emulsified in normal saline (0.5 McFarland turbidity standard) was smoothly lined from the end of the meropenem disc to the end of the culture plate. The plates were kept warm for 24 hrs at 37ºC. Afterwards, the plates were inspected for spread-out at the point of intersection of the test organism and *Escherichia coli* ATCC 25922 within the inhibition region of the meropenem antibiotic disc. *P. aeruginosa* ATCC 27853 served as control strain (Walsh *et al.*, 2011).

3.2.3.3 Combined disc test for phenotypic detection of metallo-beta-lactamases (MBLs)

Test organism equivalent to 0.5 McFarland turbidity standards was inoculated on plate of Muller Hinton agar. Two imipenem (10 µg) and meropenem (10 µg) discs were positioned on inoculated plates and 5 µL of 0.5M EDTA solution was added to one of each imipenem and meropenem disc. After 18-24 hour incubation at 37ºC, the region that does not permit the growth of the test organism all over the single imipenem and meropenem discs and the one having EDTA was noted and compared. An escalation in size of at least 7 mm at the area surrounding the imipenem-EDTA disc and meropenem-EDTA discs where no growth was observed reflected a positive result. *P. aeruginosa* ATCC 27853 served as control strain (Walsh *et al.*, 2005).

3.2.3.4 Curing of antibiotic resistance

The genetic basis of resistance to carbapenems in selected carbapenem-resistant *P. aeruginosa* was investigated by subjecting them to plasmid curing experiment. This was done to determine the location of the resistant marker in the bacteria whether it is chromosomal or plasmid mediated. Curing was carried out according to the method described by Obaseiki-Ebor (1988) with additional adjustment in the use of more than one concentration of ethidium bromide. Ten carbapenem-resistant *P. aeruginosa* strains were randomly selected. Overnight culture of each resistant strain was obtained in 5 mL nutrient broth containing 12.5, 25, 50 and 100 µg/mL of ethidium bromide and incubated at 37^{o}C for 24 hours. Then, 10^{-2} dilution of mutagen-exposed overnight culture was inoculated on Mueller Hinton agar and incubated at 37^{o}C for 24 hours. Afterwards, four colonies were selected from each of the plates which showed dispersed colonies. Each colony was incubated similarly after inocution in 5 mL nutrient broth and diluted to obtain 0.5 McFarland standard. Thereafter, 0.1 mL of the inoculum was introduced into 20 mL molten Mueller Hinton agar, whirled to mix, and then transferred into sterile Petri dish and allowed to set. A cork-borer of 8 mm width was used to bore wells in the set agar medium; wells were then occupied with different concentrations of the imipenem and meropenem. After a pre-incubation circulation period of 2 hours, plates were incubated at 37^{o}C for 24 hours, and then observed for zones of growth inhibition. Presence of inhibition zone on agar medium was suggestive of plasmid-mediated resistance.

3.2.4. Molecular methods

3.2.4.1 Isolation of plasmid DNA

The WizPrep™ Plasmid DNA Miniprep purification Kit (Wizbiosolutions) was used for plasmid DNA extraction in accordance with Manufacturer's instruction. Bacterial cells grown overnight in Luria Bertani broth were transferred into 1.5 mL spin tube and spinned for 60 seconds at 13000 rpm. The supernatant was gently discarded. Two hundred microliters of PD1 buffer containing RNase A was introduced into the Eppendorf tube and vortexed to resuspend the cell pellet. Then, 200 µL of PD2 Buffer was introduced into the suspension and slightly turned upside down for 10 times. The tube was allowed to stand on the table for 2 minutes or until the lysate was mixed. The mixture was not vortexed o avoid shearing the plasmid DNA. Three hundred microliter of PD3 Buffer was then introduced into the suspension and immediately mixed by

inverting the tubes ten times and spinned for 10 minutes at 13000 rpm. Spin Column (SC) was retained in a 2 mL Collation Tube. The supernatant was poured into the SC and spinned for 1 minute at 13000 rpm. The tube containing clear liquid was discarded and the SC was placed back into the 2 mL Collation Tube. At this point, 600 µL of Wash Buffer containing ethanol was put into the DNA SC and spinned for 1 minute at 13,000 rpm. The tube holding clear liquid was discarded and the DNA SC was placed back in the 2 mL Collation Tube. Centrifugation process was repeated for 2 minutes at 13000 rpm to make sure that the matrix is totally dried. The dried SC was transferred to a new micro tube. Then, 50 µL of elution buffer was introduced to the centre of the column matrix and allowed to stand for 3 minutes and then spinned for 1 minute at 13,000 rpm to elute the DNA.

3.2.4.2 Extraction of genomic DNA

For routine isolation of genomic DNA from *P. aeruginosa*, WizPrep™ genomic DNA purification kit was used in accordance with Manufacturer's instruction. Cells grown overnight in 5 mL LB broth ($<2x10^9$) were spinned for 10 min at 7,500 rpm. The supernatant was carefully separated from the pellet and bacterial pellet was resuspended in 180 µL of GT1 Buffer. Two hundred microliters (200 µL) of GT2 Buffer and 20 µL of Proteinase K was introduced into the solution and vortexed. The mixture was incubated at 56℃ for 10 minutes and the tube was inverted every 5 minutes. Two hundred microliters of 100% ethanol was added to the sample lysates and mixed by vortexing briefly. The suspension was poured to the Spin Column (SC) and spinned for 1 min. at 13,000 rpm. The tube containing clear liquid was thrown away and re-joined with the SC. Five hundred microliters (500 µL) of W1 Buffer was introduced into the SC and centrifuged for 1min. at 13,000 rpm. The flow-through was thrown-away and rejoined with the SC. Seven hundred microliter (700 µL) of W2 Buffer (ethanol added) was added in the Spin Column and spinned for 1 min. at 13,000 rpm. The tube containing clear liquid was discarded and rejoined with the SC and spinned for 2 min. at 13,000 rpm. The SC was connected to a new 1.5 mL tube; 100µL of Elution Buffer was introduced into the middle of the SC and let stand on the table for 1 min. and centrifuged for 1 min. at 13,000 rpm. The SC was discontinued and eluted DNA was kept at –20°C for a few days or –70°C for extended period of storage.

3.2.4.3 Extraction of total RNA

The PureLink TM total RNA purification kit (Invitrogen) was used to extract total RNA from carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) in accordance with Manufacturer's instruction. The bacterial cells were harvested from 5 mL volume of mid-log-phase culture in LB medium and collected into 1.5 mL eppendorf tube by spinning at 5000 x g for 5 minutes at 4^{o}C and the supernatant was discarded. Then, 10 mg/mL of lysozyme was introduced into TE buffer (10mM Tris-HCl + 0.1mM EDTA, pH 8.0), followed by addition of 0.5 µL of 10% sodium dodecyl sulphate. The suspension was thoroughly mixed and incubated for 5 seconds (room temperature). Then, 350 µL of RNA Lysis solution prepared by adding 1% (v/v) 2-mercaptoethanol was introduced into the mixture and vortexed vigorously. The lysate was transferred to a 15 mL round-bottomed tube and homogenised using a rotor-stator homogeniser at maximum speed for at least 45 seconds and spinned at room temperature for 5 minutes at 2,600 × g. The supernatant was decanted into a microcentrifuge tube free of RNase. To the lysate was added 250 µL of 100% ethanol and vortexed thoroughly.

The homogenised lysate was poured into the RNA spin-catridge pre-inserted in a collection tube and then spinned at room temperature for 15 seconds at 12,000 × g. To a clean, RNase-free microcentrifuge tube, 70 µL of DNase buffer plus 10 µL of DNase 1 (1 unit/µL) was mixed slightly by turning the mixture upside down and briefly spinned to bring together the contents of the tube. The solution was then transferred to the middle of the spin cartridge and incubated at room temperature for 15 minutes. Then, 350 µL of Wash Buffer I was introduced into the spin cartridge (SC) and then spinned at 12000 x g for 15 seconds at room temperature and the liquid collected inside the collection tube was discarded. Thereafter, 700 µL of wash buffer 1 was introduced into the SC and spinned at room temperature for 15 seconds at 12000 x g. Then 500 µL of wash buffer II was introduced into a clean RNA Wash tube containing the SC and spinned at 12000 x g for 15 seconds (room temperature) to wash off the column-bound RNA. Previous step was repeated. To dry the spin-column membrane with adhered RNA completely; the SC was spinned at 12,000 × g for 1 minute at room temperature. Then, 50 µL of RNase-free water was introduced to the middle of the SC inside the RNA recovery tube and incubated for 1 minute (room temperature). RNA was eluted by centrifuging the SC for 2 minutes at 12000 x g at room temperature. The eluted RNA was kept at -20^{o}C in 10 µL aliquots until used.

3.2.4.4 Quantification of DNA /RNA

The purity and concentration of DNA and RNA were checked by quantifying the absorption of ultraviolet light at 260 and 280 nm with a NanoDrop® spectrophotometer.

3.2.4.5 Gel preparation and electrophoresis

Pre-weighed agarose was suspended in 1x TAE buffer (Appendix II) to achieve 0.8% and 1.5% concentrations. The mixture was heated to melt the agarose completely. The suspension was cooled to about 54^{o}C and ethidium bromide (CARL ROTH®) was introduced to achieve a final concentration of 0.5 µg/mL. A gel tray was taped at the ends with glue tape and appropriate comb was inserted. The gel was poured to the thickness preferred and the gel was allowed to set and then immersed in an electrophoretic tank containing 1X TAE buffer.

3.2.4.6 Preparation of sample for agarose gel electrophoresis

The sample DNA /RNA (5 µL) was mixed with 6x gel-loading buffer (Thermo Scientific) in a 5:1 (vol/vol) proportion preceeding filling into wells. For PCR products, DNA loading dye was not mixed with the product prior to filling into wells because it already contains gel-loading buffer. DNA molecular weight marker (100 bp or 1 kb plus ladders) was included in a well in each gel to determine the size of the amplicons. Electrophoresis was carried out until the dye in the loading buffer migrated an appropriate distance (45 – 60 minutes) with voltage of 90 – 100 on 0.8 - 1.5% agarose.

3.2.4.7 Visualisation of DNA

The ethidium bromide stained gel was visualised using a UV transilluminator. The pictures were captured saved electronically with the Gel Documentation and Analysis System *(GenoSens* 1560). Electronic images were edited using Windows Photo Viewer and images were labeled with Microsoft word.

3.2.4.8 Molecular identification of *Pseudomonas aeruginosa*

Confirmation of identity of *P. aeruginosa* strains was done by PCR using *oprI* and *oprL* primers which anneal to the begining and the end of the exposed reading frame of the *oprI* and *oprL* genes, respectively (De Vos *et al*., 1992; Lim *et al*., 1997). A total 25 µL reaction mixture comprising 12.5 µL *WizPure*™ PCR 2X Master (Wizbiosolutions, Korea South), 1 µL of 10 µmol/L forward and reverse primers of

oprI and *oprL* (Alpha DNA) genes in a singleplex reaction, 1 µL of genomic DNA and 9.5 µL DNase/RNase-Free Distilled Water (Invitrogen) was prepared in a 0.2 mL PCR tube. The tubes were loaded into **Thermal Cycler** (Applied Biosystems).

PCR Conditions: DNA was first denatured at 94°C for 5 minutes then followed by30 cycles of denaturation at 94 °C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 50 s, and a final extension step at 72°C for 5 minutes. A positive control with *P. aeruginosa* ATCC 27853 DNA and a negative control without DNA were included. Gel electrophoresis was performed on all amplified DNA fragments with 1.5% agarose and estimated with 100 bp plus ladder as explained in sections 3.2.4.5 to 3.2.4.7.

3.2.5 Detection of carbapenemase encoding genes by PCR

3.2.5.1 Class A carbapenemases

GES, NMC-A, BIC-1 and SME: Multiplex PCR was carried out in a **Thermal Cycler** (Applied Biosystems) with a total volume of 25 µL containing 1µL of total DNA isolated in section 3.2.2.1, 12.5 µL of 2× MyTaq Red Mix (Bioline, London), 1 µL each 10 µmol/L of both forward and reverse primers and 9.5 µl DNase/RNase-Free Distilled Water (Invitrogen). The following thermal cycling conditions were used: DNA was first denatured at 94°C for 5 minutes and followed by 25 cycles of 94 °C for 30 s, 50°C for 30 s and 72°C for 1 minute of denaturing, annealing and extension, and a final extension step at 72°C for 7 minutes. A positive control with *P. aeruginosa* ATCC 27853 DNA and a negative control lacking DNA were included (Hong *et al*., 2012).

Gel electrophoresis was performed on all amplified DNA fragments with 1.5% agarose and estimated with 100 bp plus ladder as explained in sections 3.2.4.5 to 3.2.4.7.

3.2.5.2 Class B carbapenemases (MBLs)

A 25 µl volume containing 1µL of total DNA isolated in section 3.2.2.1, 12.5 µL of WizPure™ PCR 2X Master mix (Wizbiosolutions, Korea South), 1 µL each 10 µmol/L of both forward and reverse primers and 9.5 µL distilled water devoid DNase / RNase (Invitrogen) were mixed in 0,2 mL tubes. The tubes were loaded into a **Thermal Cycler instrument** (Applied Biosystems).

For the detection of $bla_{IMP,}$ bla_{VIM} and bla_{SPM}, the following thermal cycling conditions were used: DNA was first denatured at 94°C for 5 minutes then followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at specific temperatures (***bla***$_{IMP}$, ***bla***$_{SPM}$ -

52°C and ***bla***$_{VIM}$ -55°C) for 40 s and extension at 72°C for 50 s, with a final extension for 5 minutes at 72°C (Poirel *et al*., 2011).

For the detection of *bla*$_{NDM}$, the following thermal cycling conditions were used: DNA was first denatured at 95°C for 5 minutes then followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60^{o}C for 30 s, and extension at 72^{o}C for 30 s, with a single final extension for 3 minutes at 72°C.

For the detection of *bla*$_{AIM,}$ *bla*$_{DIM,}$ *bla*$_{SIM}$ and *bla*$_{GIM}$, overall capacity of 25 µL containing 1µL of total DNA isolated in section 3.2.2.1, 12.5 µL PCR 2X Master mix (Bioline, London; Wizbiosolutions, Korea South), 1 µL each of both forward and reverse primers (10 µmol/L), 3 µL of dimethyl sulfoxide and 6.5 µL distilled water devoid DNase / RNase (Invitrogen) was used. The following thermal cycling conditions were used for amplification: 10 minutes at 94^{o}C with 36 cycles of denaturation at 94^{o}C for 30 seconds, 52^{o}C at 40 seconds and 72^{o}C at 50 seconds, and a single finishing step of 5 minutes at 72°C (Poirel *et al*., 2011).
Gel electrophoresis was performed on all amplified DNA fragments with 1.5% agarose and estimated with 100 bp plus ladder or 1 kb plus ladder as explained in sections 3.2.4.5 to 3.2.4.7.

3.2.5.3 Class D carbapenemases

For the detection of *bla*$_{OXA-48}$ and *bla*$_{OXA-58}$, a 25 µL volume containing 1µL of total DNA isolated in section 3.2.2.1, 12.5 µL of WizPure™ PCR 2X Master mix (Wizbiosolutions, Korea South), 1 µL each 10 µmol/L of both forward and reverse primers and 9.5 µL distilled water devoid DNase / RNase (Invitrogen). The tubes were loaded into a **Thermal Cycler instrument** (Applied Biosystems).

PCR Conditions: The following thermal cycling conditions were used: DNA was first denatured at 94°C for 5 minutes then followed by 30 cycles at 94 °C for 35 s, 60^{o}C for 35 s, 72^{o}C for 30 s, for denaturing, annealing and extension, respectively and finally a single extension for 6 minutes at 72°C.
Gel electrophoresis was performed on all amplified DNA fragments with 1.5% agarose and estimated with 100 bp plus ladder as explained in sections 3.2.4.5 to 3.2.4.7.

3.2.6.1 Characterisation of class 1, 2 and 3 integron

One microliter of entire DNA was exposed to PCR in a 20 µL reaction capacity. The mixture contained 10 µL of 2× MyTaq Red Mix (Bioline, London; Wizbiosolutions, Korea South), 10 µmol/L of each primer and 7 µL distilled water devoid of DNase and RNase (Invitrogen). The tubes were loaded into **Thermal Cycler** (Applied Biosystems).

PCR conditions: The following thermal cycling conditions were used: DNA was first denatured at 94°C for 5 minutes and then succeeded by 30 cycles at 94 °C for 30 s, 55^{o}C for 30 s, 72^{o}C for 45 s, of denaturing, annealing and extension, respectively with a final extension for 10 minutes at 72°C (White *et al*., 2001).

Gel electrophoresis was performed on all amplified DNA fragments with 1.5% agarose and estimated with 100 bp plus or 1 kb plus ladders as explained in sections 3.2.4.5 to 3.2.4.7.

3.2.6.2 Restriction Fragment Length Polymorphism (RFLP) for differentiation of integrons

In strains that were positive for integron, integrase PCR products were analysed to determine the class of the integron possessed by each strain by digesting the PCR products with *Rsa*I (Thermo Scientific) restriction enzyme. PCR products (10 µL) were digested with 1 µL of *Rsa*I by adding 2 µL of 10x buffer and 7 µL of PCR water (UltraPure, Invitrogen) to obtain the overall capacity of 20 µL. The mix was incubated at 37^{o}C for 3 hours. To inactivate *Rsa*I enzyme, the mix was further incubated at 80^{o}C for 20 minutes. The digested amplicons were electrophoresed for 45 minutes at 90 V on 1.5% agarose. After digestion of PCR products with *Rsa*I restriction enzyme, integrase I will give only one amplicon size of 491 bp, integrase II will give two amplicon sizes of 334 bp and 157 bp, while integrase III will give rise to three fragment sizes of 97 bp, 104 bp and 290 bp if they were present (White *et al*., 2001).

All amplified DNA fragments were estimated alongside with a DNA molecular weight marker (100 bp plus and 1 kb plus ladders) as explained in sections 3.2.4.5 to 3.2.4.7.

3.2.6.3 Characterisation of cassette arrays

Strains that gave amplicon size of 491 bp were subjected to another PCR using hep58 and hep59 primers to detect class 1 gene cassette arrays (White *et al*., 2001).

PCR Conditions: the initial denaturation temperature was set at 94°C for 5 minutes, succeeded by 30 cycles of 94°C for 30 seconds, annealing at 55°C for 30 seconds followed by 72°C for 4 minutes (White *et al*., 2000).

All amplified DNA fragments were estimated alongside with a DNA molecular weight marker (1 kb plus ladder) as explained in sections 3.2.4.5 to 3.2.4.7.

3.2.7 Purification and Sequencing of PCR products

3.2.7.1 Purification of PCR products

For DNA purification purposes, a WizPrep Gel/PCR purification kit (Wizbiosolutions) was used in accordance with Manufacturer's instruction. The PCR product was transferred into 1.5ml eppendorf tube. To 1 volume of the sample, 5 volume of GP Buffer was introduced and mixed thoroughly. The mixture was removed into a DNA Spin Column (SC), spinned at 13,000 rpm for 19 minute and the filtrate was discarded. Then, 700 µL of Wash buffer containing ethanol was introduced into the DNA SC, spinned at 13,000 rpm for 30 seconds and the filtrate was discarded. The Spin Column was spinned for an additional 1 minute and the DNA Spin Column was transferred to a new 1.5 ml tube. Then, 50 µL of Elution Buffer was introduced to the centre of the column matrix and allowed to stand for 1 minute and spin for 1 minute at 13,000 rpm to elute the DNA.

3.2.7.2 Sequencing of PCR products

The purified PCR products for bla_{VIM} and bla_{NDM} were sent to First Base Laboratory in Malaysia for sequencing using both forward and reverse primers. Sequencing reactions were done with BigDye Terminator v3.1 Cycle Sequencing Kit on ABI PRISM 3730xi Genetic analyser (Applied Biosystems, USA). The resulting sequences were compared with the sequences in the NCBI GenBank database through the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.8 Transformation experiment

Preparation of competent cell: A single colony of *Escherichia coli* (DH5α) from 18-24 hour old culture was added into LB medium and incubated overnight at 37°C with shaking at 250 rpm. LB medium (100 mL) was inoculated with 1ml of the *E. coli* (DH5α) culture and incubated at 37°C for 2-3 hours with agitation until the OD reached 600 nm. The culture was put in an ice bath for 10 minutes and transferred into 50 mL falcon tubes. The pre-chilled culture was spinned at 2700x g for 10 minutes at

4°C and the supernatant was discarded. The cell pellet was mixed in 1.6 ml of cold $CaCl_2$ and incubated on ice for 30 minutes. The cell solution was spinned at 2700x g for 10 minutes at 4°C and the supernatant removed. Ice cold 100 mM $CaCl_2$ was used to liquefy the pellet and incubated on ice for 20 minutes. Then, 0.5 mL of ice cold 80% glycerol was put and dispensed in 100 µL aliquots and stored at -80°C (Chang *et al*., 2017)

Transformation protocol: DH5α cells (chemically competent) were defrosted on ice for 30 minutes. At this juncture, 2 uL of plasmid DNA was added and the cells were heat-shocked at 42^{o}C for 60 seconds. The cells were placed on ice for 5 minutes. Thereafter, 900 uL of LB broth was introduced and incubated at 37^{o}C for 2 hours at 125 rpm. The cell was centrifuged at 5000 rpm for 45 seconds. LB (600 µL) was removed and the suspension was resuspended. Then, 100 µL was spread onto the plate comprising LB agar and 100 µg/mL of ampicillin and incubated overnight. Transformants were selected from colonies grown on Luria Bertani agar containing 100 µg/mL of ampicillin. Plasmid DNA was extracted from the transformant with DNA extraction kit (Wizbiosolutions) and subjected to PCR using PCR conditions for bla_{VIM} and bla_{NDM} detection as explained in section 3.2.5.2.

3.2.9 Statistical analysis

Sensitivity and specificity, negative predictive values (NPV), and positive predictive values (PPV) were also calculated. Fisher's exact test was used to define the connection between integrons and MBL(s); association between *exoU* and *exoS*; association of clinical source with T3SS and association of T3SS and clonality. One-way analysis of variance (ANOVA) was used to determine the association between antibiotic sensitivity profile of MBL+ve and MBL-ve strains. A *P* value of < 0.05 was considered to be significant.

3.2.10 Quantification of gene expression

3.2.10.1 Removing the co-purified contaminating DNA and synthesis of First strand complementary DNA (cDNA)

To remove any trace of genomic DNA co-purified along with total RNA prior to RT-PCR. DNase 1 (Thermo Scientific) was used following manufacturer's instruction. The following components were assembled in a thin-walled 0.2 ml tube, on ice: 0.5 - 4

µL (1 µg) of RNA template, 1 µL of 10x DNase 1 buffer, 1 µL DNase 1, RNase-free water to make up 10 µL. The mixture was incubated for 30 minutes at 37°C.

cDNA was synthesised by means of the WizScript™ cDNA Synthesis Kit (Wizbiosolutions, Korea) according to the manufacturers instruction. The following components were added to the purified RNA. 1 µL of EDTA (50mM), 2 µL of Random hexamer and 1 µL of dNTP mix (2.5 mM). The mixture was incubated at 65°C for 10min and then cooled immediately on ice.

Thereafter, 2 µL of 10x buffer, 1 µL of Reverse transcriptase (200 U/µL), 0.5 µL of RNase Inhibitor (40U/µL), 1 µL of DTT (100mM) and RNase free water to make up to 20 µL were added to the template RNA and primer mix. The reaction mixture was mixed slightly and incubated at 37°C for 60 min. Reverse transcriptase was inactivated by incubation at 70°C for 10 min and cooling on ice. Synthesised cDNA was kept at -20°C and used as template for PCR

3.2.10.2 Expression levels quantification of efflux pumps, *ampC* and *oprD* transcripts

The expression levels of two genes in each of the four major *P. aeruginosa* efflux pumps; (*mexA, mexB, mexC, mexD, mexE, mexF, mexX* and *mexY*), *ampC* and *oprD* genes were analysed with Step One ® real-time reverse transcription-PCR (Applied Biosystem).

The primers for the PCR amplification of cDNA were shown in Table 3.1. Primer conditions were optimised with PCR. Real time PCR was accomplished in a set of three per sample. A total capacity of 20 µL comprised of 10 µL of SYBR green /ROX qPCR master mix (Thermo Scientific), 0.5 µL (10 pmol) each of onward and backward primers, 1 µL of the cDNA, 8 µL of PCR water, was used. PCR conditions include initial denaturation temperature at 95°C for 10 min to trigger the modified Taq polymerase, followed by 40 cycles of 20s at 95°C, 30s at 66°C (*mexA, mexB, mexC, mexX* and *mexY*), 30s at 67°C (*mexE*), 30s at 68°C (*mexF*), 30s at 64°C (*mexD*), 30s at 62°C (*ampC*), 30s at 62°C (*oprD*), 30s at 62°C (*rpsL*) and 30s at 72°C were performed. Data acquisition was performed at 72°C. At the end of the 40 cycles, a melt curve was determined to look for the existence of a single PCR product. The mean threshold cycle (ct) value of triplicate sample was taken.

To regulate the expression levels of mRNA, housekeeping gene (*rpsL* gene) was included and outcomes were referenced against *P. aeruginosa* ATCC 27853 expressions. When the equivalent mRNA level was at least 2-fold (*mexA, mexB), 4-fold (mexX and mexY*) or 100-fold (*mexC, mexD, mexE* and *mexF*) higher than that for *P. aeruginosa* ATCC 27853, strains were considered to be overexpressed (Hocquet *et al*., 2006). Control reaction lacking reverse transcriptase was set up to check for contaminating genomic DNA. Fold expression level was calculated using the formula below:

TE – HE = ΔCTE

TC – HC = ΔCTC

ΔCTE – ΔCTC = ΔΔCt

Fold change = $2^{\wedge -\Delta\Delta Ct}$

Where:

TE = Threshold cycle value obtained from amplification of test organism cDNA with resistance gene primer

HE = Threshold cycle value obtained from amplification of test organism cDNA with housekeeping gene primer

TC = Threshold cycle value obtained from amplification of control organism cDNA with resistance gene primer

HC = Threshold cycle value obtained from amplification of control organism cDNA with housekeeping gene primer

ΔCTE = Delta Ct housekeeping gene

ΔCTC = Delta Ct control

3.2.11 Detection of type III secretion system

Multiplex PCR was done to detect type III secretion systems in carbapenem non-susceptible strains of *P. aeruginosa* using primers which amplify the conserved regions of *exoS, exoU, exoT*, and *exoY* genes (Ajayi *et al*., 2003).

PCR set up included 1 µL of DNA template, 0.5 µL of each primers (Macrogen), 12.5 µL of WizPure™ PCR 2X Master mix **(**Wizbiosolutions, Korea South**),** and 7.5 µL of

DNase/RNase-Free Distilled Water (Invitrogen). *P. aeruginosa* ATCC 27853 served as a control.

PCR Conditions: The following thermal cycling conditions were used: DNA was first denatured at 94°C for 2 minutes then followed by 36 cycles at 94 °C for 30 s, 58^{o}C for 30 s, and 68^{o}C for 1 minute, of denaturing, annealing and extension, respectively with a single final extension at 68°C for 7 minutes (Ajayi *et al*., 2003).

Gel electrophoresis was performed on all amplified DNA fragments with 1.5% agarose and estimated with 100 bp plus and 1kb plus ladders as explained in sections 3.2.4.5 to 3.2.4.7.

3.2.12 PCR-based genotyping

For genotyping of CRPA, three PCR-based genotyping methods were employed. PCR set up included 1 µL of DNA template, 0.5 µL of each primer (Macrogen), 12.5 µL of WizPure™ PCR 2X Master mix **(**Wizbiosolutions, Korea South**),** and 7.5 µL of distilled water devoid of DNase and RNase (Invitrogen).

3.2.12.1 Repetitive extragenic palindromic PCR (REP PCR)

Two primers were used; REP1R-I and REP2-I (Table 3.1).

PCR Conditions: Preliminary denaturation temperature was set at 95°C for 2 min; succeeded by 35 cycles of 95°C for 30 s, 38°C for 1 minute, and 72°C for 2 minutes of denaturation, annealing and extension, respectively. A final extension step was set at 72°C for 16 minutes. The arrangement of amplified DNA fragments was scored alongside with a DNA molecular weight marker (1 kb plus ladder) as explained in sections 3.2.4.5 to 3.2.4.7. Data matrices were computed based on the presence or absence of bands at definite positions. REP-PCR profiles were compared with dice coefficient method and clustered with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using Phylotree software.

3.2.12.2 Enterobacterial Repetitive Intragenic Consensus-PCR (ERIC PCR)

Two primers were used; ERIC1R and ERIC2 (Table 3.1).

PCR Conditions: Preliminary denaturation temperature of 94°C for 2 min; followed by 35 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 2 minutes of denaturation, annealing and extension, respectively. A final extension step was set at 72°C for 10 minutes (Pinna *et al*., 2009). The arrangement of amplified DNA fragments was scored

alongside with a DNA molecular weight marker (1 kb plus ladder) as explained in sections 3.2.4.5 to 3.2.4.7. Data matrices were computed based on the presence or absence of bands at definite positions. ERIC-PCR profiles were compared with dice coefficient method and clustered with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using Phylotree software.

3.2.12.3 BOX PCR

One primer (BOX-A1R) was used for BOX-PCR typing of *P. aeruginosa* (Table 3.1).

PCR Conditions: Preliminary denaturation temperature of 94°C for 2 min; followed by 35 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 2 minutes of denaturation, annealing and extension, respectively. A final extension step set at 72°C for 10 minutes was used (Pinna *et al*., 2009). The arrangement of amplified DNA fragments was scored alongside with a DNA molecular weight marker (1 kb plus ladder) as explained in sections 3.2.4.5 to 3.2.4.7. Data matrices were computed based on the presence or absence of bands at definite positions. BOX-PCR profiles were compared with dice coefficient method and clustered with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using Phylotree software.

CHAPTER FOUR

RESULTS

4.1 Identification of clinical isolates of *Pseudomonas aeruginosa*

Four hundred and forty-seven isolates were collected on nutrient agar slants from seven tertiary hospitals in five southwestern states of Nigeria and transported to the Department of Pharmaceutical Microbiology laboratory, University of Ibadan, for further identification. Ninety (90) out of 92 isolates collected from University College Hospital, Ibadan (UCHI) were identified as *P. aeruginosa*. Among 97 isolates collected from Ogun state, 47 and 50 isolates from Olabisi Onabanjo University Teaching Hospital, Sagamu (OTHS) and Federal Medical Centre Abeokuta (FMCA), respectively, 45 isolates each were identified as *P. aeruginosa*. Out of 48 isolates collected from Ladoke Akintola University Teaching Hospital, Osogbo (LTHO), 45 were identified as *P. aeruginosa*, 45 isolates collected from Obafemi Awolowo University Teaching Hospital, Ile-Ife (OTHI) were identified as *P. aeruginosa*; of the 94 isolates collected from Federal Medical Centre, Owo (FMCO), 90 were identified as *P. aeruginosa*. Of the 72 isolates collected from Federal Medical Centre, Ido-Ekiti (FMCI), 70 were recognised as *P. aeruginosa*. the identification centered on their growth on *Pseudomonas* cetrimide agar, pigment production, motility, production of cytochrome c oxidase, catalase, urease positive, inability to produce hydrogen sulphide, ability to utilise citrate as their only carbon source, oxidative utilisation of glucose. Further confirmation at the molecular level was done at the Department of Biosciences, COMSATS University, Islamabad, Pakistan.

4.2 Distribution of clinical isolates of *P. aeruginosa* according to site of isolation in relation to hospital

Figure 4.1 illustrates the distribution of clinical isolates of *P. aeruginosa* according to their clinical sources. Out of 430 *P. aeruginosa* isolates, 298 (69.3%) isolates were from wound swab, 5 (1.2%) from wound biopsy, 49 (11.4%) from ear, 43 (10.0%) from urine, 10 (2.3%) from eye, 5 (1.2%) from ear pus, 5 (1.2%) from sputum,

4 (0.9%) from blood, 3 (0.7%) from tracheal aspirate, 3 (0.7%) from cerebrospinal fluid, 4 (0.9%) from catheter tips and 1 (0.2%) from genital discharge. Table 4.1 presents the dissemination of clinical isolates of *P. aeruginosa* according to hospital with respect to their clinical sources. Out of the 90 isolates from UCHI, 37 (41.1%) were isolated from wound swab, followed by17 (18.9%) from ear, 16 (17.8%) from urine, 5 (5.6%) from wound biopsy, 4 (4.4%) from catheter tips, 3 (3.3%) each from eye, tracheal aspirate and blood, 2 (2.2%) each from sputum and cerebrospinal fluid, and 1 (1.1%) from genital discharge and ear pus. Among the FMCA isolates, 32 (71.1%) were isolated from wound followed by 5 (11.1%) from urine, 3 (6.7%) from eye, 2 (4.4%) from ear, 2 (2.2%) from catheter tips and 1 (2.2%) from ear pus. Twenty-eight (62.2%), out of forty-five (45) isolates from OTHS were isolated from wound followed by 7 (15.6%) from urine, 5 (11.1%) from ear, 2 (4.4%) from eye, 2 (4.4%) from sputum and 1 (2.2%) from ear pus. Out of 45 isolates from LTHO, 37 (82.2%) were from wound, 2 (4.4%) isolates each were from urine, ear, ear pus while 1 (2.2%) each were from cerebrospinal fluid and blood. Out of 45 isolates from OTHI, 24 (53.3%) were from wound, 11 (24.4%) were from urine, 6 (13.3%) from ear, 2 (4.4%) were from wound biopsy, while 1 (2.2%) each were from eye and sputum. Ninety (90) isolates collected from FMCO was from wound 79 (87.8%) and ear 11 (12.2%) only. Out of 70 isolates of *P. aeruginosa* collected from FMCI, 71 (87.1%) were isolated from wound, 6 (8.6%) from ear, 2 (2.9%) from urine and 1 (1.4%) from eye.

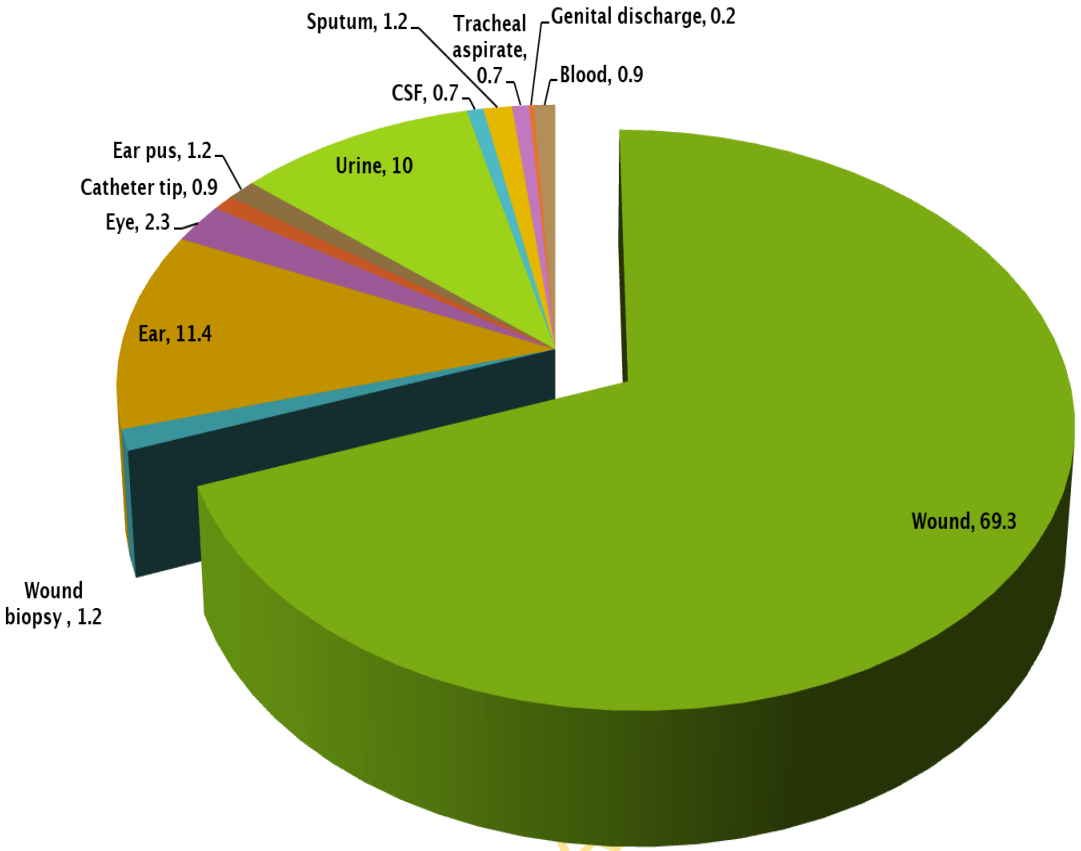

Figure 4.1. Distribution of the isolates according to clinical source

Table 4.1. Occurrence of *P. aeruginosa* strains according to hospital with respect to their clinical sources

Site of isolation	**UCHI (90)**	**FMCA (45)**	**OTHS (45)**	**LTHO (45)**	**OTHI (45)**	**FMCO (90)**	**FMCI (70)**
Wound (298)	37	32	28	37	24	79	61
Wound biopsy (5)	3	-	-	-	2	-	-
Ear (49)	17	2	5	2	6	11	6
Eye (10)	3	3	2	-	1	-	1
Catheter tips (4)	2	2	-	-	-	-	-
Ear pus (5)	1	1	1	2	-	-	-
Urine (43)	16	5	7	2	11	-	2
CSF (3)	2	-	-	1	-	-	-
Sputum (5)	2	-	2	-	1	-	-
Tracheal aspirate (3)	3	-	-	-	-	-	-
Genital discharge (1)	1	-	-	-	-	-	-
Blood (4)	3	-	-	1	-	-	-

Key:

UCHI = University College Hospital, Ibadan

FMCA = Federal Medical Centre, Abeokuta

OTHS = Olabisi Onabanjo University Teaching Hospital, Sagamu

LTHO = Ladoke Akintola University Teaching Hospital, Osogbo

OTHI = Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife

FMCO = Federal Medical Centre, Owo

FMCI = Federal Medical Centre, Ido-Ekiti

4.3 Antibiotic susceptibility profile of clinical isolates of *Pseudomonas aeruginosa*

Table 4.2 is the antibiotic susceptibility profile of 430 clinical isolates of *P. aeruginosa* against twenty-three selected antibiotics. One hundred percent (100%) of the isolates were resistant to ampicillin, cephalothin and cefuroxime followed by amoxicillin clavulanate (99.5%), ticarcillin (64.4%), ceftriaxone (54.4%), cefepime (45.8%), gentamicin (43.3%), ofloxacin (43.3%), ciprofloxacin (39.3%), ceftazidime (38.1%), levofloxacin (38.1%), cefoperaxone (33.0%), tobramycin (32.6%), amikacin (27.4%), meropenem (18.8%), piperacillin (18.6%), doripenem (17.8%), piperacillin tazobactam (17.6%), aztreonam (16.7%), imipenem (15.8%), colistin sulphate (5.1%) and polymyxin B (3.7%).

Percentage antibiotic resistance among isolates from the seven hospitals is given in figure 4.2. An equivalent proportion (100%) was observed to be resistant to amoxicillin clavulanate in all the hospitals. Highest proportion of resistance to cefepime (65.6%), ofloxacin (62.2%), levofloxacin (56.7%), ciprofloxacin (56.7%), gentamicin (56.7%), tobramycin (51.1%), piperacillin (27.8%), amikacin (42.2%), imipenem (27.8%), colistin sulphate (8.9%) and polymyxin B (7.8%) was observed among isolates from UCHI. Among isolates from the seven hospitals, OTHI isolates showed highest proportion of resistance to ticarcillin (84.4%), ceftriaxone (71.1%), cefoperaxone (66.7%), ceftazidime (60%), piperacillin tazobactam (31.1%), meropenem and aztreonam (28.9%). Isolates from FMCO gave least resistance to ciprofloxacin (28.9%) and gentamicin (35.6%), while resistance to tobramycin (13.3%), amikacin (11.1%), imipenem (2.2%), ceftriaxone (32.7%), ticarcillin (48.9%), piperacillin (8.9%) and piperacillin tazobactam (4.4) was least in isolate from FMCA.

Table 4.3 shows the antibiotic resistance profile of *P. aeruginosa* isolates. According to the criteria for classification of multidrug resistant *P. aeruginosa* described by Magiorakos and colleagues (2012), 135 (31.4%) and 50 (11.6%) of *P. aeruginosa* isolates were multidrug resistant (MDR) and extensive drug resistant (XDR), respectively. Multidrug resistance was highest among isolates from UCHI 33/90 (36.7%) followed by OTHI 16/45 (35.6%), FMCI 31/90 (34.4%), FMCA 15/45 (33.3%), FMCO 25/90 (27.8%), OTHS 12/45 (26.7%) and LTHO 10/45 (22.2%). The occurrence of extensively drug resistance (XDR) among the clinical isolates according

to the hospital was in the following descending order: UCHI 20 (22.2%) > OTHI 8 (17.8%) > LTHO 7 (15.6%) > FMCI 5 (7.1%) > FMCO 6 (6.7%) = OTHS 3 (6.7%) > FMCA 1 (2.2%).

4.4. Minimum inhibitory concentrations (MICs) of selected antibiotics against clinical isolates of *P. aeruginosa*

The MIC of five selected antibiotics together with their MIC_{50} and MIC_{90} against the 430 clinical isolates of *P. aeruginosa* is given in Table 4.4. MIC of imipenem and meropenem against all the isolates ranged from 0.12 to >64 µg/mL and 0.06 to >64 µg/mL, respectively with MIC_{50} and MIC_{90} of 1 and >64 µg/mL.

The MIC of imipenem and meropenem against clinical isolates from UCHI, FMCO and FMCI ranged from 0.12 to >64 µg/mL, each. MIC of imipenem and meropenem against clinical isolates from FMCA and OTHS state ranged from 0.12 to 32 µg/mL and 0.06 to 64 µg/mL, respectively while LTHO and OTHI isolates had MIC ranged from 0.12 to 64 µg/mL for imipenem and meropenem. MIC of cefepime against clinical isolates from UCHI, FMCA and OTHS ranged from 0.5 to >128 µg/mL while that of FMCO and FMCI ranged from 1 to >128 µg/mL. MIC of cefepime against isolates from LTHO and OTHI ranged from 1 to 64 µg/mL. UCHI and FMCI isolates had the same MIC ranged from 1 to >256 µg/mL against ceftazidime with FMCA, OTHS, LTHO, OTHI and FMCO isolates having the same MIC ranged from 0.5 to >256 µg/mL. The MIC of ciprofloxacin against clinical isolates from the seven hospitals ranged from <0.03 to >128 µg/mL

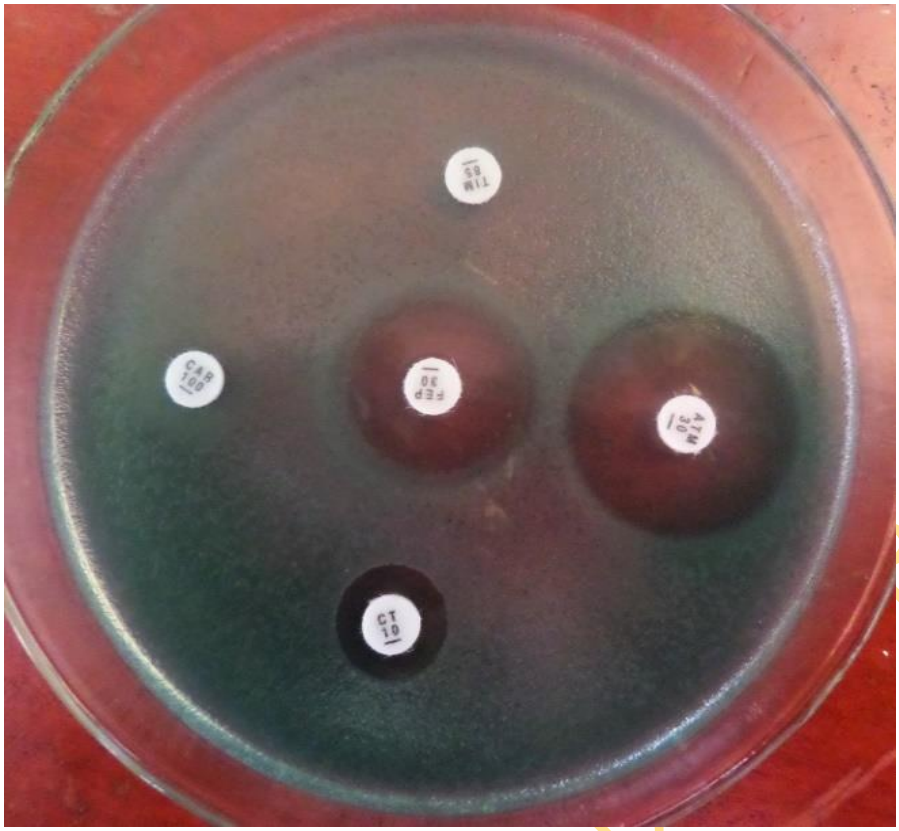

Plate 4.1. Zone of growth inhibition of *P. aeruginosa* by aztreonam (ATM), cefepime (FEP) and colistin sulphate (CT), ticarcillin clavulanic acid (TIM)

Table 4.2. Antibiotic susceptibility profile of clinical isolates of *P. aeruginosa*

Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)
Ceftazidime	214 (49.8)	47 (10.9)	164 (38.1)
Amoxicillin clavulanate	2 (0.5)	0 (0)	428 (99.5)
Piperacillin	299 (69.5)	51 (11.9)	80 (18.6)
Ticarcillin	32 (7.4)	121 (28.1)	277 (64.4)
Cephalothin	0 (0)	0 (0)	430 (100)
Cefoperazone	211 (49.1)	77 (17.9)	142 (33.0)
Piperacillin/ tazobactam	303 (70.5)	51 (11.9)	76 (17.7)
Gentamicin	214 (49.8)	30 (7.0)	186 (43.3)
Cefuroxime	0 (0)	0 (0)	430 (100)
Ampicillin	0 (0)	0 (0)	430 (100)
Imipenem	360 (83.7)	2 (0.5)	68 (15.8)
Aztreonam	212 (49.3)	146 (34.0)	72 (16.7)
Ceftriaxone	63 (14.7)	133 (30.9)	234 (54.4)
Tobramycin	284 (66.1)	6 (1.4)	140 (32.6)
Meropenem	341 (79.3)	8 (1.9)	81 (18.8)
Amikacin	29 (69.5)	13 (3.0)	118 (27.4)
Levofloxacin	259 (60.2)	7 (1.6)	164 (38.1)
Doripenem	347 (80.7)	7 (1.6)	76 (17.8)
Cefepime	203 (47.2)	30 (7.0)	197 (45.8)
Polymixin B	414 (96.3)	0 (0)	16 (3.7)
Ciprofloxacin	257 (59.8)	4 (0.9)	169 (39.3)
Colistin sulphate	408 (94.9)	0 (0)	22 (5.1)
Ofloxacin	231 (53.7)	13 (3.0)	16 (3.7)

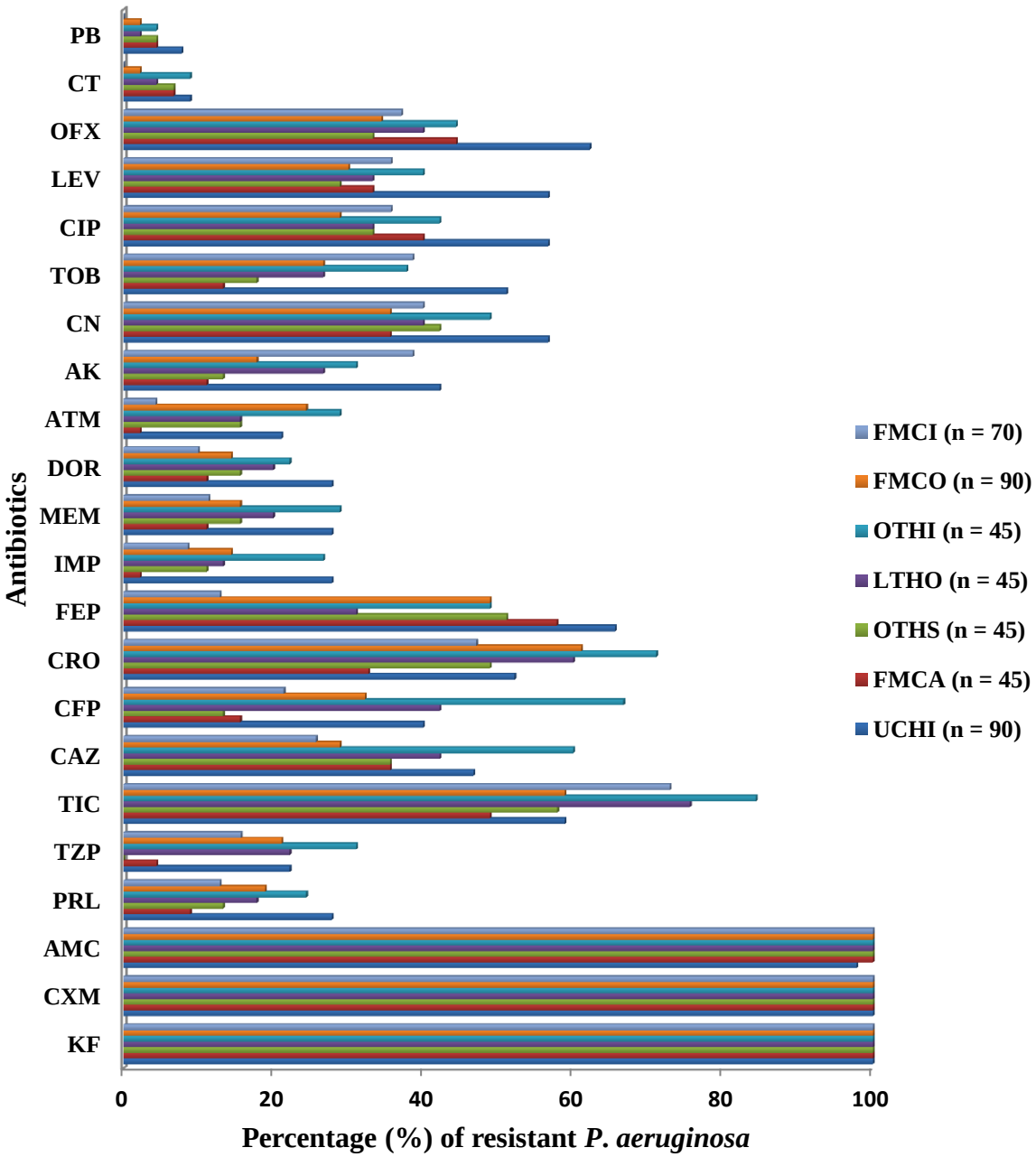

Figure 4.2. Percentage antibiotic resistance of 430 isolates of *P. aeruginosa* from seven hospitals

Key: AMC=Amoxicillin clavulanate, PRL=Piperacillin, TZP=Piperacillin-tazobactam, TIC=Ticarcillin, CAZ=Ceftazidime, PB=Polymyxin B, CFP=Cefoperazone, CRO=Ceftriaxone, FEP=Cefepime, IPM=Imipenem, CT=colistin sulphate, MEM=Meropenem, DOR=Doripenem, ATM=Aztreonam, AK=Amikacin, CN=Gentamicin, CXM=Cefuroxime, TOB=Tobramycin, KF=Cephalothin, CIP=Ciprofloxacin, LEV=Levofloxacin, OFX=Ofloxacin

Table 4.3. Classification of clinical isolates of *P. aeruginosa* based on antibiotic resistance profiles

Hospital	MDR (no (%))	XDR (no (%))	Non-MDR (no (%))
UCHI (n = 90)	33 (36.7)	20 (22.2)	37 (41.1)
FMCA (n = 45)	15 (33.3)	1 (2.2)	29 (64.4)
OTHS (n = 45)	12 (26.7)	3 (6.7)	30 (66.7)
OTHI (n = 45)	16 (35.6)	8 (17.8)	21 (46.7)
LTHO (n = 45)	10 (22.2)	7 (15.6)	28 (62.2)
FMCO (n = 90)	25 (27.8)	6 (6.7)	59 (65.6)
FMCI (n = 70)	24 (26.7)	5 (7.1)	41 (58.6)

Key:

MDR = Multidrug resistant

XDR = Extensive drug resistant

UCHI = University College Hospital, Ibadan

FMCA = Federal Medical Centre, Abeokuta

OTHS = Olabisi Onabanjo University Teaching Hospital, Sagamu

LTHO = Ladoke Akintola University Teaching Hospital, Osogbo

OTHI = Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife

FMCO = Federal Medical Centre, Owo

FMCI = Federal Medical Centre, Ido-Ekiti

Table 4.4. Antibiotic susceptibility rates of 430 clinical isolates of *P. aeruginosa* at indicated MIC in µg/mL

	Isolates number at indicated MIC (µg/mL)																
Antibiotics	0.03	0.06	.12	0.25	0.5	1	2	4	8	16	32	64	128	256	Break points	% susceptible	MIC_{50}/MIC_{90} (µg/mL)
Imipenem			**43**	**65**	**76**	**97**	**54**	16	16	16	9	36[c]			2/8	76.7	1/>64
Meropenem		**2**	**38**	**73**	**71**	**88**	**49**	20	16	11	6	48[c]			2/8	74.7	1/>64
Cefepime					**3**	**51**	**68**	**73**	**82**	49	22	31	39[c]		8/32	64.4	8/>128
Ceftazidime					**15**	**49**	**53**	**61**	**48**	42	24	13	25	85[c]	8/32	52.6	8/>256
Ciprofloxacin	**54**[b]	**39**	**61**	**37**	**22**	**33**	25	21	15	15	25	4	68[c]		1/4	57.2	1/>128

Key:

[a]The bolded fonts indicate the susceptible strains

[b]MIC ≤ the value indicated

[c]MIC ≥ the value indicated

Breakpoints according to CLSI, 2015 (susceptible ≤/resistant>).

4.5 Phenotypic detection of carbapenemase, beta lactamase and metallo beta-lactamase (MBL)

Phenotypic detection of carbapenemase using Modified Hodges test showed negative result for all the isolates (Plate 4.2). Highest prevalence of beta lactamase was observed in isolates from UCHI (81.1%) followed by OTHS (80.0%), OTHI (77.7%), FMCO (75.6%), LTHO (75.5%), FMCA (64.4%) and lastly FMCI (61.4%). Based on phenotypic MBL detection using combined disc method with EDTA soaked imipenem and meropenem discs, the prevalence of MBL in clinical isolates of *P. aeruginosa* was established to be 15.4%. These isolates exhibited a significant zone size enhancement (≥ 7mm) with the EDTA soaked imipenem and meropenem discs when compared with the plain imipenem and meropenem discs. UCHI had the highest prevalence of 22 (24.4%) followed by OTHI, LTHO, FMCO, OTHS, FMCI and FMCA states with the prevalence of 10 (22.2%), 8 (17.8%), 14 (15.6%), 5 (11.1%), 6 (8.6%) and 1 (2.2%), respectively (Table 4.5). Plates 4.3a and 4.3b shows the zone of growth inhibition between the EDTA soaked imipenem and meropenem discs and with the plain imipenem and meropenem discs.

Figure 4.3 compares the antibiotic sensitivity profile of MBL-positive *P. aeruginosa* isolates and MBL-negative isolates to different antipseudomonad antibiotics. There was significant difference in antibiotic sensitivity profile of MBL-positive *P. aeruginosa* isolates when compared with MBL-negative isolates ($p < 0.05$). MBL-negative *P. aeruginosa* isolates were more sensitive to majority of the antibiotics (17 out of 23) than MBL-positive strains. MBL-positive strains exhibit 0.0% sensitivity to ticarcillin, ceftazidime, cefoperazone, ceftriaxone, doripenem and meropenem as compared to MBL-negative strains with percentage sensitivity of 8.8, 58.8, 58.0, 17.3, 100 and 100, respectively. MBL-positive strains were more sensitive to only colistin sulphate and polymyxin B (97%) while MBL-negative strains had 96.2% and 94.5% sensitivity to colistin sulphate and polymyxin B, respectively.

4.6 Curing of antibiotic resistance in carbapenem-resistant *P. aeruginosa*

Table 4.6a and 4.6b shows the effect of different concentrations of ethidium bromide on the susceptibility of carbapenem-resistant strains of *P. aeruginosa* to imipenem and meropenem. After subjecting the ten carbapenem-resistant strains isolates to 12.5, 25, 50 and 100 µg/mL concentrations of ethidium bromide, the carbapenem-resistant strains showed varying degree of sensitivity to imipenem and meropenem at 0.5, 1, 2,

4 and 8 µg/mL concentrations after treatment with 50 and 100 µg/mL ethidium bromide while resistance was observed with untreated strains.

Table 4.5. Prevalence of beta lactamase and MBL in clinical isolates of CRPA using phenotypic method

Hospital	Beta-lactamase	MBL
UCHI (n = 90)	81.1% (73)	24.4% (22)
FMCA (n = 45)	64.4% (29)	2.2% (1)
OTHS (n = 45)	80.0% (36)	11.1% (5)
OTHI (n = 45)	77.7% (35)	22.2% (10)
LTHO (n = 45)	75.5% (34)	17.8% (8)
FMCO (n = 90)	75.6% (68)	15.6% (14)
FMCI (n = 70)	61.4% (43)	8.6% (6)
Total (n = 430)	74.0% (318)	15.4% (66)

Key:

UCHI = University College Hospital, Ibadan

FMCA = Federal Medical Centre, Abeokuta

OTHS = Olabisi Onabanjo University Teaching Hospital, Sagamu

LTHO = Ladoke Akintola University Teaching Hospital, Osogbo

OTHI = Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife

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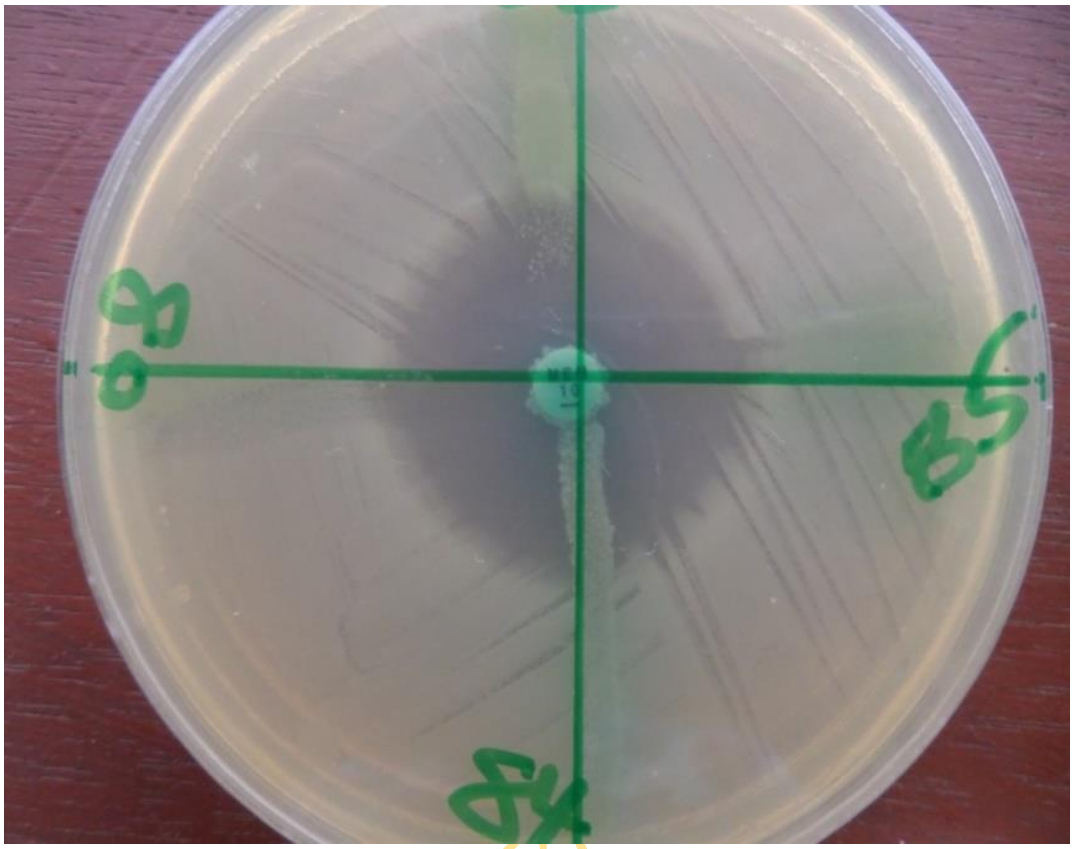

Plate 4.2. Modified Hodges test for detection of carbapenemases. There was no indentation or flattening at the intersect of control organism (*E. coli* ATCC 25922) with the test organism (*P. aeruginosa*) indicating a negative result

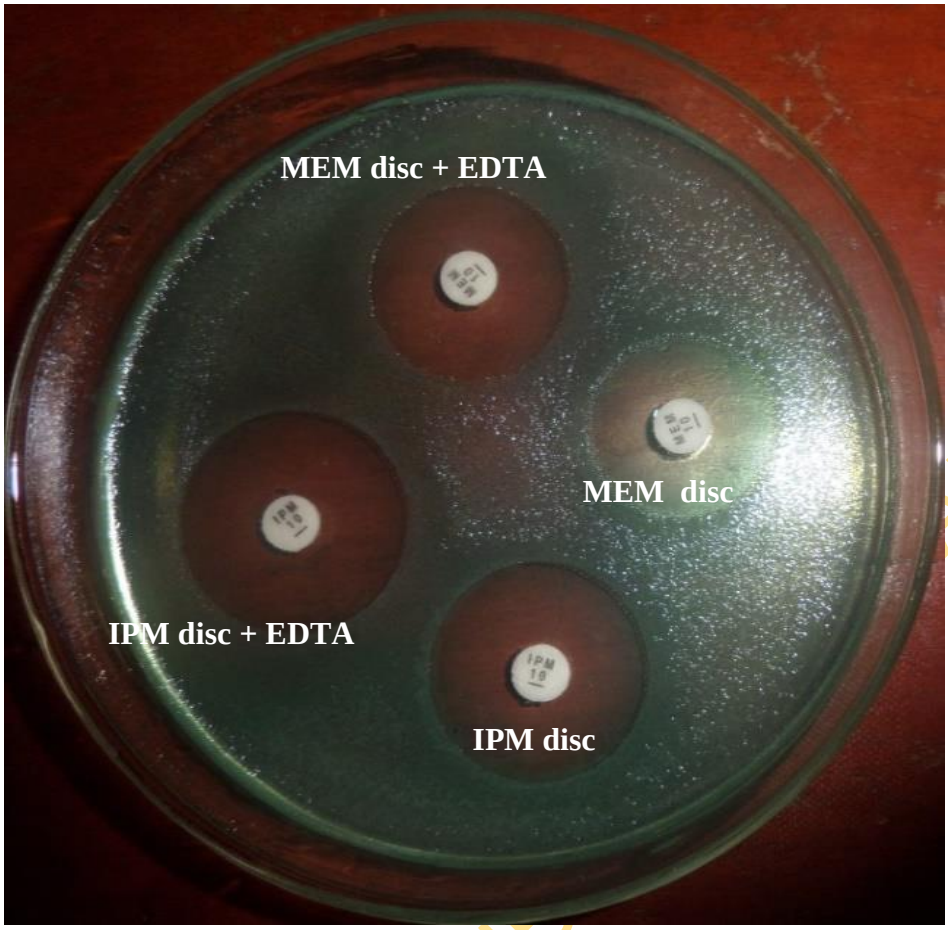

Plate 4.3a. No inhibition zone size augmentation with EDTA impregnated imipenem and meropenem discs in comparison with the plain imipenem and meropenem discs (Phenotypic MBL negative).

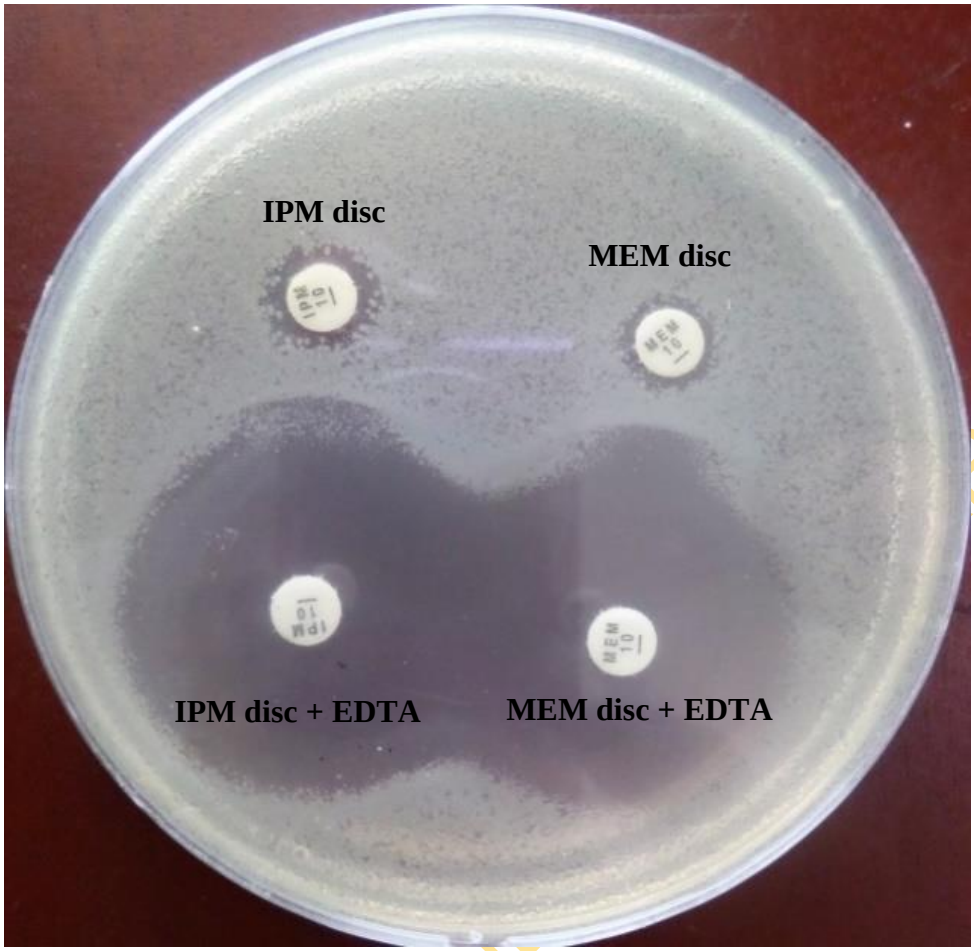

Plate 4.3b. Inhibition zone size augmentation with the EDTA soaked imipenem and meropenem discs in comparison with the plain imipenem and meropenem discs (Phenotypic MBL positive)

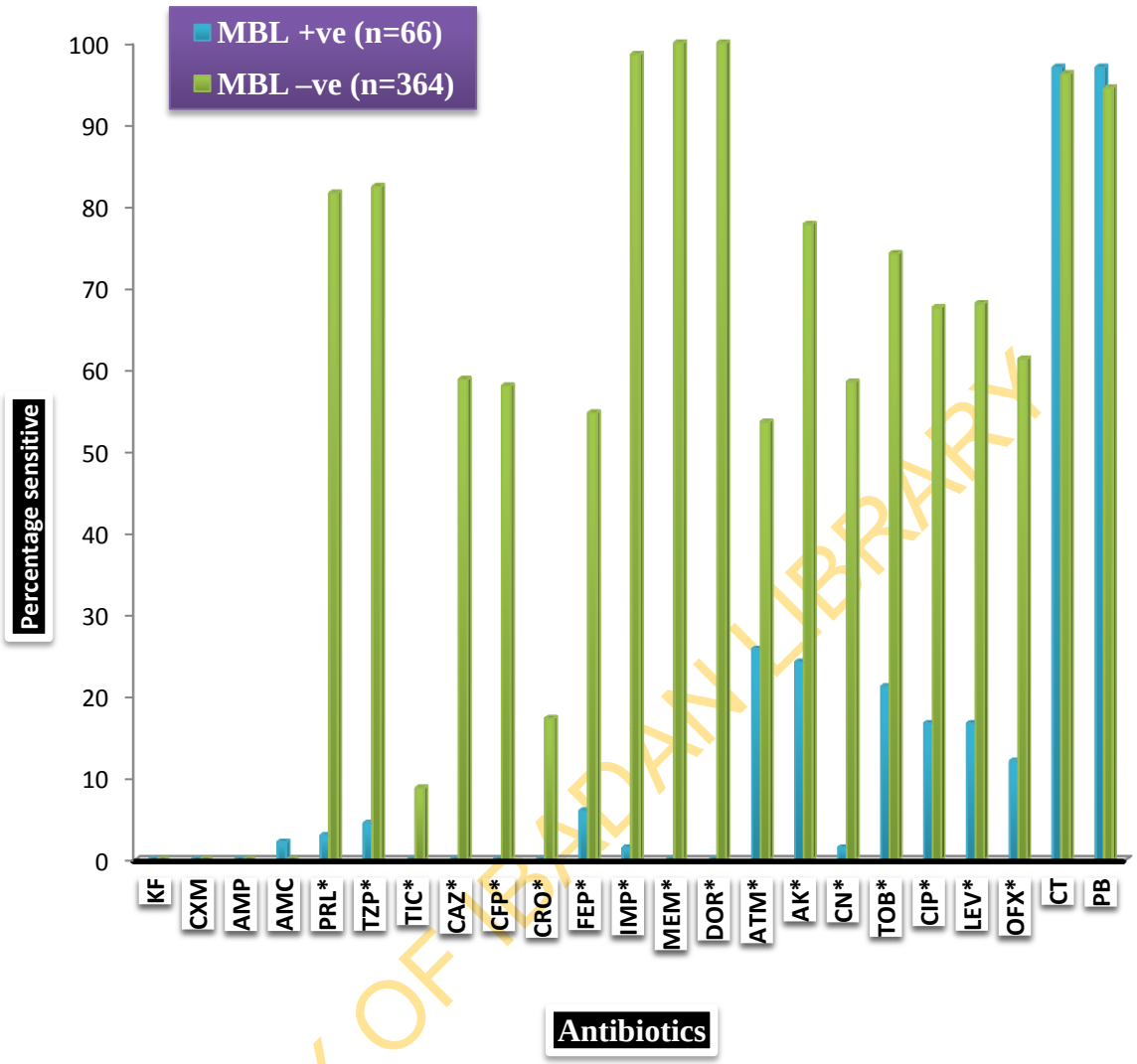

Figure 4.3. Comparison of antibiotic sensitivity among MBL-positive and MBL-negative *P. aeruginosa* isolates

Key:

* = $p<0.05$, KF = Cephalothin, CXM = Cefuroxime, AMP = Ampicillin, AMC=Amoxicillin clavulanate, PRL* = Piperacillin, TZP* = Piperacillin-tazobactam, TIC* = Ticarcillin, CAZ* = Ceftazidime, PB = Polymyxin B, CFP* = Cefoperazone, CRO* = Ceftriaxone, FEP* = Cefepime, IPM* = Imipenem, CT = colistin sulphate, MEM* = Meropenem, DOR* = Doripenem, ATM* = Aztreonam, AK* = Amikacin, CN* = Gentamicin, TOB* = Tobramycin, CIP* = Ciprofloxacin, LEV* = Levofloxacin, OFX* = Ofloxacin.

Table 4.6a. Effect of 100 µg/mL ethidium bromide on the susceptibility of carbapenem-resistant strains of *P. aeruginosa* to imipenem and meropenem

Treated strains of *P. aeruginosa*	Imipenem (µg/mL)					Meropenem (µg/mL)				
	0.5	1	2	4	8	0.5	1	2	4	8
PS093										
Colony 1	8	11	14	18	22	8	13	17	19	20
Colony 2	8	11	14	20	22	8	13	17	19	21
Colony 3	8	16	18	20	22	8	14	17	18	20
Colony 4	8	17	20	21	23	8	14	17	19	23
Control	8	8	8	8	8	8	8	8	8	8
PS146										
Colony 1	8	12	18	20	23	8	13	16	19	20
Colony 2	8	12	20	20	23	8	13	16	19	20
Colony 3	8	13	19	22	24	8	13	16	19	21
Colony 4	8	12	19	22	24	8	13	15	15	20
Control	8	8	8	8	8	8	8	8	8	8
PS150										
Colony 1	8	14	16	20	24	8	15	20	22	23
Colony 2	8	13	16	20	25	8	15	20	19	21
Colony 3	8	12	16	16	23	8	16	20	18	21
Colony 4	8	12	16	18	24	8	16	19	19	22
Control	8	8	8	8	8	8	8	8	8	8
PS154										
Colony 1	8	13	15	18	26	8	12	14	17	21
Colony 2	8	13	15	19	27	8	13	14	16	21
Colony 3	8	14	16	19	26	8	13	15	16	24
Colony 4	8	13	16	20	26	8	12	14	17	23
Control	8	8	8	8	8	8	8	8	8	8
PS168										
Colony 1	8	13	18	20	24	8	14	18	20	23
Colony 2	8	13	18	20	25	8	14	17	20	23
Colony 3	8	13	17	19	25	8	13	17	20	24
Colony 4	8	13	17	20	24	8	14	18	21	24
Control	8	8	8	8	8	8	8	8	8	8

Key: Diameter of cork borer = 8 mm

Table 4.6a. Effect of 100 µg/mL ethidium bromide on the susceptibility of carbapenem-resistant strains of *P. aeruginosa* to imipenem and meropenem (cont'd)

Treated strains of *P. aeruginosa*	Imipenem (µg/mL)					Meropenem (µg/mL)				
	0.5	1	2	4	8	0.5	1	2	4	8
PS184										
Colony 1	8	13	16	18	22	8	14	17	19	23
Colony 2	8	14	16	18	23	8	13	17	19	24
Colony 3	8	14	17	17	22	8	14	17	18	23
Colony 4	8	13	16	18	22	8	14	17	19	25
Control	8	8	8	8	8	8	8	8	8	8
PS185										
Colony 1	8	13	18	20	24	8	12	16	19	20
Colony 2	8	12	18	20	25	8	13	15	18	21
Colony 3	8	13	17	19	27	8	12	16	19	20
Colony 4	8	13	17	19	24	8	13	15	15	20
Control	8	8	8	8	8	8	8	8	8	8
PS202										
Colony 1	10	14	18	20	24	10	13	16	18	21
Colony 2	11	13	17	20	25	10	13	15	18	22
Colony 3	11	14	16	19	23	10	13	16	19	22
Colony 4	12	14	16	18	24	10	13	16	19	23
Control	8	8	8	8	8	8	8	8	8	8
PS293										
Colony 1	8	13	15	18	22	8	13	16	20	22
Colony 2	8	13	15	19	24	8	14	16	19	22
Colony 3	8	14	16	19	23	8	13	15	17	19
Colony 4	8	13	16	20	23	8	14	15	17	19
Control	8	8	8	8	8	8	8	8	8	8
PS335										
Colony 1	8	13	15	18	26	8	14	16	18	23
Colony 2	8	13	15	19	27	8	14	16	19	25
Colony 3	8	14	16	19	26	8	14	16	19	23
Colony 4	8	13	16	20	26	8	14	17	20	22
Control	8	8	8	8	8	8	8	8	8	8

Key: Diameter of cork borer = 8 mm

Table 4.6b. Effect of 50 µg/mL ethidium bromide on the susceptibility of carbapenem-resistant strains of *P. aeruginosa* to imipenem and meropenem

Treated strains of *P. aeruginosa*	Imipenem (µg/mL)					Meropenem (µg/mL)				
	0.5	1	2	4	8	0.5	1	2	4	8
PS093										
Colony 1	8	11	14	18	20	8	12	16	19	20
Colony 2	8	11	14	16	18	8	12	17	19	21
Colony 3	8	12	14	16	19	8	12	17	18	20
Colony 4	8	13	14	17	20	8	12	17	19	20
Control	8	8	8	8	8	8	8	8	8	8
PS146										
Colony 1	8	12	15	16	19	8	12	16	19	20
Colony 2	8	12	14	16	18	8	12	16	19	21
Colony 3	8	12	15	16	19	8	13	16	19	20
Colony 4	8	12	15	16	19	8	14	15	15	20
Control	8	8	8	8	8	8	8	8	8	8
PS150										
Colony 1	8	11	14	16	18	8	13	18	19	21
Colony 2	8	11	14	16	18	8	14	17	19	21
Colony 3	10	13	14	17	17	8	14	17	18	20
Colony 4	8	12	14	16	18	8	13	17	19	20
Control	8	8	8	8	8	8	8	8	8	8
PS154										
Colony 1	8	11	13	16	18	10	14	17	19	21
Colony 2	8	11	13	17	19	10	13	17	19	22
Colony 3	8	12	13	16	18	10	13	17	19	21
Colony 4	8	12	14	16	19	10	14	16	19	22
Control	8	8	8	8	8	8	8	8	8	8
PS168										
Colony 1	8	14	16	18	19	10	12	15	18	21
Colony 2	8	13	15	18	20	9	13	15	17	21
Colony 3	8	14	15	19	21	9	12	15	17	21
Colony 4	8	15	16	20	21	10	12	16	19	21
Control	8	8	8	8	8	8	8	8	8	8

Key: Diameter of cork borer = 8 mm

Table 4.6b. Effect of 50 µg/mL ethidium bromide on the susceptibility of carbapenem-resistant strains of *P. aeruginosa* to imipenem and meropenem (cont'd)

Treated strains of *P. aeruginosa*	Imipenem (µg/mL)					Meropenem (µg/mL)				
	0.5	1	2	4	8	0.5	1	2	4	8
PS184										
Colony 1	10	12	15	16	20	10	11	14	16	20
Colony 2	10	13	14	16	21	11	12	14	15	21
Colony 3	9	12	13	15	21	10	12	14	17	20
Colony 4	10	12	14	16	21	10	11	14	17	20
Control	8	8	8	8	8	8	8	8	8	8
PS185										
Colony 1	8	13	16	18	20	9	13	16	19	20
Colony 2	8	13	16	17	20	0	13	16	18	20
Colony 3	8	13	15	18	20	0	13	16	19	22
Colony 4	8	13	16	18	21	0	13	15	15	20
Control	8	8	8	8	8	8	8	8	8	8
PS202										
Colony 1	8	13	14	16	19	8	11	15	17	21
Colony 2	8	11	14	17	19	8	10	15	17	21
Colony 3	9	12	12	16	19	8	11	15	17	21
Colony4	8	11	13	16	19	8	10	15	18	21
Control	8	8	8	8	8	8	8	8	8	8
PS293										
Colony 1	9	12	15	18	23	8	13	15	18	22
Colony 2	9	13	15	19	25	9	13	15	19	24
Colony 3	9	14	16	18	25	9	14	16	19	22
Colony 4	9	13	16	20	24	8	13	16	20	24
Control	8	8	8	8	8	8	8	8	8	8
PS335										
Colony 1	8	13	15	18	23	8	13	15	18	23
Colony 2	8	13	15	18	23	8	12	15	19	23
Colony 3	8	14	16	18	23	8	12	16	19	24
Colony 4	8	13	16	18	23	8	13	16	20	24
Control	8	8	8	8	8	8	8	8	8	8

Key: Diameter of cork borer = 8 mm

4.7 Molecular identification of *Pseudomonas aeruginosa*

Molecular identification of *P. aeruginosa* was carried out by detecting *oprI* and *oprL* encoding lipoproteins which are specific for fluorescent pseudomonads and *P. aeruginosa* species, respectively using isolated DNA as a template. All the clinical isolates were positive for both *oprI* and *oprL* lipoproteins including *P. aeruginosa* ATCC 27853 which served as positive control strain while *oprI* and *oprL* genes were not amplified in *E. coli* ATCC 25922. The agarose gel electrophoresis of amplified *oprI* and *oprL* genes with amplification product of 249 and 504 bp, respectively is given in Plates 4.4a - 4.4c. Amplification of both *oprI* and *oprL* genes indicates that the isolate is *P. aeruginosa.* All the 75 isolates and standard strain (*P. aeruginosa* ATCC 27853) screened were identified as *P. aeruginosa.*

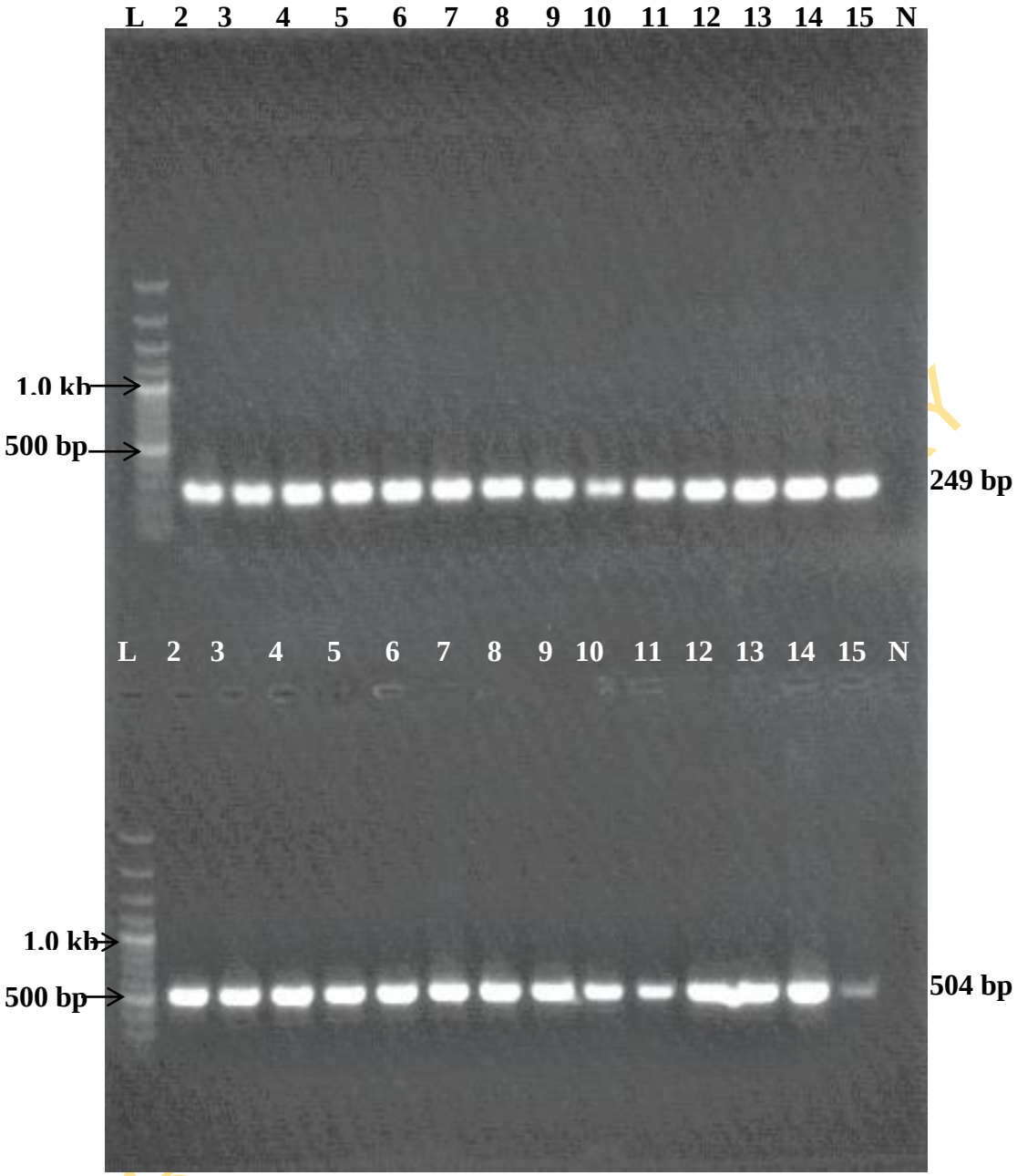

Plate 4.4a. Row 1: Agarose gel electrophoresis of PCR products (1.5%) for the identification of *Pseudomonas* spp. using *oprI* Genus specific primers L: 100 bp plus ladder; Lane 2 ATCC 27853; Lane 3-15 (PS296, PS325, PS97, PS100, PS185, PS182, PS154, PS168, PS173, PS204, PS210, PS224, PS414); N = ATCC 25922

Row 2: PCR products for the identification of *P. aeruginosa* using *oprL* species specific primers L: 100 bp plus ladder; Lane 2 ATCC 27853; Lane 3-15 (PS296, PS325, PS97, PS100, PS185, PS182, PS154, PS168, PS173, PS204, PS210, PS224, PS414), N = ATCC 25922

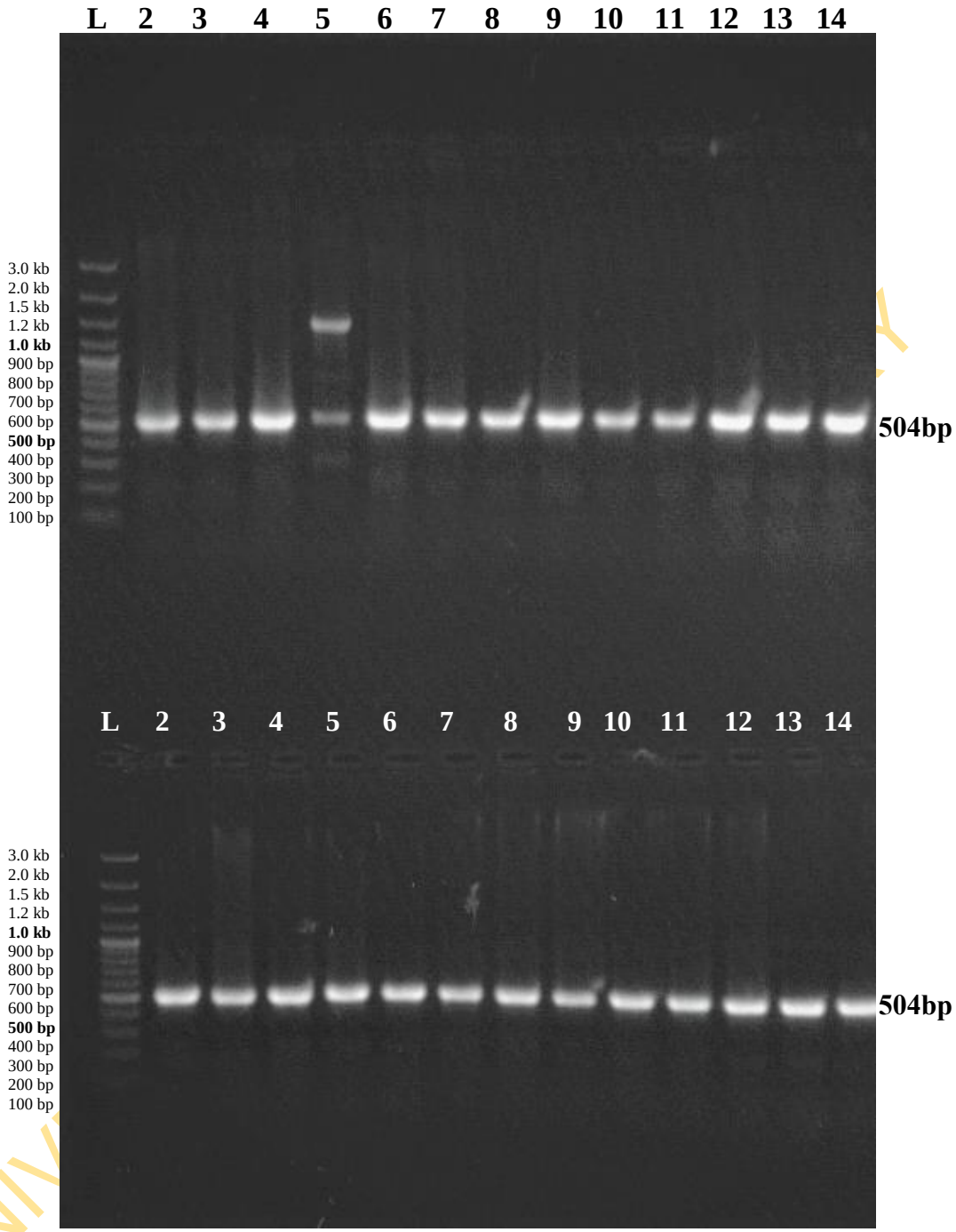

Plate 4.4b. Agarose gel electrophoresis of PCR products for the proof of identity of *P. aeruginosa* using *oprL* species specific primer L: 100 bp plus ladder; Lane 2 ATCC 27853; Row 1: Lane 3-15 (PS383, PS202, PS297, PS335, PS147, PS394, PS367, PS250, PS293, PS405, PS007, PS400, PS285); Row 2: Lane 2-14 (PS398, PS386, PS353, PS392, PS346, PS354, PS393, PS222, PS291, PS303, PS395, PS096).

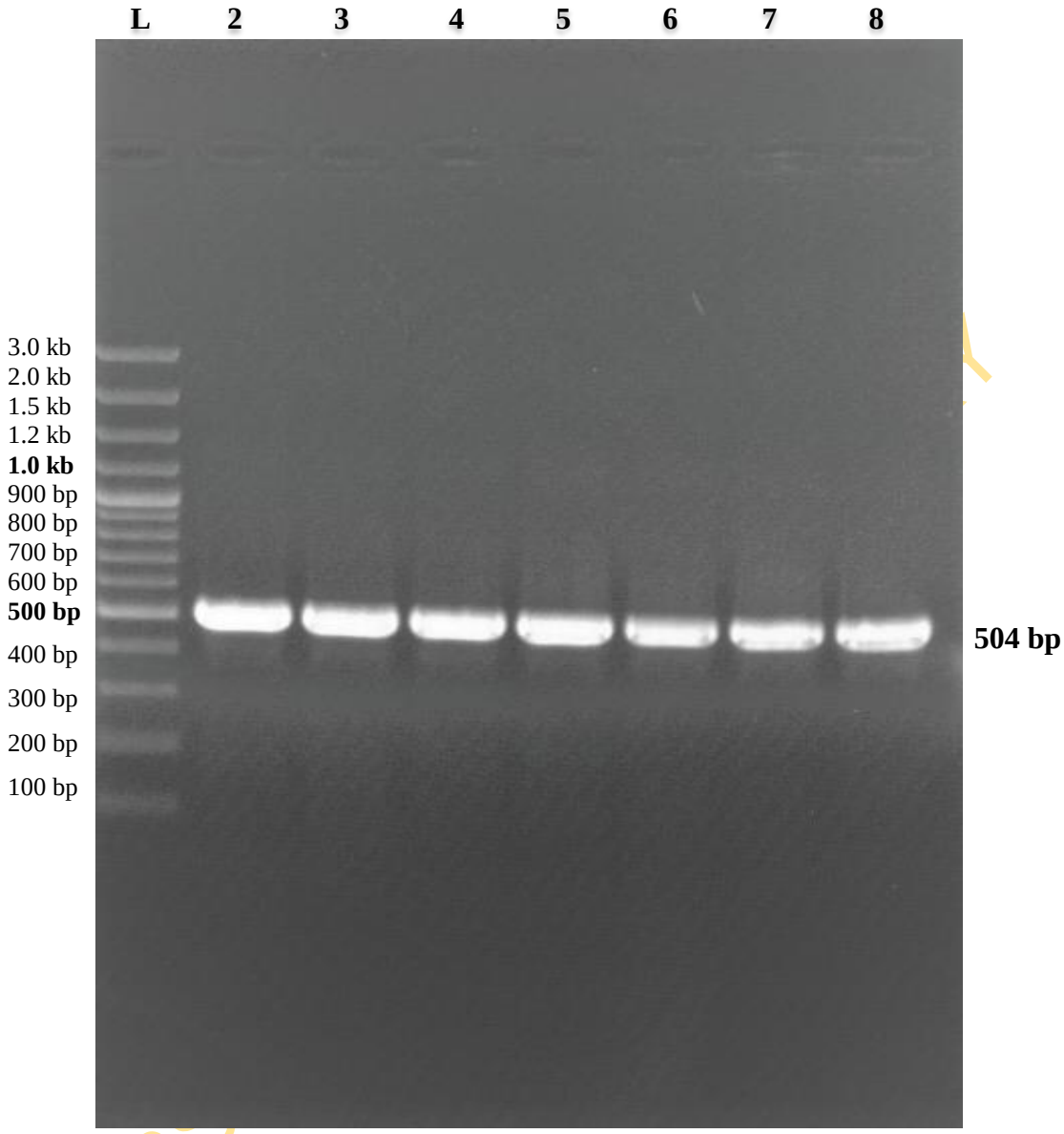

Plate 4.4c. Gel electrophoresis of PCR products for the identification of *P. aeruginosa* using *oprL* species specific primer L: 100 bp plus ladder; Lane 2-8 (PS093, PS292, PS235, PS184, PS352, PS244, PS170).

4.8 Molecular detection of class A and D carbapenemases

Carbapenem non-susceptible *P. aeruginosa* isolates were screened for the presence of class A and D carbapenemases with primers specific for bla_{SME}, bla_{NMC-A}, bla_{GES}, bla_{BIC-1} bla_{OXA-48} and bla_{OXA-58}. The result showed that none of the 73 carbapenem non-susceptible *P. aeruginosa* clinical isolates harboured at least one of bla_{SME}, bla_{GES}, bla_{NMC-A}, and bla_{BIC-1} genes. Class D carbapenemases (bla_{OXA-48} and bla_{OXA-58}) were not detected in any of the seventy-three carbapenem non-susceptible *P. aeruginosa*.

4.9 Amplification of MBL-resistance genes in carbapenem-resistant *P. aeruginosa*

Seventy-three carbapenem non-susceptible *P. aeruginosa* strains were subjected to PCR to detect genes encoding metallo beta lactamases which include bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{SPM}, bla_{SIM}, bla_{NDM}, bla_{GIM}, bla_{AIM} and bla_{DIM}. Only bla_{VIM} and bla_{NDM} genes were detected. Agarose gel electrophoresis of amplified bla_{VIM} and bla_{NDM} genes were shown in Plates 4.5a – 4.5c and 4.6a -4.6c, respectively. The prevalence of MBL genes in clinical isolates of *P. aeruginosa* was 14.7% (8.1% bla_{VIM} and 8.6% bla_{NDM}) while among carbapenem-resistant clinical isolates of *P. aeruginosa*, prevalence of 86.3% was found. The bla_{VIM} was present in 48.0% carbapenem-resistant clinical isolates of *P. aeruginosa* while 50.7% isolates possessed bla_{NDM}. Co-existence of bla_{VIM} and bla_{NDM} was found in 9 (12.3%) isolates which include 1 (4.4%) from UCHI, 1 (12.5%) from LTHO, 2 (16.7%) from OTHI, 3 (21.4%) from FMCO and 2 (25.0%) from FMCI. None of the isolates from FMCA and OTHS co-harboured bla_{VIM} and bla_{NDM}.genes (Table 4.7).

Table 4.8 shows the distribution of metallo beta-lactamase genes according to hospital. Among 23 carbapenem non-susceptible isolates from UCHI, 7 (30.4%) carried bla_{VIM} gene, 8 (34.8%) possessed bla_{NDM}, only 1 (4.4%) carried both bla_{VIM} and bla_{NDM}, while 7 (30.4%) possessed neither bla_{VIM} nor bla_{NDM}. Out of two carbapenem non-susceptible *P. aeruginosa* from FMCA, 1 (50.0%) had bla_{VIM}, the remaining one (50.0%) isolate neither possessed bla_{VIM} nor bla_{NDM}. Out of five carbapenem non-susceptible *P. aeruginosa* from OTHS, 2 (40.0%) had bla_{VIM}, 3 (60.0%) had bla_{NDM}. Among 13 carbapenem non-susceptible *P. aeruginosa* from OTHI, 4 (33.3%) possessed bla_{VIM} and 6 (50.0%) carried bla_{NDM}. No MBL gene was detected in 1 (8.3%) isolate. Out of eight carbapenem non-susceptible *P. aeruginosa* from LTHO, 1 (12.5%) possessed bla_{VIM} and 6 (75.0%) carried bla_{NDM}. Out of 14 carbapenem non-

susceptible from FMCO, *bla*$_{VIM}$ and *bla*$_{NDM}$ genes were found in 7 (50.0%) and 3 (21.4%) isolates, respectively while only 1 (7.1%) isolate was negative for MBL. Among eight carbapenem non-susceptible *P. aeruginosa* isolates from FMCI, *bla*$_{VIM}$ and *bla*$_{NDM}$ were found in 3 (37.5%) each, while 2 (25.0%) had both *bla*$_{VIM}$ and *bla*$_{NDM}$ genes. Table 4.9 shows the distribution of MBL genes in carbapenem-resistant *P. aeruginosa* in relation to clinical source. The *bla*$_{VIM}$ and *bla*$_{NDM}$ were each found in 21 (37.5%) of CRPA isolates from wound while 8 (14.3%) had both *bla*$_{VIM}$ and *bla*$_{NDM}$.

Table 4.10 shows the MIC of CRPA isolates relative to the type of MBL gene they contain. Majority of the isolates habouring MBL-resistance genes had MIC value of >64 µg/mL against imipenem and meropenem. Only three isolates out of ten isolates that do not harbour MBL-resistance genes had MIC value of >64 µg/mL towards meropenem while only one isolate with no MBL-resistance gene had MIC of >64 µg/mL against imipenem.

4.10 Analysis of sequenced PCR products

4.10.1 Alignments of *bla*$_{VIM}$ and *bla*$_{NDM}$ sequenced amplicons using Basic Local Alignment Search Tools (BLASTn)

Sequenced amplicons of nine *bla*$_{VIM}$ and nine *bla*$_{NDM}$ submitted to Basic Local Alignment Search Tools (BLASTn) on National Center for Biotechnological Information (NCBI**)** website to determine their identity with BLASTN 2.9.0+ program. The best BLAST Hits with the submitted nucleotide sequence were chosen based on the maximum similarity with the one in the GenBank database.

All the nine *P. aeruginosa* strains PS183, PS152, PS335, PS184, PS367, PS154, PS303, PS395 and PS285, *bla*$_{NDM-1}$ sequences showed 100% similarity with *Pseudomonas aeruginosa* strain Hana9 NDM family beta-lactamase (*bla*$_{NDM}$) gene, partial cds with accession number MK371546 in the GenBank with 100% query cover and E-value of 0.0 (Appendix IV).

Six out of the nine sequenced *bla*$_{VIM}$ amplicons belonging to *P. aeruginosa* strains, PS220, PS209, PS210, PS243, PS168 and PS291 gave maximum similarity of 99.73% with *bla*$_{VIM-5}$ complete coding sequence of *Pseudomonas aeruginosa* strain B43647 metallo-beta-lactamase VIM-5 (*bla*$_{VIM-5}$) gene complete cds (MK209000) in the GenBank. When compared with *bla*$_{VIM-5}$ complete coding sequence of *Pseudomonas*

aeruginosa strain B43647 metallo-beta-lactamase VIM-5 (*bla*$_{VIM-5}$) gene complete cds (MK209000) in the GenBank, there was silent mutation at only one position (321) in *bla*$_{VIM-5}$ nucleotide sequences where A was substituted with G (GT**A** to GT**G** which codes for valine).

The remaining three sequenced *bla*$_{VIM}$ amplicons belonging to *P. aeruginosa* strains PS219, PS204 and PS097 gave maximum identity of 99.48%, 99.46% and 99.23%, respectively with *bla*$_{VIM-5}$ complete coding sequence of *Pseudomonas aeruginosa* strain B43647 metallo-beta-lactamase VIM-5 (*bla*$_{VIM-5}$) gene complete cds (MK209000) in the GenBank. When compared with *bla*$_{VIM-5}$ complete coding sequence of *Pseudomonas aeruginosa* strain B43647 metallo-beta-lactamase VIM-5 (*bla*$_{VIM-5}$) gene complete cds (MK209000) in the GenBank, the following were observed:

1. Nucleotide sequence of PS209 showed silent mutations at three positions in the nucleotide sequence. The first mutation occurs at position 321 where A was replaced with G (GT**A** to GT**G** which codes for valine). The second mutation occurs at position 375 where G was substituted with T (GC**G** to GC**T** which codes for alanine) and third one occurs at position 381 where T was replaced with C (CC**T** to CC**C** which codes for proline).

2. Nucleotide sequence of PS219 also showed silent mutations in the nucleotide at two positions: 321 (GT**A** to GT**G** which codes for valine) and 375 (GC**G** to GC**T** which codes for alanine).

3. Nucleotide sequence of PS204 also showed silent mutation in the nucleotide at position 321 (GT**A** to GT**G** which codes for valine) while at nucleotide position 161, missense mutation occurs where G was substituted with T which changed the amino acid from leucine (T**T**G) to tryptophan (T**G**G) (Appendix IV).

4.10.2 GenBank accession numbers of sequenced *bla*$_{NDM-1}$ and *bla*$_{VIM-5}$ genes

The *bla*$_{NDM}$ sequences of PS303, PS184, PS395, PS152, PS335, PS154, PS285, PS367 and PS183 *P. aeruginosa* strains submitted to the GenBank were assigned the following accession numbers: MN193051, MN193052, MN193053, MN193054, MN193055, MN193056, MN193057, MN193058 and MN193059, respectively.

The *bla*$_{VIM}$ sequences of PS168, PS220, PS219, PS210, PS243, PS291, PS209, PS097 and PS204 *P. aeruginosa* strains submitted to the GenBank were assigned the following accession numbers: MN201592, MN201593, MN201594, MN201595, MN201596, MN201597, MN201598, MN201599 and MN201600, respectively.

4.11 Transformation experiments

Agarose gel electrophoresis gave bands that correspond to *bla*$_{VIM}$ and *bla*$_{NDM.}$ Therefore *bla*$_{VIM}$ and *bla*$_{NDM}$ was successfully transferred into *E. coli* DH5α (Plate 4.7).

Table 4.7. Molecular detection of Class B Carbapenemases (MBLs) in carbapenem-resistant *P. aeruginosa*

S/N	Isolate	*bla*$_{IMP}$	*bla*$_{VIM}$	*bla*$_{SPM}$	*bla*$_{SIM}$	*bla*$_{GIM}$	*bla*$_{NDM}$	*bla*$_{AIM}$	*bla*$_{DIM}$
1.	PS007	-	-	-	-	-	-	-	-
2.	PS022	-	+	-	-	-	-	-	-
3.	PS088	-	-	-	-	-	-	-	-
4.	PS093	-	+	-	-	-	-	-	-
5.	PS096	-	+	-	-	-	-	-	-
6.	PS097	-	+	-	-	-	-	-	-
7.	PS099	-	-	-	-	-	-	-	-
8.	PS100	-	+	-	-	-	-	-	-
9.	PS146	-	-	-	-	-	+	-	-
10.	PS147	-	+	-	-	-	-	-	-
11.	PS150	-	-	-	-	-	-	-	-
12.	PS152	-	-	-	-	-	+	-	-
13.	PS154	-	-	-	-	-	+	-	-
14.	PS166	-	-	-	-	-	-	-	-
15.	PS168	-	+	-	-	-	-	-	-
16.	PS170	-	-	-	-	-	-	-	-
17.	PS172	-	+	-	-	-	-	-	-
18.	PS173	-	+	-	-	-	-	-	-
19.	PS181	-	-	-	-	-	+	-	-
20.	PS182	-	-	-	-	-	-	-	-
21.	PS183	-	-	-	-	-	+	-	-
22.	PS184	-	-	-	-	-	+	-	-
23.	PS185	-	-	-	-	-	-	-	-
24.	PS202	-	+	-	-	-	-	-	-
25.	PS204	-	+	-	-	-	-	-	-

Key: + = present; - = absent

Table 4.7. Molecular detection of Class B Carbapenemases (MBLs) in carbapenem-resistant *P. aeruginosa* (Cont'd)

S/N	Isolate	*bla*$_{IMP}$	*bla*$_{VIM}$	*bla*$_{SPM}$	*bla*$_{SIM}$	*bla*$_{GIM}$	*bla*$_{NDM}$	*bla*$_{AIM}$	*bla*$_{DIM}$
26.	PS205	-	+	-	-	-	-	-	-
27.	PS209	-	+	-	-	-	-	-	-
28.	PS210	-	+	-	-	-	-	-	-
29.	PS219	-	+	-	-	-	-	-	-
30.	PS220	-	+	-	-	-	-	-	-
31.	PS222	-	+	-	-	-	-	-	-
32.	PS224	-	+	-	-	-	+	-	-
33.	PS229	-	-	-	-	-	+	-	-
34.	PS230	-	-	-	-	-	-	-	-
35.	PS235	-	-	-	-	-	-	-	-
36.	PS238	-	-	-	-	-	+	-	-
37.	PS243	-	+	-	-	-	-	-	-
38.	PS244	-	-	-	-	-	-	-	-
39.	PS246	-	+	-	-	-	-	-	-
40.	PS250	-	+	-	-	-	+	-	-
41.	PS253	-	+	-	-	-	-	-	-
42.	PS285	-	-	-	-	-	+	-	-
43.	PS291	-	+	-	-	-	-	-	-
44.	PS292	-	-	-	-	-	+	-	-
45.	PS293	-	+	-	-	-	+	-	-
46.	PS294	-	+	-	-	-	-	-	-
47.	PS296	-	+	-	-	-	+	-	-
48.	PS297	-	+	-	-	-	-	-	-
49.	PS303	-	-	-	-	-	+	-	-
50.	PS325	-	+	-	-	-	-	-	-

Key: + = present; - = absent

Table 4.7. Molecular detection of Class B Carbapenemases (MBLs) in carbapenem-resistant *P. aeruginosa* (Cont'd)

S/N	Isolate	*bla*$_{IMP}$	*bla*$_{VIM}$	*bla*$_{SPM}$	*bla*$_{SIM}$	*bla*$_{GIM}$	*bla*$_{NDM}$	*bla*$_{AIM}$	*bla*$_{DIM}$
51.	PS335	-	-	-	-	-	+	-	-
52.	PS346	-	-	-	-	-	+	-	-
53.	PS348	-	-	-	-	-	+	-	-
54.	PS349	-		-	-	-	-	-	-
55.	PS350	-	+	-	-	-	-	-	-
56.	PS352	-	-	-	-	-	+	-	-
57.	PS353	-	+	-	-	-	-	-	-
58.	PS354	-	-	-	-	-	+	-	-
59.	PS367	-	-	-	-	-	+	-	-
60.	PS383	-	-	-	-	-	+	-	-
61.	PS384	-	-	-	-	-	-	-	-
62.	PS386	-	-	-	-	-	+	-	-
63.	PS392	-	+	-	-	-	-	-	-
64.	PS393	-	-	-	-	-	+	-	-
65.	PS394	-	-	-	-	-	-	-	-
66.	PS395	-	-	-	-	-	+	-	-
67.	PS396	-	+	-	-	-	-	-	-
68.	PS397	-	-	-	-	-	-	-	-
69.	PS398	-	-	-	-	-	-	-	-
70.	PS400	-	-	-	-	-	+	-	-
71.	PS405	-	+	-	-	-	+	-	-
72.	PS409	-	+	-	-	-	-	-	-
73.	PS414	-	-	-	-	-	+	-	-
74.	PS351	-	-	-	-	-	-	-	-
75.	PS423	-	-	-	-	-	-	-	-
76.	ATCC 27853	-	-	-	-	-	-	-	-

Key: + = present; - = absent

Table 4.8. Distribution of metallo beta-lactamase (MBL) genes in carbapenem-resistant *P. aeruginosa* according to hospital

Hospital	MBL gene(s)			
	***bla*$_{VIM}$**	***bla*$_{NDM}$**	***bla*$_{VIM}$ and *bla*$_{NDM}$**	**MBL absent**
UCH (23)	7 (30.4%)	8 (34.8%)	1 (4.4%)	7 (30.4%)
FMCA (2)	1 (50.0%)	-	-	1 (50.0%)
OTHS (5)	2 (40.0%)	3 (60.0%)	-	-
OTHI (13)	4 (33.3%)	6 (50.0%)	2 (16.7%)	1 (8.3)
LTHO (8)	1 (12.5%)	6 (75.0%)	1 (12.5%)	-
FMCO (14)	7 (50.0)	3 (21.4)	3 (21.4)	1 (7.1)
FMCI (8)	3 (37.5)	3 (37.5)	2 (25.0)	-

Key:

- = absence of amplified gene

UCHI = University College Hospital, Ibadan

FMCA = Federal Medical Centre, Abeokuta

OTHS = Olabisi Onabanjo University Teaching Hospital, Sagamu

OTHI = Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife

LTHO = Ladoke Akintola University Teaching Hospital, Osogbo

FMCO = Federal Medical Centre, Owo

FMCI = Federal Medical Centre, Ido-Ekiti

Table 4.9. Distribution of metallo beta-lactamase (MBL) genes in carbapenem-resistant *P. aeruginosa* according to clinical source

MBL(s)	Clinical source (no (%))				
	Wound (56)	**Tracheal aspirate (2)**	**Ear (5)**	**Urine (8)**	**Wound biopsy (2)**
bla_{VIM}	21 (37.5)	1 (50.0)	2 (40.0)	2 (25.0)	-
bla_{NDM}	21 (37.5)	1 (50.0)	1 (20.0)	3 (37.5)	2 (100.0)
bla_{VIM} and bla_{NDM}	8 (14.3)	-	1 (20.0)		-
MBL absent	6 (10.7)	-	1 (20.0)	3 (37.5)	-

Key:

- = absence of amplified gene

Table 4.10. MIC of selected antibiotics against carbapenem-resistant clinical isolates of *P. aeruginosa* in relation to type of MBL gene possessed

S/N	Isolate	Sample	Clinical source	Ipm	Mem	Fep	Caz	Cip	MBL(s) gene
1.	PS007	UCHI	Wound	16	16	>128	>256	128	-
2.	PS022	UCHI	Tracheal aspirate	>64	>64	128	128	32	bla_{VIM}
3.	PS088	FMCA	Wound	32	16	4	128	16	-
4.	PS093	FMCA	Ear	2	8	16	128	>128	bla_{VIM}
5.	PS096	FMCO	Wound	4	16	16	>256	0.0313	bla_{VIM}
6.	PS097	FMCO	Wound	8	32	32	256	0.0313	bla_{VIM}
7.	PS099	FMCO	Wound	8	8	16	256	4	-
8.	PS100	FMCO	Wound	4	8	16	256	8	bla_{VIM}
9.	PS146	UCHI	Urine	>64	>64	>128	>256	>128	bla_{NDM}
10	PS147	UCHI	Urine	16	64	32	256	16	bla_{VIM}
11	PS150	UCHI	Urine	>64	>64	>128	>256	>128	-
12	PS152	UCHI	Tracheal aspirate	32	>64	>128	>256	>128	bla_{NDM}
13	PS154	UCHI	Urine	32	>64	1	>256	>128	bla_{NDM}
14	PS166	UCHI	Urine	16	2	1	4	>128	-
15	PS168	UCHI	Wound	4	16	16	>256	8	bla_{VIM}
16	PS170	UCHI	Ear	8	16	8	>256	32	-
17	PS172	OTHS	Ear pus	16	16	4	16	0.0625	bla_{VIM}
18	PS173	OTHS	Wound	16	16	4	128	0.0625	bla_{VIM}
19	PS181	UCHI	Wound	16	64	>128	>256	>128	bla_{NDM}
20	PS182	UCHI	Wound	4	8	4	4	16	-
21	PS183	UCHI	Wound	16	64	>128	>256	>128	bla_{NDM}
22	PS184	UCHI	Wound biopsy	16	64	>128	>256	>128	bla_{NDM}
23	PS185	UCHI	Wound	4	>64	>128	>256	>128	-
24	PS202	OTHS	Wound	8	16	16	>256	32	bla_{VIM}
25	PS204	UCHI	Wound	>64	>64	>128	>256	>128	bla_{VIM}

Key: Ipm = Imipenem, Caz = Ceftazidime, Mem = Meropenem, Fep = Cefepime, Cro = Ceftriaxone, Ciprofloxacin; - = absence of MBL genes

Table 4.10. MIC of selected antibiotics against carbapenem-resistant clinical isolates of *P. aeruginosa* in relation to type of MBL gene possessed (cont'd)

S/N	Isolate	Sample	Clinical source	Ipm	Mem	Fep	Caz	Cip	MBL(s) gene
26.	PS205	UCHI	Wound	32	>64	>128	>256	>128	bla_{VIM}
27.	PS209	OTHI	Wound	8	>64	32	128	4	bla_{VIM}
28.	PS210	OTHI	Wound	16	>64	32	256	8	bla_{VIM}
29.	PS219	FMCO	Wound	>64	64	64	>256	>128	bla_{VIM}
30.	PS220	FMCO	Wound	16	64	>128	32	>128	bla_{VIM}
31.	PS222	FMCO	Wound	16	32	>128	256	>128	$bla_{VIM,}$ bla_{NDM}
32.	PS224	FMCO	Wound	32	32	64	64	32	$bla_{VIM,}$ bla_{NDM}
33.	PS229	OTHS	Wound	32	>64	>128	>256	8	bla_{NDM}
34.	PS230	OTHS	Wound	16	>64	32	256	1	bla_{NDM}
35.	PS235	LTHO	Wound	>64	>64	>128	>256	>128	bla_{NDM}
36.	PS238	LTHO	Ear	>64	>64	>128	>256	>128	$bla_{VIM,}$ bla_{NDM}
37.	PS243	LTHO	Wound	64	>64	>128	>256	>128	bla_{VIM}
38.	PS244	UCHI	Wound	>64	>64	>128	>256	>128	-
39.	PS246	UCHI	Wound	>64	>64	>128	>256	>128	bla_{VIM}
40.	PS250	UCHI	Wound	>64	>64	>128	>256	>128	$bla_{VIM,}$ bla_{NDM}
41.	PS253	UCHI	Wound	>64	>64	>128	>256	>128	bla_{VIM}
42.	PS285	FMCI	Wound	>64	>64	>128	>256	>128	bla_{NDM}
43.	PS291	FMCI	Wound	2	4	128	8	64	bla_{VIM}
44.	PS292	FMCI	Wound	2	8	>128	8	64	bla_{NDM}
45.	PS293	FMCI	Wound	>64	>64	>128	>256	>128	$bla_{VIM,}$ bla_{NDM}
46.	PS294	FMCI	Wound	1	4	128	8	64	bla_{VIM}
47.	PS296	FMCI	Wound	4	16	128	16	128	$bla_{VIM,}$ bla_{NDM}
48.	PS297	FMCI	Wound	>64	>64	>128	>256	>128	bla_{VIM}
49.	PS303	FMCI	Wound	>64	>64	>128	>256	>128	bla_{NDM}
50.	PS325	FMCO	Wound	32	32	>128	>256	>128	bla_{VIM}

Key: Ipm = Imipenem, Caz = Ceftazidime, Mem = Meropenem, Fep = Cefepime, Cro = Ceftriaxone, Ciprofloxacin; - = absence of MBL genes

Table 4.10. MIC of selected antibiotics against carbapenem-resistant clinical isolates of *P. aeruginosa* in relation to type of MBL gene possessed (cont'd)

S/N	Isolate	Sample site	Clinical source	Ipm	Mem	Fep	Caz	Cip	MBL(s) gene
51.	PS335	FMCO	Ear	0.5	0.0625	4	8	0.0625	bla_{NDM}
52.	PS346	FMCO	Wound	4	2	64	128	1	bla_{NDM}
53.	PS348	OTHI	Urine	8	64	>128	>256	>128	bla_{NDM}
54.	PS349	OTHI	Urine	4	>64	>128	>256	>128	-
55.	PS350	OTHI	Urine	1	8	64	256	0.0625	bla_{VIM}
56.	PS352	OTHI	Wound	2	1	32	128	0.0625	bla_{NDM}
57.	PS353	OTHI	Wound	1	4	32	128	>128	bla_{VIM}
58.	PS354	OTHI	Wound	16	4	64	128	>128	bla_{NDM}
59.	PS367	FMCO	Wound	>64	>64	>128	>256	>128	bla_{NDM}
60.	PS383	UCHI	Wound	>64	>64	>128	>256	>128	bla_{NDM}
61.	PS384	UCHI	Wound	>64	>64	>128	>256	>128	bla_{NDM}
62.	PS386	LTHO	Wound	>64	>64	>128	>256	>128	bla_{NDM}
63.	PS392	LTHO	Wound	>64	>64	128	256	>128	bla_{NDM}
64.	PS393	LTHO	Wound	>64	>64	>128	>256	>128	bla_{NDM}
65.	PS394	LTHO	Wound	>64	>64	16	>256	>128	bla_{NDM}
66.	PS395	OTHI	Wound	>64	>64	>128	>256	>128	bla_{NDM}
67.	PS396	OTHI	Wound	>64	>64	16	256	>128	$bla_{VIM,}$ bla_{NDM}
68.	PS397	OTHI	Wound	>64	>64	>128	>256	>128	bla_{NDM}
69.	PS398	OTHI	Wound	>64	>64	>128	>256	>128	bla_{NDM}
70.	PS400	OTHI	Wound	>64	>64	>128	>256	>128	$bla_{VIM,}$ bla_{NDM}
71.	PS405	FMCO	Wound	>64	>64	>128	>256	>128	$bla_{VIM,}$ bla_{NDM}
72.	PS409	FMCO	Wound	>64	>64	>128	>256	>128	bla_{VIM}
73.	PS414	LTHO	Wound biopsy	>64	>64	>128	>256	>128	bla_{NDM}

Key: Ipm = Imipenem, Mem = Meropenem, Fep = Cefepime, Caz = Ceftazidime, Cro=Ceftriaxone, Ciprofloxacin; - = absence of MBL genes

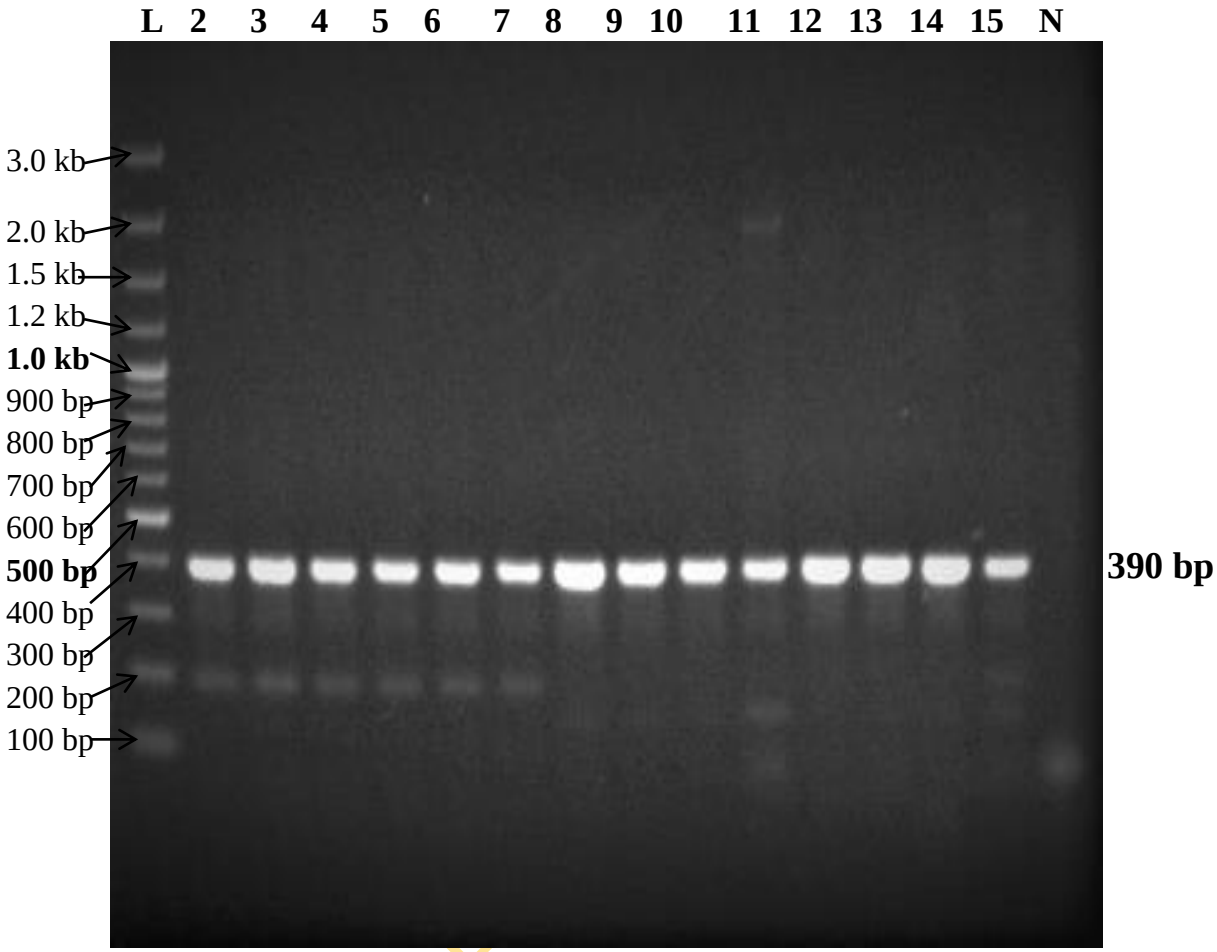

Plate 4.5a. The *bla*$_{VIM}$ PCR products on 1.5% agarose gel after electrophoresis. L = 100 bp plus ladder; Lane 2-15 shows different CRPA positive for *bla*$_{VIM}$ (PS325, PS97, PS100, PS168, PS173, PS204, PS210, PS202, PS297, PS147, PS243, PS220, PS246, PS291) N: Negative control

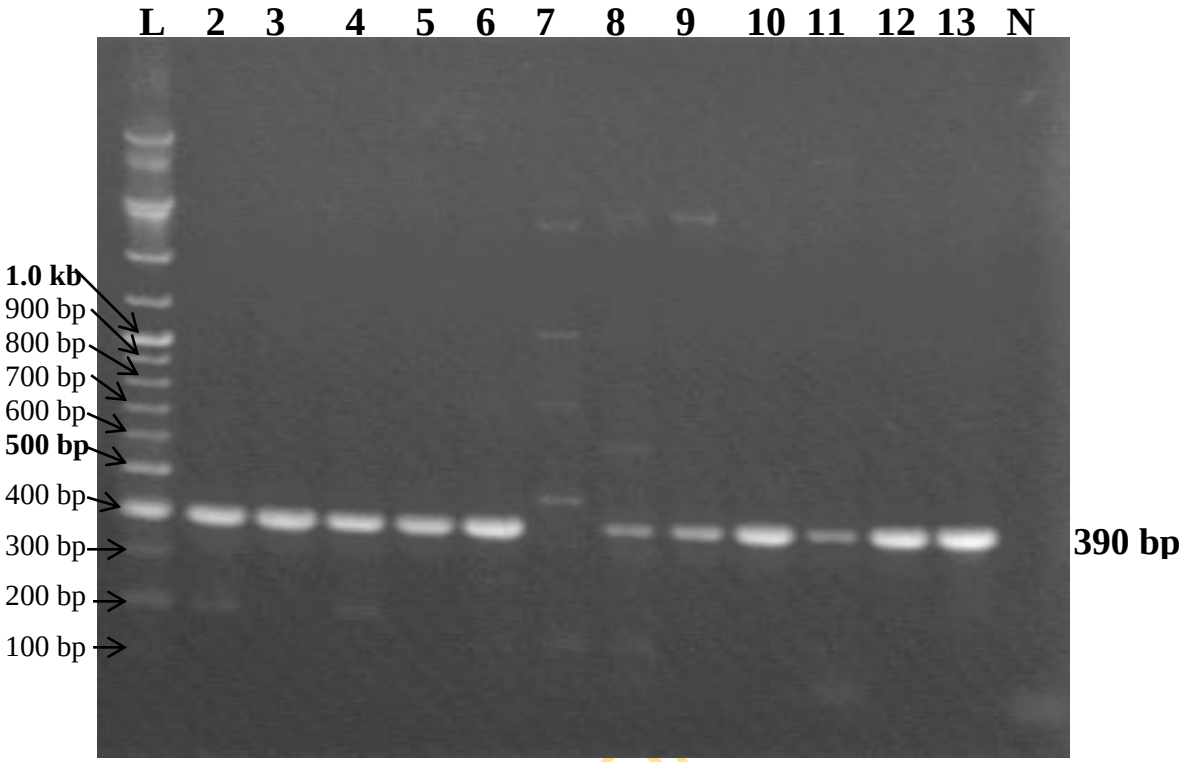

Plate 4.5b. The *bla*$_{VIM}$ PCR products on 1.5% agarose gel after electrophoresis. L = 100 bp plus ladder; Lanes 2-6,8-13 shows different CRPA positive for *bla*$_{VIM}$ (PS219, PS022, PS353, PS293, PS172, PS007, PS350, PS205, PS294, PS222, PS250); Lane 7 is a CRPA negative for *bla*$_{VIM}$ (PS185); N: Negative control

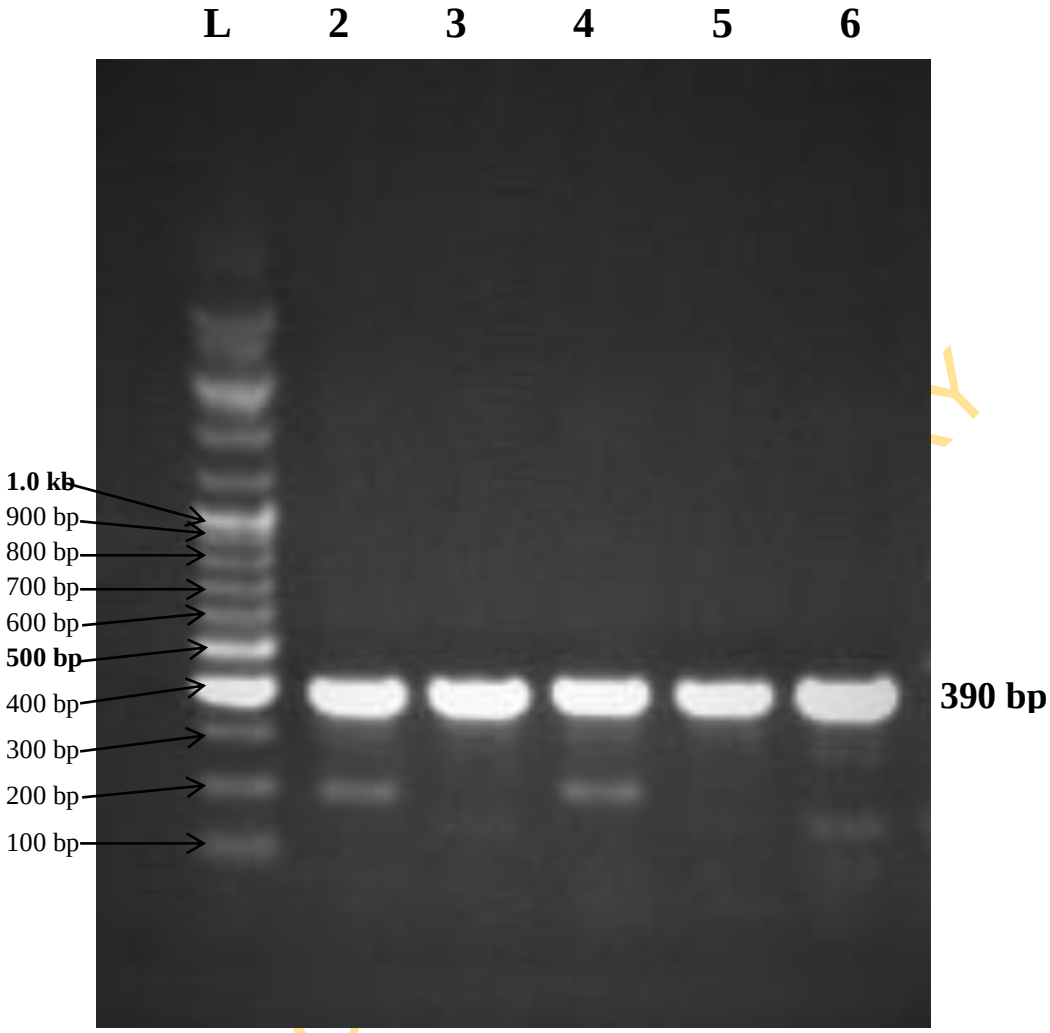

Plate 4.5c. The *bla*$_{VIM}$ PCR products on 1.5% agarose gel after electrophoresis. L = 100 bp plus ladder; Lane 2-16 shows different CRPA positive for *bla*$_{VIM}$ (PS405, PS093, PS400, PS396, PS209)

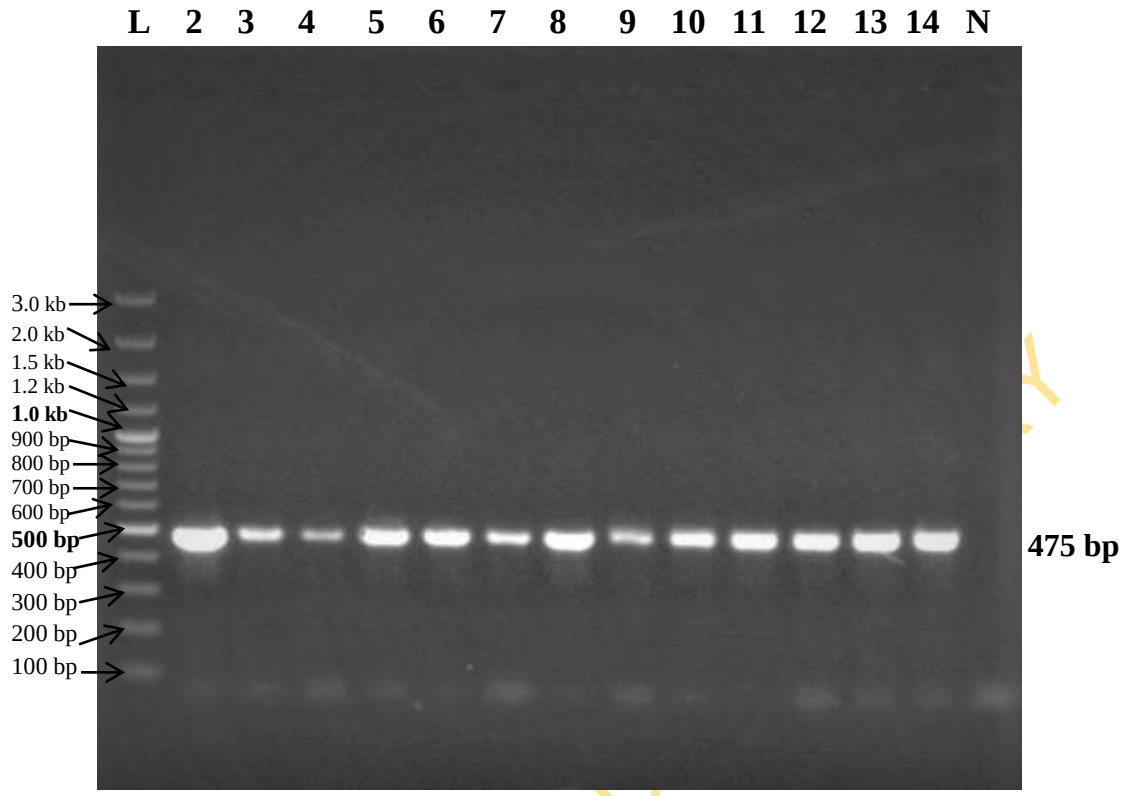

Plate 4.6a. The *bla*$_{NDM}$ PCR products on 1.5% agarose gel after electrophoresis. L = 100 bp plus ladder; Lane 2-14 shows different CRPA isolates positive for *bla*$_{NDM}$ (PS394, PS367, PS285, PS398, PS386, PS392, PS346, PS222, PS238, PS354, PS393, PS303, PS395); N: Negative control

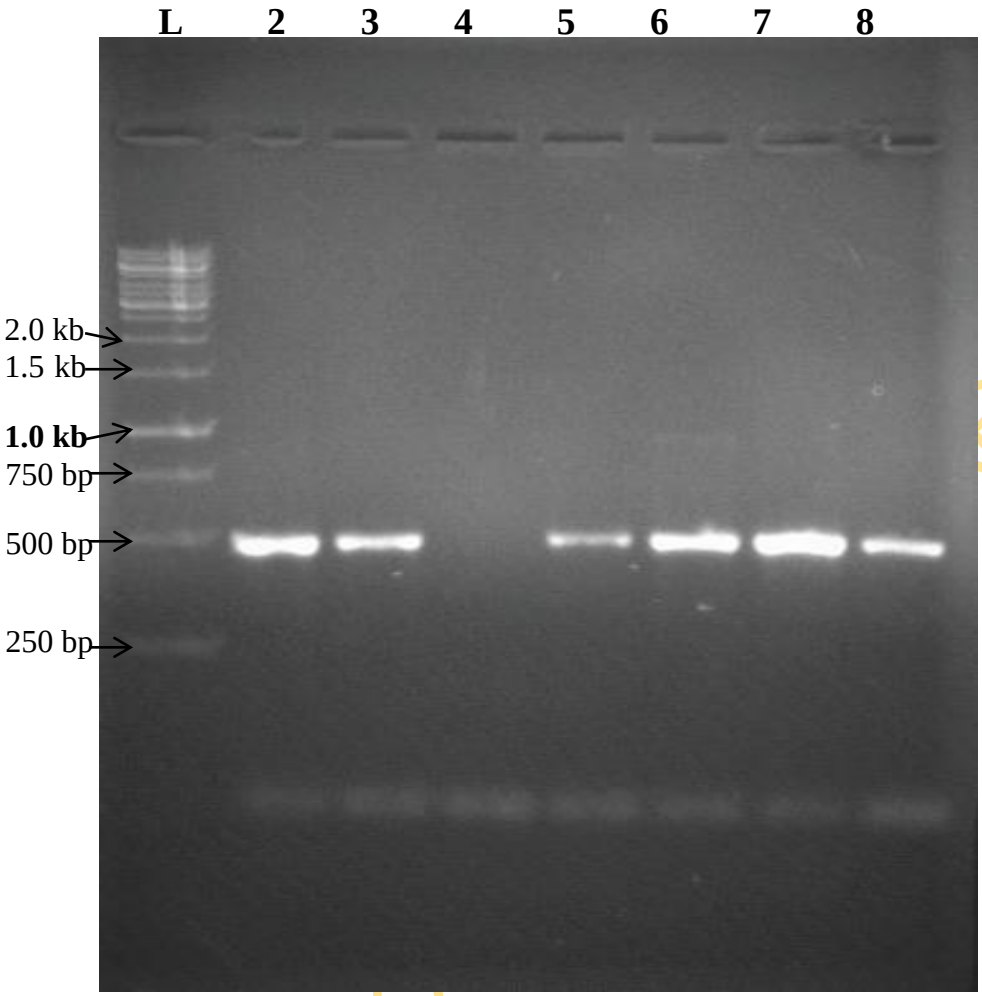

Plate 4.6b. The *bla*$_{NDM}$ PCR products on 1.5% agarose gel after electrophoresis L = 1 kb plus ladder; Lane 2, 3, 5-8 shows different CRPA isolates positive for *bla*$_{NDM}$ (PS292, PS152, PS154, PS235, PS184, PS352); Lane 4 = PS353 negative for *bla*$_{NDM}$

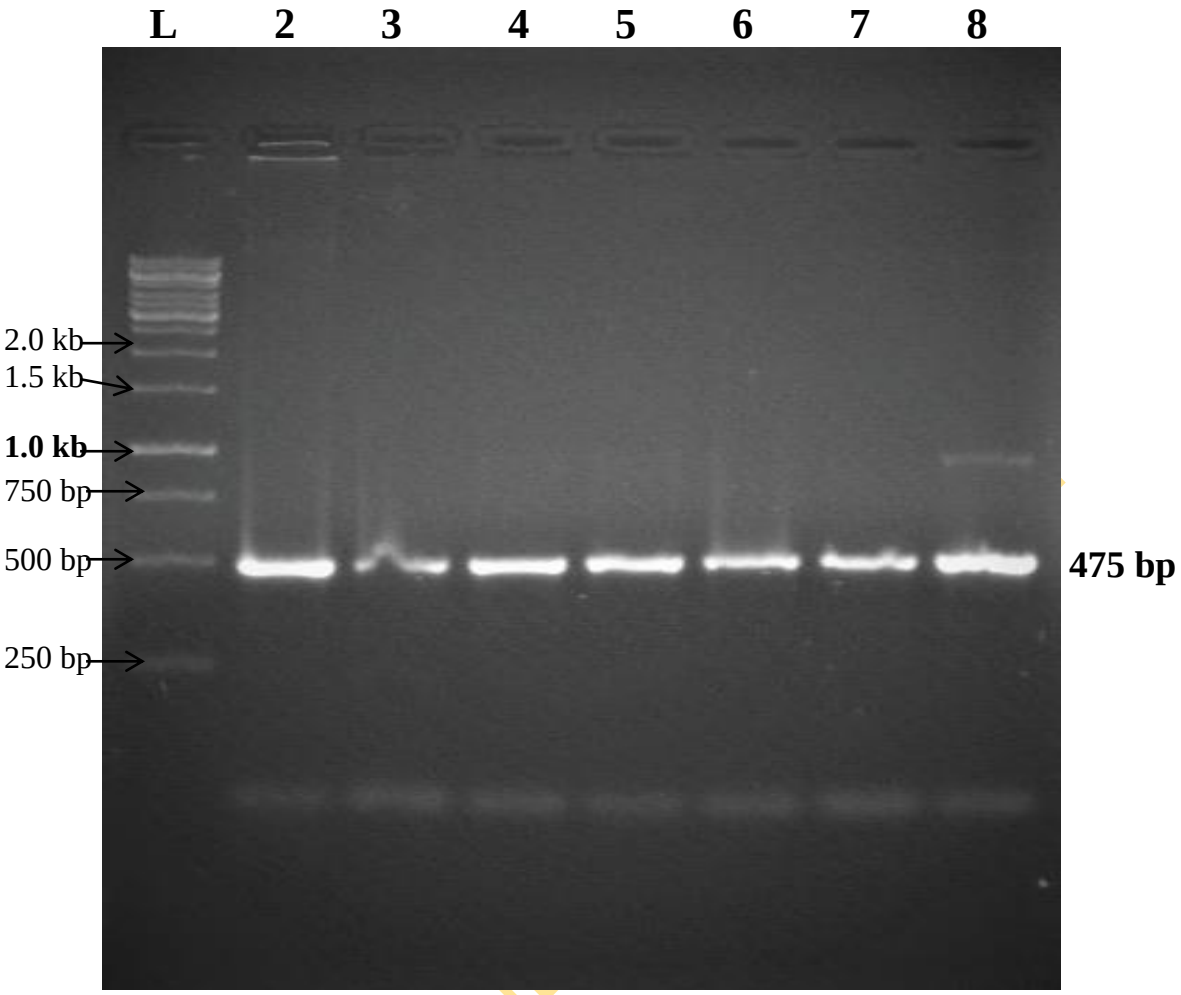

Plate 4.6c. The *bla*$_{NDM}$ PCR products on 1.5% agarose gel after electrophoresis L = 1 kb plus ladder; Lane 2-8 shows different CRPA isolates positive for *bla*$_{NDM}$ (PS181, PS296, PS230, PS397, PS348, PS293, PS396).

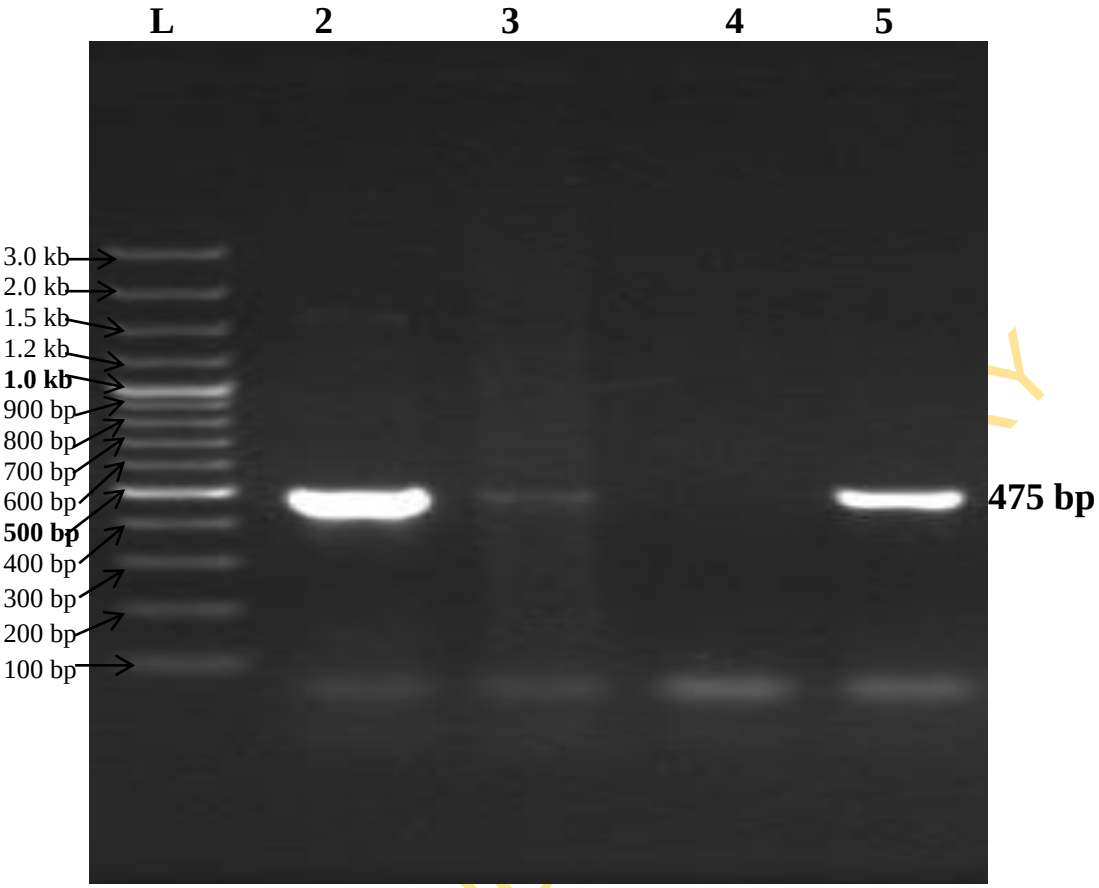

Plate 4.7. Agarose gel (1.5%) showing PCR products for *bla*$_{NDM}$ after transformation L: 100 bp plus ladder; Lanes 2,3,5 showing positive results (PS293, PS202, PS414); Lane 4 showing negative result (PS394)

4.12 Amplification of integron and integrase gene cassette

Class 1 integron only was found in 57.5% (42) carbapenem-resistant *P. aeruginosa* (CRPA) while class 1 and 2 integrons were present in 12.3% (9) (Plates 4.8a - 4.9). None of the isolates possessed class 2 integrons alone. Class 3 integrons were not found in any of the isolates. Six among ten carbapenem-resistant isolates that do not harbour MBL genes do not possess integrons while the remaining four isolates harboured class 1 integrons.

Class 1 integron was most commonly detected in 14 (69.6%) CRPA from UCHI followed by FMCI 5 (62.5%) (Table 4.11). Among the 56 CRPA isolates from wounds, 27 (48.2%) harbour only class 1 integrons while 20 (31.8%) lacked integrons. All the isolates that possessed both class 1 and 2 integrons 9 (16.1%) were from wound. Seven out of every 10 CRPA isolates from wound possessed integrons. Among CRPA isolates from urine samples, 6 (75%) out of 8 isolates harbour class 1 integrons. All CRPA from tracheal aspirate, ear and wound biopsy possessed class 1 integrons only (Table 4.12). Four (PS219, PS243, PS246 and PS396) and seven (PS230, PS346, PS384, PS386, PS392, PS397 and PS398) isolates carrying bla_{VIM} and bla_{NDM} genes, respectively, lacked integrons. Two isolates (PS396 and PS405) that harboured both bla_{VIM} and bla_{NDM} genes do not harbour integrons.

Fifty-one class 1 integron positive CRPA were further screened for class 1 integrase gene cassettes. Among these isolates with class 1 integron, gene cassettes were amplified in 34 isolates. Class 1 integrase gene could not be amplified in 17 isolates that contain class 1 integrons. Eleven of these isolates that could not amplify in class 1 integrase gene were extensive-drug resistant (XDR) while the remaining six were multidrug resistant (MDR). Amplified class 1 integrons gene cassette yielded 5 amplicon sizes approximately 250 bp to 3.5 kb (Plate 4.10). Seventeen (33.3%) of the class 1 integron positive CRPA gave a single fragment size of ~3.5 kb while 3 (5.9%) gave double fragment sizes of ~3.5 kb and 800 bp. Single fragment sizes of ~1.6 kb, 1.4 kb, 800 bp and 250 bp were detected in 4 (7.8%), 1 (2.0%), 8 (15.7%) and 1 (2.0%) isolates, respectively (Table 4.13).

4.13 Prevalence of type III effector toxins in carbapenem-resistant *P. aeruginosa*

Multiplex PCR assay was used to assess the existence of four effector toxins found in *P. aeruginosa*: *exoT*, *exoY*, *exoS* and *exoU*. *exoY* and *exoT* were found in all carbapenem-resistant *P. aeruginosa* isolates, 35 (48.0%) of carbapenem-resistant *P. aeruginosa* contained *exoU* gene while 49.3% contained *exoS* (Plates 4.11a – 4.11d). Two isolates had both *exoU* and *exoS* type III effector genes. Three (PS166, PS182, PS185) out of ten isolate that were negative for MBL genes possessed *exoU* which is known to be cytotoxic while the remaining seven isolates had *exoS*. The occurrence of *exoU* among CRPA from each hospital was in the following ascending order: FMCA 0 (0.0%) > OTHS 1 (20.0%) > FMCI 2 (25.0%) > FMCO 4 (28.6%) > UCHI 14 (60.9%) > OTHI 8 (61.5%) > LTHO 6 (75.0%) (Table 4.15). Among isolates from wound 25 (44.6%) carbapenem-resistant *P. aeruginosa* possessed *exoU*, 29 (51.8%) produced *exoS* while 2 (3.6%) had both *exoU* and *exoS*. *exoU* and *exoS* was found in 2 (40.0%) and 3 (60.0%) of CRPA from ear, respectively (Table 4.16).

4.14 Statistical analysis

Table 4.14 shows the presence of MBL gene(s) and integrons in carbapenem non-susceptible *P. aeruginosa*. The outcome of Fisher's exact test to determine the relationship between integron and MBL genes showed that there was positive association between integron and MBL genes ($p = 0.0064$).

Fisher's exact also shows that there was positive association between *exoU* and *exoS* ($p < 0.0001$)

Table 4.11. Distribution of integrase genes in carbapenem-resistant *P. aeruginosa* according to hospital

Hospital (no)	Integrons No (%)				Integron absent
	Class 1	Class 2	Class 3	Class 1 and 2	
UCHI (23)	14 (69.6)	-	-	2 (8.7)	7 (30.4)
FMCA (2)	1 (50.0)	-	-	-	1 (50.0)
OTHS (5)	3 (60.0)	-	-	-	2 (40.0)
OTH1 (13)	8 (61.5)	-	-	-	5 (38.5)
LTHO (8)	4 (50.0)	-	-	-	4 (50.0)
FMCO (14)	6 (42.9)	-	-	4 (28.6)	4 (28.6)
FMCI (8)	5 (62.5)	-	-	3 (37.5)	-

Key:

- = absence of amplified gene

UCHI = University College Hospital, Ibadan

FMCA = Federal Medical Centre, Abeokuta

OTHS = Olabisi Onabanjo University Teaching Hospital, Sagamu

OTHI = Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife

LTHO = Ladoke Akintola University Teaching Hospital, Osogbo

FMCO = Federal Medical Centre, Owo

FMCI = Federal Medical Centre, Ido-Ekiti

Table 4.12. Distribution of integrase genes in carbapenem-resistant *P. aeruginosa* according to clinical source

Clinical source (no)	Integrons No (%)				Integron absent
	Class 1	Class 2	Class 3	Class 1 and 2	
Wound (56)	27 (48.2)	-	-	9 (16.1)	20 (31.8)
Tracheal aspirate (2)	2 (100.0)	-	-	-	-
Ear (5)	5 (100.0)	-	-	-	-
Urine (8)	6 (75.0)	-	-	-	2 (25.0)
Wound biopsy (2)	2 (100.0)	-	-	-	-

Key:

- = absence of amplified gene

Table 4.13. PCR-Restriction fragment length polymorphism investigation of integrons and characterisation of cassette arrays in carbapenem-resistant *P. aeruginosa*

S/NO	Isolate	Hospital	Clinical source	Integron	Class I Cassette
1.	PS007	UCHI	Wound	NA	ND
2.	PS022	UCHI	Tracheal aspirate	*intI1*	3.5 kb
3.	PS088	FMCA	Wound	NA	ND
4.	PS093	FMCA	Ear	*intI1*	800 bp
5.	PS096	FMCO	Wound	*intI1*	3.5 kb
6.	PS097	FMCO	Wound	*intI1*	1.6 kb
7.	PS099	FMCO	Wound	NA	ND
8.	PS100	FMCO	Wound	*intI1*	3.5 kb
9.	PS146	UCHI	Urine	*intI1*	3.5 kb
10.	PS147	UCHI	Urine	*intI1*	3.5 kb
11.	PS150	UCHI	Urine	NA	ND
12.	PS152	UCHI	Tracheal aspirate	*intI1*	NA
13.	PS154	UCHI	Urine	*intI1*	3.5 kb
14.	PS166	UCHI	Urine	*intI1*	1.6 kb
15.	PS168	UCHI	Wound	*intI1*	1.4 kp
16.	PS170	UCHI	Ear	*intI1*	800 bp
17.	PS172	OTHS	Ear pus	*intI1*	800 bp; 3.5 kb
18.	PS173	OTHS	Wound	*intI1*	NA
19.	PS181	UCHI	Wound	*intI1*	3.5 kb
20.	PS182	UCHI	Wound	NA	ND
21.	PS183	UCHI	Wound	*intI1*,*intI2*	250 bp
22.	PS184	UCHI	Wound biopsy	*intI1*	3.5 kb
23.	PS185	UCHI	Wound	NA	ND
24.	PS202	OTHS	Wound	NA	ND
25.	PS204	UCHI	Wound	NA	ND

Key: NA = No amplification; ND = Not determined

Table 4.13. PCR-Restriction fragment length polymorphism investigation of Integrons and characterisation of cassette array in carbapenem-resistant *P. aeruginosa* (cont'd)

S/NO	Isolate	Hospital	Clinical source	Integron	Class I Cassette
26.	PS205	UCHI	Wound	*intI1*	NA
27.	PS209	OTHI	Wound	*intI1*	3.5 kb
28.	PS210	OTHI	Wound	*intI1*	3.5 kb
29.	PS219	FMCO	Wound	NA	ND
30.	PS220	FMCO	Wound	*Int1*	3.5 kb
31.	PS222	FMCO	Wound	*intI1*, *intI2*	800 bp
32.	PS224	FMCO	Wound	*intI1*, *intI2*	NA
33.	PS229	OTHS	Wound	*intI1*	800 bp
34.	PS230	OTHS	Wound	NA	ND
35.	PS235	LTHO	Wound	*intI1*	NA
36.	PS238	LTHO	Ear	*intI1*	3.5 kb
37.	PS243	LTHO	Wound	NA	ND
38.	PS244	UCHI	Wound	*intI1*	3.5 kb
39.	PS246	UCHI	Wound	NA	ND
40.	PS250	UCHI	Wound	*intI1*	800 bp; 3.5 kb
41.	PS253	UCHI	Wound	*intI1*	NA
42.	PS285	FMCI	Wound	*intI1*,*intI2*	NA
43.	PS291	FMCI	Wound	*intI1*	800 bp
44.	PS292	FMCI	Wound	*intI1*,*intI2*	800 bp
45.	PS293	FMCI	Wound	*intI1*,*intI2*	NA
46.	PS294	FMCI	Wound	*intI1*	NA
47.	PS296	FMCI	Wound	*intI1*	800 bp
48.	PS297	FMCI	Wound	*intI1*	NA
49.	PS303	FMCI	Wound	*intI1*	3.5 kb
50.	PS325	FMCO	Wound	*intI1*	800 bp; 3.5 kb

Key: NA = Not amplification; ND = Not determined

Table 4.13. PCR-Restriction fragment length polymorphism investigation of Integrons and characterisation of cassette array in carbapenem-resistant *P. aeruginosa* (cont'd)

S/N	Isolate	Hospital	Clinical source	Integron	Class I Cassette
51.	PS335	FMCO	Ear	*intI1*	NA
52.	PS346	FMCO	Wound	NA	ND
53.	PS348	OTHI	Urine	*Int1*	NA
54.	PS349	OTHI	Urine	NA	ND
55.	PS350	OTHI	Urine	*intI1*	1.6 kb
56.	PS352	OTHI	Wound	NA	ND
57.	PS353	OTHI	Wound	*intI1*	3.5 kb
58.	PS354	OTHI	Wound	*intI1*	NA
59.	PS367	FMCO	Wound	*intI1*,*intI2*	1.6 kb
60.	PS383	UCHI	Wound	*intI1*,*intI2*	NA
61.	PS384	UCHI	Wound	NA	ND
62.	PS386	LTHO	Wound	NA	ND
63.	PS392	LTHO	Wound	NA	ND
64.	PS393	LTHO	Wound	*intI1*	NA
65.	PS394	LTHO	Wound	*intI1*	NA
66.	PS395	OTHI	Wound	*intI1*	3.5 kb
67.	PS396	OTHI	Wound	NA	ND
68.	PS397	OTHI	Wound	NA	ND
69.	PS398	OTHI	Wound	NA	ND
70.	PS400	OTHI	Wound	*Int1*	3.5 kb
71.	PS405	FMCO	Wound	NA	ND
72.	PS409	FMCO	Wound	*intI1*,*intI2*	NA
73.	PS414	LTHO	Wound biopsy	*intI1*	NA
74.	PS351(C)	LTHO	Urine	*intI1*	800 bp
75.	PS423 (C)	OTHI	Wound	NA	ND
76.	ATCC27853			NA	NA

Key: NA = Not amplified; ND = Not determined; (C) = Carbapenem susceptible strain

Table 4.14. Presence of MBL gene(s) and integrons in carbapenem non-susceptible *P. aeruginosa*

S/NO	Isolate	Hospital	Clinical source	MBL gene(s)	Integron
1.	PS007	UCHI	Wound	-	NA
2.	PS022	UCHI	Tracheal aspirate	bla_{VIM}	*intI1*
3.	PS088	FMCA	Wound	-	NA
4.	PS093	FMCA	Ear	bla_{VIM}	*intI1*
5.	PS096	FMCO	Wound	bla_{VIM}	*intI1*
6.	PS097	FMCO	Wound	bla_{VIM}	*intI1*
7.	PS099	FMCO	Wound	-	NA
8.	PS100	FMCO	Wound	bla_{VIM}	*intI1*
9.	PS146	UCHI	Urine	bla_{NDM}	*intI1*
10.	PS147	UCHI	Urine	bla_{VIM}	*intI1*
11.	PS150	UCHI	Urine	-	NA
12.	PS152	UCHI	Tracheal aspirate	bla_{NDM}	*intI1*
13.	PS154	UCHI	Urine	bla_{NDM}	*intI1*
14.	PS166	UCHI	Urine	-	*intI1*
15.	PS168	UCHI	Wound	bla_{VIM}	*intI1*
16.	PS170	UCHI	Ear	-	*intI1*
17.	PS172	OTHS	Ear pus	bla_{VIM}	*intI1*
18.	PS173	OTHS	Wound	bla_{VIM}	*intI1*
19.	PS181	UCHI	Wound	bla_{NDM}	*intI1*
20.	PS182	UCHI	Wound	-	NA
21.	PS183	UCHI	Wound	bla_{NDM}	*intI1*,*intI2*
22.	PS184	UCHI	Wound biopsy	bla_{NDM}	*intI1*
23.	PS185	UCHI	Wound	-	NA
24.	PS202	OTHS	Wound	bla_{VIM}	NA
25.	PS204	UCHI	Wound	bla_{VIM}	NA

Key: NA = No amplification; ND = Not determined

Table 4.14. Presence of MBL gene(s) and integrons in carbapenem non-susceptible *P. aeruginosa* (cont'd)

S/NO	Isolate	Hospital	Clinical source	MBL gene(s)	Integron
26.	PS205	UCHI	Wound	*bla*$_{VIM}$	*intI1*
27.	PS209	OTHI	Wound	*bla*$_{VIM}$	*intI1*
28.	PS210	OTHI	Wound	*bla*$_{VIM}$	*intI1*
29.	PS219	FMCO	Wound	*bla*$_{VIM}$	NA
30.	PS220	FMCO	Wound	*bla*$_{VIM}$	*Int1*
31.	PS222	FMCO	Wound	*bla*$_{VIM,}$ *bla*$_{NDM}$	*intI1, intI2*
32.	PS224	FMCO	Wound	*bla*$_{VIM,}$ *bla*$_{NDM}$	*intI1, intI2*
33.	PS229	OTHS	Wound	*bla*$_{NDM}$	*intI1*
34.	PS230	OTHS	Wound	*bla*$_{NDM}$	NA
35.	PS235	LTHO	Wound	*bla*$_{NDM}$	*intI1*
36.	PS238	LTHO	Ear	*bla*$_{VIM,}$ *bla*$_{NDM}$	*intI1*
37.	PS243	LTHO	Wound	*bla*$_{VIM}$	NA
38.	PS244	UCHI	Wound	-	*intI1*
39.	PS246	UCHI	Wound	*bla*$_{VIM}$	NA
40.	PS250	UCHI	Wound	*bla*$_{VIM,}$ *bla*$_{NDM}$	*intI1*
41.	PS253	UCHI	Wound	*bla*$_{VIM}$	*intI1*
42.	PS285	FMCI	Wound	*bla*$_{NDM}$	*intI1,intI2*
43.	PS291	FMCI	Wound	*bla*$_{VIM}$	*intI1*
44.	PS292	FMCI	Wound	*bla*$_{NDM}$	*intI1,intI2*
45.	PS293	FMCI	Wound	*bla*$_{VIM,}$ *bla*$_{NDM}$	*intI1,intI2*
46.	PS294	FMCI	Wound	*bla*$_{VIM}$	*intI1*
47.	PS296	FMCI	Wound	*bla*$_{VIM,}$ *bla*$_{NDM}$	*intI1*
48.	PS297	FMCI	Wound	*bla*$_{VIM}$	*intI1*
49.	PS303	FMCI	Wound	*bla*$_{NDM}$	*intI1*
50.	PS325	FMCO	Wound	*bla*$_{VIM}$	*intI1*

Key: NA = Not amplification; ND = Not determined

Table 4.14. Presence of MBL gene(s) and integrons in carbapenem non-susceptible *P. aeruginosa* (cont'd)

S/NO	Isolate	Hospital	Clinical source	MBL gene(s)	Integron
51.	PS335	FMCO	Ear	*bla*$_{NDM}$	*intI1*
52.	PS346	FMCO	Wound	*bla*$_{NDM}$	NA
53.	PS348	OTHI	Urine	*bla*$_{NDM}$	*Int1*
54.	PS349	OTHI	Urine	-	NA
55.	PS350	OTHI	Urine	*bla*$_{VIM}$	*intI1*
56.	PS352	OTHI	Wound	*bla*$_{NDM}$	NA
57.	PS353	OTHI	Wound	*bla*$_{VIM}$	*intI1*
58.	PS354	OTHI	Wound	*bla*$_{NDM}$	*intI1*
59.	PS367	FMCO	Wound	*bla*$_{NDM}$	*intI1,intI2*
60.	PS383	UCHI	Wound	*bla*$_{NDM}$	*intI1,intI2*
61.	PS384	UCHI	Wound	*bla*$_{NDM}$	NA
62.	PS386	LTHO	Wound	*bla*$_{NDM}$	NA
63.	PS392	LTHO	Wound	*bla*$_{NDM}$	NA
64.	PS393	LTHO	Wound	*bla*$_{NDM}$	*intI1*
65.	PS394	LTHO	Wound	*bla*$_{NDM}$	*intI1*
66.	PS395	OTHI	Wound	*bla*$_{NDM}$	*intI1*
67.	PS396	OTHI	Wound	*bla*$_{VIM,}$ *bla*$_{NDM}$	NA
68.	PS397	OTHI	Wound	*bla*$_{NDM}$	NA
69.	PS398	OTHI	Wound	*bla*$_{NDM}$	NA
70.	PS400	OTHI	Wound	*bla*$_{VIM,}$ *bla*$_{NDM}$	*Int1*
71.	PS405	FMCO	Wound	*bla*$_{VIM,}$ *bla*$_{NDM}$	NA
72.	PS409	FMCO	Wound	*bla*$_{VIM}$	*intI1,intI2*
73.	PS414	LTHO	Wound biopsy	*bla*$_{NDM}$	*intI1*
74.	PS351(C)	LTHO	Urine		*intI1*
75.	PS423 (C)	OTHI	Wound		NA
76.	ATCC27853				NA

Key: NA = Not amplified; ND = Not determined; (C) = Carbapenem susceptible strain

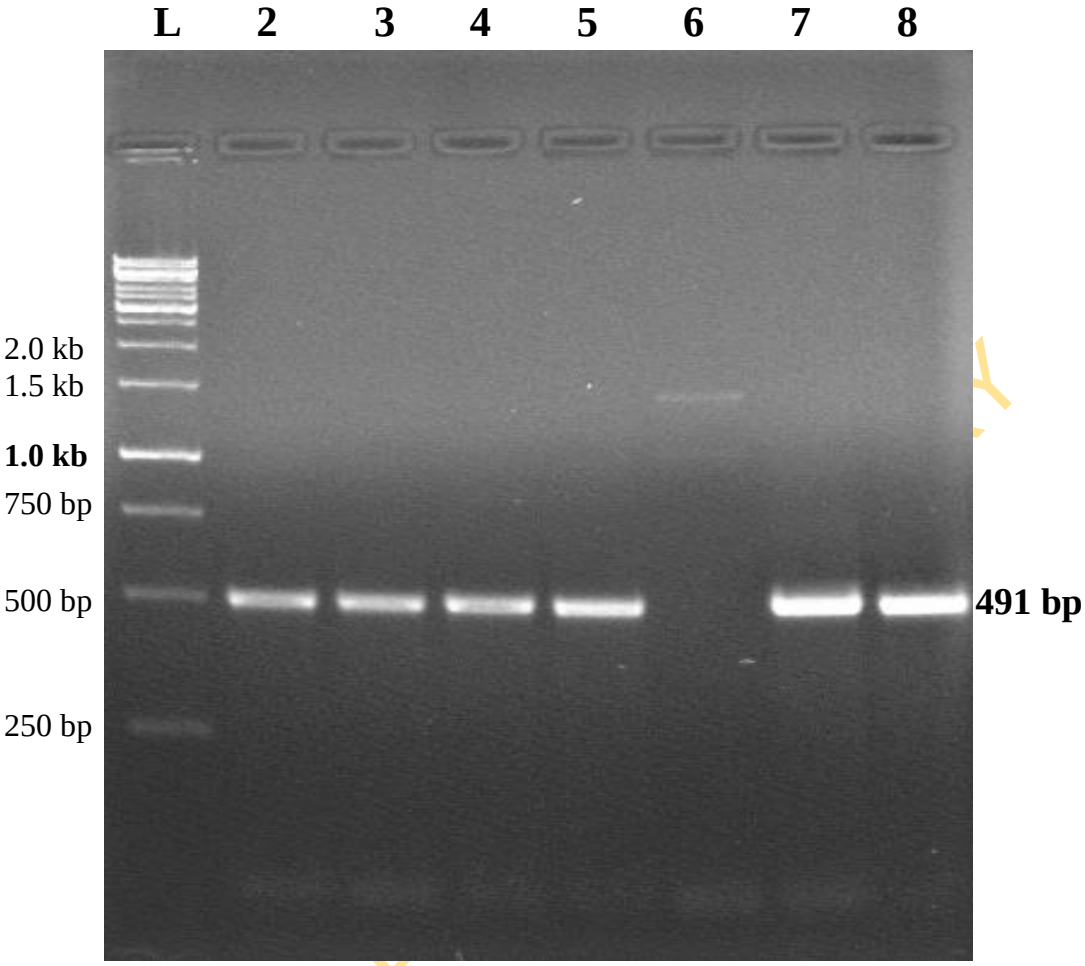

Plate 4.8a. Agarose gel (1.5%) showing PCR products for integrons before digestion with *Rsa*1 enzyme L = 1kb plus bp ladder; Lanes 2-5, 7,8 showed positive results for integrons (PS152, PS154, PS168, PS183, PS210, PS220); Lane 6 (PS204) is negative for integrons

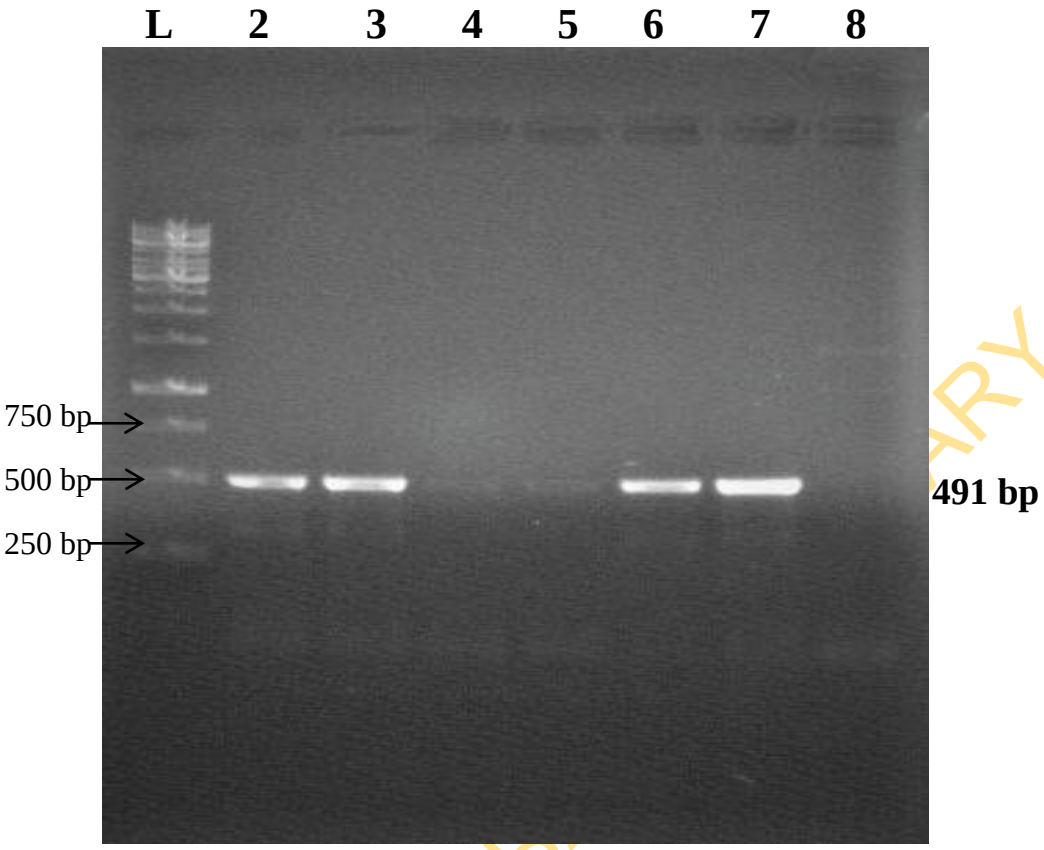

Plate 4.8b. Agarose gel (1.5%) showing PCR products for integrons before digestion with *Rsa*1 enzyme L = 1 kb plus bp ladder; Lanes 2,3,6,7 (PS181, PS184, PS209, PS205) showed positive results for integrons; Lanes 4,5,8 (PS185, PS202, PS210) showed negative results for integrons.

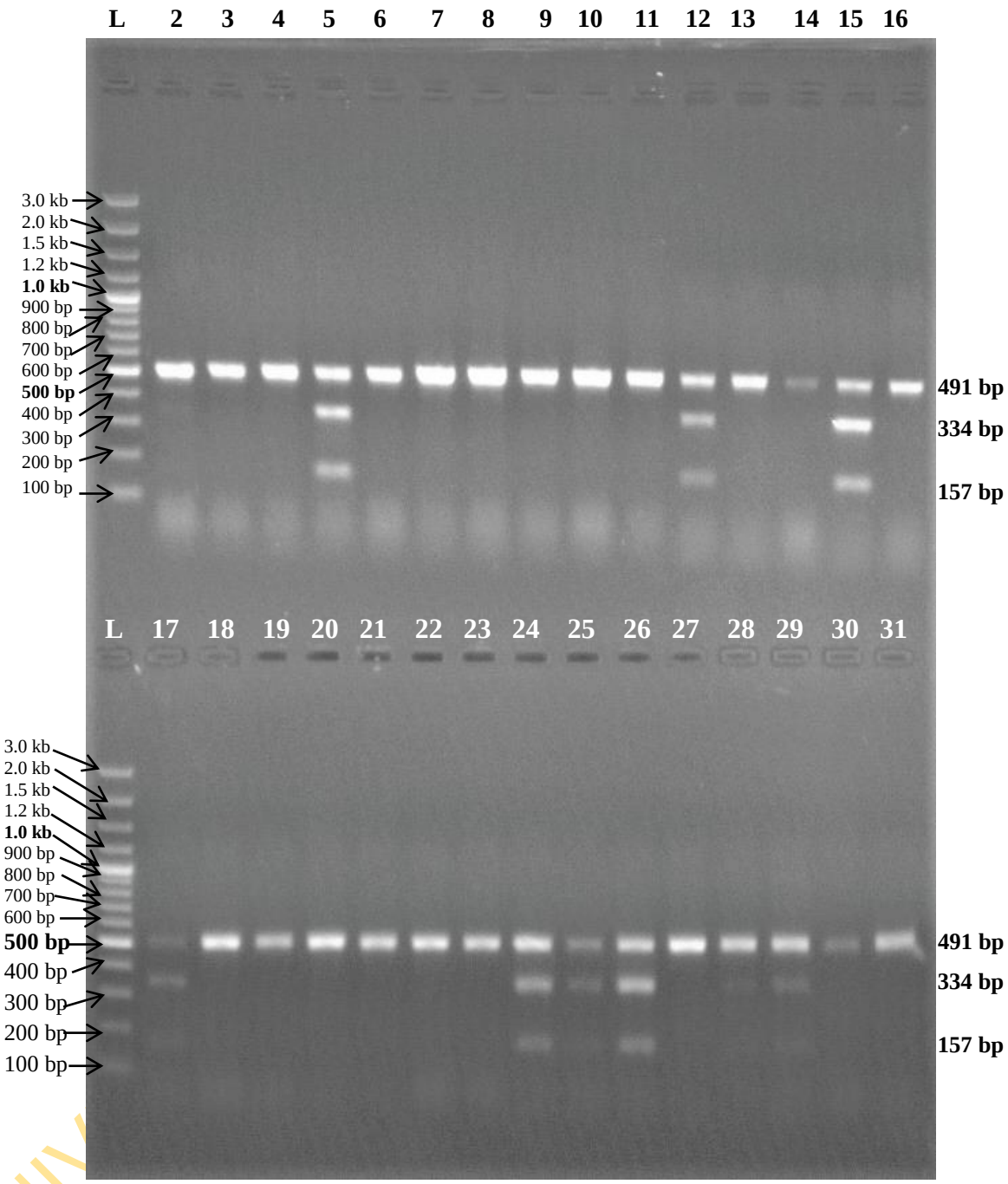

Plate 4.9a. Agarose gel (1.5%) showing PCR products for integrons L = 100 plus bp ladder; Lanes 2-4, 6-11, 13,14, 16,18-23, 27,28,30,31 shows different carbapenem-resistant *P. aeruginosa* positive for class integrons after digestion of PCR-products with *Rsa*I enzyme (PS154, PS181, PS205, PS152, PS220, PS209, PS168, PS184, PS210, PS414, PS335, PS297, PS348, PS235, PS354, PS393, PS303, PS395, PS253, PS291, PS173, PS394); Lanes: 5, 12, 15,24-26,29 shows different carbapenem-resistant *P. aeruginosa* positive for class 1 and 2 integrons after digestion of PCR-products with *Rsa*I enzyme (PS183, PS285, PS222, PS409, PS367, PS383, PS293, PS224)

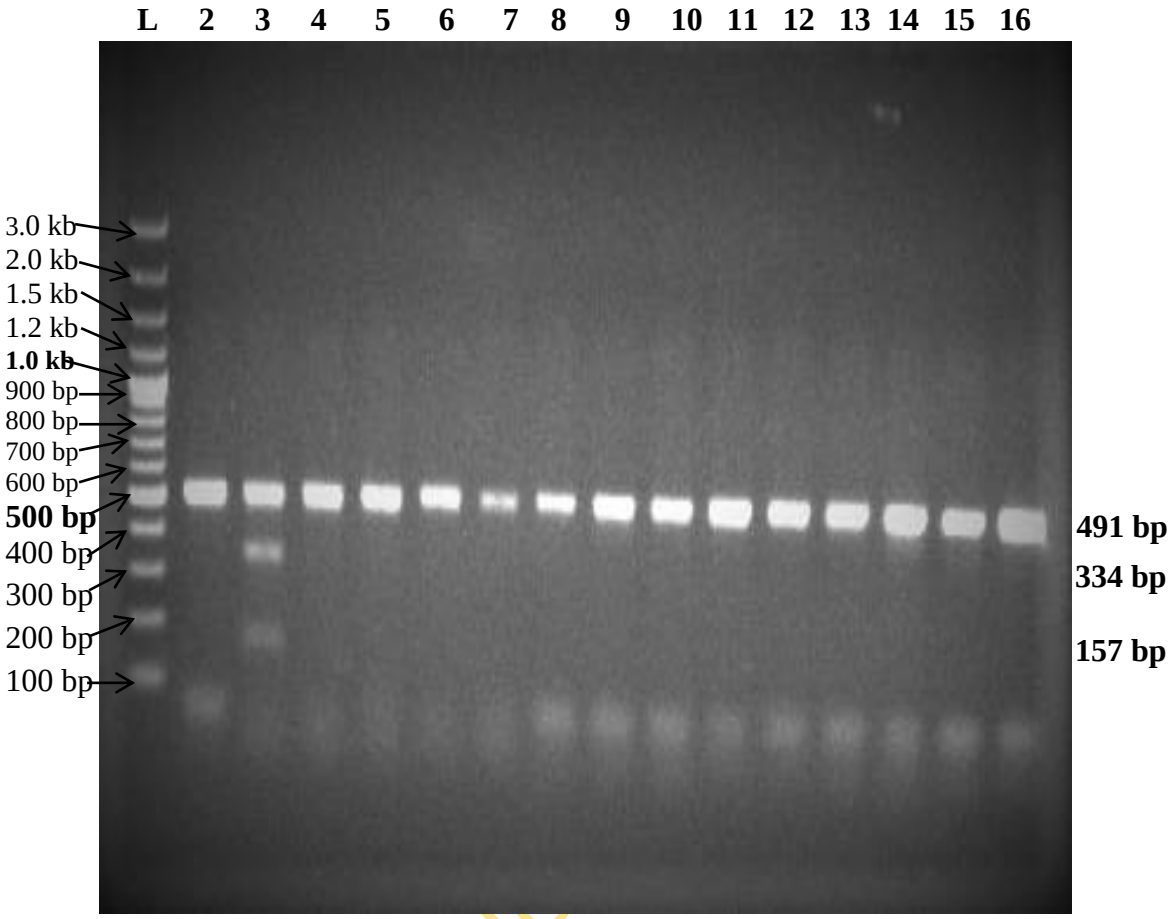

Plate 4.9b. Agarose gel (1.5%) showing PCR products for integrons. Lane 2,4-16 shows different carbapenem-resistant *P. aeruginosa* positive for class 1 integrons after digestion of PCR-products with *Rsa*I enzyme (L = 100 plus bp ladder; Lane 2-16 = PS093, PS096, PS146, PS100, PS097, PS022, PS244, PS147, PS294, PS325, PS294, PS296, PS250, PS172); Lane 3 (PS292) shows different carbapenem-resistant *P. aeruginosa* positive for class 1 and 2 integrons after digestion of PCR-products with *Rsa*I enzyme

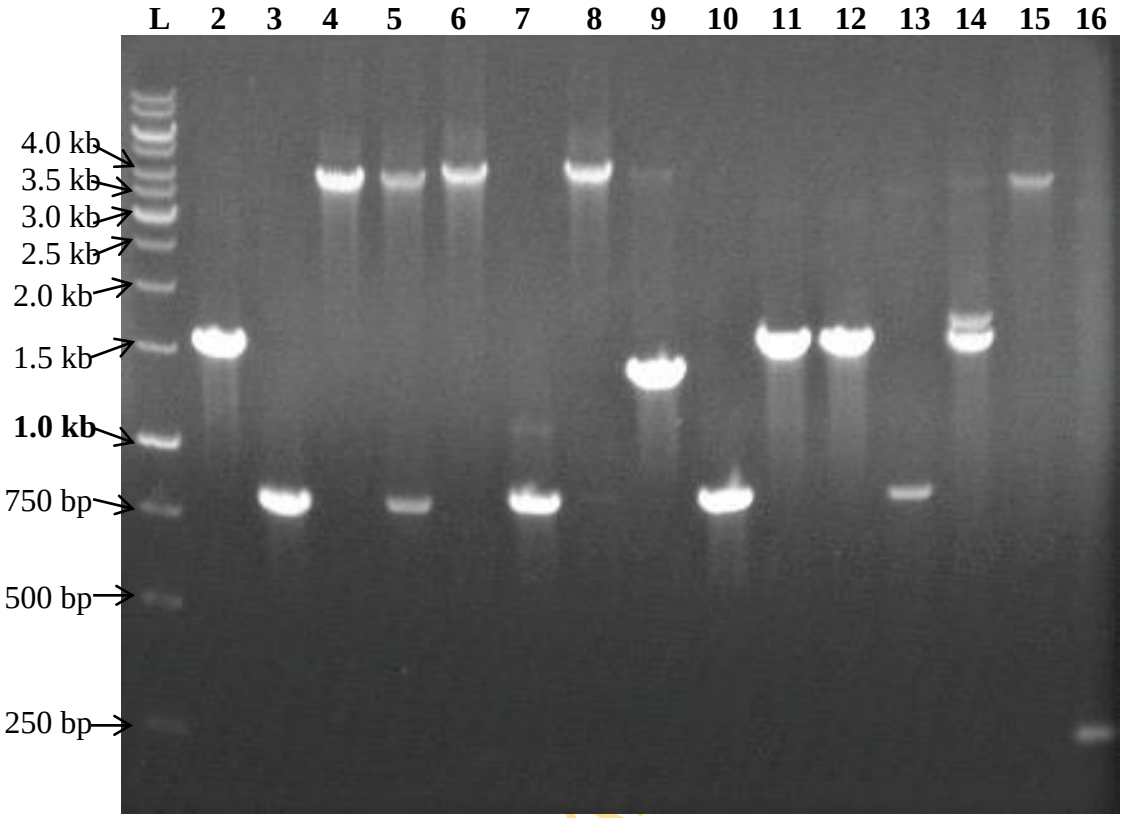

Plate 4.10. Agarose gel electrophoresis (1.5%) of class 1 integron gene cassette L = 1kb ladder; Lane 2-15 (PS097, PS093, PS220, PS250, PS209, PS244, PS210, PS168, PS291, PS160, PS367, PS222, PS350, PS303) shows different *P. aeruginosa* class 1 integrase gene cassette; N = Negative control.

Table 4.15. Prevalence of Type III effector toxins in carbapenem-resistant *P. aeruginosa* according to hospital

Hospital (no)	**T3SS toxins**				
	exoT	*exoY*	*exoU*	*exoS*	*exoU* and *exoS*
UCHI (23)	23 (100)	23 (100)	14 (60.9)	9 (39.1)	-
FMCA (2)	2 (100)	2 (100)	-	2 (100)	-
OTHS (5)	5 (100)	5 (100)	1 (20)	4 (80)	-
OTH1 (13)	13 (100)	23 (100)	8 (61.5)	4 (30.8)	1 (7.7)
LTHO (8)	8 (100)	8 (100)	6 (75.0)	2 (25.0)	-
FMCO (14)	14 (100)	14 (100)	4 (28.6)	10 (71.4)	-
FMCI (8)	8 (100)	8 (100)	2 (25.0)	5 (62.5)	1 (12.5)

Key:

- = absence of amplified gene

UCHI = University College Hospital Ibadan

FMCA = Federal Medical Centre Abeokuta;

OTHS = Olabisi Onabanjo University Teaching Hospital Sagamu;

OTHI = Obafemi Awolowo University Teaching Hospital Ile-Ife;

LTHO = Ladoke Akintola University Teaching Hospital Osogbo;

FMCO = Federal Medical Centre Owo;

FMCI = Federal Medical Centre Ido-Ekiti

Table 4.16. Prevalence of Type III effector toxins in carbapenem-resistant *P. aeruginosa* according to clinical source

T3SS toxins	**Clinical source (no (%))**				
	Wound (56)	**Tracheal aspirate (2)**	**Ear (5)**	**Urine (8)**	**Wound biopsy (2)**
exoT	56 (100)	2 (100)	5 (100)	8 (100)	2 (100)
exoY	56 (100)	2 (100)	5 (100)	8 (100)	2 (100)
exoU	24 (42.9)	1 (50.0)	2 (40.0)	6 (75.0)	2 (100)
exoS	30 (53.6)	1 (50.0)	3 (60.0)	2 (25.0)	-
exoU and *exoS*	2 (3.6)	-	-	-	-

Key:

- = absence of amplified gene

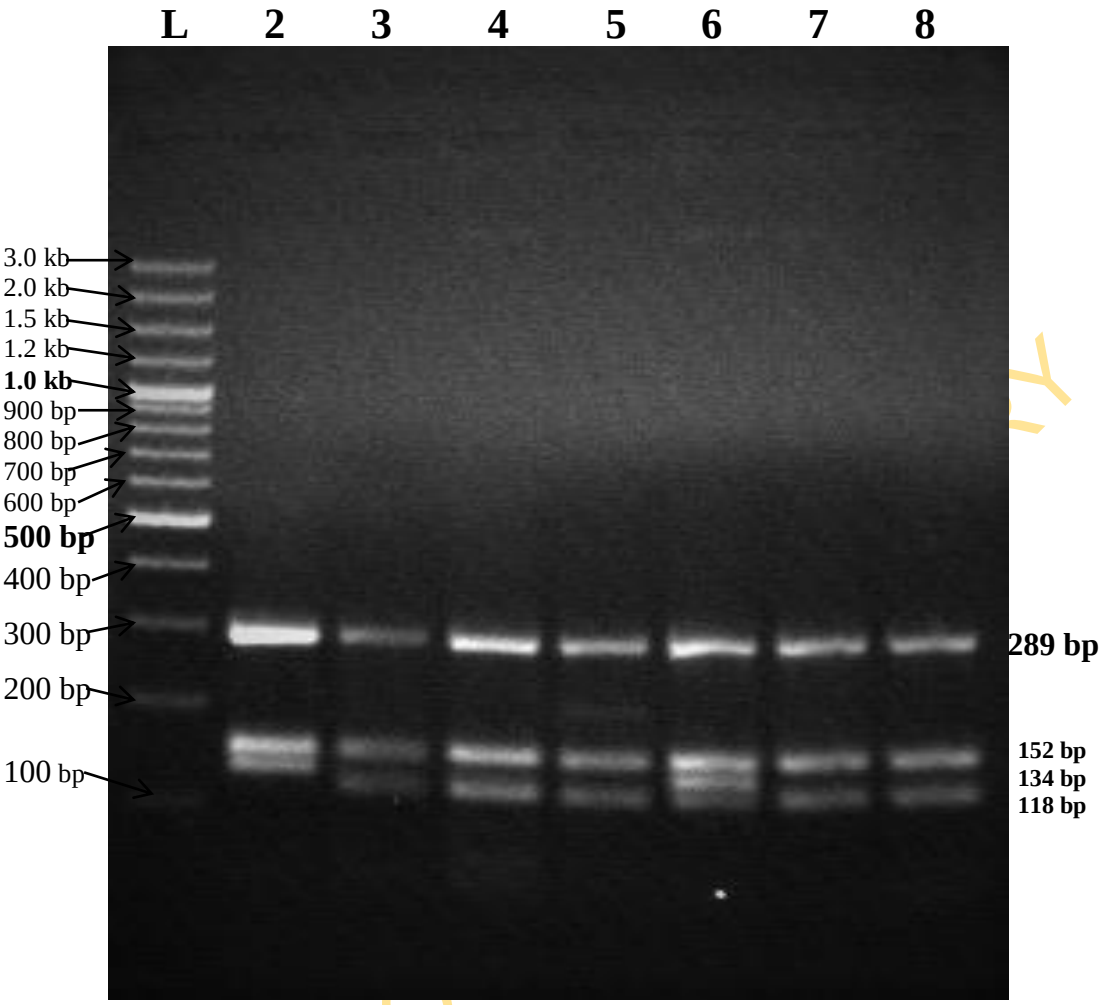

Plate 4.11a. Genotyping of *exoU*, *exoS*, *exoT* and *exoY* in carbapenem-resistant *P. aeruginosa* (CRPA) isolates with multiplex PCR. L: 100 bp plus ladder. Lane 2 (PS181) is *exoY*, *exoT* and *exoU* positive strain; Lanes 3-5, 7,8 is *exoY*, *exoT* and *exoS* positive strains (PS205, PS170, PS093, PS172, PS022). Lane 6 (PS293) is *exoY*, *exoT*, *exoU* and *exoS* positive strain (*exoY*-289bp; *exoT*-152bp; *exoU*-134bp; *exoS*-118bp)

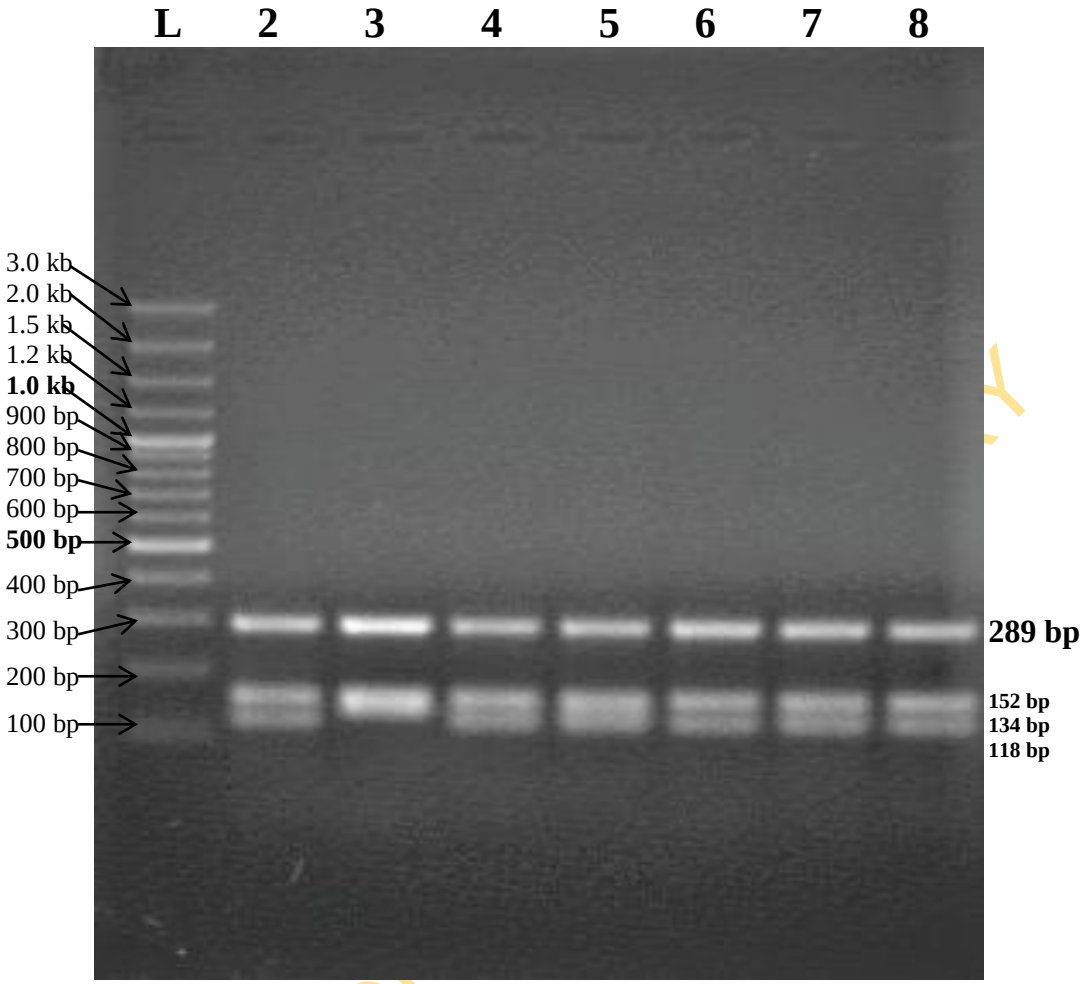

Plate 4.11b. Genotyping of *exoU*, *exoS*, *exoT* and *exoY* in carbapenem resistant *P. aeruginosa* (CRPA) isolates by multiplex PCR. Lanes 2, 4-8 is *exoY*, *exoT* and *exoS* positive strains (PS007, PS088, PS096, PS099, PS100, PS150); Lane 3 (PS335) *exoY*, *exoT* and *exoU* positive strain (*exoY*-289bp; *exoT*-152bp; *exoU*-134bp; *exoS*-118bp)

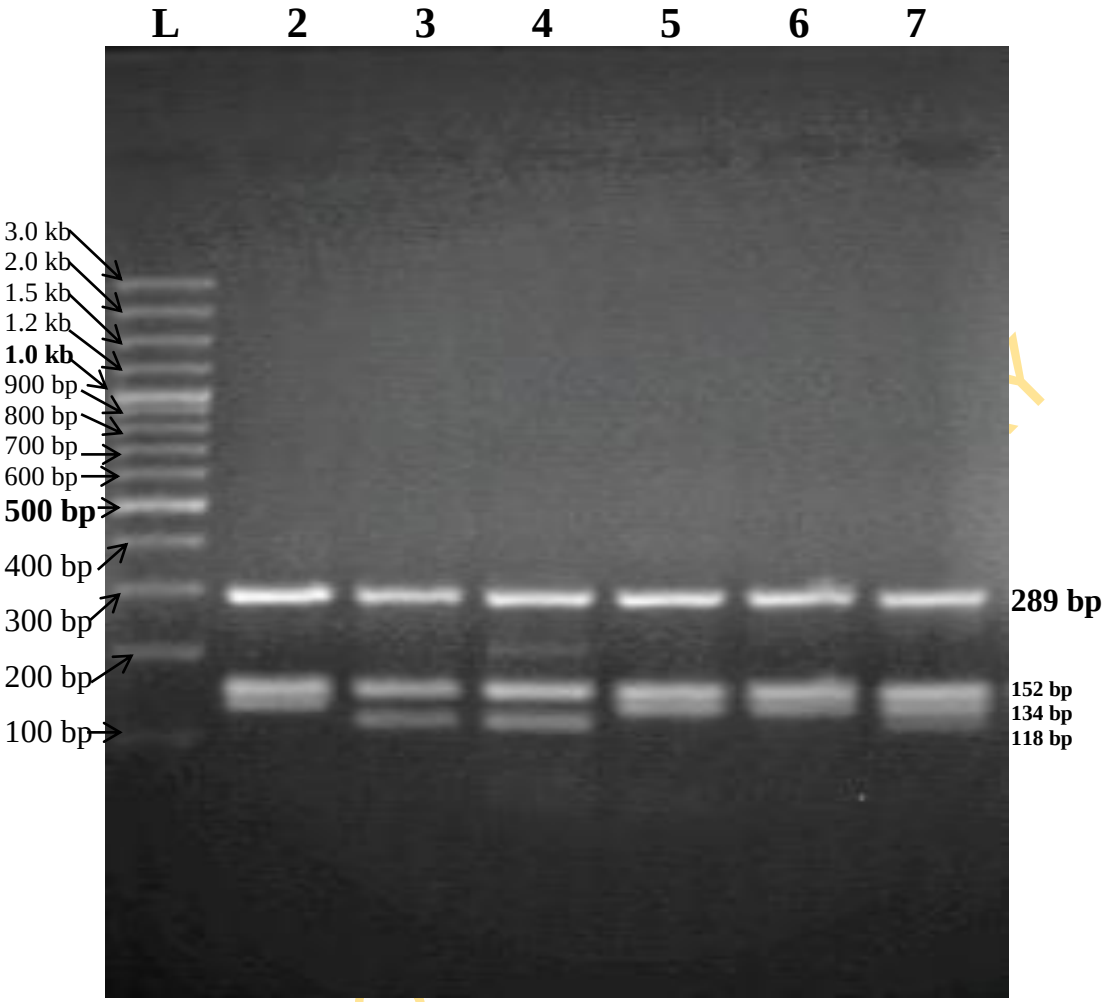

Plate 4.11c. Genotyping of *exoU*, *exoS*, *exoT* and *exoY* in carbapenem resistant *P. aeruginosa* (CRPA) isolates by multiplex PCR. Lanes 3 and 4 are *exoY*, *exoT* and *exoS* positive strains (PS291, PS292); Lanes 2,5,6 are *exoY*, *exoT* and *exoU* positive strains (PS224, PS097, PS348); Lane 8 (PS354) is *exoY*, *exoT*, *exoU* and *exoS* positive strain (*exoY*-289bp; *exoT*-152bp; *exoU*-134bp; *exoS*-118bp)

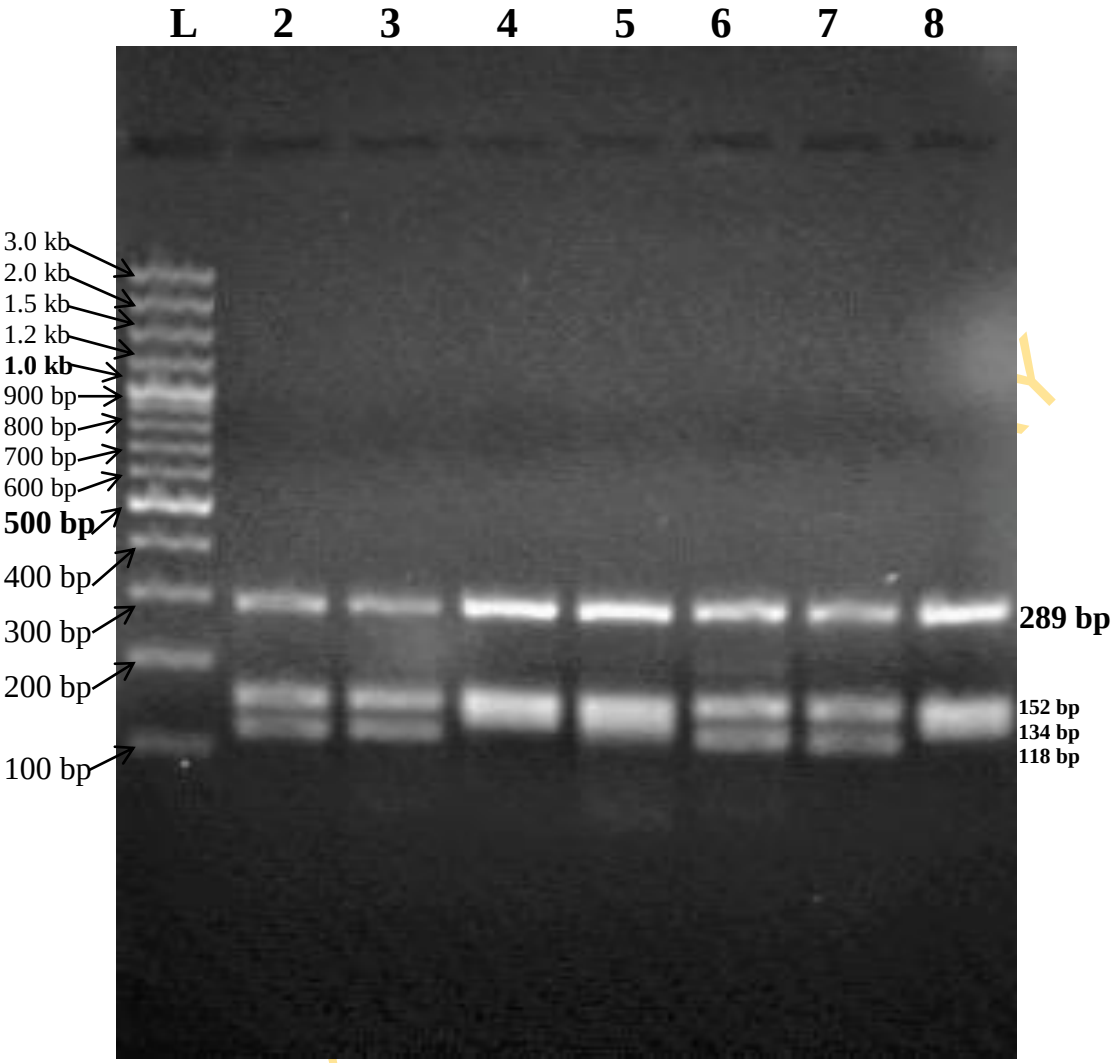

Plate 4.11d. Genotyping of *exoU, exoS, exoT* and *exoY* in carbapenem resistant *P. aeruginosa* (CRPA) isolates by multiplex PCR. Lanes 2,3,6,7 is *exoY*, *exoT* and *exoS* positive strains (PS173, PS202, PS205, PS088); Lanes 4,5,8 (PS183, PS209, PS210) *exoY*, *exoT* and *exoU* positive strain (*exoY*-289bp; *exoT*-152bp; *exoU*-134bp; *exoS*-118bp)

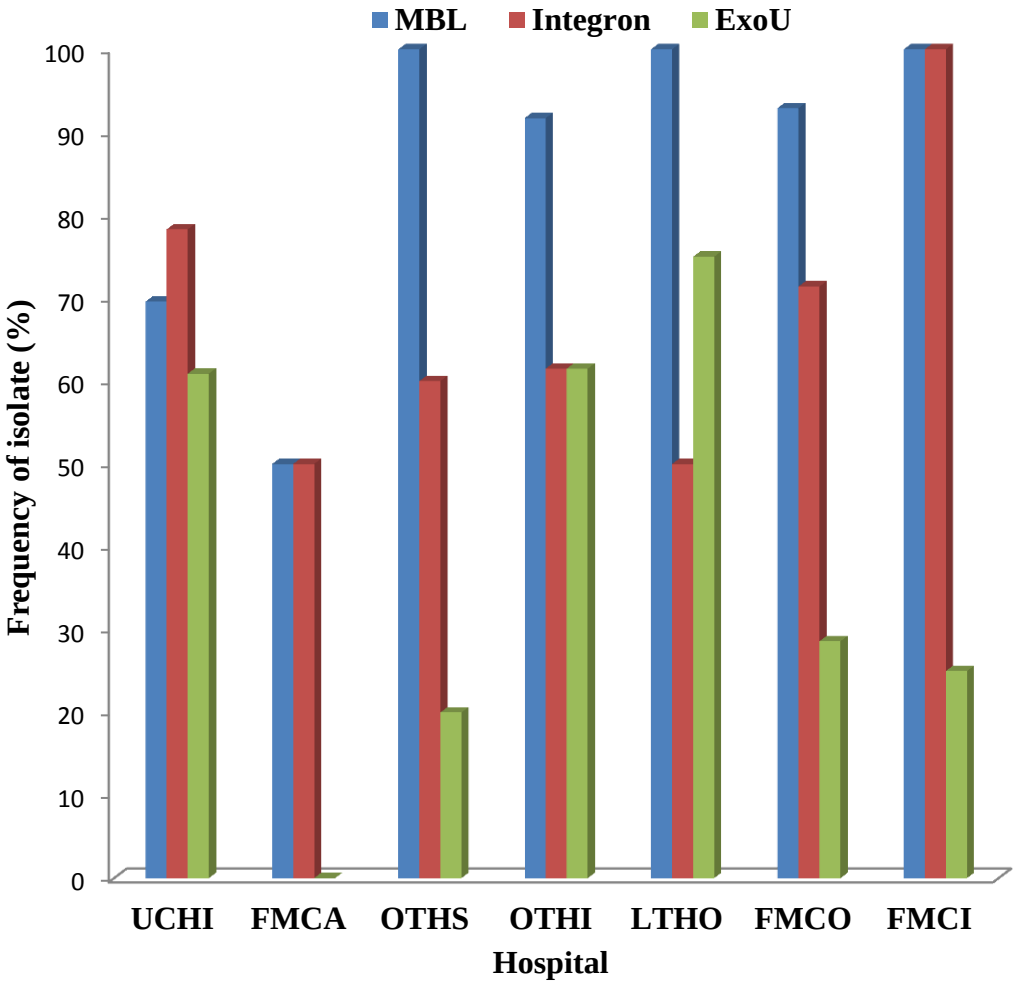

Figure 4.4. Distribution of MBL(s), integron and *exoU* in carbapenem-resistant *P. aeruginosa* (CRPA) according to hospital

Key:

UCHI = University College Hospital, Ibadan

FMCA = Federal Medical Centre, Abeokuta

OTHS = Olabisi Onabanjo University Teaching Hospital, Sagamu

LTHO = Ladoke Akintola University Teaching Hospital, Osogbo

OTHI = Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife

FMCO = Federal Medical Centre, Owo

FMCI = Federal Medical Centre, Ido-Ekiti

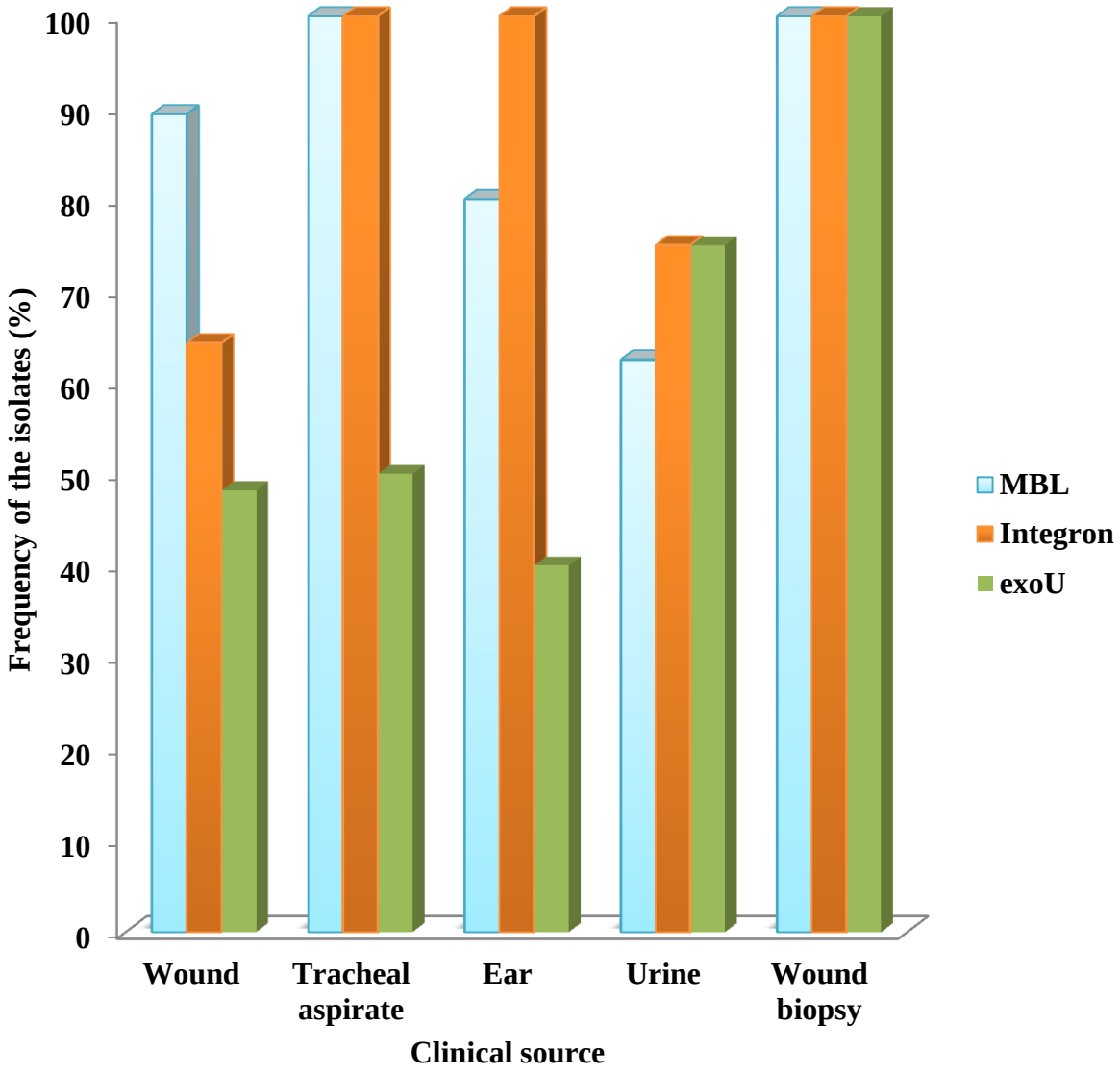

Figure 4.5. Distribution of MBL(s), integron and *exoU* in carbapenem-resistant *P. aeruginosa* (CRPA) according to clinical source

4.15 Quantification of efflux pumps expression in carbapenem-resistant *P. aeruginosa*

Plate 4.12 shows the RNA isolated from carbapenem-resistant *Pseudomonas aeruginosa* on an agarose gel. Upregulation of the four efflux systems (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) was determined in 48 randomly selected carbapenem-resistant *P. aeruginosa* and one susceptible strain by determining the relative transcription levels of *mexA, mexB, mexC, mexD, mexE, mexF, mexX* and *mexY* genes by quantitative real-time PCR. The mRNA was said to be overexpressed if the equivalent mRNA level was at least 2-fold (*mexA, mexB), 4-fold (mexX and mexY*) or 100-fold (*mexC, mexD, mexE* and *mexF*) higher than that for *P. aeruginosa* ATCC 27853 (Hocquet *et al*., 2006). The analysis of gene expression showed that 33 (68.8%) of the carbapenem-resistant *P. aeruginosa* clinical isolates overexpressed one or more efflux pump genes while 15 (31.3%) isolates showed no overexpression of any of the efflux gene. One isolate (PS293) overexpressed all the four pumps. MexXY-OprM was the most overexpressed pump occurring in 28 (58.3%) of the isolates followed by MexAB-OprM which was overexpressed in 22 (45.8%) strains, MexCD-OprJ in 5 (10.4%) strains and MexEF-OprN was found in 3 (6.25%) of the isolates (Table 4.17).

Among the carbapenem-resistant *P. aeruginosa* strains, *mexX* was the most expressed gene as observed in 26 (54.2%) strains with fold increase from 4.98 to 996.3 while *mexY* was overexpressed in 18 (37.5%) strains with fold increase from 5.3 to 643.8 compared to *P. aeruginosa* ATCC 27853. In 19 (39.6%) of the carbapenem-resistant strains, fold increase in *mexA* from 2.50 to 310.4 was observed while 18 (37.5%) showed increased mexB from 3.1 to 214.5 fold at the transcriptional mRNA levels compared to *P. aeruginosa* ATCC 27853. Increase in *mexC* was demonstrated in 4 (8.3%) strains from 133.4 to 1661.6 fold while 5 (10.4%) isolates showed an increase in *mexD* from 211.1 to 2550.9 fold levels compared to *P. aeruginosa* ATCC 27853. The *mexE* gene was overexpressed in 2 (4.2%) strains with fold increase of 108.3 and 279.9 while only one strain (2.1%) overexpressed *mexF* with a fold increase of 101.3 compared to *P. aeruginosa* ATCC 27853 (Table 4.19).

In 16 (33.3%) isolates, both *mexX* and *mexY* genes were overexpressed while *mexX* and *mexY* were overexpressed seperately in 10 (20.8%) and 2 (4.2%) isolates, respectively. Both *mexA* and *mexB* genes were overexpressed in 14 (29.2%) of isolates while *mexA* and *mexB* genes were overexpressed individually in 5 (10.4%) and 4

(8.3%) isolates, respectively. Both *mexC* and *mexD* genes was overexpressed in 4 (8.3%) of the strains. None of the isolates overexpressed both *mexE* and *mexF* genes (Figure 4.6).

4.16 Quantification of *ampC* overexpression and diminished expression of *oprD* porin

Table 4.18 shows the relative fold expression of *ampC* and *oprD* porin. The *ampC* gene was overexpressed in 13 (27.1%) strains with fold increase of between 50.2 and 5086.7 compared to *P. aeruginosa* ATCC 27853 (Table 4.19). Underexpressed outermembrane porin (*oprD*) was observed in 37 (77.1%) carbapenem-resistant *P. aeruginosa* strains. Among the ten carbapenem-resistant *P. aeruginosa* that lacked MBL genes, the only mode of resistance in four isolates was attributable to efflux pump overexpression (PS007, PS088, PS099, PS349) while defective *oprD* alone was responsible for carbapenem resistance in three isolates (PS150, PS166, PS185). Carbapenem resistance in PS170 and PS244 was due to efflux pump and *ampC* overexpression while in strain PS414, carbapenem resistance results from overexpression of efflux pump and underexpression of *oprD*. In this study, only ten isolates had both reduced *oprD* mRNA transcription and *ampC* overexpression.

4.17 MIC of antibiotics against carbapenem-resistant *P. aeruginosa* isolates in relation to MBL and efflux pump genes

Table 4.20 compares the MIC of selected antibiotics against CRPA isolates relative to the presence or absence of MBL efflux pump overexpression. It was observed that isolates that lack MBL genes but overexpressed efflux pump gene had reduced MIC against imipenem, meropenem, ceftazidime, cefepime and ciprofloxacin when compared with isolates having combination of MBL genes and efflux pump.

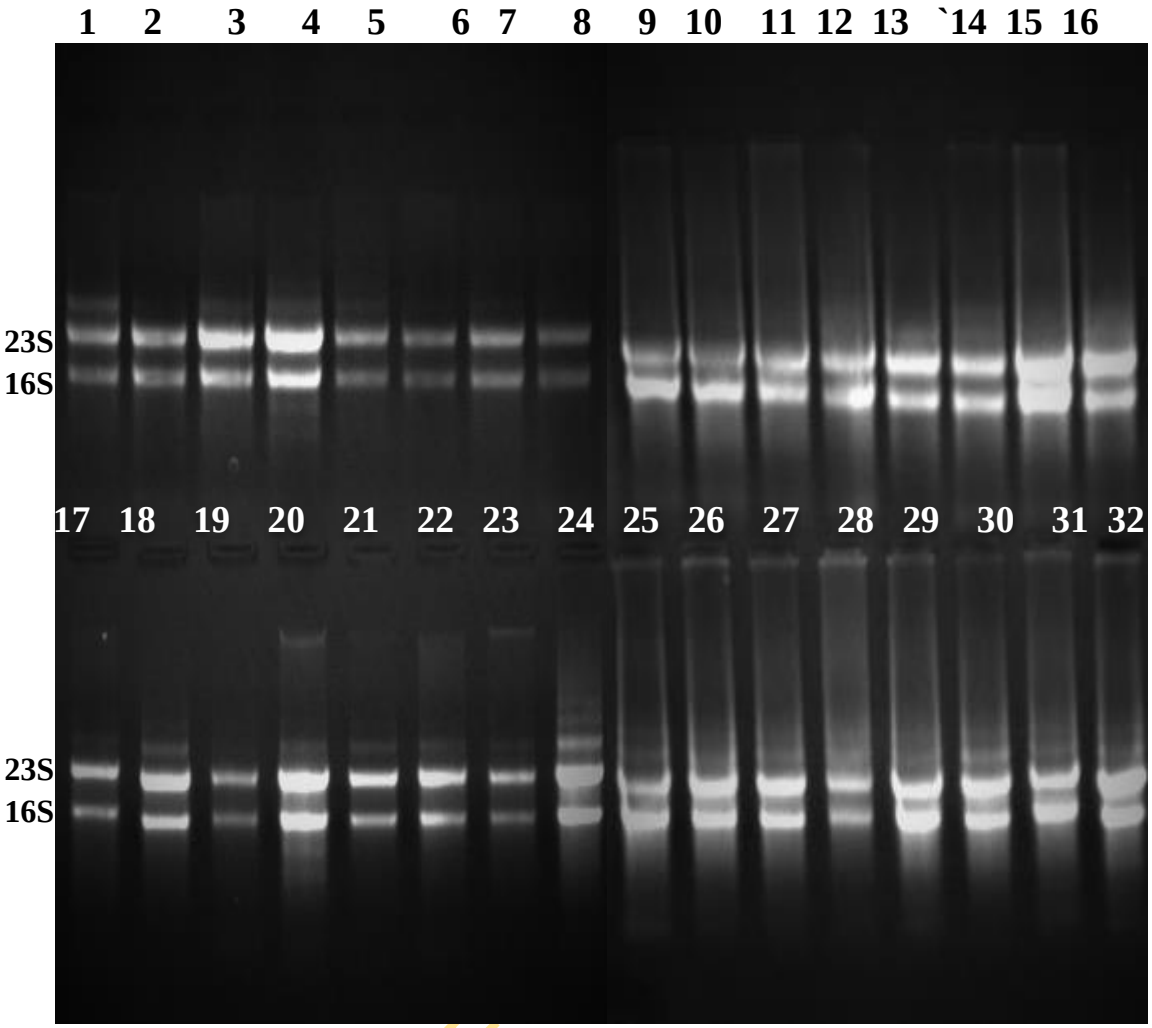

Plate 4.12. Agarose gel (1.0%) showing total RNA extracted from carbapenem-resistant *P. aeruginosa* with PureLinkTM Micro-to-Midi Total RNA Extraction System (Invitrogen). Lane 1-32 carbapenem-resistant *P. aeruginosa* total RNA (PS429, PS099, PS349, PS397, PS170, PS250, PS007, PS230, PS246, PS292, PS022, PS405, PS224, PS173, PS202, PS222, PS100, PS398, PS394, PS166, PS384, PS210, PS296, PS185, PS291, PS414, PS183, PS238, PS243, PS400, PS152, PS088

Table 4.17. Relative fold expression of efflux pump genes in carbapenem-resistant *P. aeruginosa* clinical isolates compared to *P. aeruginosa* ATCC 27853

S/N	Isolate	Efflux pump genes							
		mexA	***mexB***	***mexC***	***mexD***	***mexX***	***mexY***	***mexE***	***mexF***
1.	PS007	2.9	1.8	2.5	0.6	5.3	1.3	1.4	1.0
2.	PS022	0.5	0.3	1.8	0.7	4.4	1.7	0.5	0.4
3.	PS088	1.7	0.8	1.5	0.3	21.3	1.3	3.1	0.9
4.	PS093	4.3	2.5	10.9	7.5	16.2	93.0	17.9	4.4
5.	PS097	0.8	0.4	0.4	0.2	1.3	0.1	0.2	0.1
6.	PS099	2.9	3.7	29.0	8.9	4.9	1.2	1.0	1.1
7.	PS100	2.8	25.3	21.4	0.7	17.7	1.6	4.5	3.2
8.	PS150	0.2	0.5	0.7	0.2	1.1	0.3	0.4	0.2
9.	PS152	0.6	0.4	0.03	0.3	9.2	0.6	0.3	0.01
10.	PS166	0.9	1.2	0.4	0.5	0.7	3.4	0.6	0.2
11.	PS170	6.8	2.0	59.8	55.0	166.3	256.9	108.3	41.6
12.	PS173	0.5	0.6	0.4	0.2	1.0	2.9	0.4	0.1
13.	PS181	0.5	0.7	0.8	0.3	34.9	49.6	0.3	0.01
14.	PS182	7.9	67.6	42.1	39.2	176.9	171.7	42.0	101.3
15.	PS183	1.6	0.9	1.1	1.1	78.9	10.4	0.5	0.3
16.	PS185	1.7	1.1	1.1	0.5	2.3	0.2	0.6	1.6
17.	PS202	31.5	6.6	425.5	2550.9	740.7	580.5	23.1	1.3
18.	PS204	3.8	2.0	9.2	14.3	37.2	43.3	12.4	3.0
19.	PS209	0.5	13.7	0.4	0.3	51.5	65.5	2.5	2.3
20.	PS210	3.3	5.3	0.3	0.7	2.6	1.5	0.4	0.6
21.	PS219	310.4	214.5	1661.6	112.0	245.7	332.5	47.8	101.6
22.	PS220	23.59	23.3	48.4	244.2	643.2	3.4	4.2	3.5
23.	PS222	1.94	1.1	1.2	2.5	0.9	3.5	0.8	0.5
24.	PS224	0.85	1.6	1.0	0.0064	2.0	11.4	0.3	0.2
25.	PS230	0.67	0.5	0.7	0.5	1.7	0.7	0.2	0.4

Table 4.17. Relative fold expression of efflux pump genes in carbapenem-resistant *P. aeruginosa* clinical isolates compared to *P. aeruginosa* ATCC 27853 (cont'd)

S/N	Isolate	Efflux pump genes							
		mexA	***mexB***	***mexC***	***mexD***	***mexX***	***mexY***	***mexE***	***mexF***
26.	PS238	0.1	3.3	0.4	0.3	10.6	24.2	0.1	0.01
27.	PS243	0.3	0.7	2.6	0.4	34.7	46.1	0.7	0.6
28.	PS244	141.6	54.2	323.4	276.3	276.4	113.4	11.8	32.4
29.	PS246	0.5	0.8	0.5	0.1	0.9	1.6	0.3	0.4
30.	PS250	0.9	1.3	0.8	0.3	2.4	1.2	0.2	0.1
31.	PS291	1.7	0.1	0.1	0.1	3.4	0.04	0.1	0.05
32.	PS292	3.5	5.3	0.6	0.2	7.0	3.3	0.3	0.2
33.	PS293	75.8	20.2	133.4	211.1	996.3	2430.8	279.9	41.7
34.	PS296	1.0	0.2	1.6	0.02	3.3	0.2	1.1	1.0
35.	PS303	0.1	0.1	0.2	0.1	17.1	0.4	0.1	0.01
36.	PS349	0.2	2.3	5.5	1.8	25.6	0.5	0.03	0.7
37.	PS367	2.5	0.2	1.8	0.2	91.3	2.3	0.5	0.1
38.	PS384	0.4	0.6	0.2	0.1	0.6	0.6	0.5	0.1
39.	PS392	0.6	0.4	11.8	8.1	40.4	54.4	8.1	5.3
40.	PS393	0.3	0.2	0.4	0.1	12.1	2.9	0.1	0.01
41.	PS394	1.8	2.3	2.3	0.8	2.9	1.0	0.02	0.5
42.	PS395	8.1	0.01	21.5	26.6	11.1	8.5	1.0	0.9
43.	PS397	3.1	1.4	1.5	0.5	1.6	0.7	0.1	0.2
44.	PS398	4.5	2.0	0.7	0.5	1.3	2.3	0.4	0.7
45.	PS400	1.5	0.7	5.9	3.2	24.1	5.3	0.9	1.4
46.	PS405	2.2	3.1	0.2	0.1	2.7	1.2	0.04	0.3
47.	PS409	0.1	0.8	5.2	0.2	1.2	1.4	0.2	0.2
48.	PS414	0.4	0.7	0.3	0.3	3.3	31.2	0.01	0.03
49.	PS351 (nonMDR)	0.8	0.4	2.4	0.4	0.2	0.4	0.03	0.3

Table 4.18. Relative fold expression of *ampC* and outermembrane porin (*oprD*) in carbapenem-resistant *P. aeruginosa* clinical isolates compared to *P. aeruginosa* ATCC 27853

S/N	Isolate	*oprD*	*ampC*
1.	PS007	5.4	0.8
2.	PS022	0.0002	1.1
3.	PS088	4.1	1.9
4.	PS093	0.02	405.2
5.	PS097	0.001	1.3
6.	PS099	2.0	2.2
7.	PS100	0.5	1.9
8.	PS150	0.002	2.0
9.	PS152	0.001	1.6
10.	PS166	0.01	13.1
11.	PS170	1.6	510.6
12.	PS173	0.2	8.3
13.	PS181	0.01	0.7
14.	PS182	1.8	2823.7
15.	PS183	0.3	113.8
16.	PS185	0.003	3.7
17.	PS202	0.2	5086.7
18.	PS204	1.1	217.4
19.	PS209	0.0	0.4
20.	PS210	0.004	0.3
21.	PS219	0.2	464.1
22.	PS220	1.1	7.1
23.	PS222	0.08	0.2
24.	PS224	0.03	1.6
25.	PS230	0.001	0.6

Table 4.18. Relative fold expression of *ampC* and outermembrane porin (*oprD*) in carbapenem-resistant *P. aeruginosa* clinical isolates compared to *P. aeruginosa* ATCC 27853 (cont'd)

S/N	Isolate	*oprD*	*ampC*
26.	PS238	0.001	0.2
27.	PS243	0.1	118.5
28.	PS244	1.5	50.3
29.	PS246	0.4	0.8
30.	PS250	0.003	137.2
31.	PS291	0.02	0.3
32.	PS292	0.3	0.1
33.	PS293	0.3	1767.5
34.	PS296	0.3	2.4
35.	PS303	0.001	0.1
36.	PS349	1.5	0.6
37.	PS367	0.1	8.0
38.	PS384	0.5	1.0
39.	PS392	0.002	460.3
40.	PS393	0.002	6.0
41.	PS394	0.01	0.4
42.	PS395	0.1	9.4
43.	PS397	1.3	2.0
44.	PS398	1.1	0.9
45.	PS400	0.02	50.2
46.	PS405	0.2	0.7
47.	PS409	0.4	0.2
48.	PS414	0.002	0.3
49.	PS351(nonMDR)	0.7	8.4

Table 4.19. MDR efflux gene overexpression, *ampC* overexpression and *oprD* loss in carbapenem-resistant *P. aeruginosa*

Efflux gene	No (%) of isolates with overexpression	Range of fold increase
mexA	19 (39.6)	2.5 - 310.4
mexB	18 (37.5)	3.1 - 214.5
mexC	4 (8.3)	133.4 - 1661.6
mexD	5 (10.4)	211.1 – 2550.9
mexE	2 (4.2)	108.3 - 279.9
mexF	1 (2.1)	101.3
mexX	26 (54.2)	5.0 - 996.3
mexY	18 (37.5)	5.3 - 2430.8
oprD	37 (77.1)	0.001 - 0.5
ampC	13 (27.1)	50.2 – 5086.7

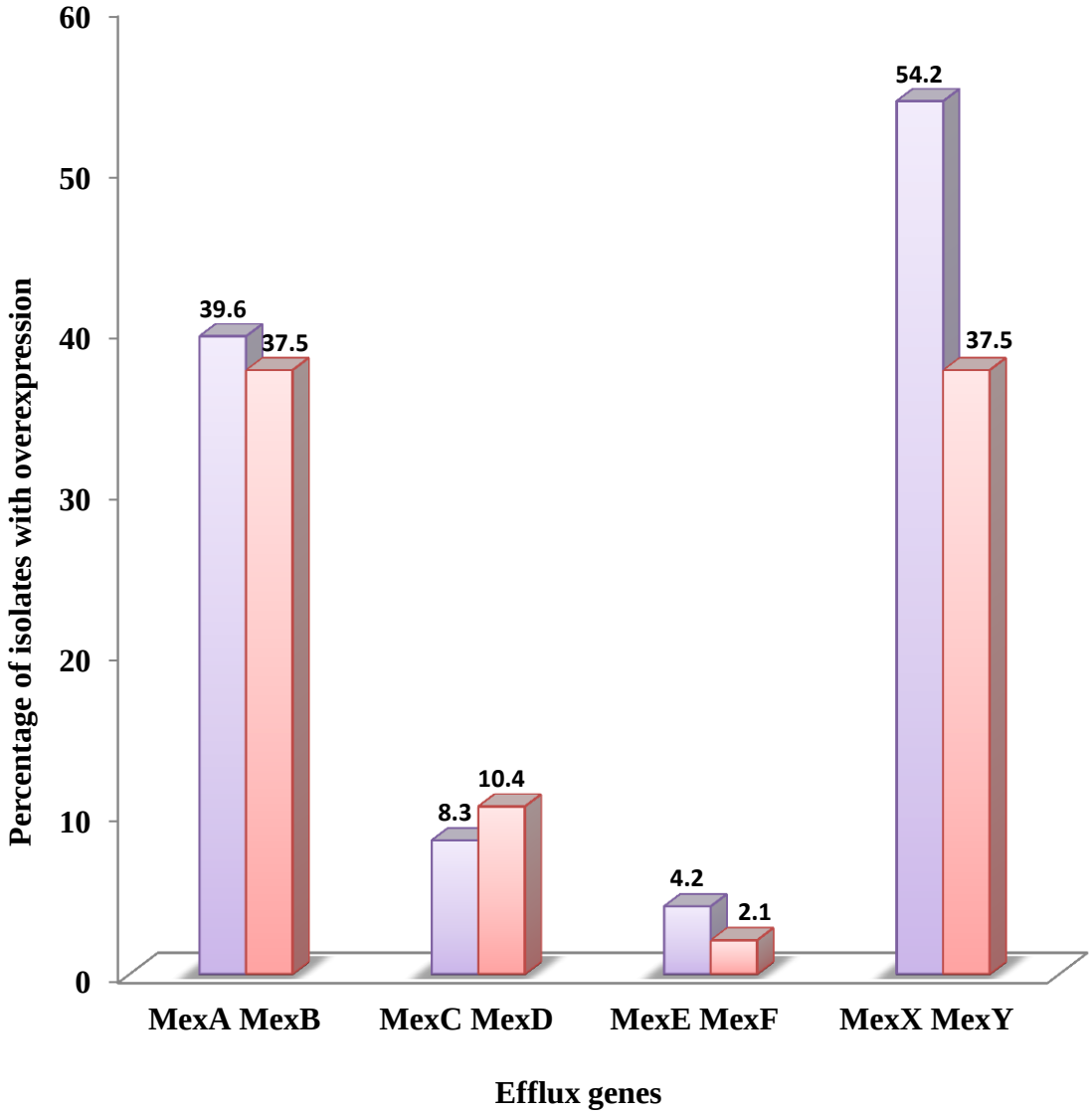

Figure 4.6. Comparison of proportion of two efflux genes overexpressed in each of the four efflux pump system in carbapenem-resistant *P. aeruginosa*

Table 4.20. MIC of antibiotics against carbapenem-resistant *P. aeruginosa* isolates in relation to MDR efflux gene overexpressed and MBL

S/N	Isolate	Sample	Ipm	Mem	Fep	Caz	Cip	Effux gene	Efflux pump	MBL
1.	PS007	UCHI	16	16	>128	>256	128	*A*	MexAB-OprM	-
2.	PS088	FMCA	32	16	4	128	16	*X*	MexXY-OprM	-
3.	PS093	FMCA	2	8	16	128	>128	*A, B, X, Y*	MexAB-OprM, MexXY-OprM	+
4.	PS099	FMCO	8	8	16	256	4	*A, B, X*	MexAB-OprM, MexXY-OprM	-
5.	PS100	FMCO	4	8	16	256	8	*A, B, X*	MexAB-OprM, MexXY-OprM	+
6.	PS152	UCHI	32	>64	>128	>256	>128	*X*	MexXY-OprM	+
7.	PS170	UCHI	8	16	8	>256	32	*A, X, Y, E*	MexAB-OprM, MexXY-OprM, MexEF-OprN	-
8.	PS182	UCHI	4	8	4	4	16	*A, B, X, Y, F*	MexAB-OprM, MexXY-OprM, MexEF-OprN	-
9.	PS183	UCHI	16	64	>128	>256	>128	*XY*	MexXY-OprM	+
10.	PS202	OTHS	8	16	16	>256	32	*A, B, C, D, X, Y*	MexAB-OprM, MexCD-OprJ,	+
11.	PS204	UCHI	>64	>64	>128	>256	>128	*A, B, X, Y*	MexAB-OprM, MexXY-OprM	+
12.	PS209	OTHI	8	>64	32	128	4	*B, X, Y*	MexAB-OprM, MexXY-OprM	+
13.	PS210	OTHI	16	>64	32	256	8	*A, B*	MexAB-OprM	+
14.	PS219	FMCO	>64	64	64	>256	>128	*A, B, C, D, X, Y*	MexAB-OprM, MexCD-OprJ, MexXY-OprM	+
15.	PS220	FMCO	16	64	>128	32	>128	*A, B, D, X*	MexAB-OprM, MexCD-OprJ, MexXY-OprM	+
16.	PS224	FMCO	32	32	64	64	32	*Y*	MexXY-OprM	+
17.	PS238	LTHO	>64	>64	>128	>256	>128	*B, X, Y*	MexAB-OprM, MexXY-OprM	+

Key: Ipm = Imipenem, Mem = Meropenem, Fep = Cefepime, Caz = Ceftazidime, Cro = Ceftriaxone, Cip = Ciprofloxacin, + = MBL present, - = MBL absent, *A* = *mexA*, *B* = *mexB*, *C* = *mexC*, *D* = *mexD*, *E* = *mexE*, *F* = *mexF*, *X* = *mexX*, *Y* = *mexY*

Table 4.20. MIC of selected antibiotics against carbapenem-resistant *P. aeruginosa* isolates in relation to MDR efflux gene overexpressed and MBL (cont'd)

S/N	Isolate	Sample	Ipm	Mem	Fep	Caz	Cip	Efflux gene	Efflux pump	MBL
18.	PS243	LTHO	64	>64	>128	>256	>128	*X, Y*	MexXY-OprM	+
19.	PS244	UCHI	>64	>64	>128	>256	>128	*A, B, C, D, X, Y*	MexAB-OprM, MexCD-OprJ, MexXY-OprM	-
20.	PS292	FMCI	2	8	>128	8	64	*A, B, X*	MexAB-OprM, MexXY-OprM	+
21.	PS293	FMCI	>64	>64	>128	>256	>128	*A, B, C, D, X, Y, E*	MexAB-OprM, MexCD-OprJ, MexXY-OprM, MexEF-OprN	+
22.	PS303	FMCI	>64	>64	>128	>256	>128	*X*	MexXY-OprM	+
23.	PS349	OTHI	4	>64	>128	>256	>128	*B, X*	MexAB-OprM, MexXY-OprM	-
24.	PS367	FMCO	>64	>64	>128	>256	>128	*A, X*	MexAB-OprM, MexXY-OprM	+
25.	PS392	LTHO	>64	>64	128	256	>128	*X, Y*	MexXY-OprM	+
26.	PS393	LTHO	>64	>64	>128	>256	>128	*X*	MexXY-OprM	+
27.	PS394	LTHO	>64	>64	16	>256	>128	*B*	MexAB-OprM	+
28.	PS395	OTHI	>64	>64	>128	>256	>128	*A, X, Y*	MexAB-OprM, MexXY-OprM	+
29.	PS397	OTHI	>64	>64	>128	>256	>128	*A*	MexAB-OprM	+
30.	PS398	OTHI	>64	>64	>128	>256	>128	*A, B*	MexAB-OprM	+
31.	PS400	OTHI	>64	>64	>128	>256	>128	*X, Y*	MexXY-OprM	+
32.	PS405	FMCO	>64	>64	>128	>256	>128	*A, B*	MexAB-OprM	+
33.	PS414	LTHO	>64	>64	>128	>256	>128	*Y*	MexXY-OprM	+

Key: Ipm = Imipenem, Mem = Meropenem, Fep = Cefepime, Caz = Ceftazidime, Cro = Ceftriaxone, Cip = Ciprofloxacin, + = MBL present, - = MBL absent, *A* = *mexA*, *B* = *mexB*, *C* = *mexC*, *D* = *mexD*, *E* = *mexE*, *F* = *mexF*, *X* = *mexX*, *Y* = *mexY*

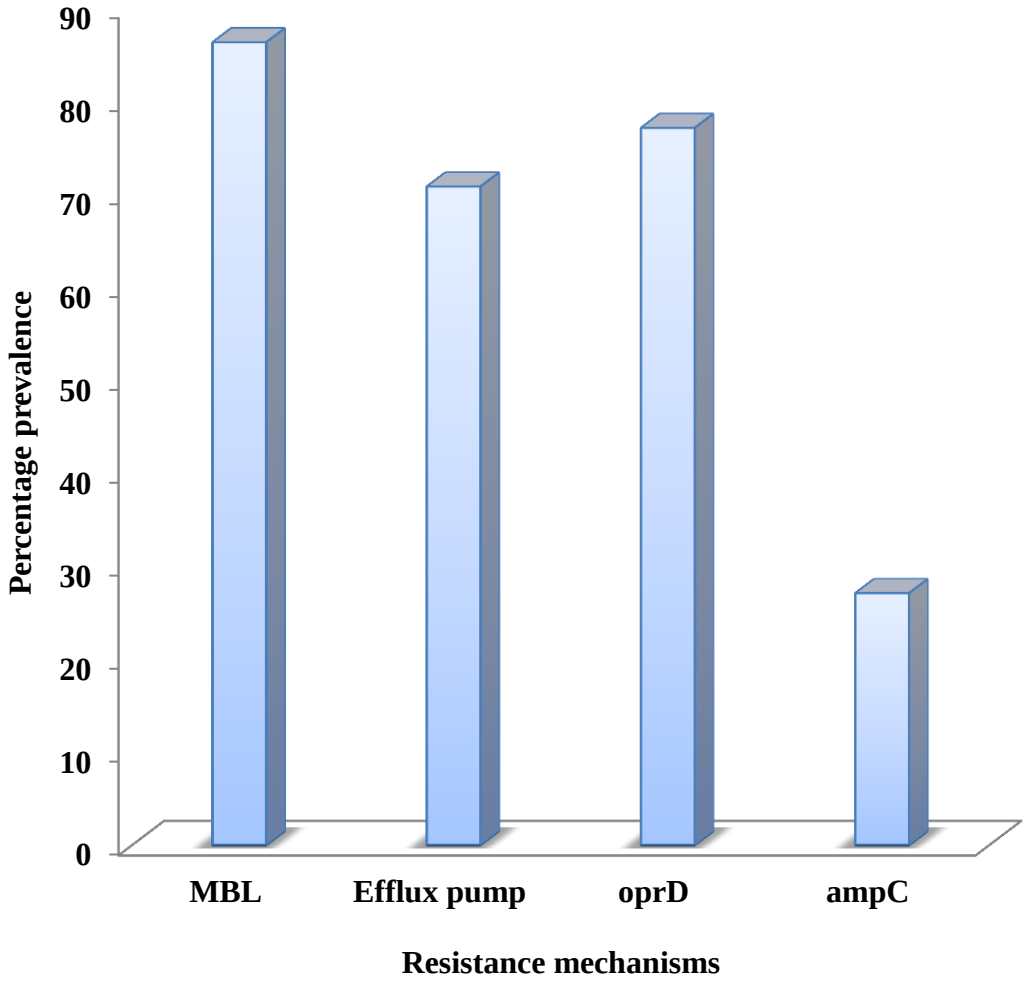

Figure 4.7. Occurrence of various carbapenem resistance mechanisms in carbapenem-resistant *P. aeruginosa*

Table 4.21. Singles and combinations of resistance mechanisms in CRPA

Resistance mechanisms	**Number of isolates**
MBL, Efflux pump, *oprD*, *ampC*	8
MBL, Efflux pump, *oprD*	13
MBL, Efflux pump, *ampC*	1
MBL, *oprD*	8
MBL, Efflux pump	3
Efflux pump, *ampC*	2
Efflux pump, *oprD*	1
MBL, *oprD*, *ampC*	1
MBL	4
Efflux pump	4
oprD	3

4.18 Molecular typing of carbapenem-resistant *Pseudomonas aeruginosa*

The three rep-PCR methods used for typing revealed a lot of genetic diversity among the isolates. Amplification of genomic DNA of seventy-three carbapenem-resistant and two carbapenem-sensitive strains of *P. aeruginosa* with REP1R and REP2 primers produced 48 fingerprinting patterns with 27 common types and 21 single types from the dendrogram. ERIC-PCR primers (ERIC1R and ERIC2) gave 50 fingerprinting patterns containing 25 common types and 25 unique patterns were found. Fifty-two (52) fingerprinting profiles were obtained with BOXAIR primer, 22 of which were common types while the remaining 30 were unique types (Table 4.22). REP-PCR produced 2 to 14 bands with molecular weight ranging from 200bp to 4000bp. ERIC-PCR gave 4 to 15 bands with band sizes ranging from 100bp to 3500bp while BOX-PCR produced 7 to 15 bands with molecular weight of between 250bp and 3500bp (Plates 4.13 – 4.15).

Cluster analysis of ERIC-PCR type at 0.15 distances revealed twenty two (22) types containing sixteen (16) clusters and six (6) single isolates with discriminatory index of 0.934. The prevalent cluster was type C consisting of eleven (11) isolates followed by type B with ten (10) isolates. BOX-PCR type produced twenty three (23) types containing twelve (12) clusters with 11 single isolates with discriminatory index of 0.896 at 0.15 distances. Type B was the most common cluster consisting of eighteen isolates followed by types E and I with thirteen (13) and eight (8) isolates, respectively. REP-PCR type gave twelve (12) types consisting of eight (8) clusters with four (4) single isolates having discriminatory index of 0.854. Type A was the prevalent consisting of eighteen (18) isolates followed by types F and B with fifteen (15) and fourteen (14) isolates, respectively (Figures 4.8 - 4.10).

REP-PCR type could not differentiate between pairs of seven set of isolates (PS303 = PS395; PS235 = PS184; PS296 = PS351; PS205 = PS088; PS100 = PS022; PS166 = PS350; PS168 = PS209). PS303 and PS395 strains obtained from different hospitals (FMCI and OTHI, respectively) could not be distinguished with REP-PCR. The two isolates harboured *exoU* and bla_{NDM} and also belong to the same cluster C and E on ERIC- and BOX- PCR. PS235 and PS184 were obtained from LTHO and UCHI, respectively, however, these strains could not be differentiated with REP-PCR but belong to the same cluster F on ERIC-PCR and cluster I on BOX- PCR dendrogram. Both isolates harboured bla_{NDM} and have the *exoU* gene. REP-profile was not able to

distinguish between PS296 and PS351 isolates that PS296 was resistant to all the three carbapenem (imipenem, meropenem and doripenem) as well as harboured *bla*$_{VIM}$ and *bla*$_{NDM}$ while PS351 was susceptible to the three carbapenems and do not possess neither *bla*$_{VIM}$ nor *bla*$_{NDM}$ although they both have *exoS* gene. PS296 and PS351 belong to different clusters A and I on ERIC-profile as well as clusters A and L on BOX-profile dendrograms, respectively. PS205 and PS088 obtained from different hospitals (UCHI and FMCA respectively) are not distinguishable on REP-PCR dendrogram but belong to different clusters C and D on ERIC-profile as well as clusters E and F on BOX-profile, respectively. PS205 contain *bla*$_{VIM}$ which was not present in PS088 but both have *exoS* toxin. PS100 and PS022 was another pair of two different strains obtained from different hospitals (FMCI and UCHI, respectively) that could not be distinguished with BOX-profile. The two isolates both belong to cluster B by ERIC-profile but cluster differs (clusters B and G) by BOX-profile. They both contain *bla*$_{VIM}$ contain *exoS* gene. PS166 and PS350 were also obtained from different hospitals (UCHI and OTHI, respectively) but were indistinguishable by REP-profiling. PS350 belongs to cluster P while PS166 does not cluster with any isolate on ERIC-profile while on BOX-profile, PS166 and PS350 belong to clusters G and H, respectively. PS350 contain *bla*$_{VIM}$ which was not present in PS166 but they both contain *exoU* gene. PS168 and PS209 was another pair of epidemiologically unrelated isolates obtained from different hospitals (UCHI and OTHI, respectively) but was not distinguishable by REP-profiling. PS168 and PS350 belong to different clusters B and P by ERIC-profile and clusters J and G by BOX-profile, respectively. Both contain *bla*$_{VIM}$ and *exoU* toxin (Table 4.23).

BOX-profiling was unable to differentiate between PS297 and PS285 isolates from the same hospital (FMCI). Both isolates have different type 3 effector toxin and also contain different MBL gene (*bla*$_{VIM}$:*exoS* and *bla*$_{NDM}$:*exoU*, respectively). Another set of isolates that could not be differentiated with BOX-PCR was PS202, PS383 and PS250. PS202 isolate was obtained from OTHS and was carrying *bla*$_{VIM}$ and also contain *exoS* gene; for PS383 and PS250 obtained from UCHI, PS383 harboured *bla*$_{NDM}$ only while PS250 harboured both *bla*$_{VIM}$ and *bla*$_{NDM}$ even though both isolates have *exoU* type III effector toxin. Likewise, BOX-profile could not distinguish between PS303 and PS354 obtained from FMCI and OTHI, respectively. These isolates belong to the same cluster (B) on ERIC- and REP- PCR dendrograms, both

harboured *bla*$_{NDM}$ and contain *exoU* effector toxin but PS354 also possessed *exoS* alongside *exoU* toxin. Similarly, PS238 and PS152 obtained from LTHO and UCHI, respectively could not be differentiated with BOX-PCR and also belong to the same clusters C and I on REP- and ERIC- PCR. Although both PS238 and PS152 contain *exoU* gene, PS152 harboured *bla*$_{NDM}$ only while PS238 harboured both *bla*$_{VIM}$ and *bla*$_{NDM}$. Only BOX-PCR was able to group the two carbapenem susceptible isolates into the same cluster L (Table 4.24).

Table 4.25 gave the genotypic characteristic of four clones confirmed by two or more rep-PCR methods. Two strains in clone 01 both possessed *bla*$_{VIM}$, class 1 integrons with approximately 3.5 kb cassette, *exoS* type III effector gene, *ampC* overexpression absent, underexpressed *oprD*, while PS100 overexpressed *mexA*, *mexB* and mexX, PS022 do not overexpress any of the efflux pump genes.

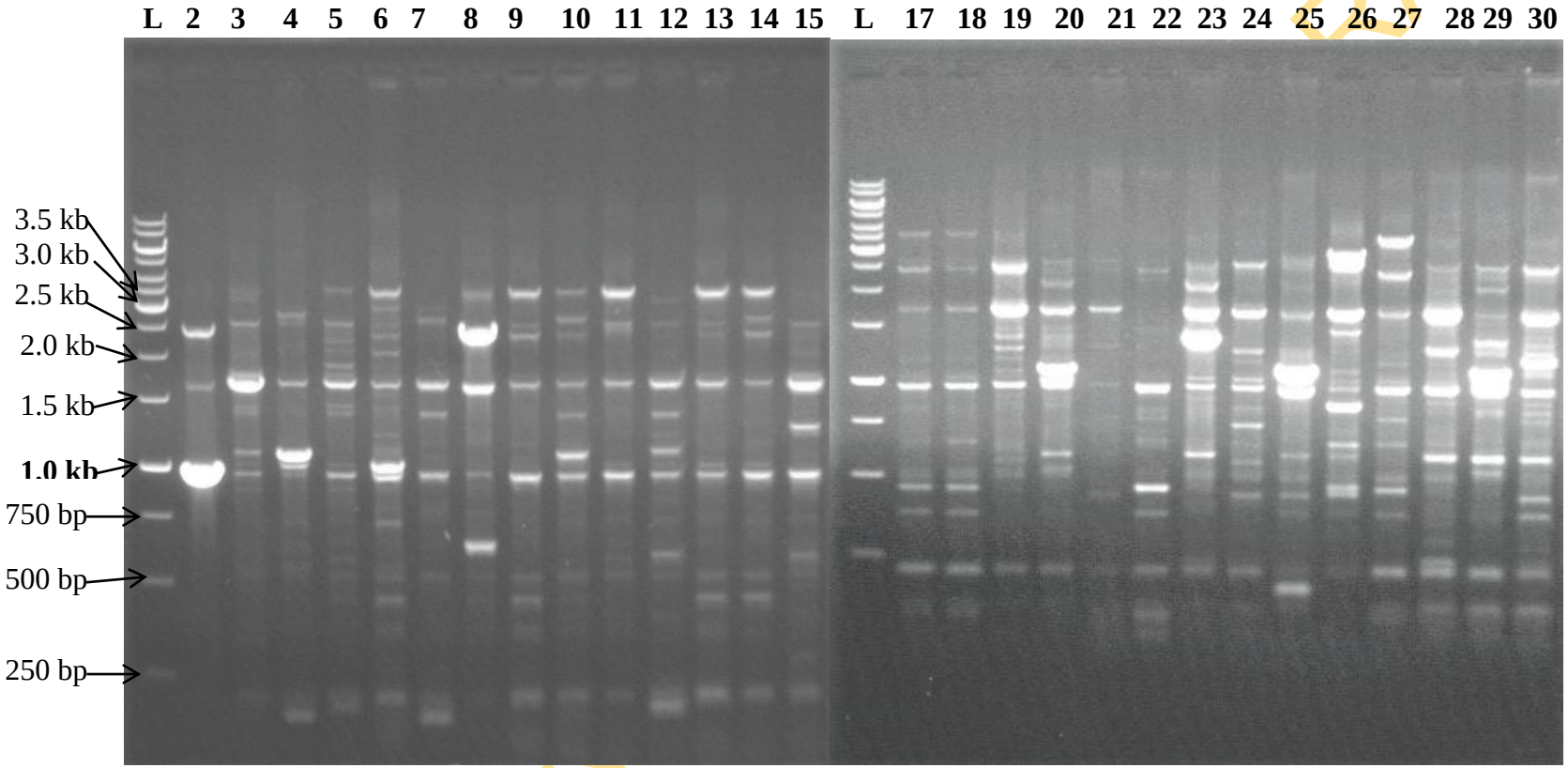

Plate 4.13. Representative image of fingerprinting patterns of carbapenem-resistant *P. aeruginosa* strains by gel electrophoresis after REP-PCR; Lanes L = 1 kb plus ladder, Lanes 2-15, 17-30 = PS383, PS202, PS297, PS335, PS147, PS394, PS367, PS250, PS293, PS405, PS007, PS400, PS285, PS398, PS235, PS184, PS352, PS244, PS170, PS181, PS230, PS397, PS253, PS409, 348, PS243, PS220 and PS246.

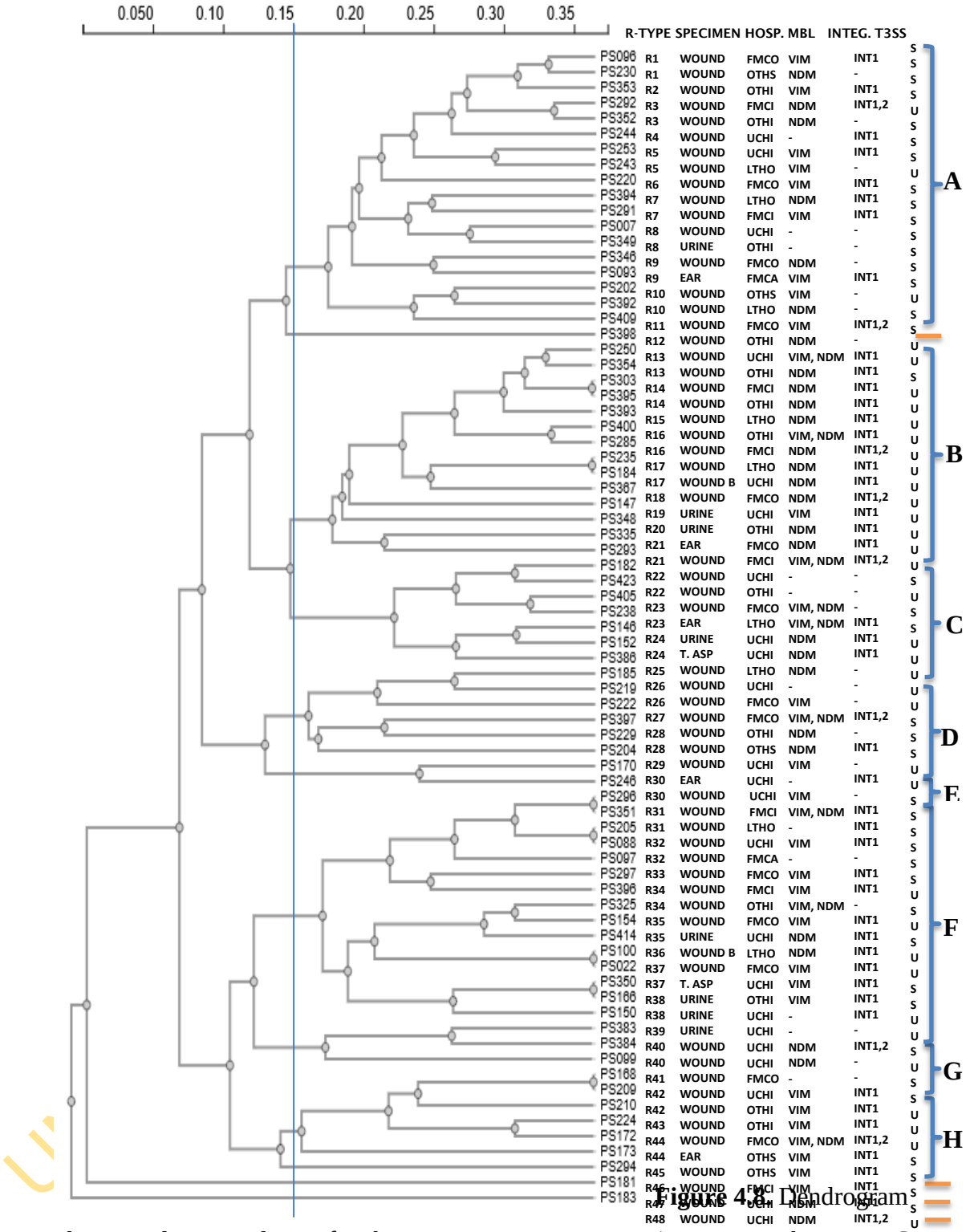

Figure 4.8. Dendrogram showing cluster analysis of carbapenem-resistant *P. aeruginosa* strains by REP-PCR using phylotree software and Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Key: U= *exoU*; S= *exoS*

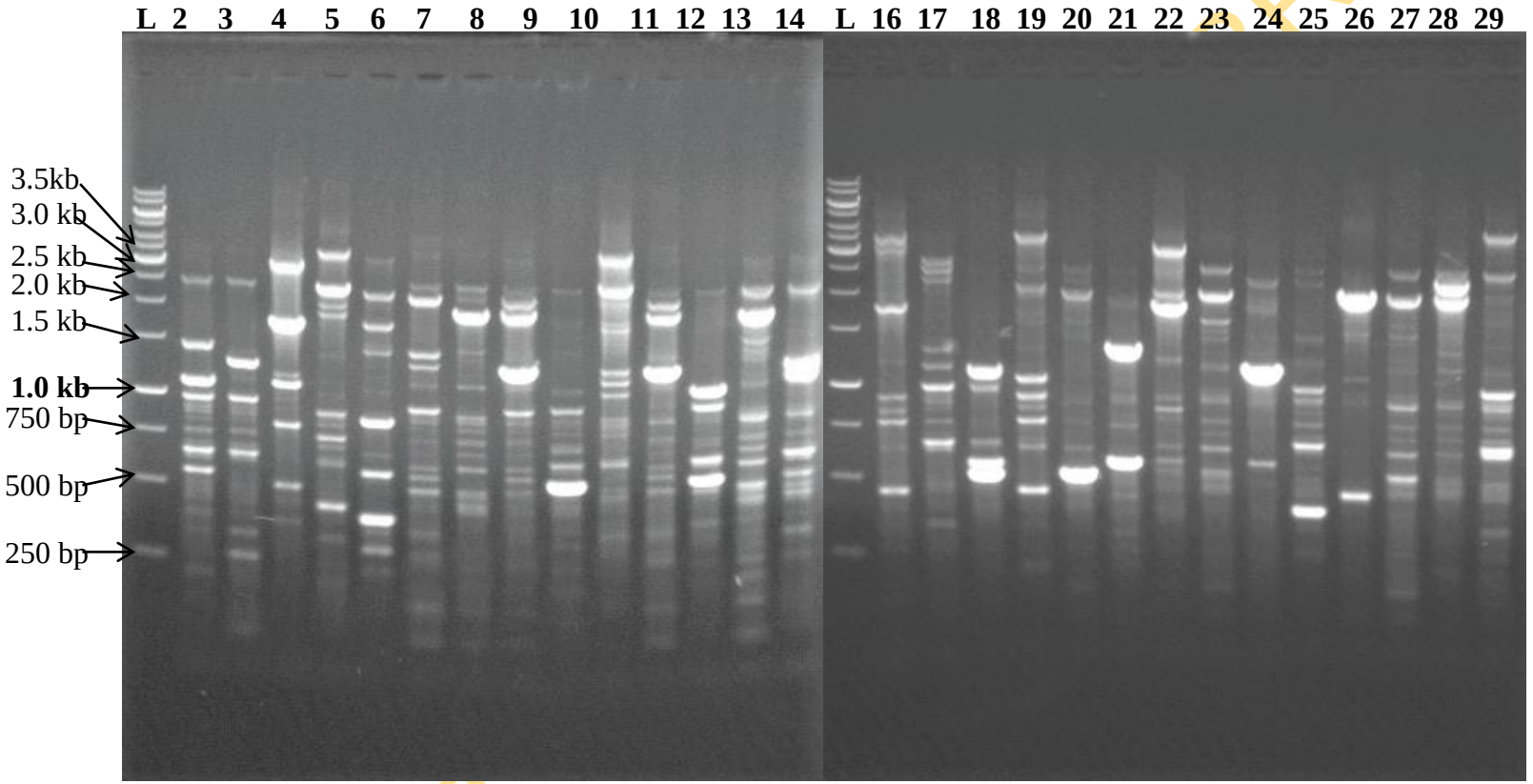

Plate 4.14. Representative image of fingerprinting patterns of carbapenem-resistant *P. aeruginosa* strains by gel electrophoresis following ERIC-PCR; Lanes L = 1 kb plus ladder, Lanes 2-14, 16-29 = PS296, PS325, PS97, PS100, PS185, PS182, PS154, PS168, PS173, PS204, PS210, PS224, PS414, PS383, PS202, PS297, PS335, PS147, PS394, PS367, PS250, PS293, PS405, PS007, PS400, PS285 and PS398.

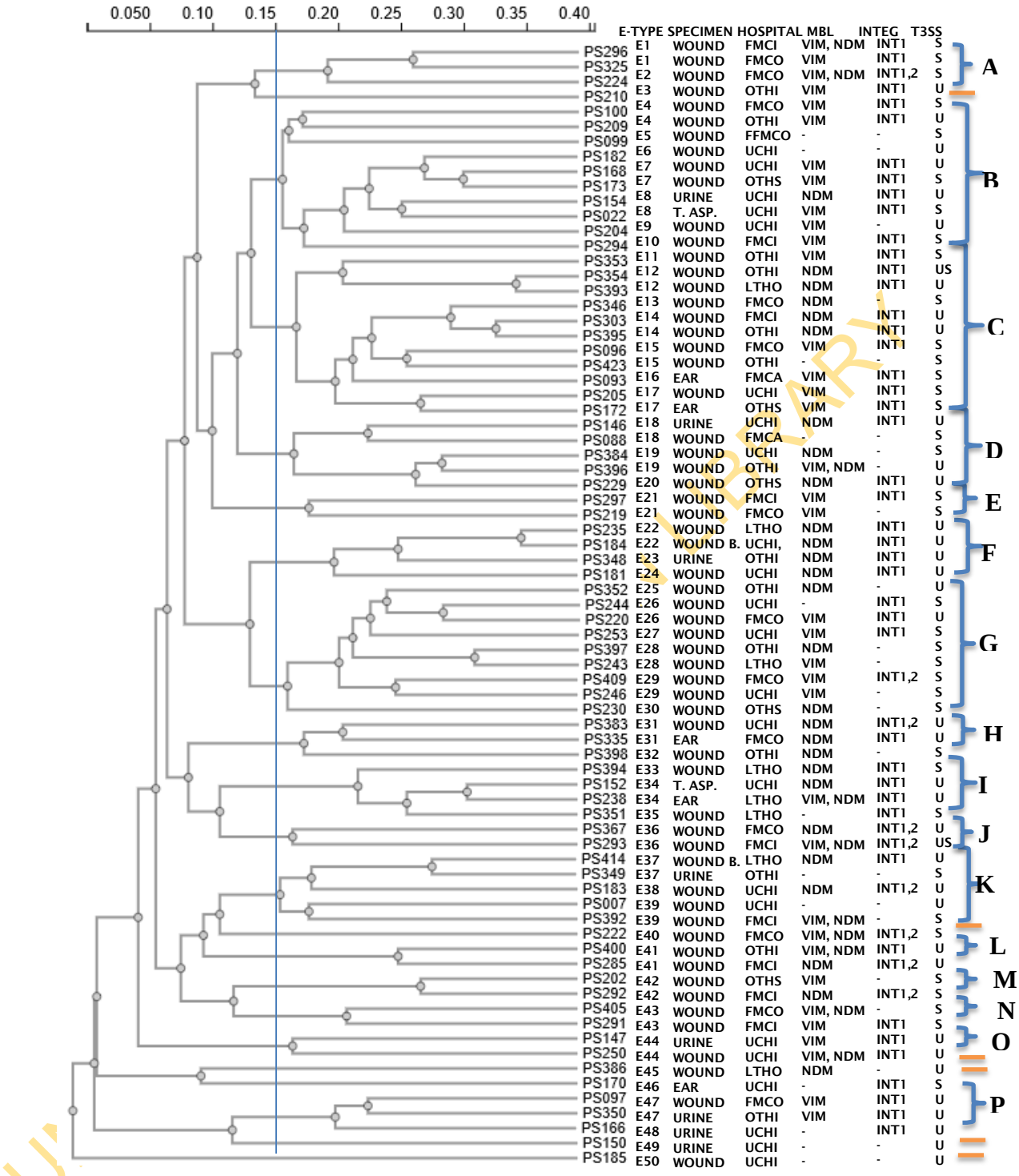

Figure 4.9. Dendrogram showing cluster analysis of carbapenem-resistant *P. aeruginosa* strains by ERIC-PCR using Phylotree software and Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Key: U= *exoU*; S= *exoS*

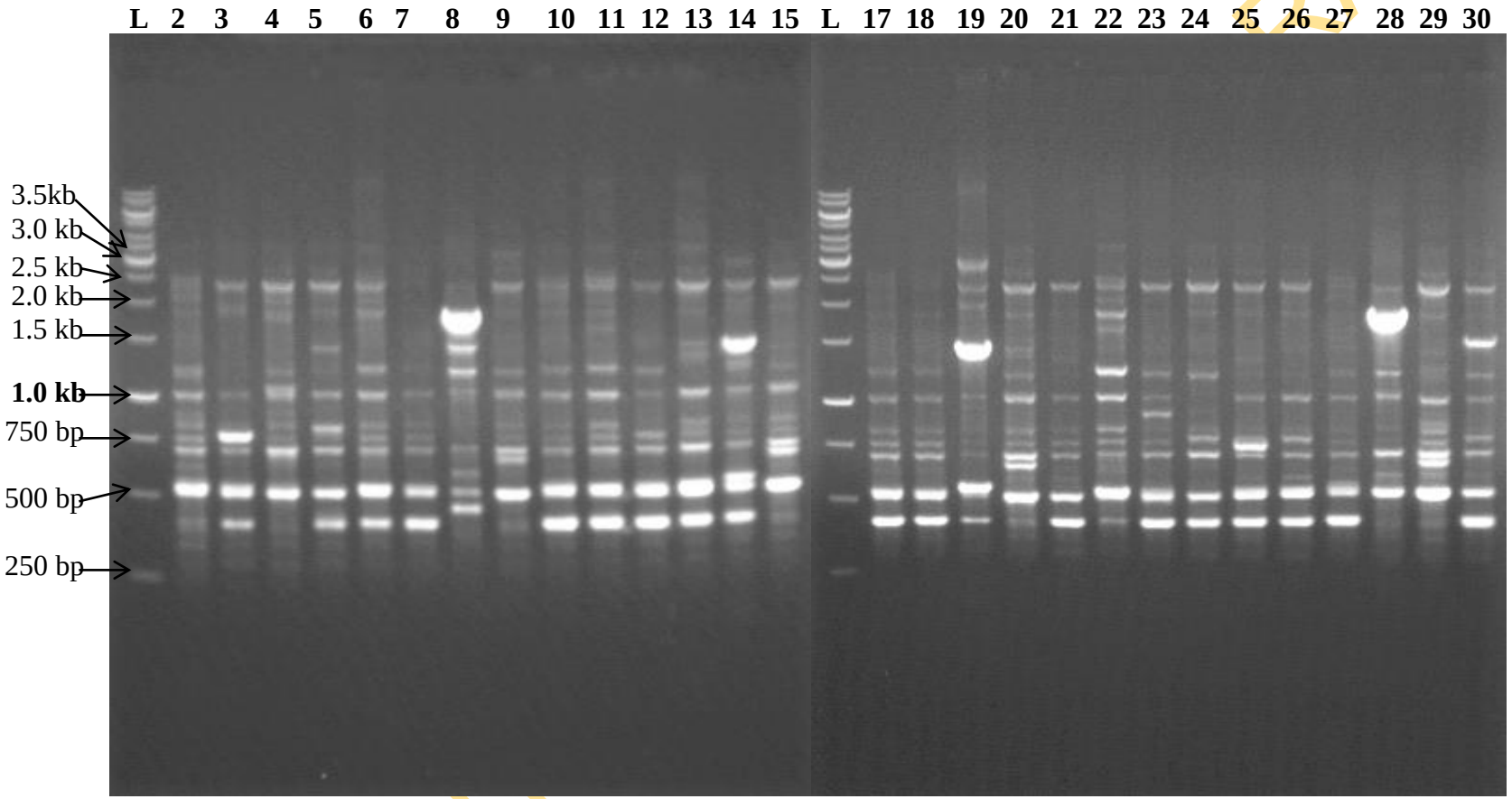

Plate 4.15. Representative image of fingerprinting patterns of carbapenem-resistant *P. aeruginosa* strains by gel electrophoresis following BOX-PCR; Lanes L = 1 kb plus ladder, Lanes 2-15, 17-30 = PS386, PS353, PS392, PS346, PS354, PS393, PS222, PS291, PS303, PS395, PS096, PS349, PS093, PS292, PS235, PS184, PS352, PS244, PS170, PS181, PS230, PS397, PS253, PS409, 348, PS243, PS220 and PS246.

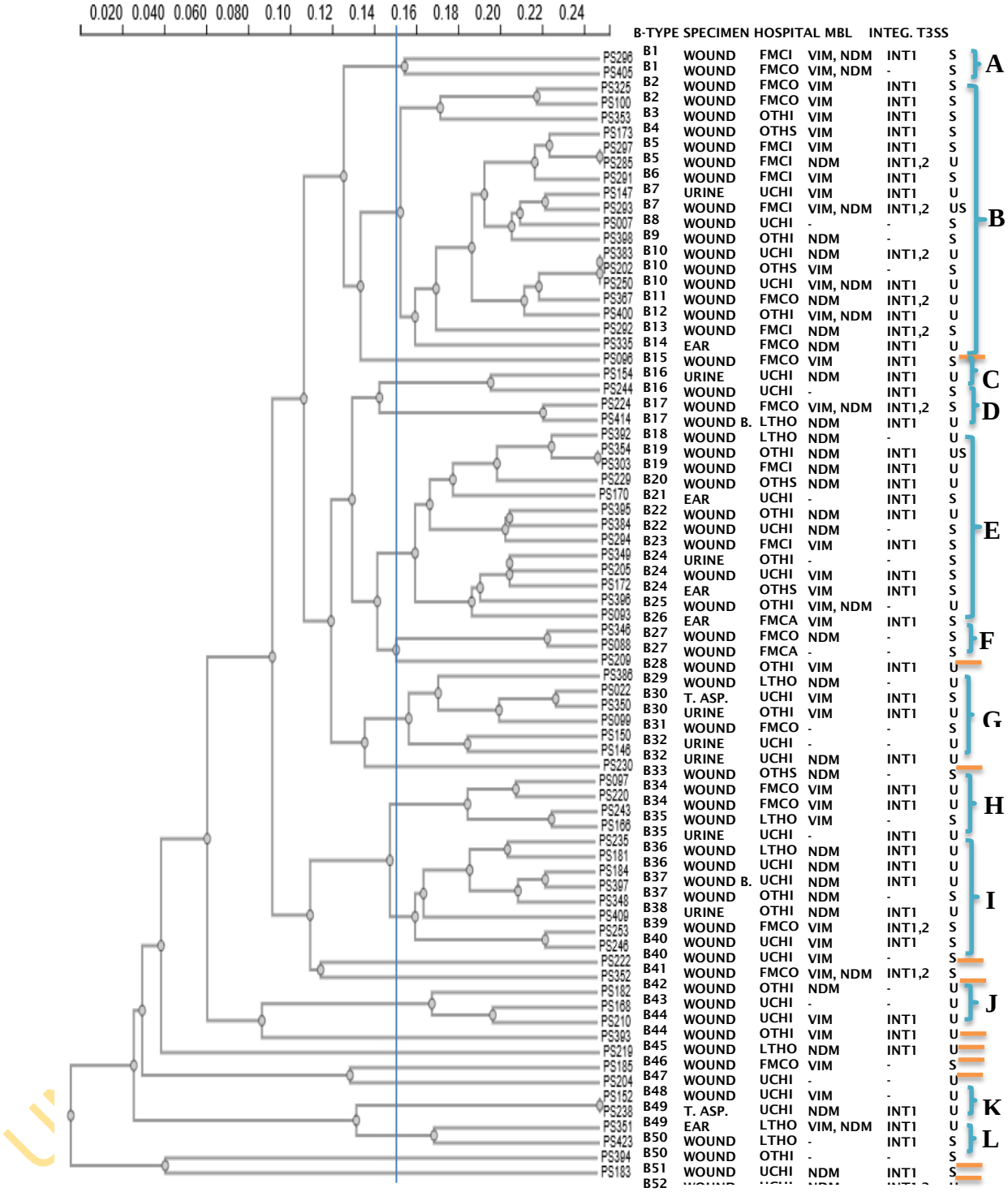

Figure 4.10. Dendrogram showing cluster analysis of carbapenem resistant *P. aeruginosa* by BOX-PCR using Phylotree software and Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Key: U = *exoU*, S = *exoS*

Table 4.22. Comparison between REP-, ERIC- and BOX- PCR

Characteristic	**REP-PCR**	**ERIC-PCR**	**BOX-PCR**
Fingerprint pattern	48 (27 CT, 21 ST)	50 (25CT, 25 ST)	52 (22 CT, 30 ST)
No of bands	2 – 14	4 – 15	7 – 15
Molecular weight	200 – 4000 bp	100 – 3500 bp	250 – 3500 bp
Discriminatory index *(D)*	0.854	0.934	0.896
Typeable strains	61/75 strains	75/75 strains	66/75 strains
Clusters	8	16	12
Single isolates	4	6	11
Prevalent cluster	A (18 isolates)	C (11 isolates)	B (18 isolates)

Key:

CT = Common type
ST = Single type

Table 4.23. Indistinguishable isolates with REP-PCR

Clone	Isolate	Hospital	T3SS	MBL	ERIC-PCR cluster	BOX-PCR cluster
01	PS303	FMCI	*exoU*	bla_{NDM}	C	E
	PS395	OTHI	*exoU*	bla_{NDM}	C	E
02	PS235	LTHO	*exoU*	bla_{NDM}	F	I
	PS184	UCHI	*exoU*	bla_{NDM}	F	I
03	PS296	FMCI	*exoS*	bla_{VIM}, bla_{NDM}	A	A
	PS351	LTHO	*exoS*	-	I	L
04	PS205	UCHI	*exoS*	bla_{VIM}	C	E
	PS088	FMCA	*exoS*	-	D	F
05	PS100	FMCI	*exoS*	bla_{VIM}	B	B
	PS022	UCHI	*exoS*	bla_{VIM}	B	G
06	PS166	UCHI	*exoU*	-	P	G
	PS350	OTHI	*exoU*	bla_{VIM}	-	H
07	PS168	UCHI	*exoU*	bla_{VIM}	B	J
	PS209	OTHI	*exoU*	bla_{VIM}	P	G

Table 4.24. Indistinguishable isolates with BOX-PCR

Clone	Isolate	Hospital	T3SS	MBL	ERIC-PCR cluster	REP-PCR cluster
01	PS297	FMCI	*exoS*	bla_{VIM}	E	F
	PS285	FMCI	*exoU*	bla_{NDM}	L	B
02	PS202	OTHS	*exoS*	bla_{VIM}	M	A
	PS383	UCHI	*exoU*	bla_{NDM}	H	G
	PS250	UCHI	*exoU*	bla_{VIM}, bla_{NDM}	O	B
03	PS303	FMCI	*exoU*	bla_{NDM}	C	B
	PS354	OTHI	*exoU*, *exoS*	bla_{NDM}	C	B
04	PS238	LTHO	*exoU*	bla_{VIM}, bla_{NDM}	I	C
	PS152	UCHI	*exoU*	bla_{NDM}	I	C

Table 4.25. Genotypic characteristic of four clones confirmed by two or more methods

Clone	Isolate	Clinical source	MBL(s)	Integron	Class 1 integrase	T3SS	Efflux gene(s)	*ampC*	*oprD*
01	PS022	Tracheal aspirate	bla_{VIM}	*intI1*	3.5 kb	*exoS*	-	-	+
	PS100	Wound	bla_{VIM}	*intI1*	3.5 kb	*exoS*	*mexA, mexB, mexX*	-	+
02	PS303	Wound	bla_{NDM}	*intI1*	3.5 kb	*exoU*	*mexX*	-	+
	PS395	Wound	bla_{NDM}	*intI1*	3.5 kb	*exoU*	*mexA, mexX, mexY*	-	+
	PS354	Wound	bla_{NDM}	*intI1*	NA	*exoU, exoS*	ND	ND	ND
03	PS235	Wound	bla_{NDM}	*intI1*	NA	*exoU*	ND	ND	ND
	PS184	Wound biopsy	bla_{NDM}	*intI1*	3.5 kb	*exoU*	ND	ND	ND
04	PS238	Ear	bla_{VIM}, bla_{NDM}	*intI1*	3.5 kb	*exoU*	*mexB, mexX, mexY*	-	+
	PS152	Tracheal aspirate	bla_{NDM}	*intI1*	NA	*exoU*	*mexX*	-	+

Key: + = present; - = absent; ND = Not determined; NA = Not amplified

CHAPTER FIVE
DISCUSSION

5.1 Distribution of isolates in clinical samples

Pseudomonas aeruginosa is frequently recovered from wound infection. Wound isolates were predominant (69.3%) in this study followed by isolates from ear (11.4%) which was in agreement with the report by Oladipo *et al*. (2015) and Brown and Izundu, (2004). A study from Iran also documented highest prevalence of *P. aeruginosa* in wound samples with the percentage (69.9%) that was similar to what was obtained from this study (Khosravi *et al*. 2017). Studies from Southeastern and Central Nigeria have also documented highest prevalence of *P. aeruginosa* from wound sample (Eyo *et al*., 2015; Zubair and Iregbu, 2018). However, a report from Southwestern Nigeria with highest prevalence of *P. aeruginosa* from urine sample does not agree with findings from this study (Odumosu *et al*., 2012).

5.2 Antibiotic susceptibility profile of clinical isolates of *P. aeruginosa*

Pseudomonas aeruginosa is a recognised Gram-negative bacterium which is inherently resistant to many antibiotics due to collection of resistance mechanisms such as efflux pumps and antibiotic hydrolyzing enzymes that are disseminated through mobile genetic elements. In this present study, all the isolates were resistant to more than four antibiotics. Total resistance to ampicillin, cephalothin and cefuroxime was observed in this present study while a study in Egypt reported 95.8% resistance to cephalothin and cefuroxime (Afifi *et al*., 2013). Fluoroquinolones such as ciprofloxacin and levofloxacin are known to be potent extensive-spectrum antibiotics with efficacy on an extensive collection of bacteria including *P. aeruginosa*. This study reported higher resistant rates of 39.3% and 38.1% to ciprofloxacin and levofloxacin, respectively when compared to the report of the study from Southwestern Nigeria with resistance rate of 27.8% and 37.0%, respectively (Odumosu *et al.*, 2012), while higher resistance rate of 42.9% and 47.6% to ciprofloxacin and ofloxacin was reported by another study from southwestern Nigeria (Igbalajobi *et al*., 2016). Resistance to ofloxacin observed in this study (43.3%) was lower than the report by Olayinka *et al*. (2009) with

resistance rate of 82.6%. Gentamicin is considered as the first drug to select when it comes to treatment of *P. aeruginosa* infections (Oduyebo *et al.*, 1997), nonetheless, this study reports that 43.3% of *P. aeruginosa* isolates were not sensitive to gentamicin. This frequency was in range with earlier reports in which 40.7% resistant strains were observed (Odumosu *et al*., 2012) but higher than the report from Egypt with 21.1% resistant strains (Afifi *et al*., 2013). This study showed high resistance rate of 32.6% and 27.4% to tobramycin and amikacin correspondingly. Amikacin is considered as a replacement in the treatment of Gram-negative bacterial infections that are resistant to tobramycin, but has now been observed to have almost the same level of resistance with tobramycin (Brown and Izundu, 2004).

This present study reports higher resistance rate to third and fourth generation cephalosporins with percentage resistance of 45.8, 38.1 and 54.4 to cefepime, ceftazidime and ceftriaxone, respectively in contrast with the report of Odumosu *et al*. (2012) from the same region who recorded resistance rate of 9.3%, 14.8% and 27.8% to cefepime, ceftazidime and ceftriaxone, respectively. Higher resistance to cefepime was noticed- in this study when linked with prior information from the same region (Oladipo *et al*., 2015) where 6.0% were resistant. Increasing resistance to cephalosporins most especially the fourth generation cephalosporins necessitates proper guidelines for antibiotic prescriptions. The rate of resistance of *P. aeruginosa* to aztreonam (16.7%) found in this study was less than the percentage reported from Nigeria (33.3%), Egypt (46.1%) and Turkey (48.0%) (Odumosu *et al*., 2012; Afifi *et al*., 2013; Guvensen *et al*., 2017). In this study, only 16 (21.9%) CRPA were susceptible to aztreonam which is the only beta lactam with efficacy against MBL-producing Gram-negative bacteria but cannot resist hydrolysis by ESBLs, suggesting the presence of ESBLs among carbapenem resistance *P. aeruginosa*.

Carbapenems are potent and are used as last-line antibiotics in the therapy of Gram-negative bacterial infections especially the ESBLs. However, resistance to carbapenems is on the rise (Nabarro *et al*., 2017). Carbapenem resistance as recorded in this study was defined as non-susceptibility to at least one carbapenem (imipenem, meropenem and doripenem). Overall prevalence of 18.8% CRPA described in this study was higher than the report of 15.2% from Nigeria in 2015 and lower than 19.6% from India in 2008 (Javiya *et al*., 2008; Eyo *et al*., 2015). In the systematic evaluation on carbapenemase-producing Gram-negative bacteria in clinical settings in Africa by

Manenzhe *et al*. (2015), the overall prevalence ranged from as low as 2.3% and 9.0% to as high as 60.0% and 67.7% in sub-Saharan Africa and North Africa, respectively. Among the carbapenems, reduced resistance to imipenem (15.8%) was observed compared with meropenem (18.8%) and doripenem (17.8%) in this study. In a previous work on multidrug resistant *P. aeruginosa* from southwest Nigeria, Odumosu *et al*. (2012) reported the prevalence of 1.9% resistance to imipenem, much lower than the 15.8% reported in this study. This suggests a massive increase in the emergence of imipenem resistance. This study observed higher resistance to meropenem (18.8%) compared to imipenem (15.8%) as reported in a study from Calabar, Nigeria (Eyo *et al*., 2015) but contrary to the report from Egypt with higher resistance rate of 41.4% to imipenem than 18.0% resistance to meropenem (Afifi *et al*., 2013). The explanation for the high variation in resistance observed among carbapenems could probably be due to differences in prescription among hospitals since imipenem has been linked with progression of resistance in the course of treatment (Carmeli *et al*., 1999). High resistance to doripenem was observed in this study. This is because doripenem is structurally related to imipenem leading to cross resistance to this antibiotic. This also calls for concern as this antibiotic is not in clinical use in Nigeria.

The result of this study revealed that polymyxin B and colistin sulphate were the only antibiotics that were active against majority of carbapenem-resistant *P. aeruginosa* in this study. Majority of carbapenem resistant isolates (68.5%) were extensively drug resistant, showing resistance to virtually all the antipseudomonal antibiotics except colistin sulphate and polymyxin B. This is in conformity with the report by Palavutotai *et al*. (2018), where all the extensively drug resistant strains of *P. aeruginosa* were susceptible to colistin. Carbapenem resistant Gram-negative bacteria showing resistance to all antibiotics apart from colistin have been reported. In carbapenem-sensitive strains, lower resistance rates of 6.2% and 4.9% were recorded to colistin and polymyxin B, respectively as experimental in last studies (Pitout *et al*., 2005; Odumosu *et al*., 2012; Guvensen *et al*., 2017). This emerging resistance to polypeptides also calls for serious intervention because it is the only antibiotic that was active on greater number of MBL-producing Gram-negative bacteria.

5.3 Evaluation of antibiotic susceptibility profile of phenotypically detected MBL-producing and non-MBL-producing strains

Statistical analysis revealed significant difference in activity of seventeen (17) antibiotics against MBL-positive and MBL-negative *P. aeruginosa* isolates ($p < 0.05$) while in six antibiotics statistical analysis showed no significant difference in sensitivity profile of MBL-positive and MBL-negative *P. aeruginosa* isolates ($p < 0.05$). Varaiya *et al*. (2008) have also reported statistically sisgnificant difference in sensitivity profile of cefepime, ceftazidime ceftriaxone, and ciprofloxacin as observed in this study. Both MBL-positive and MBL-negative strains were 100% unaffected by ampicillin, cephalothin and cefuroxime while only MBL-positive strains showed 100% resistance to ticarcillin, ceftazidime, ceftriaxone, doripenem and meropenem. Across the seven hospitals, only colistin sulphate and polymyxin B exhibited highest activity against MBL-positive strains when compared with MBL-negative strains. This implies that MBL-genes do not confer resistance to colistin sulphate and polymyxin B. However, MBL-positive strains remained totally resistant to imipenem, meropenem and doripenem across six hospitals (UCHI, FMCA, OTHS, LTHO, OTHI and FMCI) while 7.1% sensitivity to imipenem was observed among MBL-positive strains from FMCO as compared to 100% sensitivity exhibited by MBL-negative strains. This is also an indication that MBL production significantly affects the efficacy of carbapenems (imipenem, meropenem likewise doripenem).

5.4 Molecular detection of carbapenemases

Genes encoding bla_{SME}, $bla_{\text{NMC-A}}$, bla_{GES} and $bla_{\text{BIC-1}}$ class A carbapenemases were investigated in this study, none of the isolates was positive for bla_{SME}, $bla_{\text{NMC-A}}$, bla_{GES} and $bla_{\text{BIC-1}}$ enzymes. However, only bla_{GES} has been reported in *P. aeruginosa* from several countries like Brazil, China, Korea, Poland and Saudi Arabia (Wang *et al*., 2006; Ahmed and Asghar, 2017). bla_{KPC} is also a class A carbapenemase commonly reported in Enterobacteriaceae especially in *K. pneumoniae* but has also been described in *P. aeruginosa* (Mushi *et al*., 2014; Galetti *et al*., 2016). CRPA isolates were not screened for bla_{KPC} in this study.

Among the class D carbapenemases, $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-58}}$ were sought but not detected in this study. There has heretofore been only one report of $bla_{\text{OXA-48}}$ in Enterobacteriaceae from Nigeria (Jesumirhewe *et al*., 2017). Although there are

several reports of *bla*$_{OXA-48}$ in Gram-negative bacteria including *P. aeruginosa* from African countries, *bla*$_{OXA-58}$ was not common (Manenzhe *et al*., 2015).

The genes encoding class B carbapenemases (MBLs) were investigated by means of primers specific for *bla*$_{IMP,}$ *bla*$_{VIM,}$ *bla*$_{SIM,}$ *bla*$_{SPM,}$ *bla*$_{SIM,}$ *bla*$_{NDM,}$ *bla*$_{GIM,}$ *bla*$_{AIM}$ and *bla*$_{DIM.}$ Only *bla*$_{VIM}$ and *bla*$_{NDM}$ were amplified. The prevalence of *bla*$_{VIM}$ and *bla*$_{NDM}$ in carbapenem-resistant *P. aeruginosa* in this study was 86.3%, a rate which was extremely higher than that documented by Mohanam and Menon (2017) who reported that *bla*$_{VIM}$ and *bla*$_{NDM}$ were prevalent in 55% carbapenem-resistant *P. aeruginosa* from India. The whole incidence of MBL-genes in *P. aeruginosa* from southwest Nigeria was found to be 14.7%, a rate which was higher than that of the study by Zubair and Iregbu (2018) from Central Nigeria with the prevalence rate of 2.5% but almost agrees with the report from Thailand with overall MBL prevalence rate of 17.3% in clinical isolates of *P. aeruginosa* (Piyakul *et al*., 2012). The prevalence of *bla*$_{VIM}$ and *bla*$_{NDM}$ in this study were 8.1% and 8.6% respectively, while the co-existence of *bla*$_{VIM}$ with *bla*$_{NDM}$ was 2.1%.

Sequence analysis of nine randomly selected amplicons for *bla*$_{VIM}$ revealed 99.46 – 99.73% identity with *bla*$_{VIM-5}$ while sequence analysis of nine randomly selected *bla*$_{NDM}$ amplicons revealed that the isolates were 100% identical to *bla*$_{NDM-1}$. Several studies from Africa have also reported the presence of *bla*$_{VIM-2}$ in *P. aeruginosa* (Manenzhe *et al*., 2015). In Africa, *bla*$_{VIM-2}$ has been confined to *P. aeruginosa* with the exception of the report of *bla*$_{VIM-2}$ producing *E. coli* from Tunisia (Manenzhe *et al*., 2015). Zubair and Iregbu (2018) have also reported the presence of *bla*$_{VIM-2}$ in clinical isolates of *P. aeruginosa* from Nigeria, there is no single report of *bla*$_{VIM-5}$ (i. e. variant number 5) in clinical isolates of *P. aeruginosa* from Africa. However, a recent study by Adelowo *et al.* (2018) has reported the occurrence of *bla*$_{VIM-5}$ in environmental strains of *Pseudomonas putida* from Nigeria. This study is the first report of *bla*$_{VIM-5}$ (i. e. variant number 5) in clinical isolates of *P. aeruginosa* from Nigeria. In Africa, *bla*$_{VIM-4}$ was first reported in *Pseudomonas aeruginosa* Northeastern Algeria (Mellouk *et al*., 2016), followed by a report from Egypt (Hashem *et al*., 2017). Another report from Algeria has recently documented the presence of *bla*$_{VIM-4}$ in *Pseudomonas aeruginosa* (Merradi *et al*., 2019).

The *bla*$_{NDM-1}$ gene was first discovered in Enterobacteriaceae from Swedish patient who received treatment in a hospital in New Delhi, India (Pitout *et al*., 2005). Since then, majority of reported cases of *bla*$_{NDM-1}$ have been linked with international travel or hospitalisation in India or Pakistan (Govind *et al*., 2013). In South Africa, *bla*$_{NDM-1}$ was discovered in *Enterobacter cloacae* isolated from urine of a patient in South Africa. The patient had been on admission in India prior to this discovery (Govind *et al*., 2013). In Africa, *bla*$_{NDM-1}$ was first noticed in *K. pneumoniae* isolated from urine sample of a patient in Kenya (Poirel *et al*., 2011). Several studies have also documented cases of *bla*$_{NDM-1}$ in *P. aeruginosa* (Jovcic *et al*., 2011; Liew *et al*., 2018; Mohamed *et al*., 2018). Nevertheless, there is limited data with respect to the incidence of *bla*$_{NDM-1}$ in Gram-negative bacteria from Nigeria. Abdullahi *et al*. (2017) have documented the presence of *bla*$_{NDM-1}$ in Enterobacteriaceae from Northwestern Nigeria. Emergence of *bla*$_{NDM-1}$ in Enterobacteriaceae from poultry in Nigeria has also been documented (Ogunleye *et al*., 2016). Zubair and Iregbu (2018) have reported the presence of *bla*$_{VIM-2}$ and the absence of *bla*$_{NDM-1}$ in clinical isolates of *P. aeruginosa*. To the best of my understanding, this represents the first report of *bla*$_{NDM-1}$ in clinical isolates of *P. aeruginosa* from Nigeria. This is also the first report of co-existence of *bla*$_{NDM-1}$ with *bla*$_{VIM-5}$ and the first detection of *bla*$_{VIM-5}$ (variant 5) in clinical isolates of *P. aeruginosa* from Africa. Mohanam and Menon (2017) have also documented the co-existence of *bla*$_{VIM}$ with *bla*$_{NDM}$ in clinical isolates of *P. aeruginosa* from India. There have been several reports of *bla*$_{NDM-1}$ co-existing with other carbapenemases in Gram-negative bacteria. The concurrence of *bla*$_{NDM-1}$ with *bla*$_{OXA-23}$ in *Acinetobacter baumannii* from India and Nepal has been reported (Karthikeyan *et al*., 2010; Joshi *et al*., 2017). Also, the coexistence of *bla*$_{NDM-1}$ with *bla*$_{OXA-48}$ in Enterobacteriaceae from India and *K. pneumoniae* from China has been reported (Karthikeyan *et al*., 2010; Xie *et al*., 2017). Likewise, coexistence of *bla*$_{NDM-1}$ with *bla*$_{KPC}$ in *Escherichia coli* from Southern Vietnam was recently documented (Hoang *et al*., 2019)

The presence of MBL genes on plasmids is an evidence of parallel transmission of these resistance genes among bacterial species (Karthikeyan *et al*., 2010). Amplification of transformant plasmid DNA with primers specific for *bla*$_{VIM}$ and *bla*$_{NDM}$ gave amplicon sizes that correspond to the expected products. This confirms that both *bla*$_{VIM}$ and *bla*$_{NDM}$ are carried on resistance plasmids which are capable of

parallel transmission of resistance genetic material between bacterial genera and species. This indicates a serious threat to our health care delivery system.

Modified Hodge Test (MHT) was not successful at detecting carbapenemase production in clinical isolates of *P. aeruginosa* in this study. This is in conformity with the report that MHT was previously recommended for screening carbapenem-resistant Enterobacteriaceae and not Gram-negative non-fermenters of which *P. aeruginosa* is among (Gniadek *et al*., 2016) but is no longer recognized as screening method for carbapenemase production because of ambiguity about the isolates real susceptibility to carbapenems (Humphries *et al*., 2019). Combined disc test (CDT) phenotypically detected 66 (15.4%) isolates as MBL-producers while MBL-genes were truly present in 58 of the 66 isolates; that is, CDT wrongly identified 8 isolates as MBL-producers with PCR as the 'gold standard'. CDT also failed to detect MBL in 5 isolates that in reality carried bla_{VIM} and bla_{NDM}. The sensitivity and specificity of CDT was 92.1% and 33.3%, respectively while positive predictive values (PPV) and negative predictive values (NPV) of 87.9% and 55.6%, respectively were obtained. This is also in line with the report that EDTA-based assay produced false-positive results as observed in this study (Gniadek *et al*., 2016). The prevalence of MBL in *P. aeruginosa* by CDT test (phenotypic) 15.4% versus genotypic 14.7% shows that CDT may be also used to detect MBL in *P. aeruginosa* where molecular screening methods are not available.

Among the MBLs, bla_{IMP}, bla_{VIM} and bla_{NDM}, are the most commonly reported enzymes from Africa while bla_{DIM} was reported in a study from Sierra Leone (Manenzhe *et al*., 2015). Though bla_{IMP} was among the most commonly reported MBL from Africa, only a study from Nigeria has documented $bla_{\text{IMP-1}}$ in *E. coli* from Abattoir (Chika *et al*., 2017). This study did not detect bla_{IMP}, bla_{SIM}, bla_{SPM}, bla_{GIM}, bla_{AIM} and bla_{DIM}. There have been no reported cases of bla_{SIM}, bla_{SPM}, bla_{GIM}, bla_{AIM} and bla_{DIM} MBL-encoding genes from Nigeria. The bla_{AIM} has not been reported again since its discovery in *P. aeruginosa* (Yong *et al*., 2006). IMP is more prevalent in Europe and Asian countries (Nordmann *et al*., 2011).

All the isolates that possessed MBL(s) gene were resistant to the three antipseudomonad carbapenems with the exclusion of a strain, PS219, which was resistant to only meropenem and doripenem. Among the ten carbapenem-resistant clinical isolates that were negative for all the MBL genes tested, eight were resistant to

all the three carbapenems used in this study while one isolate (PS088) was sensitive to imipenem but resistant to meropenem and doripenem and the remaining one of the ten isolate was sensitive to imipenem and doripenem but showed resistance to only meropenem.

The fact that 13.7% of carbapenem resistant *P. aeruginosa* do not possess any MBL-encoding gene as observed in this present study confirms that carbapenem resistance is not attributable to the existence of known carbapenemases alone. Other resistance mechanisms such as efflux pump overexpression, *ampC* cephalosporinase and deficient outer membrane porin also perform a contributory role in the resistance observed towards carbapenems and other antibiotics in this study. There may also be previously undescribed mechanisms.

5.5 PCR-RFLP analysis of integrons in carbapenem-resistant *P. aeruginosa*

Integrons are heritable elements that are capable of acquiring gene cassettes that carry antibiotic resistance genes that can be transferred from one bacterial species to the other (Poirel *et al*., 2001). The role of integrons in the distribution of antibiotic resistance genetic material in Gram-negative bacteria is well documented (Mohanam and Menon, 2017). Dissemination of class 1 integron-carrying bla_{VIM} has been described (Touati *et al*., 2013). Quite a lot of studies have described the prevalence of class 1 and 2 integrons in Gram-negative bacteria (Odumosu *et al*., 2013; Alabi *et al*., 2017; Izadi *et al*., 2017). Class 1 integrons alone was detected in 57.5% of carbapenem-resistant *P. aeruginosa* in this study. This correlates with the work of Odumosu *et al*. (2013) who stated the prevalence of 57% in clinical isolate of *P. aeruginosa* from the same region but less than the report of Hassuna *et al*. (2015) who detected class 1 integrons in 71% *P. aeruginosa* from wound patients in Egypt. However, Odumosu and his co-workers did not detect class 2 integrons while this study found co-existence of class 1 and 2 integrons in 12.3% CRPA. To the best of my understanding, this is the first documentation of class 2 integrons and co-existence of class 1 and 2 integrons in *P. aeruginosa* from Nigeria, though class 2 integrons have been documented in Enterobacteriaceae from Southwest Nigeria (Alabi *et al*., 2017; Odetoyin *et al*., 2017). Khosravi *et al*. (2017) have documented the co-existence of class 1 and 2 integrons in *P. aeruginosa* from burn wound in Iran.

In this study, two strains (PS396 and PS405) with co-existence of bla_{VIM-5} and bla_{NDM-1} genes did not harbour integrons. This result corresponds with the report by Zubair and Iregbu (2018) that all the bla_{VIM-2} containing isolates did not harbour integrons. These reports imply that bla_{VIM} and bla_{NDM} containing *P. aeruginosa* isolates may not contain class 1 integrons as well as other integron classes. Although statistical analysis revealed positive association between integrons and MBL genes ($p = 0.0064$).

This study was able to amplify cassette region that was not greater than 3.6 kb in size but gene cassette could not be amplified in 33.3% of the integron-containing isolates. This report is compatible with the study of Odetoyin *et al*. (2017), which were able to amplify integrase of not greater than 4.0 kb with some unamplifiable integrase 1 gene cassette in *E. coli* from Nigeria.

5.6 MDR efflux pump overexpression, *ampC* overexpression and o*prD* underexpression in carbapenem-resistant *P. aeruginosa*

Natural expression of efflux pumps in unmutated strains of *P. aeruginosa* plays a worthwhile part in moderately reduced susceptibility to antibiotics but overexpression of these efflux pumps in mutants leads to raised level of resistance to antibiotics (Lister, 2002). Efflux pumps have capacity to eject numerous categories of antibiotics from the cytoplasm and the periplasmic space, subsequently leading to cross-resistance to other classes of antibiotics because inhibitory concentration of antibiotic could not be achieved at the target site of action (Lister, 2002). Out of the 12 RND type efflux pumps so far identified, four are of clinical importance. These include MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Mesaros *et al*., 2007). MexXY-OprM exports fluoroquinolones, aminoglycosides, and cefepime while MexAB-OprM exports beta-lactams, macrolides, fluoroquinolones, novobiocin, sulfonamides, trimethoprim, tetracycline, chloramphenicol and meropenem (Koo, 2015). In this study, expression of two efflux genes was determined in each of the four efflux systems. Efflux pump overexpression was detected in 68.8% of CRPA in this study. MexXY-OprM was the most expressed efflux pump in this study with 58.3% prevalence. This is in support of the work of Xavier *et al*. (2010) who also reported highest prevalence of MexXY-OprM pump with prevalence of 50.8% in *P. aeruginosa* from Brazil. MexAB-OprM was overexpressed in 47.9% of isolates in this study. This was lower than the report by Pourakbari *et al*. (2016) which showed that 62% of *P. aeruginosa* overexpressed MexAB-OprM. Both MexAB-OprM and MexXY-OprM

were overexpressed simultaneously in 18 (37.5%) isolates. Co-expression of two or more efflux pumps in clinical strains of *P. aeruginosa* has been documented. For example, concomitant overexpression of MexAB-OprM, and MexXY-OprM showing resistance to multiple antibiotics in *P. aeruginosa* was reported from France (Llanes *et al*., 2004). MexAB-OprM and MexXY-OprM were naturally expressed in 'wild type' cell. MexCD-OprJ and MexEF-OprN were not active in unmutated cell but overexpressed in mutants (Koo, 2015). Since all these carbapenem-resistant strains were also impervious to many other classes of antibiotics together with fluoroquinolones, aminoglycosides, beta-lactams and monobactam, which are substrates for these efflux pumps, this underscores the task of efflux pumps in antibiotic resistance.

Overexpression of efflux pump genes has been associated with antibiotic resistance in *P. aeruginosa*. However, high level of resistance may not result from efflux pump expression alone. In this study, majority of the isolates with >64 µg/mL to imipenem and meropenem had combination of MBL-encoding genes, efflux pump overexpression and /or *oprD* down-regulation. Resistance to carbapenems could be linked to efflux pump overexpression alone in four isolates. Overexpression of MexAB-OprM was the resistant mechanism identified in one stran (PS007), which had MIC of 16 µg/mL against imipenem and meropenem while MexXY-OprM overexpression was the only resistance mechanism identified in strain PS088 with MIC of 32 and 16 µg/mL against imipenem and meropenem, respectively. Consistent with the previous report by Hocquet *et al*. (2006) simultaneous overexpression of MexAB-OprM and MexXY-OprM was found in two strains (PS182 and PS349). These isolates had intact *oprD*, lacked *ampC* overexpression and MBL genes with MIC of 4 and 8 µg/mL against imipenem with MIC of 8 and >64 µg/mL against meropenem, respectively. This study also observed that isolates with efflux pump alone had reduced MIC against imipenem, meropenem, ceftazidime, cefepime and ciprofloxacin when compared with isolates showing combination of MBL resistance genes and efflux pump.

This study also observed that 77.1% of CRPA had *oprD* down-regulation. OprD porin is the only porin through which carbapenems enter the bacterial cell. Mutation or alteration in genes that code for OprD protein results in resistance to carbapenems and

reduced expression of *oprD*. Deficient OprD porin is mainly seen in carbapenem-resistant *P. aeruginosa* (Livermore, 1992). Deficient OprD as a result of mutations has been reported to confer high resistance to imipenem than meropenem and doripenem (Livermore, 1992; Quale *et al*., 2006). Carbapenem resistance in three strains was due to loss of OprD porin alone. MBL-encoding genes, efflux pump and *ampC* overexpression were not present in these isolates. This shows that deficient *oprD* porin alone could contribute to carbapenem resistance.

Increased *ampC* expression was found in 13 (27.1%) isolates. This outcome was in concordance with the report of Cabot *et al*. (2011) who stated that 24.2% of the isolates overexpressed *ampC* but higher than the report of Xavier *et al*. (2010) who documented that 11.9% of *P. aeruginosa* isolates from Brazil overexpressed *ampC*. The overexpression of *ampC* as a resistance mechanism could not be linked with carbapenem resistance in this study, because all the isolates which overexpressed this gene also have other carbapenem resistant mechanisms. This was backed-up with the report that a*mpC* overexpression alone in *P. aeruginosa* could only lead to insignificant reduction in the activity of carbapenems but combination of other mechanisms of resistance could have a great impact in lowering the activity of carbapenems (Quale *et al*., 2006). Therefore, *ampC* overexpression alone may not necessarily be responsible for carbapenem resistance. This study for the first time in Nigeria has quantified gene expression (efflux pumps, *oprD* and *ampC*) in *P. aeruginosa*. This study also identified the entire four clinically important efflux pumps in *P. aeruginosa.*

5.7 Association of carbapenem resistance with increased expression of *ampC*, efflux pump and *oprD* underexpression

Concomitant presence of MexAB-OprM, MexXY-OprM and MexEF-OprN was found in two carbapenem-resistant isolates (PS170 and PS182) that have increased expression of *ampC*, lack carbapenemase genes and have intact *oprD* porin suggesting the impact of efflux pumps and AmpC in antibiotic resistance of these isolates. PS351 which was susceptible to all the three carbapenems tested in this study and other antibiotics did not overexpress any of the efflux genes, *ampC* and also had intact *oprD* porin. Co-existence of MexAB-OprM, MexXY-OprM, MexCD-OprJ, *ampC* and *oprD* deficient was also observed in two strains (PS202 and PS219). This shows that the part involvement of OprD loss, AmpC and efflux pumps overexpression in antibiotic

resistance could not be neglected and there is interconnection among various mechanisms of carbapenem resistance with a significant effect on resistance to carbapenem. Gomaa *et al*. (2013) documented the presence of all the four efflux pumps (MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN) in 15% of *P. aeruginosa,* however, one (2.1%) isolate was found to overexpress all the four efflux pumps in this study. This isolate had deficient *oprD*, increased expression of *ampC* found to possess all the four efflux pumps suggesting multiple mechanisms of resistance. This isolate was also resistant to all of the antipseudomonal antibiotics used in this study except colistin and Polymyxin B.

5.8 Combination of resistance mechanisms in CRPA

Eight different combinations of resistance mechanisms were observed with combinations of MBL, Efflux pumps overexpression and *oprD* loss being the most prevalent. The combinations of all the four resistance mechanisms (MBL, efflux pump overexpression, *oprD* deficient and *ampC* overexpression) were exhibited in 8 (16.7%) strains while MBL and *oprD* deficient alone was found also in 8 (16.7%) strains. Combination of efflux pumps and *ampC* was detected in 3 (6.3%) strains while MBL, *oprD* and *ampC* was present simulteneously in 1 (2.1%) strain only (Table 4.20). Among the ten isolates that lack MBL-resistance genes, four demonstrated efflux pump overexpression alone, three isolates showed *oprD* loss alone while the remaining three isolates had the combination of efflux pump and *ampC* overexpression. In this study, *ampC* overexpression alone was not detected as the only mechanism of resistance to carbapenems as opposed to the report by Castanheira *et al*. (2014) where overexpression of *ampC* alone was observed as the sole resistance mechanism in twenty-one isolates followed by combination of *ampC* overexpression and *oprD* deficient. In this present study, combination of *ampC* overexpression and *oprD* deficient as carbapenem resistance mechanism was not observed.

5.9 Prevalence of type III effector toxins in CRPA

It is believed that type III effector toxins help *P. aeruginosa* to escape host immune response, stimulate proliferation of the organism at the infection site and also inhibit host DNA synthesis resulting into death of the host cell (Jabalameli *et al*., 2012). According to Feltman *et al*. (2001), most clinical isolates of *P. aeruginosa* encode genes for type III effector toxins, but not all clinical strains are capable of secreting

effector toxins. Data on the prevalence of type III effector toxins in *P. aeruginosa* from West Africa are lacking. This study therefore, has made a novel report of the prevalence of the four type III effector toxins in *P. aeruginosa* from Nigeria. All the carbapenem-resistant *P. aeruginosa* isolates screened in this study have both *exoT* and *exoY*. This result justifies the report by Roy-Burman *et al*. (2001) that most strains of *P. aeruginosa* have *exoT* and *exoY* toxins. While several studies have reported the presence of *exoT* in all the isolates of *P. aeruginosa, exoY* was not always found in all of the isolates (Jabalameli *et al*., 2012; Adwan, 2017). This study also described the occurrence of *exoU* in strains that lack *exoS* and vice versa except in two strains that have both *exoU* and *exoS*. This conforms to the report of other studies which also document the concurrent presence of *exoU* and *exoS* in a few number of *P. aeruginosa* strains (Feltman *et al*., 2001; Jabalameli *et al*., 2012; Gawish *et al*., 2013). In this study, prevalence of *exoS* (49.3%) in strains not having *exoU* was a little higher than the prevalence of *exoU* (48.0%) in strains not having *exoS*. This agrees with the work of Adwan (2017) who also reported high prevalence of e*xoS* but absence of *exoU* in all of the clinical isolates thereby disagreeing with the report by Gawish *et al.* (2013) where 64.7% and 38.2% of the isolates produced *exoU* and *exoS*, respectively. There was statistical significant difference in the dissemination of *exoU* and *exoS* genes in CRPA isolates ($p<0.0001$). There was no statistically significant difference in the distribution of *exoU* and *exoS* among wound and non-wound isolates ($p = 0.399$).

It has been established that e*xoS*, heightens the capability of *P. aeruginosa* to pass over the epithelial obstacle of the host cell (Soong *et al*., 2008). The e*xoU* toxin is the most destructive of the type III effector proteins with phospholipase A_2 activity which is only expressed by a few number of hospital isolates (Shaver and Hauser, 2004). In this present study, 48.0% of CRPA strains have *exoU* toxin, showing 48.0% of CRPA in this study to be highly cytotoxic. The health implication of being infected with cytotoxic strains that have acquired resistance to virtually all beta-lactam antibiotics is deadly. Therefore, proper implementation of appropriate strategies to curtail the transmission of these antibiotic resistant strains is a matter of urgency and of utmost importance.

5.10 Molecular typing of CRPA with three repetitive sequence-based PCR methods

The three repetitive sequence based PCR methods; REP-, ERIC- and BOX- PCR have been successfully used by various researchers for typing *P. aeruginosa* (Syrmis *et al*., 2004; Wolska *et al*., 2012; Al-Haik *et al*., 2016). Syrmis *et al*. (2004) reported both ERIC- and BOX- PCR as potent tool for typing clinical isolates of *P. aeruginosa*. However, this study reports ERIC-PCR as the most reliable typing method among the three repetitive sequence-based PCR methods because ERIC-PCR type was able to discriminate all the unrelated 75 isolates and also have higher discriminatory index *D* (0.934) than BOX- and REP- PCR method with discriminatory index of 0.896 and 0.854, respectively.

Out of the twelve clusters identified on BOX-profile, four clusters comprised at least two or more strains displaying 100% BOX-profile (B5, B10, B19, B49), three of which (B5, B19, B49) contained two indistinguishable strains each while the remaining one contained three indistinguishable strains. The remaining eight groups clustered two or more isolates that were related to each other with identical strains. Out of the four sets of indistinguishable isolates with BOX-PCR, two pair of strains (PS238:PS152 and PS303:PS354) were recognised as clonal complex based on REP-PCR profile, while ERIC-profile identified PS238:PS152 and PS303:PS354 as a clone and clonal complex, respectively.

Out of eight clusters identified on REP-profile, seven sets of indistinguishable strains (R14, R17, R31, R32, R37, R38, R42), found on three clusters comprised at least two or more strains, displaying 100% REP-profile. These groups clustered two or more isolates that were related to each other with identical strains. Out of the seven pair of indistinguishable isolates on REP-profile, PS303:PS395 and PS235:PS184 was identified as clone and clonal complex on ERIC- and BOX-profile, respectively while the third pair of indistinguishable strains, PS100:PS022 was identified as clonal complex on ERIC-profile but exist on distant clusters on BOX-profile.

Among the three PCR-based typing methods used in this study, ERIC-PCR was able to distinguish all the seventy-five unique strains of *P. aeruginosa* unlike REP- and BOX-PCR that could not distinguish between some set of closely related isolates from different hospitals. However, some isolates which clustered together with ERIC-profile

were also found in the same cluster on REP- and BOX- profile. For instance, PS147 and PS250 which belong to group O of ERIC-profile was found as part of clonal complex in group B of REP- and BOX- profile. They both contained *exoU* and class 1 integron with integrase gene of~3.5 kb but PS147 strain carried bla_{VIM} while PS250 co-harboured bla_{VIM} and bla_{NDM}. PS147 was isolated earlier than PS250 from the same hospital; it appeared PS250 strain had later acquired bla_{NDM} through horizontal transfer of plasmid containing bla_{NDM}. Consequently, confirming cross-transmission within hospital. Also, PS235 (LTHO), PS184 (UCHI) and PS348 (OTHI) strains were part of clonal complex. These strains belong to group F on ERIC-profile and were also found together in the same cluster B and I of REP- and BOX- profile, respectively. The three strains possess $bla_{NDM,}$ class 1 integron and *exoU*. Integrase 1 gene found on PS184 was~3.5 kb while integrase 1 gene on PS235 and PS348 could not be amplified. Likewise, PS152 (UCHI) and PS238 (LTHO) were clonal and belong to group I and K of ERIC- and BOX- profile, respectively. These strains were part of clonal complex present on cluster C of REP-profile. PS152 harboured bla_{NDM} only while PS238 co-harboured bla_{VIM} and bla_{NDM} but they both contain class 1 integron and *exoU*. PS238 integrase gene was~3.5 kb while PS152 integrase 1 gene could not be amplified. In the same way, PS250 (UCHI), PS335 (FMCO), PS293 (FMCI), PS400 (OTHI) were four strains from different hospitals that were part of clonal complex. These strains were grouped together on REP- and BOX- profile. Three of these strains (PS293, PS335 and PS400) co-harboured bla_{VIM} and bla_{NDM}; two strains (PS250 and PS400) harboured integrase 1 gene of~3.5 kb while integrase gene of the remaining two strains (PS335 and PS293) could not be amplified.

5.11 Relationship between Type Three Secretion System (T3SS) and Repetitive Element Sequence-Based PCR (rep-PCR)

Sixteen out of eighteen strains that clustered into group A of REP-PCR type contain *exoS* type III effector gene while all the fourteen strains that clustered into group B contain *exoU* type III effector gene. The only two strains (PS293, PS354) out of seventy-three CRPA strains which produced both *exoS* and *exoU* also clustered in this group. On ERIC-PCR dendrogram, all strains in clusters A, E, M, N which comprised 3, 2, 2, 2 isolates, respectively contained *exoS* gene while all strains in clusters F, L, O, P which contained 4, 2, 2, 3 strains, respectively contained *exoU*. On BOX-PCR dendrogram, all the strains in clusters A, F, L which comprised 2, 2, 2 strains,

respectively contain *exoS* gene while all strains in clusters J, L which contained 3, 2 strains, respectively contained *exoU* toxin. It appears there is association between *exoU* type III effector gene and clonality. This is because majority of the strains found in clones and part of clonal complex contained *exoU* toxin. This correlates with the report by Wiehlmann *et al*. (2007) that *exoU* containing strains dominated clonal complex. However, there was no statistically significant difference in the presence of *exoU* and *exoS* genes among clonal and unrelated isolates ($p = 0.351$).

5.12 Limitations of this study

1. Whole genome sequence (WGS) and multilocus sequence typing (MLST) which are highly discriminatory sequence-based methods as well as pulsed-field gel electrophoresis (PFGE) which is the 'gold standard' for typing *P. aeruginosa* genome were not used in this study. Therefore, global spread could not be ascertained because clones obtained in this study could not be compared with international clones
2. Some isolates may be left out as it was not certain that all *P. aeruginosa* isolated during the period of sample collection were given.
3. This study employed Miniprep method which is not suitable for isolation of large low copy number plasmids
4. T3SS effector toxins were not sought in carbapenem-sensitive strains

5.13 Recommendations

1) High level of resistance demonstrated to most of antibiotics tested in this study could be linked with unnecessary use of expanded spectrum antibiotics. Public enlightenment of populace on the consequences of inappropriate use of antibiotics and implementation of proper surveillance strategy to encourage antibiotic stewardship is required in order to curb the menace of antibiotic resistance.
2) Imipenem and meropenem are already in clinical use in Nigeria because they are very stable agents against the ESBLs produced by Gram-negative bacteria. However, bacteria are already developing resistance to these carbapenems. Phenotypic detection of MBL-producing strains should be included in the routine screening of bacteria in clinical laboratories

3) Since imipenem spontaneously selects mutants during treatment, susceptibility testing of antibiotics before carbapenems prescription is suggested for proper management of antibiotic therapy within healthcare settings
4) The presence of different combinations of efflux pumps, MBLs, deficient *oprD*, and *ampC* resistance mechanisms is a cause calls for concern. The use of antibiotics as growth promoters in animals and livestocks should be totally eradicated.

5.14 Contributions to knowledge

This study has made contributions to the body of knowledge in the area of bacterial drug resistance as follows:

i. Prevalence of resistance to carbapenems and other antibiotics in *P. aeruginosa* from southwest Nigeria was reported.
ii. Novel report has been made on the presence of $bla_{\text{VIM-5}}$ and $bla_{\text{NDM-1}}$ and also on the coexistence of $bla_{\text{NDM-1}}$ with $bla_{\text{VIM-5}}$ in clinical isolates of *P. aeruginosa* from southwest Nigeria.
iii. Class 2 integrons and coexistence with class 1 integrons in clinical isolates of *P. aeruginosa* from southwest Nigeria was reported for the first time in this study.
iv. This study for the first time in Nigeria has quantified resistance gene expression (efflux pumps, *oprD* and *ampC*) in clinical isolates of *P. aeruginosa* from southwest Nigeria as well as reporting the entire four clinically important efflux pumps (MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN) in *P. aeruginosa* from southwest Nigeria.
v. This study has bridged a knowledge gap on type III effector toxins by providing a novel report of the prevalence of the four type III effector toxins in *P. aeruginosa* from southwest Nigeria.
vi. This study has reported for the first time, typing of *P. aeruginosa* from southwest Nigeria using repetitive element sequence-based PCR methods (REP-, ERIC- and BOX- PCR).

5.15 Conclusion

This study identified highly resistant *Pseudomonas aeruginosa* strains with 50 out of 63 MBL-containing isolates being extensively drug resistant. Colistin sulphate and polymyxin B were the only antibiotics that were active against most of these resistant

strains. This study reports high incidence of transmissible MBL genes, presence of co-occurring $bla_{\text{VIM-5}}$ and $bla_{\text{NDM-1}}$ genes, and co-existence of class 1 and 2 integrons in carbapenem-resistant *P. aeruginosa* isolates from Nigeria. Also, noticeable is the high expression levels of all the four clinically relevant multidrug efflux pumps, MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM as well as *oprD* and *ampC* at mRNA transcription level in *P. aeruginosa* from Nigeria. This study also observed that there was co-occurrence among these resistance mechanisms.

Transformation experiment also indicated that these resistant plasmids carrying MBL-encoding genes are on potentially mobile elements. The introduction of a plasmid carrying MBL-encoding genes into susceptible strains might result in the emergence of highly resistant strains. This study has discovered the emergence of extensively resistant *P. aeruginosa* isolates exhibiting resistance to majority of antibiotics tested with high prevalence of transmissible MBL-resistance genes among the isolates thereby posing threat to health care settings. The outcome of this research is of great health implication, and as such proper strategy has to be put in place to reduce the spread of antibiotic resistance.

Repetitive element sequence-based PCR revealed a great level of genetic diversity among carbapenem-resistant *Pseudomonas aeruginosa* strains. ERIC-PCR showed good discriminatory ability in comparison with other two methods (REP- and BOX-PCR) that could not differentiate some highly similar but unrelated strains. Since ERIC-PCR is not expensive and the methodology involved is not difficult, this method may be employed in monitoring outbreak of *P. aeruginosa* infections. No outbreak of carbapenem-resistant *P. aeruginosa* carrying MBL genes was apparent but there was evidence of cross-transmission within the hospital settings. This is a serious hazard to health care system and underscores the need for regular surveillance and monitoring resistance to carbapenems and associated resistance genes. Moreover, presence of integrons may heighten the ability of these strains to acquire multidrug resistance genes, which has enabled their persistence under the selective pressure of antibiotic and may spread in due course within the hospital setting. Therefore, implementation of active measures to control the spread of these strains in our hospitals is highly imperative.

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LIST OF AWARDS

The World Academy of Science (TWAS) Postgraduate Fellowship 2016

University of Ibadan Postgraduate College Teaching and Research Assistantship 2016

Appendix I

Media and antibiotics

***Pseudomonas* cetrimide agar (Oxoid)**

Weigh 45.3g of powder and dispense in 1000 ml of purified water with addition of 10 ml glycerol. Steam to soften the contents of the medium totally and sterilize at 121^{O}C for 15 minutes.

Formulation	**gram/litre**
Magnesium chloride	1.4
Gelatin peptone	20.0
Cetrimide	0.3
Potassium sulphate	10.0
Agar	13.6
pH 7.2 ± 0.2	

Tryptone soy broth (Oxoid)

Weigh 30g of powder and dispense in 1000 ml of purified water. Steam to soften the contents of the medium totally and sterilize at 121^{O}C for 15 minutes.

Formulation	**gram/litre**
Potassium hydrogen phosphate	2.5
Pancreatic digest of casein	17.0
Sodium chloride	5.0
Glucose	2.5
Enzymatic digest of soya bean	3.0
pH 7.3 ± 0.2	

Nutrient agar (Lab M)

Weigh 28g of powder and dispense in 1000 ml of purified water. Steam to soften the contents of the medium totally and sterilize at 121^{O}C for 15 minutes.

Formulation	**gram/litre**
Beef extract	3.0
Peptone	5.0
Agar No 2	12.0

NaCl	8.0

pH 7.3 ± 0.2

Nutrient broth (Lab M)

Weigh 13g of powder and dispense in 1000 ml of purified water, stir to mix the medium completely and sterilize at 121^{O}C for 15 minutes.

Formulation	**gram/litre**
Peptone	5.0
Beef extract	1.0
NaCl	5.0
Yeast	2.0

pH 7.4 ± 0.2

Mueller Hinton agar (Oxoid)

Weigh 38g of powder and dispense in 1000 ml of purified water. Swir to mix and steam to soften the contents of the medium totally and sterilize at 121^{O}C for 15 minutes.

Formulation	**gram/litre**
Casein hydrolysate	17.5
Agar	17.0
Beef dehydrated infusion from	300.0
Starch	1.5

Final pH: 7.3 ± 0.1

Luria Bertani broth (Lab M)

Weigh 25g of the powder and dissolve in 1000 ml of purified water and distribute into test tubes and sterilize at 121^{o}C for 15 minutes.

Formulation	**gram/litre**
Yeast extract	5.0
Tryptone	10.0
Sodium chloride	10.0

Agar technical (Agar no. 3) Oxoid

Weigh 12g of the powder and dissolve in 1000 ml of purified water. Steam to soften the contents of the medium totally and sterilize at 121^{O}C for 15 minutes.

Sulphide indole motility (SIM) medium (Oxoid)

Weigh 50g of powder and dispense in 1000 ml of purified water. Stir to mix and steam to soften the contents of the medium totally and sterilize at 121^{O}C for 15 minutes.

Formulation	**gram/litre**
Ferrous ammonium sulphate	0.2
Tryptone	20.0
Sodium thiosulphate	0.2
Peptone	6.1
Agar	3.5

Final pH: 7.3 ± 0.2

Motility indole urea (MIU) base medium (Biomark^{TM})

Suspend 18g in 950ml purified water. Steam to soften the contents of the medium totally and dispense in 95ml amount into flasks. Sterilize at 121^{O}C for 15 minutes. Cool to 50-55^{o}C and aseptically add 5ml sterile 40% urea solution per 95 ml medium.

Formulation	**gram/litre**
Dextrose	1.0
Sodium chloride	5.0
Casein enzymic lysate	10.0
Agar	2.0
Phenol red	0.01

Final pH: 6.8 ± 0.2

Motility indole ornithine (MIO) fluid medium (Scharlau Chemie)

Weigh 31.5g of powder and dispense in 1000 ml of purified water. Swirl to mix and steam to soften the contents of the medium totally and sterilize at 121^{O}C for 15 minutes.

Formulation	**gram/litre**
Gelatin peptone	10.0
Dextrose	1.0
L-ornithine hydrogen chloride	5.0
Yeast extract	3.0
Bromocresol purple	0.02
Agar	2.5

Final pH: 6.6 ± 0.2

Koser's citrate medium (Oxoid CM60)

Weigh 5.2g of powder and dispense in 1 litre of distilled water. Swirl to mix and boil to dissolve the medium completely, then sterilize by autoclaving at 121^{O}C for 15 minutes.

Formulation	**gram/litre**
Bromothymol blue	0.016
Potassium dihydrogen phosphate	1.0
Sodium ammonium phosphate	1.5
Magnesium sulphate	0.2
Sodium citrate	2.5

pH: 6.8 (approx.)

Preparation of media for oxidative utilization of sugar test

One gram of glucose, sucrose or mannitol was added into 100ml of peptone water, followed by the addition of 0.25g of NaCl and 0.025g of phenol red as indicator. The mixtures was dispensed in 5ml portions into test tubes, durham tube was then introduced into each of the tube tubes to trap the gas which could be produced during the process of oxidation.

Preparation of 0.5 M EDTA

A 0.5 M EDTA solution was made ready by introducing 18.6 g of disodium EDTA into 100 mL of purified water and pH was attuned to 8.0 with sodium hydroxide and sterilized.

Preparation of stock of antibiotics suspension

1. **Imipenem** (Bacqure)

   Manufacturer: Sun Pharmaceutical Ind. Ltd. Dewas- 455 001, India

   Indicated weight = 500 mg imipenem and 500 mg cilastatin

   Weight of vial + powder = {a}g = 23.706 g

   Weight of empty vial = {b}g = 22.641 g

   Weight of powder = {c}g = {a-b}g = 1.065 g

   Weight of excipient = {c}g - indicated weight

   = 1.065 g -1.0 g

   = 0.065 g or 65 mg

Therefore, 500 mg of imipenem is contained in 1.065 g of the powder

6.4 mg of imipenem is contained in $\frac{1065 \times 6.4}{500}$ = 13.63 mg of powder

13.63 mg of powder in 10ml of sterile distilled water = 640 µg/mL stock

2. **Ceftazidime** (Betazim) NAFDAC No.: A4-1494

Manufacturer: Strides Arcolab Limited, India. For Strides Vital Nigeria Limited

Indicated weight = 1000 mg = 1.0 g

Weight of vial + powder = {a}g = 14.32 g

Weight of empty vial = {b} = 13. 04 g

Weight of powder = {c} = {a-b} = 1.28 g

Weight of excipient = {c}g - indicated weight

= 1.28 – 1.00 g

=0.28 g or 280 mg

Therefore, 1000 mg of ceftazidime is contained in 1.28 g of the powder

= 1.28 g – 1.0 g

= 0.28 g or 280 mg

1000 mg of ceftazidime is contained in 1280 mg of the powder

51.2 mg of ceftazidime is contain in $\frac{1280 \times 51.2}{1000}$ = 65.54 mg

65.54 mg of powder in 10 ml sterile distilled water = 5120 µg/mL

3. **Ceftriaxone** (Haflone) NAFDAC No.: B4-0331

Manufacturer: NCPC Hebei Huamin Pharmaceutical Co., Ltd. Shijiazhuang, India

Indicated weight = 1000 mg = 1.0 g

Weight of vial + powder = {a}g = 15.85 g

Weight of empty vial = {b} = 14. 71 g

Weight of powder = {c} = {a-b} = 1.14 g

Weight of excipient = {c}g - indicated weight

= 1.14 – 1.00 g

=0.14 g or 140 mg

Therefore, 1000mg of ceftriaxone is contained in 1.14 g of the powder

= 1.14 g – 1.0 g

= 0.14 g or 140 mg

1000 mg of ceftriaxone is contained in 1140 mg of the powder

5.12 mg of ceftriaxone is contain in $\frac{1140 \times 5.12}{1000}$ = 5.83 mg

58.3mg of powder in 10 ml sterile distilled water = 5120 µg/mL

4. **Cefepime** (Fortsporine) NAFDAC No.: A4-6768

Manufacturer: Intracin Pharmaceutical Pvt. Ltd. Gujarat, India

Indicated weight = 1000 mg = 1.0 g

Weight of vial + powder = {a}g = 31.88 g

Weight of empty vial = {b} = 29.85 g

Weight of powder = {c} = {a-b} = 2.03 g

Weight of excipient = {c}g - indicated weight

= 2.03 – 1.00 g

= 1.03 g or 1030 mg

Therefore, 1000mg of cefepime is contained in 2.03 g of the powder

= 2.03 g – 1.0 g

= 1.03 g or 1030 mg

1000 mg of cefepime is contained in 2030 mg of the powder

5.12 mg of cefepime is contain in $\frac{2030 \times 1.28}{1000}$ = 2.60 mg

26.0 mg of powder in 10 ml sterile distilled water = 1280 µg/mL

5. **Penicillin G** (Philopen) NAFDAC NO: A4-2115

Manufacturer: Shijazhang Pharma Group, China. For Nwaeze pharmaceutical co. Limited.

Indicated weight = 600 mg

Weight of vial + powder = 16.28 g

Weight of empty vial = 15.68 g

Weight of powder = 16.28 g – 15.68 g

= 0.60 g

Appendix II

Chemicals, Enzymes, Master mix and DNA isolation kits

Molecular biology chemicals

Tris base (Phyto Technology Laboratories)

Glacial acetic acid (Scharlau)

Disodium EDTA (BDH Laboratory Supplies, Poole England)

2-mercaptoethanol

Ethanol

Sodium dodecyl sulphate

Lysozyme

Calcium chloride

Enzymes

DNase 1 (Thermo Scientific)

RNase A (Thermo Scientific)

Rsa 1 (Thermo Scientific)

Master mix

WizPure™ PCR 2X Master (Wizbiosolutions, Korea South)

2X MyTaq Red Mix (Bioline, London)

SYBR Green /ROX qPCR master mix (Thermo Scientific).

DNase/RNase-Free Distilled Water (Invitrogen)

Ethidium bromide (CARL ROTH®)

Agarose gel (HydraGene)

100 bp ladder, 100 bp plus ladder and 1 kb plus ladder (Thermo Scientific)

Kits

WizPrep™ genomic DNA purification kit

WizPrep™ Plasmid DNA Miniprep purification Kit (Wizbiosolutions)

PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen)

WizScript™ cDNA Synthesis Kit (Wizbiosolutions, Korea)

10x Tris acetate EDTA Buffer

Tris base (48.4 g)

Glacial acetic acid (11.4 ml)

Disodium EDTA (3.7 g)

Tris base, glacial acetic acid and EDTA were dissolved in 800 ml of sterile distilled water and make up to 1 litre

Appendix III

Statistical analysis

Fisher's exact test for association of integron with MBLs

	Integron			
MBL	Integron-positive	Integron-negative	**Total**	***p*-value**
MBL-positive	48	15	63	
MBL-negative	3	7	10	0.0064
Total	51	22	73	

https://www.socscistatistics.com/tests/fisher/Default2.aspx

Fisher's exact test for association between *exoU* and *exoS*

	T3SS			
T3SS	*exoU*-positive	*exoU*-negative	**Total**	***p*-value**
exoS-positive	2	35	37	
exoS-negative	36	0	36	< .0001
Total	38	35	73	

https://www.socscistatistics.com/tests/fisher/Default2.aspx

Fisher's exact test for association of clinical source and T3SS

	Clinical source			
T3SS	Wound	Non-wound	**Total**	***p*-value**
exoU	28	9	37	
exoS	32	6	38	0.399
Total	60	15	75	

https://www.socscistatistics.com/tests/fisher/Default2.aspx

Fisher's exact test for association of T3SS and clonality

CLONALITY				
	T3SS		**Total**	***p*-value**
	exoU	*exoS*		
Clonal	17	13	30	0.351
Non-clonal	20	25	45	
Total	37	38	75	

www.graphpad.com/quickcalcs/contingency1.cfm

One-Way Analysis of variance (ANOVA) to determine the association between antibiotic sensitivity profile of MBL+ve and MBL-ve strains

ANOVA Formulas

One-Way ANOVA Table					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	$k - 1$	SS_B	$MS_B = SS_B / (k - 1)$	$F = MS_B / MS_W$	Right tail of F(k-1,N-k)
Within Groups	$N - k$	SS_W	$MS_W = SS_W / (N - k)$		
Toal:	$N - 1$	$SS_T = SS_B + SS_W$			

Between Groups Degrees of Freedom: $\mathbf{DF = k - 1}$, where **k** is the number of groups

Within Groups Degrees of Freedom: $\mathbf{DF = N - k}$, where **N** is the total number of subjects

Total Degrees of Freedom: $\mathbf{DF = N - 1}$

Sum of Squares Between Groups: $\mathbf{SS_B = S^{k}_{i=1} n_i (x_i - x)^2}$, where $\mathbf{n_i}$ is the number of subjects in the i-th group

Sum of Squares Within Groups: $\mathbf{SS_W = S^{k}_{i=1} (n_i - 1) S_i^2}$, where $\mathbf{S_i}$ is the standard deviation of the i-th group

Total Sum of Squares: $\mathbf{SS_T = SS_B + SS_W}$

Mean Square Between Groups: $\mathbf{MS_B = SS_B / (k - 1)}$

Mean Square Within Groups: $\mathbf{MS_W = SS_W / (N - k)}$

F-Statistic (or F-ratio): $\mathbf{F = MS_B / MS_W}$

Piperacillin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	24.2253	6.1928	0.3246
MBL +ve	66	9.2273	13.692	1.6854

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	12567.3457	12567.3457	206.0305	0
Within Groups	428	26106.9363	60.9975		
Total:	429	38674.282			

Piperacillin tazobactam

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	24.2253	6.1928	0.3246
MBL +ve	66	9.2273	7.8483	0.9661

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	14956.6288	14956.6288	357.1223	0
Within Groups	428	17925.058	41.881		
Total:	429	32881.6868			

Ceftazidime

Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	25.6319	5.2415	0.2747
MBL +ve	66	4.3485	5.8533	0.7205

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	25308.0613	25308.0613	887.8719	0
Within Groups	428	12199.7888	28.5042		
Total:	429	37507.8502			

Cefoperazone

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	25.1374	5.8801	0.3082
MBL +ve	66	4.3485	5.8533	0.7205

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	24145.7046	24145.7046	699.3116	0
Within Groups	428	14777.9069	34.5278		
Total:	429	38923.6116			

Imipenem

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	364	25.1374	5.8801	0.3082
MBL +ve	66	4.3485	5.8533	0.7205

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	24145.7046	24145.7046	699.3116	0
Within Groups	428	14777.9069	34.5278		
Total:	429	38923.6116			

Meropenem

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	364	25.2885	5.7031	0.2989
MBL +ve	66	4.3485	5.8533	0.7205

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square	F-Stat	P-Value
Between Groups	1	24497.9768	24497.9768	747.141	0
Within Groups	428	14033.6748	32.789		
Total:	429	38531.6515			

Doripenem

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	364	25.2143	5.7341	0.3005
MBL +ve	66	4.3485	5.8533	0.7205

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	24324.6693	24324.6693	735.1137	0
Within Groups	428	14162.3776	33.0897		
Total:	429	38487.0469			

Cefepime

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	364	21.9286	10.1211	0.5305
MBL +ve	66	4.3485	5.8533	0.7205

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	17267.1056	17267.1056	187.517	0
Within Groups	428	39411.4823	92.0829		
Total:	429	56678.588			

Ampicillin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	364	0.2418	1.4476	0.0759
MBL +ve	66	0	0	0

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	3.2666	3.2666	1.8379	0.1759
Within Groups	428	760.6831	1.7773		
Total:	429	763.9497			

Amoxicillin-clavulanic acid

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	364	0.4615	2.0358	0.1067
MBL +ve	66	0	0	0

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	11.8993	11.8993	3.3852	0.0665
Within Groups	428	1504.4468	3.5151		
Total:	429	1516.3461			

Cefuroxime

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	0.4615	2.0358	0.1067
MBL +ve	66	0.3636	1.6792	0.2067

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	0.5355	0.5355	0.1358	0.7127
Within Groups	428	1687.7282	3.9433		
Total:	429	1688.2636			

Cephalothin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	0.4615	2.0358	0.1067
MBL +ve	66	0.7879	2.5269	0.311

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	5.9522	5.9522	1.3272	0.2499
Within Groups	428	1919.4864	4.4848		
Total:	429	1925.4386			

Ticarcillin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	12.4725	9.3143	0.4882
MBL +ve	66	1.1061	3.548	0.4367

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	7218.0973	7218.0973	95.6136	0
Within Groups	428	32310.7347	75.4924		
Total:	429	39528.8321			

Ceftriaxone

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	11.9753	9.4442	0.495
MBL +ve	66	1.1061	3.548	0.4367

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	6600.4269	6600.4269	85.102	0
Within Groups	428	33195.2674	77.559		
Total:	429	39795.6943			

Aztreonam

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	21.9286	10.1211	0.5305
MBL +ve	66	7.0909	8.4065	1.0348

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	12300.1395	12300.1395	126.0103	0
Within Groups	428	41778.0102	97.6122		
Total:	429	54078.1497			

Gentamicin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	21.8736	10.1014	0.5295
MBL +ve	66	5.6364	7.3456	0.9042

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	14729.8778	14729.8778	155.4829	0
Within Groups	428	40547.1559	94.7363		
Total:	429	55277.0337			

Amikacin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	21.5495	10.4114	0.5457
MBL +ve	66	5.6364	7.3456	0.9042

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	14147.7197	14147.7197	141.2941	0
Within Groups	428	42855.4613	100.1296		
Total:	429	57003.181			

Tobramycin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	364	20.3571	11.1994	0.587
MBL +ve	66	7.5152	7.6986	0.9476

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	9213.7289	9213.7289	79.8561	0
Within Groups	428	49382.2901	115.3792		
Total:	429	58596.0191			

Ciprofloxacin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	364	19.7445	11.1218	0.5829
MBL +ve	66	6.9394	6.9035	0.8498

ANOVA Sumary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	9160.9985	9160.9985	81.6875	0
Within Groups	428	47998.8703	112.1469		
Total:	429	57159.8688			

Levofloxacin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	364	19.467	11.294	0.592
MBL +ve	66	7.8485	7.4529	0.9174

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	7541.8343	7541.8343	64.671	0
Within Groups	428	49912.732	116.6185		
Total:	429	57454.5663			

Ofloxacin

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL –ve	366	19.0301	11.5551	0.604
MBL +ve	66	6.5152	6.9332	0.8534

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	8757.8205	8757.8205	72.6167	0
Within Groups	430	51859.4247	120.6033		
Total:	431	60617.2452			

Colistin sulphate

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
Group 1	364	12.2005	1.6848	0.0883
Group 2	66	11.7273	2.853	0.3512

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	12.5103	12.5103	3.4335	0.0646
Within Groups	428	1559.4686	3.6436		
Total:	429	1571.9789			

Polymixin B

Data Summary				
Groups	N	Mean	Std. Dev.	Std. Error
MBL -ve	366	14.5984	1.9217	0.1005
MBL +ve	64	15.5781	12.832	1.604

ANOVA Summary					
Source	Degrees of Freedom DF	Sum of Squares SS	Mean Square MS	F-Stat	P-Value
Between Groups	1	52.2852	52.2852	1.9091	0.1678
Within Groups	428	11721.5139	27.3867		
Total:	429	11773.7991			

https://goodcalculators.com/one-way-anova-calculator/

Appendix IV

Selected genbank flatfiles and BLASTn of *bla*$_{VIM}$ and *bla*$_{NDM}$ sequences

```
Preliminary genbank flatfile(s:

LOCUS       Seq1               343 bp    DNA     linear   BCT 21-JUL-2019
DEFINITION  metallo-beta-lactamase VIM-5 (blaVIM) gene, partial cds.
ACCESSION   MH201592
VERSION
KEYWORDS
SOURCE      Pseudomonas aeruginosa
  ORGANISM  Pseudomonas aeruginosa
            Bacteria; Proteobacteria; Gammaproteobacteria;
Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 365)
  AUTHORS   Olaniran,O.B., Adeleke,O.E. and Bukhari,S.H.
  TITLE     Pseudomonas aeruginosa VIM-5 metallo beta-lactamase
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 365)
  AUTHORS   Olaniran,O.B., Adeleke,O.E. and Bukhari,S.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JUL-2019) Pharmaceutical Microbiology,
University
            of Ibadan, Ibadan, Nigeria, Ibadan, Oyo +234, Nigeria
COMMENT     Bankit Comment: LocalID:Seq1.
            Bankit Comment: BankIt2246580.

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Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 454)
  AUTHORS   Olaniran,O.B., Adeleke,O.E. and Bukhari,S.H.
  TITLE     Pseudomonas aeruginosa NDM-1 metallo beta-lactamase
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 454)
  AUTHORS   Olaniran,O.B., Adeleke,O.E. and Bukhari,S.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-JUL-2019) Pharmaceutical
Microbiology,University
            of Ibadan, Ibadan, Nigeria, Ibadan, Oyo +234, Nigeria
COMMENT     Bankit Comment: LocalID:Seq10.          Bankit Comment:
BankIt2246115.

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