PATIENTS' DEMOGRAPHIC FEATURES AND MOLECULAR CHARACTERIZATION OF β-LACTAMS RESISTANCE IN *PSEUDOMONAS AERUGINOSA* ISOLATED FROM CLINICAL SOURCES AT THE NAIROBI HOSPITAL IN KENYA

ARMSTRONG NDIHOKUBWAYO

MASTER OF SCIENCE

(Medical Microbiology)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2019

Patients' Demographic Features and Molecular Characterization of β-lactams Resistance in *Pseudomonas aeruginosa* Isolated from Clinical Sources at The Nairobi Hospital in Kenya

Armstrong Ndihokubwayo

A Thesis Submitted in Partial Fulfillment for the Degree of Master of Science in Medical Microbiology in the Jomo Kenyatta University of Agriculture and Technology

2019

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

Signature ……... Date……...

Armstrong Ndihokubwayo

This thesis has been submitted for examination with our approval as University Supervisors

Signature …….. Date……...

Dr. Andrew Kimang'a Nyerere, PhD

JKUAT, Kenya

Signature ……... Date……...

Dr. Caroline Wangari Ngugi, PhD

JKUAT, Kenya

Signature ……... Date……...

Dr. Paul Makau

The Nairobi Hospital, Kenya

DEDICATION

This thesis is dedicated to my wife Igiraneza Emerancienne and my son Ineza Erez Axel who have tirelessly supported me all the way since the beginning of my studies. Their prayers and moral support have been the driving momentum that has enabled me to achieve the targeted goal in my studies.

ACKNOWLEDGEMENT

Foremost, I would like to acknowledge the Almighty God for having given me the strength, knowledge, intelligence, understanding and resilience to undertake this project. I extend my sincere gratitude to my supervisors, Dr. Andrew Kimang'a Nyerere and Dr. Caroline Wangari Ngugi for their great assistance, insightful criticisms, valuable advice, patient encouragement and support throughout the study. I would like also to express my gratitude to my advisor Dr. Paul Makau, whose steadfast support of this project and thoughtful advice was greatly needed and deeply appreciated. He shared with me a lot of his expertise and research insight. I am greatly indebted to Dr. Christine Macharia, who has been a great source of encouragement and inspiration and has given me a lot of support throughout the study. I thank also Mr. David Yala and Ms. Felister Musyoki for their guidance and assistance in providing laboratory materials and procedures. I am deeply grateful to The Nairobi Hospital Bioethics and Research Committee for the approval of my thesis and The Nairobi Hospital Administration through the Chief Executive Officer for having allowed me to do my project in their Hospital. I express my gratitude to all the staff of The Nairobi Hospital and PAUSTI Laboratories, for their support and encouragement in the laboratory work. I also thank my fellow classmates and lecturers for their encouragement and moral support. More thanks go to the administration of JKUAT for their general support in my Master of Science course. I express my deepest gratitude to the Government of Burundi through EAPHLNP Scholarship for financing my studies and for the research grant without which it would not be possible to do this work. I cannot finish without appreciating my family who gave me the moral support and loving conducive environment in those days of my studies. Most importantly, I wish to thank my wife who always supported and encouraged me to do my best in all matters of life.

To those who tirelessly supported me morally, socially and any other sought of input; **MAY GOD BLESS YOU ALL!**

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ABBREVIATIONS AND ACRONYMS

ADP	Adenine Dinucleotide Phosphate
AIDS	Acquired Immunodeficiency Syndrome
AMP	Ampicillin
AmpC	Ampicillinace C
AMS	Ampicillin/Sulbactam
AK	Amikacin
AST-GN	Antimicrobial Susceptibility Testing-Gram-negative Card
AUG	Augmentin
ATCC	American Type Culture Collection
AZM/AZT	Aztreonam
BAA	Business Associate Agreement
Bla	β-lactamase
BHI	Brain Heart Infusion
CAZ	Ceftazidime
CD	Combined Disk
CFM	Cefexime
CLED	Cysteine Lactose Electrolyte Deficient medium
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamycin
CRPA	Carbapenem-Resistance *Pseudomonas aeruginosa*
CRO	Ceftriaxone
CTX	Cefotaxime
CTX-M	Cefotaxime hydrolyzing abilities (Cefotaximase)
CXM	Cefuroxime
DDST	Disk Diffusion Synergy Test
DNA	Deoxyribonucleic Acid
ESBL	Extended-Spectrum β-Lactamase
EDTA	Ethylenediaminetetraacetic acid
EAPHLNP	East African Public Health Laboratory Network Project

ESKAPE	*Escherichia coli-Staphylococcus aureus-Klebsiella pneumoniae-Acinetobacter baumanii-Pseudomonas aeruginosa-Enterobacter*
F	Nitrofurantoin
FEP	Cefepime
FIM	Florence Imipenemase
FOX	Cefoxitin
3GC	Third Generation Cephalosporin
GES	Guiana Extended Spectrum
GIM	German Imipenemase
HDU	High Dependency Unit
ICU	Intensive Care Unit
ID-GN	Identification-Gram-negative Card
IMP	Imipenemase
IPM	Imipenem
JKUAT	Jomo Kenyatta University of Agriculture and Technology
LasB	Elastase B
LPS	Lipopolysaccharide
MBL	Metallo-β-Lactamase
MDR	Multi-drug Resistance
MDDT	Modified Disk Diffusion Test
MER	Meropenem
MIC	Minimal Inhibitory Concentration
MH	Mueller-Hinton
NDM	New Delhi Metallo-β-Lactamase
Non-ESBL	Non-Extended Spectrum β-Lactamase
OprD	Outer membrane Porin D
OXA	Oxacillin Hydrolyzing Abilities (Oxacillinase)
PAUSTI	Pan African University Institute for Basic Sciences, Technology and Innovation
PBP	Penicillin-Binding Protein
PCR	Polymerase Chain Reaction

PDR	Pandrug Resistant
PER	*Pseudomonas* Extended Resistant
PLC	Phospholipase
QS	Quorum Sensing
SHV	Sulfhydryl Variable
SPM	Sao Paulo metallo-β-lactamase
SPSS	Statistical Package for Social Sciences
SPP	Species
STL	Saint Lukes Ward
SXT	Trimethoprim/Sulfamethazole
TAE	Tris Acetate EDTA
TAT	Twin Arginine Translocate
TEM	Temoniera
TNH	The Nairobi Hospital
TZP	Piperacillin-tazobactam
UK	United Kingdom
USA	United States of America
USD	United States Dollars
µg	microgram
VEB	Vietnam Extended Spectrum β-lactamases
VIM	Verona Integron-encoded Metallo-β-lactamase
ZX/FEP	Cefepime
X^2	Chi-square

ABSTRACT

The increase of β-lactamases producing-*Pseudomonas aeruginosa* has led to major therapeutic failures and continues to pose a significant clinical challenge in healthcare settings. This study aimed determining the molecular characterization of β-lactams resistance in *P. aeruginosa* isolates obtained from clinical sources at The Nairobi Hospital in Kenya. A Laboratory based cross-sectional descriptive design was used with the laboratory analysis of the samples being carried out in the Microbiology Section of the Department of Pathology. Phenotypic characterization of Extended Spectrum β-Lactamases (ESBL) and Metallo-β-Lactamases (MBL)-producing isolates was done using Vitek 2 System and antimicrobial susceptibility profiles. Genotypic characterization was done using Polymerase Chain Reaction assays and gel electrophoresis. Patients' demographic data were collected from laboratory request forms. Data analysis was done using SPSS version 21.0 and Chi-square test. The high rate of *P. aeruginosa* isolates was found in indoor patients (74.1 %) than outdoor patients (25.9 %) with a high prevalence of this bacterium in males (61.1 %) than females (38.9 %). The predominance of this organism was also observed in the older age groups (64.3 %) comprising the patients above 45 years old. Phenotypical investigation of ESBL enzymes did not reveal a positive result. Overall the prevalence of MBL producers (22.7 %) was of serious concern as compared to non-MBL isolates (77.3 %); since all MBL isolates were resistant to the antibiotics tested. There was a significant association ($p<0.001$) in terms of antibiotic resistance, between MBL and non-MBL producing isolates for gentamycin, amikacin, ciprofloxacin, piperacillin/tazobactam, cefepime, ceftazidime, meropenem, and aztreonam. The highest rate of MBL producers was obtained from Intensive Care Unit (45.2 %) and High Dependency Unit (28.6 %) with higher distribution in pus swab (38.1 %); and sputum (33.3 %). There were two predominant genes bla_{VIM-2} (28.57 %) and bla_{NDM-1} (66.67 %) among MBL isolates. The rate of MBL producing isolates reported in this hospital suggests the existence of resistance emergence among the population of nosocomial bacteria. The early detection of β-lactamases producing isolates and the correct use of β-lactam antibiotics including carbapenems may help in the treatment of pseudomonal infections and avoid further spread of these multidrug resistant *P. aeruginosa*.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Pseudomonas aeruginosa is ranked second among Gram-negative bacteria isolated from hospital environment, and leading cause of nosocomial infections responsible for high rate of morbidity and mortality (Al-Zaidi, 2016). It is one of the most common non-fermenting opportunistic pathogen implicated in acute and chronic nosocomial infections (Meletis & Bagkeri, 2013; Mesquita *et al*., 2013; Sandhya Rani & Swathi, 2015). This bacterium is the leading cause of a wide range of nosocomial infections, including urinary tract infections, respiratory system infections, skin and soft tissue infections, bone and joint infections, bacteraemia and a variety of systemic infections particularly in people with decreased immune systems including cancer, burn wounds, and HIV/AIDS patients (Mesquita *et al.*, 2013). Furthermore, *P. aeruginosa* is considered the prime lung pathogen of cystic fibrosis patients, contributing to the progression and mortality rate of the disease (Mesquita *et al.*, 2013). It has been identified as the 2nd most frequent organism causing ventilator-associated pneumonia, the 4th most common causing catheter-associated urinary tract infections, the 5th cause of surgical site infections and the 7th cause of central-line-associated bloodstream infections (Wassef *et al.*, 2015). Pseudomonal infections are more prevalent among patients with immunity-impaired conditions (Sandhya Rani & Swathi, 2015). Patients suffering from ventilator-associated pneumoniae and burn wound infections face high rates of over 30 % (Klitzing *et al.*, 2018). *P. aeruginosa* is mostly commonly associated with hospital-acquired infection but community-acquired infection of this nosocomial pathogen has also been documented (Wahab & Rahman, 2013). Elderly patients are considered to be at high risk of nosocomial infections, due to a higher disease prevalence in this population (Wang *et al.*, 2019).

This bacterium is naturally resistant to many antibiotics and can produce various virulence factors (Akhi *et al.*, 2012). These virulence factors include mucoid exopolysaccharide, lipopolysaccharide, biofilm, pili, exotoxin A, pigments, lipase, protease, hemolysin, histamine, exoenzyme S, leukocidin, and rhamnolipids (Khalil *et al.*, 2015; Van't Wout, *et al.*, 2015). These factors help the bacteria to be attached to and invade their host by damaging the host's immune responses and blocking the passage of antibiotics (Van't Wout, *et al.*, 2015). The secreted virulence factors are encoded on plasmids or chromosomal genes (Khalil *et al.*, 2015). In this regard, bacterial strains have developed different strategies to overcome the antibiotics; a major factor in the development of antibiotic resistance world-wide (Dantas *et al.*, 2014; Pathmanathan *et al.*, 2009). *P. aeruginosa* is responsible for 10–15 % of the nosocomial infections worldwide (Nickerson *et al.*, 2016). Often these infections are hard to treat due to the innate resistance of the species, as well as to its extraordinary capacity of acquiring further mechanisms of resistance to many groups of antibiotics (Strateva *et al.*, 2009). Multidrug and pandrug resistant bacterial infections are closely associated with high mortality, prolonged hospitalization, multiple morbidities, and increased cost due to the limited antimicrobial therapeutic management options for patient infected with *P. aeruginosa* (Hong *et al.*, 2015).

P. aeruginosa represents a phenomenon of antimicrobial resistance, which can exhibit all known enzymatic and mutational mechanisms of microbial resistance (Strateva *et al.*, 2009). *P. aeruginosa* develops resistance by different types of mechanisms, such as biofilm formation, multi-drug resistance efflux pumps, production of β-lactamases and aminoglycoside modifying enzymes (Mahmoud *et al.*, 2013; Wolter *et al.*, 2013). The treatment and control of pseudomonal infections acquired in the community and healthcare centers is a serious public concern, for which the choice of effective drug for initiation of treatment is essential (Ding *et al.*, 2016; Toval *et al.*, 2015). The global emergence of *P. aeruginosa* resistance to multiple antimicrobial agents including β-lactams represents a considerable menace to public health (Toval *et al.*, 2015). The major problem of the resistant bacteria emergence is associated with overuse and misuse of antibiotics by patients, doctors

and may be linked to random use of antibiotics without antibiotic sensitivity test and laboratory diagnosis (Al-Zaidi, 2016).

The selective pressure which is generated by the indiscriminate use of the β-lactam antibiotics such as monobactans, cephalosporins and carbapenems has led to the selection of mutated forms of β-lactamases including extended-spectrum β-lactamases (ESBLs), Amp C β-lactamases and metallo-β-lactamases (MBLs) which appear to be the most problematic resistance mechanism as it poses a significant treatment failure in the health care settings (Oberoi *et al.*, 2013). In this context, infections due to *P. aeruginosa* harbor acquired mechanisms of resistance, such as production of β-lactamases, such as MBLs and ESBLs; and have high clinical impact in healthcare settings; therefore these enzymes are capable of hydrolyzing a wide range of β-lactam antibiotics, notably the extended-spectrum penicillins and the higher generation cephalosporins, and the carbapenems (Ahmad *et al.*, 2016; Oberoi *et al.*, 2013).

Extended spectrum β-lactamases (ESBL), a type of β-lactamase, are typically inhibitor-susceptible β-lactamase that hydrolyze penicillins, cephalosporins and aztreonam, and are also encoded by mobile genes (Jobayer *et al.*, 2017; Ogefere *et al.*, 2015; Vala *et al*., 2014). The Extended spectrum β-lactamases are yet inhibited by β-lactamase inhibitors such as clavulanic acid and have been increasingly described among the members of *Enterobacteriaceae*; and so far, ESBL producing *P. aeruginosa* has been identified and progressively observed in most parts of the world (Nithyalakshmi *et al*., 2016; Pitout *et al.*, 2005). Extended-spectrum cephalosporins and fluoroquinolones are widely used as broad-spectrum antibiotics and remain the effective drugs used in the treatment of infections caused by a variety of Gram-negative bacteria (Pitout *et al.*, 2005; Ogefere *et al*., 2015). Resistance to Carbapenems is predominantly mediated by MBLs, a class B type β-lactamases (Pitout *et al.*, 2005; Wadekar *et al.*, 2013). These enzymes require divalent cations, usually zinc, as metal cofactors for their catalytic activity and are inhibited by metal chelators such as ethylenediamine tetra acetic acid (EDTA) (Sheikh *et al.*, 2014). These enzymes, commonly codified by genes linked to mobile genetic elements, are

problematic with regard to the future of antimicrobial chemotherapy because of their extraordinary ability to disseminate (Polotto *et al.*, 2012; Zafer *et al.*, 2014). Genes encoding MBLs were shown to be carried on large transferable plasmids or were associated with transposons, allowing horizontal transfer of these MBL genes among different bacterial genera and species (Zafer *et al.*, 2014). Various genes of MBLs including bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM} have been known to be clinically important (Mathlouthi *et al.*, 2015; Vala *et al.*, 2014). The VEB and PER types were found to be the most common ESBL in *P. aeruginosa* in several countries, contrasting to the dominance of CTX-M, SHV, and TEM ESBL in *Enterobacteriaceae* (Nithyalakshmi *et al.*, 2016). Several oxacillinases (bla_{OXA-2} and bla_{OXA-10} derivatives and bla_{OXA-18}) that have extended substrate profiles, including extended spectrum cephalosporin, have been reported in *P. aeruginosa*. The OXA-ESBLs are mutants of bla_{OXA-2} and bla_{OXA-10}, belonging to the molecular class D of Ambler's scheme and 2nd of functional group under Bush-Jacoby-Medeiros classification whereas the other ESBL belongs to class A (Nithyalakshmi *et al*., 2016).

Recently, *P. aeruginosa* producing bla_{OXA-4} ESBL for the first time in India was also observed (Nithyalakshmi *et al*., 2016). Several genotypes of ESBLs, including bla_{SHV}, bla_{TEM}, bla_{PER}, bla_{OXA} and bla_{CTX-M} types have been also reported to be clinically significant (Vala *et al.*, 2014; Zafer *et al.*, 2014). While IMP and VIM variants have been reported worldwide, members of SPM, GIM, and SIM are limited to certain geographical regions as in Malaysia (Zafer *et al.*, 2014). The dissemination of MBLs genes (bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{GIM}, and bla_{SIM}) are thought to be driven by regional extended spectrum antibiotics including β-lactams and other antibiotics (Sacha *et al.*, 2008). Emergence of MBLs producing-*P.aeruginosa* in tertiary care hospitals is alarming and reflects a wide use of carbapenems (Jayarani *et al.*, 2014). The ESBL enzymes encoded by the genes, bla_{SHV} and bla_{TEM} have been found in *P. aeruginosa*, which suggests that these organisms are widespread reservoir of the ESBL enzymes since their discovery in the early 1980's; and shortly after the discovery and introduction of the cephalosporins into clinical medicine (Ejikeugwu *et al.*, 2015). ESBL-producing bacteria have turn out to be a worldwide public health

problem and the emergence of these microbes in the community including those that produce the CTX-M enzymes has been added to this problem (Ejikeugwu *et al.*, 2015). MBLs are class B enzymes which hydrolyze carbapenems and are encoded by genes like bla_{IMP}, bla_{VIM}, bla_{GIM}, and bla_{SPM} (Peshattiwar & Peerapur, 2011). The early detection of MBL producing isolates is important to limit the transmission of the organism within and between hospitals, and to accurately treat infections caused by this pathogen (Sheikh *et al.*, 2014).

Standard therapy for *P. aeruginosa* infections includes broad-spectrum β-lactamases-resistant penicillins, cephalosporins, carbapenems, and monobactams; and selected fluoroquinolones such as ciprofloxacin previously offered a reasonable alternative for treating pseudomonal infections (Sepehriseresht *et al.*, 2012). These infections have a high rate of morbidity and mortality; nevertheless, today β-lactams are the most drug of choice against this bacterium (Sepehriseresht *et al.*, 2012). Resistance to β-lactams is mostly challenging in healthcare settings because they are the mainstays for treatment of multidrug resistant *P. aeruginosa* (Hong *et al.*, 2015). Effective identification and intervention to control drug resistance is required in order to avoid the emergence of species possessing β-lactamases (Cho *et al.*, 2015).

1.2 Statement of the problem

P. aeruginosa is responsible for 10-15 % of the nosocomial infections worldwide. This bacterium is the most common cause of hospital-acquired infections which occur particularly in patients with immunocomprised conditions; but community-acquired infections have been reported (Somayeh & Fereshteh, 2013). Elderly patients are generally considered to be at high risk of nosocomial infections, due to a higher disease prevalence in this population (Wang *et al.*, 2019). Multi-drug resistance in *P. aeruginosa* isolates obtained from clinical sources is increasingly Worldwide (Aloush *et al.*, 2006). In healthcare Centers, patients' deaths resulting from antimicrobial resistance are projected to reach 10 million annually by 2050 and a cumulative 100 trillion USD of economic outputs are at risk to the rise of drug resistant infections. Today, 700,000 people die of resistant infections every year

(O'Neill, 2014). The World Health Organization (WHO) has published a global priority pathogens list to focus attention on most significant resistant pathogens Gram-negative bacteria that are resistant to 3rd Generation Cephalosporins and Carbapenems (WHO, 2017). MBL producing *P. aeruginosa* organisms are resistant to different antimicrobial agents and responsible for several nosocomial outbreaks from different parts of the World, illustrating the necessity for proper infection control measures (Pitout *et al.*, 2008). Carbapenems, mainly imipenem and meropenem, are powerful agents for the treatment of infections due to multidrug-resistant *P. aeruginosa* (Basak *et al.*, 2012). Its immunoevasive nature as well as its ability of acquiring multi-drug resistance make elimination of this pathogen a particular challenge in the community and hospitals (Craciunas *et al.*, 2010).

The presence of ESBL has led to the widespread use of Carbapenems but the emergence of MBLs and their broad spectrums and unrivalled drug resistance is creating a therapeutic challenge for clinicians and microbiologists in hospital (Peshattiwar & Peerapur, 2011). ESBLs have an extended substrate profile that cause hydrolysis of cephalosporins, penicillins; but are yet inhibited by β-lactam β-lactamase inhibitors, such as clavulanate, tazobactam and sulbactam (Ciocan *et al.*, 2015). ESBLs mediate resistance to a variety of broad-spectrum cephalosporins, including cefotaxime, ceftriaxone, ceftazidime and aztreonam (Alikhani *et al.*, 2014). The emergence of *P. aeruginosa* harboring β-lactamases is a major public health problem that require proper identification and intervention to control drug resistance (Sivaraman & Noyal, 2011). In Kenya, multi-drug resistance rate due to Metallo-β-Lactamases that has been documented amongst *P. aeruginosa* is 14 % in a tertiary care hospital (Pitout *et al.*, 2008). These resistant strains are particularly problematic leading to increased mortality, longer hospital stays and higher hospital costs for patients infected with this pathogen (Hong *et al.*, 2015). Therefore, it is important to determine the presence of the β-lactamases genes implicated in β-lactams resistance used for chemotherapy of infectious diseases caused by *P. aeruginosa* species.

1.3 Justification of the study

P. aeruginosa has become an important cause of healthcare-associated infections in hospitalized patients with decreased immunity; mostly older patients (>50 years old) with underlying diseases (Palavutitotai *et al.*, 2018). WHO has identified *P. aeruginosa* as one of a group of pathogens named ESKAPE and these pathogens have become today resistant or persistent to antibiotics treatment in clinical practice (Santajit & Indrawattana, 2016). As Gram-negative bacteria with emerging antimicrobial resistance, *P. aeruginosa* species are among several multi-drug resistant bacteria that can be found in a global priority pathogen list issued by the World Health Organization (WHO) in order prioritizing the novel antimicrobial strategies (Klitzing *et al.*, 2018).

P. aeruginosa is commonly described to be resistant to multiple classes of antimicrobial agents with carbapenems being the strongest inhibitors of *P. aeruginosa* and considered as the last drug of choice (EL-Domany *et al.*, 2017). The emergence of newer β-lactamases such as ESBLs is a significant mechanism by which pathogenic bacteria develop resistance to some frequently accessible drugs; and this trend is of a major public concern due to the multidrug resistant nature of such microorganisms (Ejikeugwu *et al.*, 2015). ESBLs are plasmid-mediated β-lactamases capable of hydrolyzing oxyimino third-generation cephalosporins and monobactans but are yet inhibited by clavulanic acid (Zhilong *et al.*, 2015). MBLs are important mechanisms of carbapenem resistance among *P. aeruginosa* isolates (Mahmoudi *et al.*, 2016). Carbapenems are relatively stable to be hydrolysed by most β-lactamases; but MBLs are able to efficiently hydrolyze all β-lactams with the exception to monobactams (Sheikh *et al.*, 2014). Carbapenems are the drug of choice for treatment of serious infections due to ESBL-producing organisms including *P. aeruginosa* but yet, carbapenem-resistant *P. aeruginosa* isolates have been reported (Ejikeugwu *et al.*, 2012; Paterson & Bonomo, 2005).

The increase of β-lactamases producing-*P.aeruginosa* can cause major therapeutic failure and poses a significant clinical challenge (Gupta *et al.*, 2016). In Kenya, few studies have been done on molecular characterization of β-lactams resistance of *P. aeruginosa* isolates in hospitals and limited information is available on β-lactamases producing isolates. This study aims to determine genes encoding-MBLs and ESBLs in *P. aeruginosa* isolates obtained from clinical sources at The Nairobi hospital. The data generated from this study will help in the management of nosocomial infections caused by *P. aeruginosa* species. This should reduce morbid-mortality rate, longer hospital stays and high hospital costs by ensuring that only effective drugs are prescribed and implemented infection control measures are followed by the medical staff, visitors and patients. It will also contribute to control antibiotic resistance by preventing the dissemination of *P. aeruginosa* resistant strains possessing β-lactamases.

1.4 Research questions

In order to formulate the succinct objectives for the study, the following research questions were set.

1. What are the demographic characteristics of patients infected with *P. aeruginosa* species from clinical samples at The Nairobi Hospital?
2. What are the antimicrobial susceptibility profiles of *P. aeruginosa* isolates from clinical sources at The Nairobi hospital?
3. What proportion of *P. aeruginosa* isolates from clinical specimen are ESBL and MBL producers at The Nairobi Hospital?
4. What is the distribution of ESBL and MBL producers according to the demographic characteristics and specimen type of patients with *P. aeruginosa* infections at The Nairobi Hospital?
5. Which genes encoding-MBLs and ESBLs are detected among β-lactams-resistant *P. aeruginosa* isolates from clinical samples at The Nairobi Hospital?

1.5 Research objectives

1.5.1 General objective

To determine demographic characteristics of patients, antimicrobial susceptibility profiles, and molecular characterization of β-lactams resistance in *P. aeruginosa* isolates obtained from clinical samples at The Nairobi Hospital in Kenya.

1.5.2 Specific objectives

1. To determine demographic characteristics of patients infected with *P. aeruginosa* isolates from clinical specimens at The Nairobi Hospital.
2. To determine antimicrobial susceptibility profiles of *P. aeruginosa* isolates from clinical samples at The Nairobi hospital.
3. To determine ESBL and MBL producers among *P. aeruginosa* isolates from clinical specimens at The Nairobi Hospital.
4. To determine the distribution of ESBL and MBL producers based on demographic characteristics and specimen type of the patient with *P. aeruginosa* infections at The Nairobi Hospital.
5. To determine genes encoding-MBLs (VIM, IMP, NDM types) and ESBLs among β-lactams-resistant *P. aeruginosa* isolates from clinical specimens at The Nairobi hospital.

1.6 Limitations of the study

The study did not investigate the clonality of the isolates and the sequence of the genes. The study did not also use primers to target all known β-lactamases genes. Despite these limitations, the study has provided the distribution of the common MBLs genes and the magnitude of β-lactams resistance in this tertiary care hospital.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Pseudomonas species*

Pseudomonas is a Gram-negative, aerobic, and facultative anaerobe bacillus which maintains a significant metabolic versatility, achieving anaerobic growth by using nitrogen as a terminal electron acceptor in the absence of oxygen (Mesquita *et al.*, 2013). The genus *Pseudomonas* comprises more than 120 species that are ubiquitous in moist environments such as water and soil ecosystems, and pathogenic to animals and humans; and most frequently associated with human infections (Klrissa & Mohammad, 2016; Mesquita *et al.*, 2013). *Pseudomonas* species are easily identifiable on agar plates because of pigments production such as pyoverdine which is a yellow green, fluorescent pigment, and pyocanin that is a blue-green pigment (Klrissa & Mohammad, 2016).

P. aeruginosa is the most opportunistic nosocomial organism, Gram-negative motile rod-shaped bacterium, which is aggressive, toxigenic and often multi-drug resistant, leading to complications during therapy (Filiatrault *et al.*, 2006; Ramprasad *et al.*, 2010). The main mechanism of resistance in *P. aeruginosa* is provided by β-lactamase and changes in membrane permeability given by the presence of efflux pumps and mutations of the transmembrane porins (Barrios *et al.*, 2014). *P. aeruginosa* is a leading cause of nosocomial infections, being responsible for urinary tract infections, soft tissue and skin infections, joint and bone infections, respiratory tract infections, bacteraemia and a variety of systemic infections mostly in people with decreased immune condition including burn wounds, cancer and HIV/AIDS patients (Filiatrault *et al.*, 2006; Mesquita *et al.*, 2013). Moreover, this lung pathogen is considered as the leading cause of cystic fibrosis, contributing to the progression and mortality rate of the disease in children and adults (Crull *et al.*, 2016; Mesquita *et al.*, 2013). It is most commonly associated with hospital-acquired infection but

community-acquired infection of this nosocomial pathogen has also been documented (Meletis & Bagkeri, 2013; Wahab & Rahman, 2013).

2.2 Epidemiology of *Pseudomonas aeruginosa*

P. aeruginosa is a gram-negative aerobic and facultative anaerobic rod-shaped bacterium, belonging to the *Pseudomonadaceae* family (Filiatrault *et al.*, 2006; Mesquita *et al.*, 2013). It is a small bacterium which does not ferment glucose, straight or slightly curved, non-spore-forming, and motile due to a unipolar and twitching flagellum (Barrios *et al.*, 2014). Various cultures of this pathogen produce a blue green pigment pyocyanin; which is very useful for isolation and identification. This bacterium has a characteristic of copper oxide in color, which has suggested the origin of the name *aeruginosa* (Barrios *et al.*, 2014). *P. aeruginosa* is reported to be ubiquitous in humans, animals, and the natural environment (Khalil *et al.*, 2015). The nutrients required by *Pseudomonas* species are very simple, and the genus is found in natural habitats like fresh water, soil and marine environment (Franzetti & Scarpellini, 2007).

This common opportunistic pathogen in humans cause a broad range of acute and chronic infections in the community and hospitals (Sandya Rani & Swathi, 2015). The most serious manifestations of infection include bacteraemia (particularly in neutropenic patients), pneumonia (particularly in cystic fibrosis patients and critically ill patients), urinary tract infections and wound infections (especially in patients with burn injuries) (Snyder *et al.*, 2013). The incidence rate of infections produced by Multi-drug resistant (MDR) *P. aeruginosa* strains may vary from 5.5 to 14 cases per 10,000 patients admitted per year (Aloush *et al.*, 2006; Harris *et al.*, 1999). A study developed in a hospital in Rome, Italy revealed the first case of MDR *P. aeruginosa* infection in a hematologic patient in 1992 (Tacconelli *et al.*, 2002). After that, the prevalence of MDR *P. aeruginosa* increased from 8 % in 1993 to 17 % in 1999, related to that hospital. Of 358 cases of bloodstream infections due to *P. aeruginosa*, 14 % (51 cases) were caused by strains of MDR *P. aeruginosa*. In these cases, 96 % (46 infections) were nosocomial. The overall mortality rate due to *P. aeruginosa*

infection is greater than 20 %, which increases when the infection is due to MDR *P. aeruginosa* strains (Barrios *et al.*, 2014). *P. aeruginosa* is the most common cause of all hospital-acquired infections which occur particularly in patients with immunocomprised conditions; suffering from pulmonary diseases, cancer and burn wounds as well as cystic fibrosis with a high rate of morbidity and mortality (Somayeh & Fereshteh, 2013). Multidrug and pandrug resistant bacterial infections are closely linked to increased mortality, longer hospitalization, multiple morbidities, and higher hospital cost due to the limited therapeutic options for infected patients with *P. aeruginosa* (Hong *et al.*, 2015).

The ubiquitous nature of the bacteria and irrational use of antibiotics via prescribers, dispensers and patients provide an appropriate ground for spread of multidrug resistant strains in the community and hospitals (Ameen *et al.*, 2015). Different reports have also shown that *P. aeruginosa* is capable of also affecting healthy immunocompetent members (Vives-Flórez & Diana, 2006). In a literature survey of infections due to *P. aeruginosa* species in healthy people, *P. aeruginosa* was reported to cause an epidemic folliculitis and dermatitis in two persons that used the same bathtub (Vives Florez & Diana, 2006). *P. aeruginosa* was also reported to cause septicemia in healthy children, ecthyma gangrenosum and infection in a child without known risk factors, liver abscess in a healthy child, and community-acquired sacro-iliitis in a young healthy man (Vives Florez & Diana, 2006). The dissemination of *P. aeruginosa* strains was observed in a Cystic fibrosis patient to healthy relatives, community-acquired fatal bacteraemia in two previously healthy patients, and community-acquired pneumonia (Vives Florez & Diana , 2006).

P. aeruginosa is able to tolerate various physical conditions and can survive with simple nutritional requirements. The bacterium is also able to survive in whirlpools, home humidifiers, swimming pools, soil and rhizosphere, contact lens solution, hot tubs and vegetables (Mesquita *et al.*, 2013). Tap water and sinks have also been shown to be typical reservoirs of *P. aeruginosa* (Mesquita *et al.*, 2013; Trautmann *et al.*, 2009). Moreover, the colonization of respiratory therapy equipment (ventilators), aggressive devices and intravenous lines (catheters) have also been reported to harbor

P. aeruginosa. This is the cause of infection outbreaks in hospital settings; because this pathogen can also be disseminated through contact with infected patients, visitors or medical staff (Mesquita *et al.*, 2013). The increase in incidence of infection associated with invasive and aggressive devices in hospitals could prompt the selection of nosocomial resistant strains and leads to major therapeutic failures.

2.3 Pathogenesis of *Pseudomonas aeruginosa*

The pathogenesis of *P. aeruginosa* has been extensively studied and proven to be a multifactorial process, mediated by two quorum sensing systems that facilitate cell to cell communication through production of signalling molecules termed auto-inducers to target specific receptors for activation (Klrissa & Mohammad, 2016). However, a high population density is necessary for the concentration of inducers to go beyond threshold (Klrissa & Mohammad, 2016). Maximal receptor activation induces gene expression of several virulence factors and biofilm formation (Ben *et al.*, 2011). These virulence factors include mucoid exopolysaccharide, lipopolysaccharide, biofilm, pili, exotoxin A, pigments, lipase, protease, hemolysin, histamine, exoenzyme S, leukocidin, and rhamnolipids (Khalil *et al.*, 2015). These factors help the bacteria to make attachment and invasion of their host by damaging their immune responses and forming an obstacle to different antimicrobial agents (Ben *et al.*, 2011). The virulence factors and associated cell secreted by *P. aeruginosa* can be encoded on plasmids or chromosomal genes (Khalil *et al.*, 2015).

In addition, flagellins of this pathogen can perform several functions during host attack, apart from enabling motility, the flagellum of *P. aeruginosa* plays an indirect role in the permeabilization of the membrane and surfactant protein-mediated bacterial clearance (Porras-Gómez *et al.*, 2012). Similarly, the pili is involved in this mechanism and play an important role during inflammation due to glycosylation in the interface between pili and host cells (Ben *et al.*, 2011; Porras-Gómez *et al.*, 2012). Adhesion of bacteria to the host cell surface is the first step in disease colonization and initiation (Klrissa & Mohammad, 2016). Type IV pili are the filamentous appendages adhered to the host cell surface of the bacterium (Ben *et al.*,

2011; Gerald *et al.*, 2007). *P. aeruginosa* also secretes different virulence factors such as extracellular polysaccharides; polysaccharide synthesis locus, alginate and polysaccharide-encoding locus that play a role in forming the biofilm matrix embedded around microcolonies (Klrissa & Mohammad, 2016). Biofilms protect *P. aeruginosa* bacterium from the host immune system elements, as well as resistance to antimicrobial agents (Ben *et al.*, 2011). In addition to type IV pili and biofilm formation, adhesion is also mediated by other various cell surface features, including lipopolysaccharides (LPS) (Gerald *et al.*, 2007). LPS are a major factor in mediating both host responses and bacterial virulence; and are involved in immunity and pathogenesis; varying depending on the underlying patient basis for the isoform of the lipopolysaccharide, particularly the lipid A component, the increased susceptibility to infection and structural variation in the antigen O side chain that influences host immunity (Gerald *et al.*, 2007).

These bacteria can also secrete, a linear polymer of mannuronic acid and glucoronic acid, which is known to stimulate production of immunoglobulin G (IgG) and immunoglobulin A (IgA) antibodies and is thought to work as an adhesin that help *P. aeruginosa's* attaching to the respiratory epithelium (Mesquita *et al.*, 2013). Studies on overexpressed alginate have reported to protect *P. aeruginosa* from phagocytosis and antibodies (Ben *et al.*, 2011). *P. aeruginosa* reacts with the host cell via a protein, as appendage recognized as the type III secretion system (T3SS) which injects toxic effector proteins into the cytosol of cells of eukaryote inhibiting the function of the cell for microbial survival (Klrissa & Mohammad, 2016). Four effector toxins have been observed and reported in *P. aeruginosa* and comprise exoenzymes Y, U, S, and T. ExoT and ExoS have GTPase activating protein activity and ADP-ribosyltransferase activity (Klrissa & Mohammad, 2016). The exoenzyme S and other adhesins strengthen the attachment to epithelial cells (Ben *et al.*, 2011). The pathogenic role of exoenzyme S is associated with the destruction of immunoglobulin G and A, the disruption of normal cytoskeletal organization, leading to depolymerization of actin filaments and resistance to macrophages (Ben *et al.*, 2011; Mesquita *et al.*, 2013). The ExoU is the major cytotoxin in *P. aeruginosa* species; and has been demonstrated that ExoU is involved in killing various

mammalian cells, adapting the host susceptible to the secondary infections (Mesquita *et al.*, 2013).

P. aeruginosa may also produce, during infection, an exotoxin A that can cause cytotoxicity by inhibiting elongation factor and inactivating protein synthesis via ADP-ribosylation. It can cause local tissue damage such as pulmonary haemorrhage, liver cell and renal necrosis, and also responsible for immunosuppression and bacterial invasion in the Cystic fibrosis lung (Mesquita *et al.*, 2013). *P. aeruginosa* and other bacterial pathogens express one or more homologous extracellular phospholipases C (PLC) that are secreted through the inner membrane via the twin arginine translocate (TAT) pathway (Barker, 2004). *P. aeruginosa* produces a hemolytic and a non-hemolytic phospholipase C (PLC) (Ben *et al.*, 2011). Studies have shown that non-hemolytic phospholipase C has no pathogenic activity, but a hemolytic phospholipase C play a role in the degradation of phosphatidylcholine and sphingomyelin that are usually found within host lung surfactant and eukaryotic membranes, generally to cause lung injury (Klrissa & Mohammad , 2016).

In addition, *P. aeruginosa* can produce elastase B (lasB) (exoproteases) which is the most elastolytic zinc metalloproteinase encoded by the lasB gene of *P. aeruginosa* species (Kuang, 2011). LasB, well-known pseudolysin can cause damage the host tissues through hydrolysis of the constituents of extracellular matrix and by breaching the endothelial and epithelial barriers by attacking tight junctions; and LasB is implicated in the destruction of different type of components of innate and adaptive immune systems (Kuang, 2011).

P. aeruginosa is the only gram-negative rod-shaped bacterium capable of producing the pyocyanin pigment (Lau & Hasset, 2004). Pyocyanin is a blue redox-active secondary metabolite that is involved in the production of a blue-green pigment, used in rapid identification of *P. aeruginosa* strains (Klrissa & Mohammad, 2016). Previous researches have shown that pyocyanin is a principal virulence component, interacting with various cell functions (Lau & Hasset, 2004; Klrissa & Mohammad, 2016). *P. aeruginosa* might also secrete two siderophores to accumulate iron;

pyoverdine and pyochelin, helping bacteria to colonise niches with limited iron availability (Mesquita *et al.*, 2013). Iron is also required for bacterial growth and affects *P. aeruginosa* pathogenesis, where an increase in iron concentration aids this pathogen to cause persistent infections (Mesquita *et al.*, 2013). Pyoverdine is a fluorescent pigment which is yellow green; and also a water-soluble fluorescein siderophore with strong iron chelating ability from the Gram-negative organism *P. aeruginosa* (Klrissa & Mohammad, 2016; Yuan & Gao, 2017). Multiple virulence factors secreted by *P. aeruginosa* can cause persistent infections in hospitals by damaging the host's immune system and blocking the passage to antimicrobial agents including β-lactams.

2.4 Antibiotic resistance of *Pseudomonas aeruginosa*

P. aeruginosa develops resistance by various mechanisms like multi-drug resistance efflux pumps, biofilm formation, production of β-lactamases and aminoglycoside modifying enzymes (Mahmoud *et al.*, 2013; Sandhya Rani & Swathi, 2015; Wolter & Lister, 2013). The risk for acquiring Multi-drug resistant organisms may be related to the number of carriers in the same ward as well as to individual risk factors, such as patient characteristics and in-hospital events (Mahmoud *et al.*, 2013).

Due to the production of a variety of β-lactamases, alterations in the penicillin-binding proteins and outer membrane permeability, and combinations of multiple mechanisms of resistance, the Gram-negative bacteria have acquired resistance to antibiotics such as extended-spectrum cephalosporins, monobactams, carbapenems and β-lactam-β-lactamase inhibitor combinations (Nasreen *et al.,* 2015). Therefore, due to its intrinsic and acquired antimicrobial resistance, only limited classes of antibiotics are effective for the treatment of *P. aeruginosa* infections (Hong *et al.*, 2015). All of the major classes of antibiotics used to treat *P. aeruginosa* infections have to cross the cell wall to reach their targets (Lambert, 2002). The β-lactams including piperacillin, ceftazidime, imipenem, meropenem and aztreonam inhibit the peptidoglycan-assembling transpeptidases located on the outer face of the

cytoplasmic membrane (Zeng & Lin , 2013). Failure of antibiotics to accumulate within the organism is due to a combination of restricted permeability of the outer membrane and the efficient removal of antibiotic molecules by the action of efflux pumps (Lambert, 2002; Sepehriseresht, *et al.*, 2012).

Therefore, previous reports have shown that various mechanisms are involved; including utilization of efflux pumps and chromosomal mutations, target site modification and the production of inactivating enzymes (Mahmoud *et al.*, 2013; Meletis & Bagkeri, 2013). *P. aeruginosa* produces β-lactamases; enzymes that hydrolyze the peptide bond of the β-lactam ring to inactivate β-lactams antibiotics (Mahmoud *et al.*, 2013; Meletis & Bagkeri, 2013; Moyo, *et al.*, 2015). The bacterium is also able to produce different types of β-lactamases such as Metallo-β-Lactamases (MBLs), Extended-Spectrum β-Lactamases (ESBL), and chromosomal cephalosporinase (AmpC) (Klrissa & Mohammad, 2016). Therefore, antibiotic resistance in *P. aeruginosa* is due to both intrinsic and acquired resistance (Kamel *et al.*, 2011). Intrinsic resistance is caused by the multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes and the lower permeability of the bacterial membranes of the cells (Hassanein *et al.*, 2009; Meletis & Bagkeri, 2013). Acquired resistance resulted either to the horizontal gene transfer of antibiotic resistance determinants or mutations in chromosomally-encoded antibiotic resistance genes (Kamel *et al.*, 2011). In Kenya, limited information on antibiotic resistance in *P. aeruginosa* is available but the resistance rate due to β-lactamases producers is scarcely documented.

2.4.1 Resistance by altered outer-membrane permeability

P. aeruginosa is a ubiquitous bacterium which can survive in the environment with minimal requirements and has the ability to adapt to various environmental challenges (Dantas *et al.*, 2014). This pathogenic plasticity is attributed to the massive genome associated with flexible metabolism and the lower permeability of the bacterial cell envelope, making organism resistant to a wide range of antimicrobial agents, including antibiotics (Dantas *et al.*, 2014). The majority of

carbapenem-resistant *P. aeruginosa* clinical isolates exhibit a porin OprD deficiency (Pechere & Thilo, 1999). OprD is a specific channel facilitating the entry of basic amino acids and carbapenems including imipenem and meropenem (Meletis & Bagkeri, 2013; Pechere & Thilo, 1999).

OprD deficiency produces resistance only when chromosomal β-lactamase is expressed, exemplifying the interplay between the two resistance mechanisms (Pechere & Thilo, 1999). Impaired penetration of different substances through the membrane including imipenem is due to diminished expression of specific OM protein (Porras-Gómez *et al.*, 2012). It has been shown that OM permeabilizers such as EDTA increase susceptibility to antibiotics, indicating that the lack of OprD protein leads to a reduction of active antibiotic molecules capable of reaching the target penicillin-binding-proteins (PBP) of the bacterial cell membrane (Porras-Gómez *et al.*, 2012).

2.4.2 Resistance by active efflux pumps

The resistance of MDR strains may be mediated by the active export of the antibiotics out of the bacterial cell by efflux pumps (Meletis & Bagkeri, 2013). Evidence from diverse bacterial genomes indicates that approximately 5%-10% of genes are involved in transport, with a large proportion of them encoding efflux pumps (Porras-Gómez *et al.*, 2012).

The multidrug efflux systems are composed of three protein components, an energy-dependent pump located in the cytoplasmic membrane, an outer membrane porin and a linker protein which couples the two membrane components together (Meletis & Bagkeri, 2013). This tripartite arrangement forms an efficient extrusion system for toxic molecules present in the cytoplasm, the cytoplasmic membrane or the periplasm, including the region between the outer and cytoplasmic membrane (Lambert, 2002). Four different antibiotic efflux systems have been described in *P. aeruginosa*: mexAB-oprM, mexXY- oprM, mexCD-oprJ and mexEF-oprN. MexAB-oprM is responsible for extrusion of β-lactams, quinolones and a range of

disinfectants (Lambert, 2002; Meletis & Bagkeri, 2013). However, most often, drug efflux appears to be insufficient by itself to confer clinically relevant levels of resistance to important β-lactams (Cavallo *et al.*, 2002).

OprM overproduction is associated with cross-resistance to unrelated antibiotics such as tetracycline, chloramphenicol and quinolones (Cavallo *et al.*, 2002). The enhanced levels of resistance to these antibiotics encountered in AmpC overproducers and penicillinases-producing strains that do not overexpress OprM are probably due to unrelated mechanisms (Alcalde-Rico *et al.*, 2016; Cavallo *et al.*, 2002).

2.4.3 Resistance by altered target

The persistent exposure of bacterial strains to a multitude of β-lactams result in mutation of β-lactamases and a dynamic and continuous production in the bacteria, expanding their activity even against higher generation cephalosporins and against aztreonam (Khalil *et al.*, 2015; Pechere & Thilo, 1999). Altered penicillin-binding proteins (PBP) appears to be rarely linked to the emergence of resistance during β-lactam treatment, but modified penicillin binding protein-4 (PBP-4) has been observed after therapy with imipenem; and after high doses of piperacillin in a cystic fibrosis patient, the decrease in PBP affinity for penicillin G was correlated with high resistance to β-lactam and increased expression of PBP-6 (Pechere & Thilo, 1999). It was reported that *P. aeruginosa* overproducing PSP-3 exhibited lower susceptibility to β-lactam antibiotics (Lambert, 2005).

2.4.4 Resistance by β-lactamase production

The common use of drug has contributed to the emergence of antibiotic resistance and the production of a variety of β-lactamases amongst *P. aeruginosa* strains, an important resistance mechanism reported in many bacteria (Inacio *et al.*, 2014). The β-lactamases are the enzymes that are encoded by the chromosomal and plasmid genes of many bacteria (Ansari *et al.*, 2016). The β-lactamases are produced as a form of metabolic by-products that have the ability to hydrolyze and destroy the β-lactam antibiotics (Ahmad *et al.*, 2016). Resistance mediated by extended spectrum

β-lactamases (ESBLs), metallo-β-lactamases (MBLs), and AmpC β-lactamase (AmpC) enzymes to broad-spectrum β-lactam antibiotics is a global concern for the antimicrobial therapy (Ansari *et al.*, 2016; Klrissa & Mohammad , 2016). Consequently, the high rate of antibiotic resistance due to β-lactamase enzymes to commonly used antibiotic for the management of pseudomonal infections and its recent influence on antimicrobial therapy failure encouraged us to find out the resistance rate due to β-lactamases and current therapeutic options for effective treatment of this pathogen (Ansari *et al.*, 2016).

2.4.4.1 Extended spectrum β-lactamase enzymes

ESBLs are plasmid-mediated enzymes that hydrolyze broad spectrum β-lactams and monobactams, and are strongly inhibited by clavulanate, which are transferable on plasmids among bacterial species and between hospitals (Laudy *et al.*, 2017). When inappropriate antimicrobial therapy is used to treat infections due to ESBL-producing isolates, failure in the clinical treatment will occur commonly (Zhilong *et al.*, 2015). ESBL producing-*P. aeruginosa* has been observed and reported in different regions of the world (Okesola & Oni, 2012). In *P. aeruginosa* strains, the ESBL enzymes from both A and D classes are reported, primary β-lactamases from the PER, GES, VEB, BEL and PME family and from the OXA family, named extended-spectrum class D β-lactamases (ES-OXA) (Laudy *et al.*, 2017). Nevertheless, ESBLs such as TEM, SHV, VEB, GES and more recently, CTX-M variants have been observed to increase in *P. aeruginosa* species in various areas (Okesola & Oni, 2012). VEB and PER types were found to be the most common ESBL in *P. aeruginosa* in several countries, contrasting to the dominance of CTX-M, SHV, and TEM ESBL in *Enterobacteriaceae* (Nithyalakshmi *et al*., 2016).

ES-OXA (class D) are less well inhibited by the known β-lactamases inhibitors than ESBLs from class A (Laudy et *al.*, 2017). Most of ESBLs are usually able to hydrolyze extended spectrum cephalosporins and monobactams (Thierry *et al.*, 1999). For the first time class A enzymes were reported in *Enterobacteriaceae* family but since 1990, the presence of these enzymes has been frequently found in *P.*

aeruginosa (Akhi *et al*., 2012). *Bla$_{PER-1}$*-type ESBLs were the first reported ESBLs ones in *P. aeruginosa* and like most other ESBLs such as *bla$_{TEM}$* and *bla$_{SHV}$* they can hydrolyze different types of β-lactam antibiotics except for carbapenem and cephamycin (Akhi *et al*., 2012). The ESBLs genes often code resistance to cephalosporins and other antibiotics such as aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulfamethoxazole-trimetroprim (Ogefere *et al.*, 2015). In contrast, *P. aeruginosa* possesses inducible, naturally occurring cephalosporinases which confer low-level resistance to aminopenicillins, narrow-spectrum cephalosporins such as cephalothin, and cephamycins such as cefoxitin, but these Ambler class C β-lactamases are not inhibited by clavulanic acid (Thierry *et al.*, 1999). The rarity of reports of *P. aeruginosa* strains harboring genes for TEM and SHV-type enzymes may have several explanations (Weldhagen *et al.*, 2003). First, the rarity of narrow-spectrum TEM-type enzymes may limit antibiotic selection of TEM and SHV-type enzymes with an expanded spectrum of hydrolysis; and second, a high prevalence of chromosome-encoded oxacillinase and carbenicillinase genes may explain why narrow-spectrum enzymes of the TEM type are rare in *P. aeruginosa* (Weldhagen *et al.*, 2003).

Several different methods have been suggested for the detection of ESBLs in clinical isolates, such as disk approximation or double disk synergy, modified double disc test, CLSI phenotypic confirmatory method, E-test ESBL strips, three dimensional test, and automated systems including Vitek 2 System (Begum *et al.*, 2013). While each of these tests has merit, none are able to detect all of the ESBLs encountered. Disk approximation or double disk synergy is one of the currently available and most widely used techniques for the detection of ESBLs (Begum *et al.*, 2013). In Kenya, infections by *P. aeruginosa* due to ESBLs are scarcely investigated and recent studies have shown a limited information on genes encoding ESBLS including *bla$_{VEB}$*, *bla$_{PER}$*, and *bla*$_{GES}$ (Gachuki *et al.*, 2019; Kilivwa *et al.*, 2018).

2.4.4.1.1 PER, VEB and GES ESBL types of *P. aeruginosa*

A variety of ESBL enzymes within Ambler class A (PER, VEB, GES, and OXA) have been observed in *P. aeruginosa* from certain geographic regions (Freshteh *et al.*, 2009). The presence of less-studied types of ESBLs, including OXA, VEB, PER, and GES, has been reported to spread genes encoding ESBLs that may play role in the spread of antibiotic resistance and may complicate the treatment of infections due to *P. aeruginosa* organism because of limitations therapeutic options (Amirkamali *et al.*, 2017). Bla_{PER-1}, the most common ESBL found among *P. aeruginosa*, demonstrates high level of catalytic activity against aztreonam and cefotaxime (Freshteh *et al.*, 2009). Bla_{VEB-1} has the highest homology with bla_{PER} and confers higher resistance to ceftazidime, cefotaxime, and aztreonam, which is reversed by clavulanate, a β-lactam β-lactamase inhibitor (Freshteh *et al.*, 2009). The gene, bla_{GES-1}, conferred an extended-spectrum cephalosporin resistance profile, such as clavulanic acid, tazobactam and imipenem (Pellegrino *et al.*, 2006). The resistance genes in *P. aeruginosa* strains is yet to be determined.

2.4.4.2 Metallo- β-lactamases enzymes

MBLs are broad-spectrum β-lactamases that are able to hydrolyse most of the β-lactam antibiotics with exception to monobactams and are not reserved by β-lactamase β-lactam inhibitors, including clavulanic acid or sulbactam (Jayarani *et al.*, 2014). The presence of MBL enzymes is influenced by the fact that MBLs are affected by the elimination of zinc from the active site of the enzyme (Sniha *et al.*, 2018). These MBL enzymes need zinc for their enzymatic activity and are so far inhibited by EDTA and thiol compounds, metal chelators (Sood, 2014). The increased diversity, the rapid dissemination of these enzymes is due to the fact that they are frequently encoded on mobile genetic components such as integrons, transposons, plasmids together with other resistance genes; MBL-producers belong to the set of clinically multidrug-resistant microorganisms (Jayarani *et al.*, 2014).

MBLs producing isolates were first observed from Japan in 1991 and found after from different parts of the world (Sood *et al.*, 2014). MBLs belonging to the Ambler class B, have the ability to inactivate a wide range of β-lactam agents, including penicillins, cephalosporins and carbapenems but not monobactams (Sniha *et al.*, 2018). MBLs can be distributed into six groups of based on their molecular structure namely IMP, VIM, GIM, SIM, SPM and AIM (Ellabib *et al.*, 2013). *P. aeruginosa* is exclusively challenging due to its capability to acquire resistance to a variety of antimicrobial classes via mutation to all applicable treatments (Ellabib *et al.*, 2013). Moreover, other main MBL types such as bla_{SPM}, bla_{GIM}, and bla_{SIM} genes are involved in the emergence of antibiotic resistance worldwide (Sniha *et al.*, 2018).

The β-lactam agents comprise a wide family of antibiotic with a common characteristic of harboring β-lactam ring in their molecules (Jayarani *et al.*, 2014). Acquired MBL genes will consistently confer resistance to broad-spectrum β-lactam in *P. aeruginosa* (Sako *et al.*, 2014). The genes encoding MBLs are usually carried by class 1 integrons, which are found in transposons, resulting in extremely transferable genetic elements (Sako *et al.*, 2014). The MBL production, as a common mechanism of the acquired mechanism of resistance to carbapenems, is mediated by plasmidic genes like bla_{IMP} and bla_{VIM} (Bejestani *et al.*, 2015). A worldwide increase in the distribution of resistance to carbapenem is reported and the development of this multi-drug resistant pathogen has become problematic in healthcare settings (Khosravi *et al.*, 2013). Among Carbapenems, imipenem is the appropriate antibiotic against this opportunistic bacterium, but *P. aeruginosa* can hydrolyze it through MBLs (Sepehriseresht, *et al.*, 2012). The imipenem-resistance allele was found on transmissible conjugative plasmid that might be freely mobilized to other circulating strains (Sako *et al*., 2014).

The detection of β-lactamase production has been accomplished in the past by calculating the production of penicilloic acid, which is produced when benzyl penicillin is inactivated (Basak *et al.,* 2012). The acid production can be identified by acidometric method, iodometric method and chromogenic cephalosporin (nitrocephin) method (Basak *et al.,* 2012). The most common used tests are the

double-disk synergy test (DDST), the combined disk (CD) assay, and the MBL E-test (Lucena *et al.*, 2014). Nevertheless, these methods have reported discordant results according to the employed methodology, β-lactam substrates used, presence of MBL inhibitors (IMBL), bacterial genus tested and local prevalence of MBL types (Lucena *et al.*, 2014). The early detection of MBL producing *P. aeruginosa* may help in proper management of the bacterial infections and avoid further spread of these multidrug resistance strains (Rajput *et al.*, 2012). In the absence of novel antimicrobial agents for the therapy of infections due to the multi-resistant Gram-negative pathogen, the uncontrolled spread of MBL producers may lead to a therapeutic failure with high rate of morbidity and mortality (Devi *et al.*, 2015). Few studies have been done on Metallo-β-lactamases-producing *P. aeruginosa* in Kenya. Therefore, it is important to determine the presence of β-lactamases enzymes implicated in β-lactams resistance used for chemotherapy of infectious diseases caused by *P. aeruginosa* species.

2.4.4.2.1 VIM, IMP, and NDM MBL types of *P. aeruginosa*

The most significant way for carbapenem resistance is metallo-β-lactamases (MBL) production, of which the VIM and IMP MBL families are more predominant between Gram-negative bacilli (Sepehriseresht, *et al.*, 2012). The occurrence of VIM-type and IMP-type MBLs between the isolates of different bacteria including *P. aeruginosa* has been reported in different studies (Sepehriseresht, *et al.*, 2012). The bla_{IMP} genes were first observed in Japan, while the VIM enzymes were first shown in Europe, but both categories are increasing in Asia, Europe, and America as acquired resistance factors in nosocomial pathogens of *P. aeruginosa* and other non-fastidious, Gram-negative and non-fermenting rods (Lagatolla, *et al.*, 2004). The VIM enzyme is <40% amino acid homologous with the $bla_{VIM\text{-}1}$variant and 90% amino acid homologous with the IMP enzymes (Lagatolla, *et al.*, 2004). Both types of resistance genes are carried on mobile gene cassettes inserted into plasmid or chromosomal borne integrons, a location that finally assists horizontal transfer among different strains (Lagatolla, *et al.*, 2004). MBL genes were reported to be carried on great transferable plasmids or were linked to transposons, allowing horizontal spreading of these MBL

genes among different bacterial genera and species (Zafer *et al*., 2014). While IMP and VIM variants have been reported worldwide, other members of MBL families are limited to certain geographical areas (Zafer *et al*., 2014). However, the clinically important MBLs genes are encoded in mobile genetic components and include bla_{VIM}, bla_{IMP} and the most emerged bla_{NDM} (Lee *et al.*, 2003).

Because of its aptitude to disseminate, carbapenem resistance associated to IMP and VIM β-lactamase production has become a major public concern (Lee, *et al.*, 2003). Actually, VIM-2 has developed as a dominant MBL variant in North Africa and worldwide (Najla, *et al.*, 2015). The VIM types have been shown in carbapenem-resistant isolates of *P. aeruginosa* from African countries including Algeria, Tunisia, and Egypt; and from countries in the Mediterranean basin such as Spain, Greece, Lebanon, Italy, and France (Najla, *et al.*, 2015). Though different inhibitors have been analysed in vitro, there is no clinical compound which is capable of inhibiting any of the MBLs; which exhibited an extended substrate spectrum, including not only carbapenems, but also penicillins family and the higher generation of cephalosporins (Meini *et al.*, 2014). Though they do not hydrolyze aztreonam, they are commonly associated with serine-β-lactamases that reduce the susceptibility of the bacteria towards this drug (Meini *et al.*, 2014). The emergence of bla_{VIM}, bla_{IMP}, and bla_{NDM} genes play a crucial role in antibiotic resistance particularly carbapenem resistance. The detection of MBL genes may help in appropriate antimicrobial therapy and avoid further spread of the resistant strains in healthcare settings. Moreover, the prevalence of these genes among *P. aeruginosa* isolates obtained from clinical sources is yet to be determined.

2.5 β-lactams resistance

P. aeruginosa is a well-known cause of severe and potentially life-threatening infections including bacteremia, skin and wound infections, pulmonary disease, especially among individuals with cystic fibrosis, nosocomial urinary tract infections, endocarditis and meningitis (Aktas *et al.*, 2012). The β-lactam group of antibiotics which include penicillins, cephalosporins, monobactams and carbapenems are mainly

used to treat infections caused by Gram-negative bacteria including *P. aeruginosa* (Basak *et al.*, 2012). The widespread use of antibiotics put tremendous selective pressure on bacteria which develop new mechanisms to escape the lethal action of the antibiotics (Basak *et al.*, 2012). These infections are difficult to treat because of emergence of newer β-lactamases such as Extended Spectrum β-lactamases (ESBL), AmpC β-lactamases and Carbapenemases (Basak *et al.*, 2012). Failure to detect these enzymes producing strains has contributed to their uncontrolled spread in Health Care setup and therapeutic failure (Basak *et al.*, 2012).

Class A β-lactamases confer resistance to a wide range of β-lactam compounds, including cephalosporins and monobactams but are yet inhibited by β-lactam β-lactamase inhibitors combinations, including clavulanate, sulbactam and tazobactam (Jayarani et *al.*, 2014; Devi *et al*., 2015). Class B MBLs have the ability to hydrolyse a wide variety of β-lactam agents such as penicillins, cephalosporins and carbapenems (Devi *et al*., 2015). Carbapenems are among the most reliable therapeutic options for treating *P. aeruginosa* infection, the finding of carbapenem resistance is an ominous development that challenges this "last resort antibiotic" because the production of MBLs is the most common mechanism of carbapenem resistance (Mano *et al.*, 2015).

Carbapenem-resistant *P. aeruginosa* is associated with the MBL production and has the ability to hydrolyze all β-lactam antibiotics with exception to aztreonam (Ochoa *et al.*, 2013). It is accountable for nosocomial outbreaks in tertiary care hospitals (Ochoa *et al.*, 2013). Three groups of MBL have been observed, including class A which is serine dependent and partially inhibited by clavulanic acid, inducible and nontransferable; class B which is zinc dependent, inhibited by EDTA, inducible or associated with conjugative plasmids; and class C (oxacillinase) (Ochoa *et al.*, 2013). Nevertheless, β-lactam resistance of *P. aeruginosa* can be acquired either by natural or inducible cephalosporinase produced due to chromosomal origin, either due to reduced permeability of the antibiotic in the bacteria (Wateba *et al.*, 2014).

ESBLs are plasmid mediated β-lactamases that are capable of hydrolyzing the oxyimino β-monobactams but have no outcome on the cephamycin and carbapenems but they are strongly inhibited by clavulanic acid and tazobactam (Ahmed *et al.*, 2015). Lower permeability and efflux pumps are common mechanisms of the resistance phenotype for β-lactams and other antibiotics including aminoglycosides and quinolones which are basically important properties of this pathogen (Pramodhini *et al.*, 2015). The innate resistance to antibiotics of *P. aeruginosa* results from the limited permeability of the cell envelope and is increased by the activity of efflux systems (Pramodhini *et al.*, 2015). The development of β-lactam resistance in *P. aeruginosa* can be caused by several mechanisms such as genetic mutations that lead to stable overexpression of AmpC, a chromosome-mediated cephalosporinase, acquisition of transferable genes that code for a variety of β-lactamases, overproduction of efflux systems, and reduced permeability (Figure 2.1) (Ahmed *et al.*, 2015; Nasreen *et al.*, 2015). Antibiotic resistance mechanisms in *P .aeruginosa* strains appear to be complex and determination of the resistance mechanisms and susceptibility rates will guide the effectiveness of antimicrobial treatment, reducing the development and dissemination of resistant strains (Aktas *et al.*, 2012).

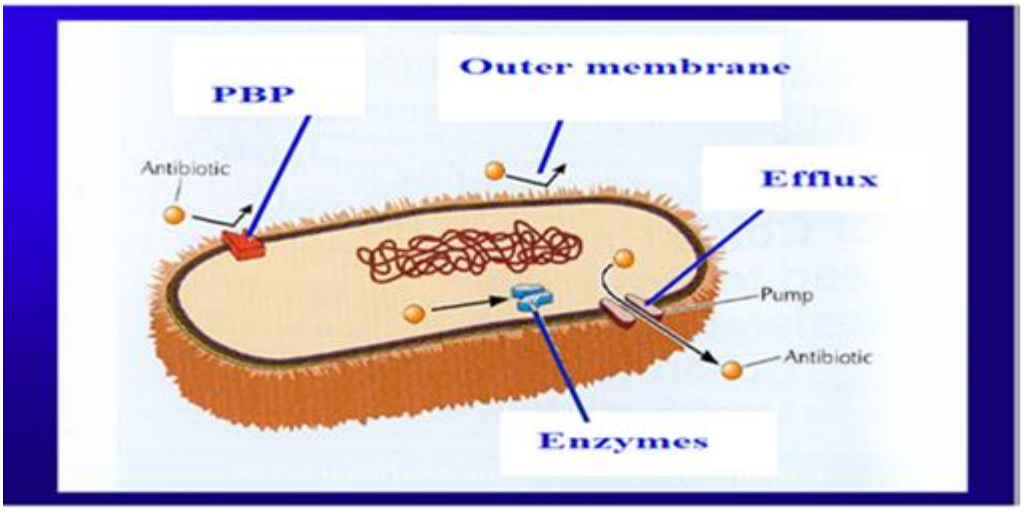

Figure 2.1: Mechanisms of antibiotics resistance of *P. aeruginosa*

Production of various **enzymes**, impaired penetration to the target (**PBP**), reduced **outer-membrane** permeability and active export of antibiotic molecules outside the

bacterial cell wall (**Efflux pump** system) are mechanisms of **antibiotic** resistance in *P. aeruginosa* (Mesquita *et al.*, 2013; Nordmann, 2005).

This study is focused on β-lactams resistance, β-lactams degrading enzymes (β-lactamases)**.**The most common used tests for detection of MBL enzymes are the double-disk synergy test (DDST), the combined disk (CD) assay, and the MBL E-test (Lucena *et al.*, 2014). Nevertheless, these methods have reported discordant results according to the employed methodology, β-lactam substrates used, presence of MBL inhibitors (IMBL), bacterial genus tested and local prevalence of MBL types (Lucena *et al.*, 2014). Several different methods have also been suggested for the detection of ESBLs in clinical isolates, such as disk approximation or double disk synergy test, modified double disc test, CLSI phenotypic confirmatory method, E-test ESBL strips, three dimensional test, and automated systems including Vitek 2 System. While each of these tests has merit, none are able to detect all of the ESBLs encountered. Disk approximation or double disk synergy is one of the currently available and most widely used techniques for the detection of ESBLs (Begum *et al.*, 2013).

2.6 Automated Antimicrobial Susceptibility Testing Systems

Several automated and semi-automated systems for antimicrobial susceptibility testing (AST) and identification of clinically relevant bacteria have been widely used. Most of them are commercially available. Each of these comes with well-known inherent strengths and performance specifications (Mwinyikombo, 2018). Examples of these methods include Vitek 2 system (BioMérieux), Phoenix Automated Microbiology system (BD Diagnostic Systems), and MicroScan WalkAway Microbiology System (Dade Behring). The VITEK system is an automated microbiology system using growth-based technology. The Vitek system originated in the 1970s as an automated system for identification and antimicrobial susceptibility testing (AST) and has evolved today into the Vitek 2 System, which performs automatically all of the steps required for identification and AST after a primary inoculum that has been prepared and standardized (Ligozzi *et al.*, 2002). The

databases of the Vitek 2 system for identification of bacterial products are made with large strain sets of well-characterized microorganisms experienced under many culture factors. These strains are prepared from variety of clinical and industrial sources as well as from public and university culture collections (ATCC). An unknown microorganism is compared to the database of reactions for each taxon and a numerical probability calculation is performed giving various levels of identification (Pincus, BioMérieux, USA).

The system uses colorimetric reagents cards, which after inoculation with the suspected bacteria are incubated in the Vitek 2 system and interpreted. Each card contains at least one positive control well with no antibiotic (growth-promoting broth only) and multiple wells with increasing concentrations of various antibiotics in the broth. The Vitek 2 AST cards for Gram-negative bacilli contains wells that test for antimicrobial agents at different concentrations and result in a Minimum Inhibitory Concentration (MIC) value (Mwinyikombo, 2018). The MIC is determined by comparing the growth of the isolate to the growth of isolates with known MICs. The Vitek 2 AST can report doubling dilution MICs for antibiotic concentration range of ≤0.25 µg/ml to ≥8 µg/ml (Michalik, 2017). Expression of ESBLs is made by comparison of logarithmic reduction in growth with the wells containing clavulanic acid and cephalosporin (cefotaxime, ceftazidime, cefepime) in combinations to the cephalosporin alone (not containing clavulanic acid) (Ampaire *et al.*, 2017).

2.7 Molecular detection of β-lactamases

These include DNA probes, Polymerase Chain Reaction (PCR), oligotyping, PCR-RFLPs and Nucleotide Sequencing. PCR is a molecular technique that amplifies target genes. It offers rapid and sensitive methods to identify the presence of resistant genes in bacteria and is crucial in the elucidation of genetic elements responsible for antibiotic resistance. This method is used also to characterize target organisms and is useful for investigating non culturable or slow growing organisms (Mwinyikombo, 2018; Vaez *et al.*, 2015). This chapter on screening for β-lactam antibiotic resistance in clinical samples is also focused on the application of PCR amplification using

specific primers for detection of specific β-lactamases genes in *P. aeruginosa* in order to understand the spread of resistance genes in clinical environment.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The Nairobi Hospital is a private tertiary hospital opened on 9th April 1954, as a totally European Hospital, which was the colony of the United Kingdom. On October 1961, it started to serve non-Europeans and the name was transformed to The Nairobi Hospital. The Nairobi Hospital has a capacity of 750 beds and offers quality general medical services, specialized diagnostic services using advanced technology in atmosphere of safety, trust and comfort. It is a tertiary care and receives transfers for specialized medical care from various healthcare Centers in the region. The hospital is in Nairobi Town, Capital city of Kenya on Argwings Kodhek Road in the Neighborhood of Upper Hill along Valley Road; approximately 4.5 km west of Kenyatta International Convention Centre (The Nairobi Hospital, 2017).

The study was carried out at The Nairobi Hospital in the Department of Pathology in Kenya. *P. aeruginosa* isolates were obtained from clinical samples which were tested positive with standard laboratory techniques in the Microbiology Section including Anderson and Upper Hill Medical Center laboratories belonging to The Nairobi Hospital Main Laboratory. A map indicating the location of the Hospital where the samples were obtained and some specialized care centers within the Hospital is shown (Appendix IX).

3.2 Study design

This study is a laboratory based cross-sectional descriptive design.

3.3 Study population

The study involved all positive clinical samples from in-and-out patients of all age groups and both sexes admitted at The Nairobi Hospital with *P. aeruginosa* infections. Additionally, demographic characteristics (age, sex, origin) and specimen

type from laboratory request forms of the patient were also included in the study. The positive clinical samples of patients suffering from different pseudomonal infections were collected during May 2017 to April 2018 for data processing.

3.4 Sample size determination

In Kenya, the MDR resistance due to MBL that has been documented amongst *P. aeruginosa* is 14 % whereas the MDR resistance rates due to ESBL production is scarcely documented (Pitout, *et al.*, 2008). The burden of MDR resistance due to ESBL-producing *P. aeruginosa* is probably large in some African countries (Cholley *et al.*, 2014; Olowo-Okere *et al.*, 2018). The estimated prevalence of this characteristic (p) which is beta-lactamase resistance among *P. aeruginosa* isolates is assumed to be 14 % in Kenya (Pitout *et al.*, 2008). The sample size was determined using the Fisher formula (Fisher *et al*., 1998) as shown below.

$$n = \frac{Z^2_{(1-\alpha)}PQ}{d^2}$$

Where;

n= minimum sample size
Z= Standard normal derivative that corresponds to 95 % confidence interval (1.96)
α = the level of significance (95 %)
P = Prevalence of previous study in Kenya (14 %)
Q= 1-P
$\mathbf{d^2}$= Absolute precision (0.05)
n=185.01

The minimum sample size of positive clinical samples of *P. aeruginosa* isolates required to be screened for β-lactamases production were 185.

3.5 Inclusion and Exclusion criteria

3.5.1 Inclusion criteria

The study included all positive *P. aeruginosa* clinical specimens from in-and-out patients of all age groups and both sexes admitted in a tertiary care Centre, The Nairobi Hospital.

3.5.2 Exclusion criteria

The repeat positive clinical samples of *P. aeruginosa* from the same patients were excluded in the study**.**

3.6 Sampling technique

The sample was obtained by consecutive sampling whereby different clinical samples which were already tested positive with standard laboratory techniques (growth characteristics, gram staining, and pigment production) for *P. aeruginosa* were selected for the study. Within this sampling technique, all positive clinical specimens meeting the criteria of inclusion were recruited until the required sample size was achieved. The isolates from studied positive clinical samples were stored without any patient identifiers but recorded as *P. aeruginosa* with only allocated numbers, date of isolation and type of specimen.

3.7 Methodology of data collection

3.7.1 Collection of demographic data

The research did not involve sampling patients from the hospital directly. However, demographic data for the patients (age, sex, origin) was collected from laboratory request forms. These characteristics of patients with *P. aeruginosa* infections were immediately recorded in the notebook and transferred after in the Microsoft Excel 2013 for further data analyses.

3.7.2 Laboratory Procedures

The following laboratory procedures were used in this study:

3.7.2.1 Isolation of *Pseudomonas aeruginosa* species

The samples were collected under aseptic conditions and immediately transported under appropriate environment to the Department of pathology, Microbiology Laboratory for analyses. The samples were cultured on different media, incubated at 37°C for 18-24 hours and stored at -20°C in the freezer for further application.

The clinical samples (pus and wound swabs, respiratory secretions, blood, body fluids, tissue or biopsy and catheter tips) which tested positive for *P. aeruginosa* were selected for the study and cultured onto MacConkey agar or CLED plates using streak plate method, and incubated aerobically at 42°C for 18-24 hours, in preparation for further analyses. Presumptive identification of organisms was done based on colony morphology, Gram staining, pigment production, and growth at 42°C. The clinical isolates from studied positive samples were subcultured on Nutrient Agar for further analyses. The media were prepared according to manufacturer's instructions (Appendix I). The ability to produce gas and acid, and oxidation of glucose among others were assessed by Automated Microbiology Vitek 2 System (Appendix II).

3.7.2.2 Identification of the isolates by Vitek 2 system

The bacterial isolates from nutrient agar colonies were analysed using Vitek 2 System according to the manufacturer's instructions (BioMérieux, USA). A sufficient number of similar colonies from a pure culture was transferred using a sterile applicator stick; and then the bacterial organism was suspended in 3.0 ml of sterile normal saline (aqueous 0.45 % to 0.50 % NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic test tube. The turbidity of the solution was adjusted consequently and calculated using the DensiCheck, a turbidity meter. A density of 0.5 to 0.63

McFarland solution was calculated for *P. aeruginosa*. Identification cards (ID-GN) with microbial suspensions were inoculated using an integrated vacuum apparatus.

The bacterial suspension contained in a test tube was placed into a cassette and the identification card was positioned in the adjacent slot. However, the transfer tube was introduced into the equivalent suspension tube. The cassette was filled and then positioned either by hand into a vacuum chamber station. After the vacuum was applied and air is re-introduced into the station, the microbial suspension was forced through the transfer tube into micro-channels that filled all the test well. The cassette was engaged in the Filler box on the left side of the Vitek 2 system unit and hit start Fill button on the instrument. Filling the cards was taken approximately 70 seconds for cassette according to the number of cards in the cassette container. The Vitek 2 system instrument will beep when the filling cycle is complete.

The Vitek 2 system was loaded within 10 minutes from the completion of filling the cards in order to start loading the cards to avoid the cards from being rejected. When the cards were ended, the Load Door was automatically unlocked; and then the cassette was positioned in the Load Door. The Vitek 2 system machine will be checking the scanned barcodes against the virtual cassette. Cards were sealed, straws were cut and the cards were automatically loaded into carousel incubator. When all the cards were loaded, the cassette was removed and disposed of the tubes and straws in a biohazard container. The Vitek 2 system automatically proceeded to processing the cards once all the cards were loaded (Abd EL-Salam *et al.*, 2017).

The **Vitek 2 system** accommodate colorimetric reagents cards with 64 wells of biochemical tests (Appendix III) that are incubated and interpreted automatically. Interpretation of test reactions was allowed by a transmittance optical system using different wavelengths in the visible spectrum. During incubation, each test reaction was read every 15 minutes to measure either turbidity or colored products of substrate metabolism (Appendix III). On the Vitek 2 system, test reaction results appear as "+"," –", "(–)" or "(+)". An unknown microorganism was compared to the database of reactions for each taxon (a well-identified *P. aeruginosa* ATCC 27853 in

this case) (Appendix V). The different levels of identification were interpreted according to the manufacturer's instructions where the probability of ≥95 % indicated a well-identified *P. aeruginosa* (Table 3.1) (Pincus, BioMérieux, USA).

Table 3.1: Identification messages and their interpretations

ID Message	Choices	% Probability	Comments
Confidence Level			
Excellence	1	96 to 99	Isolate is reported
Very good	1	93 to 95	Isolate is reported after interpretation
Good	1	89 to 92	Isolate is reported after interpretation
Acceptance	1	85 to 88	Isolate is reported after interpretation
Low Discrimination	2 to 3	Total choices=100. After resolution to one choice, % probability reflects the number linked to the selected choice	2 to 3 taxa show the same biopattern. Separate by supplemental testing.
Unidentified	>3 or 0		Checking a gram stain and purity is a must.

This **table 3.1** indicates that **≥95 %** probability calculation (**Excellence confident level**) performed from a pure culture was interpreted as a well-characterized *P. aeruginosa* isolate. From **85 to 95 %** probability calculation (**Very good, good, acceptance Confident level)**, the result was reported after interpretation based on colonies morphology, pure culture, and clinical signs. **Low discrimination confident level** required a supplemental testing and for **unidentified** organism, to repeat a gram stain and purity checking was a must.

3.7.2.3 Antimicrobial susceptibility testing using Vitek 2 system

This procedure was done according to the Standard Operating Procedures (SOPs) of The Nairobi Hospital Laboratory (Microbiology Section) and manufacturer's instructions (BioMérieux, USA). Isolates on MacConkey Agar (Oxoid, UK) were subcultured on Nutrient Agar plate (Oxoid, UK) and incubated at 35^{o}C overnight. Inoculums were prepared from a pure culture, according to Good Laboratory Practices (GLP). A plate for purity check was recommended for the confirmation of the purity of the culture before testing. The 0.5McFarland bacterial suspension was diluted with 1.5x10^{7}/ml in 0.45 % normal saline and measured with DensiCheck. Aseptically, 3.0 ml of sterile normal saline (0.45 %) to 0.5 % NaCl, pH 4.5 to 7.0) were transferred into a clear plastic (polystyrene) test tube (12 mm x 75 mm). A sterile stick or swab was used to transfer a sufficient number of morphologically similar colonies to the saline tube. In a second tube containing 3.0 ml of saline, 145 µl of the suspension was transferred for AST-GN cards. Then this tube was placed in the cassette with a susceptibility card. All the cards were automatically filled, sealed, and loaded into the Vitek 2 system for incubation and reading.

The AST-GN83 card contained ampicillin (AMP, 10 µg), ampicillin/clavulanic acid (AMC, 20 µg), ampicillin/sulbactam (AMS, 20 µg), piperacillin/tazobactam (PTZ, 110 µg), aztreonam (AZM, 30 µg), cefazolin (CFZ, 30 µg), cefuroxime (CFX, 30 µg), CFX Axetill, cefoxitin (FOX, 30 µg), cefepime (FEP, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), meropenem (MER, 10 µg), gentamicin (CN, 10 µg), amikacin (AK, 30 µg), ciprofloxacin(CIP, 5 µg), nitrofurantoin (F, 300 µg), and trimethoprim/Sulfamethoxazole (SXT, 25 µg). The Minimum Inhibitory concentrations (MICs in µg/ml) of each antimicrobial agent tested for *P. aeruginosa* were generated by the Vitek 2 system and interpreted to resistant (R), intermediate (I) or sensitive (S) according to manufacturer's recommendations (Table 3.2) (BioMérieux, USA). In this case, the isolates were tested to three categorical antimicrobial agents including β-lactams (piperacillin/tazobactam (PTZ, 110 µg), aztreonam (AZT, 30 µg), cefepime (FEP, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), meropenem (MER, 10 µg)

aminoglycosides such as gentamycin (CN, 30 µg), amikacin (AK, 30 µg); and fluoroquinolones such as ciprofloxacin (CIP, 5 µg)). Cefotaxime was tested for monitoring. Other antibiotics listed above in the AST-GN83 card such as AMP, AMC, AMS, CFX, CFX Axetill, Fox, CRO, F, and SXT are not recommended by Clinical Laboratory Standards Institute (Appendix VI) to be tested against *P. aeruginosa* isolates due to their natural resistance to those antibiotics. The various concentrations of different antibiotics listed above (AST-GN83) were not recorded from Vitek 2 manufacturer's instructions (BioMérieux, USA) but from CLSI recommendations (27th Edition).

Table 3.2: MIC Interpretation standard

Categories	**Antibiotics**	**MIC Value (µg/ml)**		
β-lactams		**R**	**I**	**S**
β-lactamase inhibitor	PTZ 110 µg	≥8	4-8	≤4
Cephalosporin 4th Generation	FEP 30 µg	≥8	1-8	≤1
Cephalosporin 3rd Generation	CAZ 30 µg	≥4	1-4	≤1
Monobactams	AZM 30 µg	≥8	2-8	≤2
Carbapenem	MER 10 µg	≥1	0.25-1	≤0.25
Non-β-lactams		**R**	**I**	**S**
Aminoglycosides	CN 10 µg	≥2	1-2	≤1
	AK 30 µg	≥4	2-4	≤2
Fluoroquinolones	CIP 5 µg	≥1	0.25-1	≤0.25

Key: µg: microgram,

β-lactamase inhibitor: β-lactam β-lactamase inhibitor combinations

I: MIC µg/ml of each antibiotic interpreted to Intermediate isolate

R: MIC µg/ml of each antibiotic interpreted to Resistant for resistant isolate

S: MIC µg/ml of each antibiotic interpreted to Sensitive for susceptible isolate

3.7.2.4 Detection of Extended Spectrum β-lactamases

The Vitek 2 system for ESBL test is a new tool for rapid detection of ESBL production which is based on simultaneous assessment of the inhibitory effects of Cefepime, cefotaxime and Ceftazidime, alone and in the presence of Clavulanic acid

according to manufacturer's instructions (Spanu *et al.*, 2006). In this case, each isolate was tested using the Vitek 2 System with the ESBL test panel (Jamal *et al.*, 2005). The cards were inserted in the respective test tubes and loaded into the Vitek 2 system. While in the Vitek 2 System, the cards were filled, sealed and incubated in the Vitek 2 System incubator until results were interpreted by the Advanced Expert System of the Vitek 2 system and generated for the ESBL phenotype. An optical scanner determine quantitatively the growth in each well; and the proportional reduction in growth in wells comprising Cephalosporin plus Clavulanic acid associated with those comprising the Cephalosporin alone were interpreted as indicative of ESBL production. The quality Controls strains used for detection of ESBLs were *Escherichia coli* ATCC 25922, *P.aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603.

3.7.2.5 Screening of Metallo-β-lactames Carbapenemases

This procedure was done according to the Standard Operating Procedures (SOPs) of The Nairobi Hospital Laboratory (Microbiology Section) and manufacturer's instructions (BioMérieux, USA). The colonies from an overnight nutrient agar plate culture of each isolate were suspended in 3 ml of 0.45 % normal saline and adjusted to a turbidity of 0.5-0.63 McFarland standard with Vitek DensiCheck (BioMérieux, USA), using AST-GN83 cards comprising meropenem in the same time with antimicrobial susceptibility testing. Then, the cards were automatically filled, sealed, and loaded into the Vitek 2 system for incubation and reading (Sun *et al.*, 2015). The MIC correlates are reported and the advanced expert system interprets the results. Meropenem-resistant *P. aeruginosa* isolates were analysed by the expert system as carbapenem resistance mechanism for production of Carbapenemase. The results were finally interpreted by the Advanced Expert System (AES) for MBL phenotype. But the Vitek 2 System does not identify accurately MBL-producing strains. Alternative test methods such as targeted PCR amplification were required to confirm these MBL isolates (Sun *et al.*, 2015). The resistance to MER was shown by Vitek 2 system in *P. aeruginosa* isolates (Table 3.3).

Table 3.3: Clinical Laboratory Standard Institute breakpoints shifts

Micro-organism	Meropenem (µg/ml)
Pseudomonas (**S**/**R**)	≤2/≥8

S: Sensitive (MIC ≤ 2) **R**: Resistance (MIC ≥ 8)

P. aeruginosa isolates were stocked in 1.5 ml capacity vials containing Brain Heart Infusion broth (BHI, Himedia, India) with 30 % glycerol for preservation and stored at -20°C after identification of the isolates, antimicrobial susceptibility testing and phenotypical investigation of ESBL and MBL carbapenemases.

3.7.2.6 DNA extraction

3.7.2.6.1 Revival of the stored isolates

Frozen stocks of the isolates were removed from the freezer at -20°C. The isolates were cultured on MacConkey agar (Oxoid Lt, UK) by carefully spreading over the agar plates. The agar was then incubated at 37°C overnight and the frozen stock was returned to the freezer. The next day, a sterile wire loop was used to inoculate bacterial cells from about one colony on Nutrient Agar or Muller Hinton Agar (Oxoid Lt, UK) in order to get pure culture. Three to five colonies for DNA extraction were picked using sterile applicator stick.

3.7.2.6.2 DNA extraction by Mechanical Method

From ≤$5x10^6$ bacterial cells; Quick-DNA Mini-Prep plus Kit (Zymo Research, USA) was used in order to isolate total DNA. A total of 1,060 µl proteinase K storage Buffer was added to each tube of proteinase K (20 mg) prior to use. The final concentration of proteinase K (mixture) ~20 mg/ml was stored at -20°C. The cultured cells or bacterial pellets (1-$5x10^6$ cells) were re-suspended using sterile applicator or stick in 200 µl DNA Elution Buffer. To a solution of 200 µl sample was added 200 µl of BioFluid and Cell Buffer with 20 µl proteinase K for lysing the bacterial cell. The bacterial cells mixture was vortexed thoroughly 10-15 seconds and then

incubated at 55°C for 10 minutes. A volume of 420 µl Genomic Binding Buffer was added to the digested sample. The mixture was transferred to a Zymo-Spin TM IIC-XL column, and centrifuged at ≥12,000xg for 1 minute. A volume of 400 µl DNA pre-wash Buffer was added to the spin column in a new collection tube and then centrifuged at ≥12,000xg for 1 minute as recommended. After, the volume of 700 µl and 200 µl-DNA Wash buffer was added to the spin column and centrifuged each at ≥12,000xg for 1 minute respectively.

Lastly, the collection tube was removed from the spin column and transferred to a clean microcentrifuge tube and approximately 50 µl DNA Elution Buffer was directly added onto the matrix. It was then incubated for 5 minutes at room temperature and centrifuged at maximum speed for 1 minute to elute the DNA required. The eluted DNA was kept at -20°C for further application. The concentrations of DNA from 2 µl of sample were determined using NanoDrop Spectrophotometer at 260 nm. The purity of the DNA was checked by measuring the absorbance from 230 nm to 320 nm in order to detect other possible contaminants. Good quality DNA was found by dividing the ratio of absorbance at 260 nm by the reading at 280 nm (A260/A280). The calculated ratio between 1.7 and 2.0 was indicative of pure DNA. Finally, the purity was confirmed by running DNA on gel in order to observe the presence of bands.

3.7.2.7 Detection of genes-encoding Metallo-β-lactames by PCR assays

The DNA extract was used as template in the specific PCR amplifications for detection of MBL genes according to manufacturer's instructions (BioLabs, UK). The isolates resistant to meropenem and MBL carbapenemases producers were subjected to PCR assays for detection of MBLs genes; $\boldsymbol{bla_{IMP-1}}$, $\boldsymbol{bla_{IMP-2}}$, $\boldsymbol{bla_{VIM-1}}$, $\boldsymbol{bla_{VIM-2}}$, and $\boldsymbol{bla_{NDM-1}}$ using four pair primers experienced by Shibata *et al*.(2003) and one pair primer bla_{NDM-1} used by Mushi *et al.*(2014).

A total volume of 50 µl master mix primer reaction was diluted as follows: (PCR mix, one tube) starting with 18 µl of Nuclease free water followed by Forward-

primer (20 pmol/µl) 1 µl and Reverse-primer (20 pmol/µl) 1 µl then Taq 2x master mix (Thermo) 25.0 µl and lastly the DNA template 5.0 µl was added. The PCR was performed with 5 µl DNA from the sample and master mix comprising Taq 2x master mix (BioLabs, UK) with standard buffer (Taq polymerase 50 U/ml, dNTPs 600 µM, $MgCl_2$ 6 mM, KCl 100 mM, TrisCl PH 8.6 2 0 mM, Glycerol 10 %, Trehalose 200 mM, BSA 0.4 mg/ml, and Detergents 0.26 %), Nuclease-free water, and five pairs primers as listed in the Table 3.4 (Peymani *et al*., 2017).

The PCR conditions were as follows: 5 minutes at 94°C and 35 cycles of 1 minute at 94°C for the denaturation of the template, annealing temperatures were primer-specific (Table 3.4) for 45 seconds and short extension times were fragment-size dependent (Table 3.4) at 72°C before a final extension of 7 minutes at 72°C for Bla_{IMP-1}, Bla_{IMP-2} and Bla_{NDM-1}. The final extension times were 5 minutes for bla_{VIM-1} and 10 minutes for bla_{VIM-2} (Mushi *et al.*, 2014; Shibata *et al.*, 2003).

Table 3.4: Primers sequences used for detection of MBL genes

Genes	Oligonucleotides sequences (5'-3')	E. T (sec)	A.T (°C)	Product size
bla_{VIM-1}	F: AGT GGT GAG TAT CCG ACA G	30	55	261
	R: ATG AAA GTG CGT GGA GAC			
bla_{VIM-2}	F: ATG TTC AAA CTT TTG AGT AAG	60	51	801
	R: CTA CTC AAC GAC TGA GCG			
bla_{IMP-1}	F: ACC GCA GCA GAG TCT TTG CC	45	51	587
	R: ACA ACC AGT TTT GCC TTA CC			
bla_{IMP-2}	F: GTT TTA TGT GTA TGC TTC C	45	51	678
	R: CGG AAT GGC TCA TCA CGA TC			
bla_{NDM-1}	F:GGT TTG GCG ATC TGG TTT TC	45	54	621
	R: CGG AAT GGC TCA TCA CGA TC			

A.T (°C): Annealing temperatures in **degree Celsius** were specific for each primer used for detection of Metallo-β-lactames genes

E.T (sec): Short extension time in **seconds** is Fragment-size dependent (depending on the product size of a complementary copy strand of DNA)

3.7.2.8 Agarose Gel Electrophoresis

Gel electrophoresis was done according to manufacturer's instructions (AMRESCO, USA). Agarose gel (2 % w/v) was prepared by suspending 2.0 grams of agarose powder (AMRESCO, USA) in 100 ml of Tris acetate EDTA (TAE) buffer (40 mM Tris acetate, 0.05 M EDTA, PH 8.2-8.4). The agarose was dissolved by bringing the suspension to maintained homogeneity of the solution. The agarose solution was boiled at high temperature and then cooled to 55^{o}C in a water bath maintained at this temperature. Five microliters of E-Z vision, in gel solution staining (AMRESCO, USA) were added into the agarose solution. The cooled agarose solution was placed into a plastic electrophoresis tray in a horizontal position, and then placed in the proper position in the electrophoresis tank. The electrophoresis buffer (TAE buffer 0.5 %) was poured into the tank until the surface of the gel was covered and the comb was positioned into the agarose solution. The samples of DNA were prepared for electrophoresis by mixing 5 µl of DNA with 5 µl of loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 30 % w/v glycerol in water) and then loaded into the wells. Each gel run included a molecular weight DNA marker from the *Escherichia coli* which was loaded into the first well of the gel. The gel electrophoresis for the PCR products included the Quick-load™ 2-log DNA Ladder 100 bp (BioLabs, UK) that had a well-known length of fragments ranging from 0.1-1.5 kb. Electrophoresis was done at 110 volts for 30 minutes. Separated DNA was visualised by placing the gel on an ultraviolet trans-illuminator, and photographed using gel imaging system. The length of the DNA fragments from samples was determined using a DNA marker containing fragments of known length (Alshalah *et al.* , 2017). All reagents used for molecular level were prepared according to manufacturer's instructions (Appendix III).

3.8 Data management and analysis

Laboratory results were recorded in the laboratory note book. All demographic data and laboratory results were transferred to Microsoft office Excel 2013 linked software and saved in the hard disk drive and flash disks. The notebook was kept securely in a lockable cabinet and a protected password was used for the data in the

computer hard disk drive. Data presentation was done by use of graphs and tables. Analysis of the collected data was done by use of Statistical Package for Social Sciences (SPSS) software version 21.0. Chi-square (X^2) or Fisher's exact where applicable were used for the test of association between β-lactamases production and antibiotic resistance or demographic characteristics ($\alpha=0.05$, $p\leq0.05$). Therefore, efficient detection of β-lactamases genes and intervention to control β-lactams resistance were recommended to prevent the dissemination of resistant strains.

3.9 Ethical approval

Institutional approval to carry out this study was sought from JKUAT. Ethical clearance was approved by Bioethics and Research Committee of The Nairobi Hospital (Appendix VIII). The investigation did not involve sampling patients from the hospital directly. The bacterial isolates were collected from positive clinical samples of The Nairobi Hospital Pathology Department. The demographic data and specimen type of patients were collected from laboratory request forms. The isolates from studied positive samples were stored without any patient identifiers but recorded as *P. aeruginosa* with only allocated numbers, date of isolation and type of specimen.

3.10 Expected outcomes

It was first expected to determine demographic characteristics for patients infected with *P. aeruginosa*, antimicrobial susceptibility profiles of *P. aeruginosa* isolates and the proportion of β-lactamases genes among β-lactams-resistant *P. aeruginosa* strains isolated from clinical specimens at The Nairobi Hospital. Second, I was expected to publish in a peer-reviewed journal (Appendix VII) and get an Award of a MSc. degree.

CHAPTER FOUR

RESULTS

4.1 Identification of *P. aeruginosa* isolates from positive clinical samples

The colonies of *P. aeruginosa* isolates on MacConkey agar (MCA) were colorless and non-lactose fermenters with a characteristic "grape-like" or the colonies were flat with a characteristic of fruity smell. Two pigments; pyocyanin which is blue green in color and pyoverdine which is yellow green in color, were expressed on agar plates (Figure.4.1)

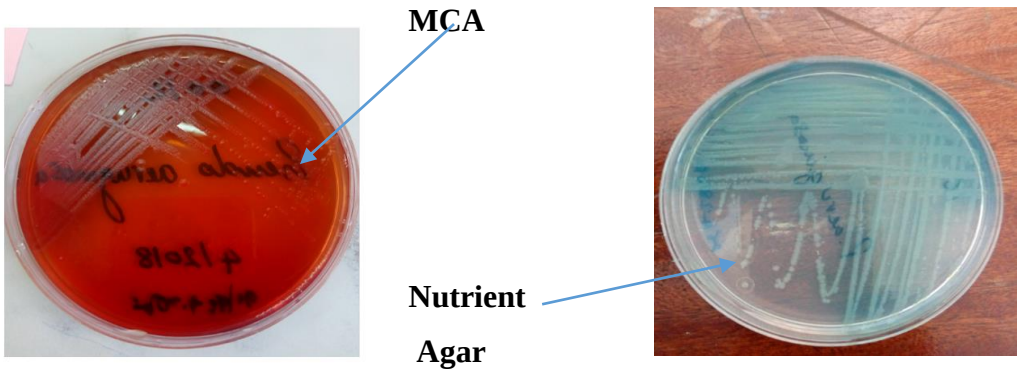

Figure 4.1: Colonies of *P. aeruginosa* isolates on MacConkey and Nutrient agar

A total of 185 isolates from positive clinical samples presumptively identified as *P. aeruginosa* based on their growth characteristics on agar plates were subcultured on Nutrient Agar and confirmed using Vitek 2 System. Among *P. aeruginosa* isolates, 73 isolates (39.5 %) were obtained from pus and wound swabs, 48 (25.9 %) from sputum and tracheal aspirates, 35 (18.9 %) from urine, 11 (5.9 %) from catheter tips, 9 (4.9 %) from body fluids, 6 (3.2 %) from tissue or biopsy, 3 (1.6 %) from blood.

4.2. Demographic characteristics of patients with *P. aeruginosa* infections

The prevalence of the bacterial isolates was higher among the male (61.1 %) than female (38.9 %) patients. The high rate of *P. aeruginosa* prevalence was observed among the age groups above 45 years (64.3 %) including age group 46-60 (20.0 %), age group 61-75 (21.1 %), age group 76-90 (20.5 %) and age group >90 (2.7 %) with

the mean age of patients of 56.1±21.6 years old, ranging from 4 to 100 years old (Table 4.1).

Table 4.1: Age and gender distribution of patients infected with *P. aeruginosa*

Age/Year	**Number of isolates**	**%**
Age group <15	4	2.2
Age group 15-30	23	12.4
Age group 31-45	39	21.1
Age group 46-60	37	20.0
Age group 61-75	39	21.1
Age group 76-90	38	20.5
Age group >90	5	2.7
Gender	**Number of isolates**	**%**
Female	72	38.9
Male	113	61.1
Total	**185**	**100.0**

Out of 185 participants who showed the growth of *P. aeruginosa* isolates, 137 (74.1 %) were inpatients and 48 (25.9 %) were outpatients (Table 4.2).

Table 4.2: *P. aeruginosa* isolates distribution by the origin of the patient

Type of patient	**Number of isolates**	**%**
Outpatient	48	25.9
Inpatient	137	74.1
Total	**185**	**100.0**

4.3. Antimicrobial susceptibility profiles of *P. aeruginosa* isolate

The susceptibilities to piperacillin/tazobactam (55.1 %), cefepime (67.6 %), ceftazidime (68.1 %), meropenem (65.9 %), gentamycin (67.6 %), amikacin (68.1 %), ciprofloxacin (68.1 %), and aztreonam (57.8 %) are shown (Table 4.3). The resistant rates of β-lactam antibiotics including cefepime (28.1%), ceftazidime (28.1 %), meropenem (31.4 %), were lower than those of non-β-lactam antibiotics,

including amikacin (31.9 %), gentamycin (31.9 %), and ciprofloxacin (31.4 %) with exception to aztreonam (40.0 %) and piperacillin/tazobactam (37.3 %) (Table 4.3).

Table 4.3: Antimicrobial susceptibility profiles of *P. aeruginosa* isolates

Sensitive	(%)	Intermediate	(%)	Resistant	(%)	Antibiotics
102	55.1	14	7.6	69	37.3	PTZ 110 µg
125	67.6	8	4.3	52	28.1	FEP 30 µg
126	68.1	7	3.8	52	28.1	CAZ 30 µg
122	65.9	5	2.7	58	31.4	MER 10 µg
125	67.6	1	0.5	59	31.9	CN 10 µg
126	68.1	0	0	59	31.9	AK 30 µg
126	68.1	1	0.5	58	31.4	CIP 5 µg
107	57.8	4	2.2	74	40.0	AZM 30 µg

Key: **µg**: microgram, **(%)**: percentage

PTZ 110µg-piperacillin/tazobactam, FEP 30 µg-cefepime, CAZ 30 µg-ceftazidime, MER 10 µg-meropenem, CN 10 µg-gentamycin, AK 30 µg-amikacin, CIP 5 µg-ciprofloxacin, AZM 30 µg-aztreonam.

4.4 Detection of β-lactamases producers among the isolates

Of the one hundred and eighty five (185) *P. aeruginosa* isolates, forty two (42-22.7%) exhibited MBL Carbapenemases enzymes (Figure 4.2) but phenotypical identification of ESBLs enzymes did not reveal a positive result in *P. aeruginosa* isolates obtained from clinical specimens in this hospital.

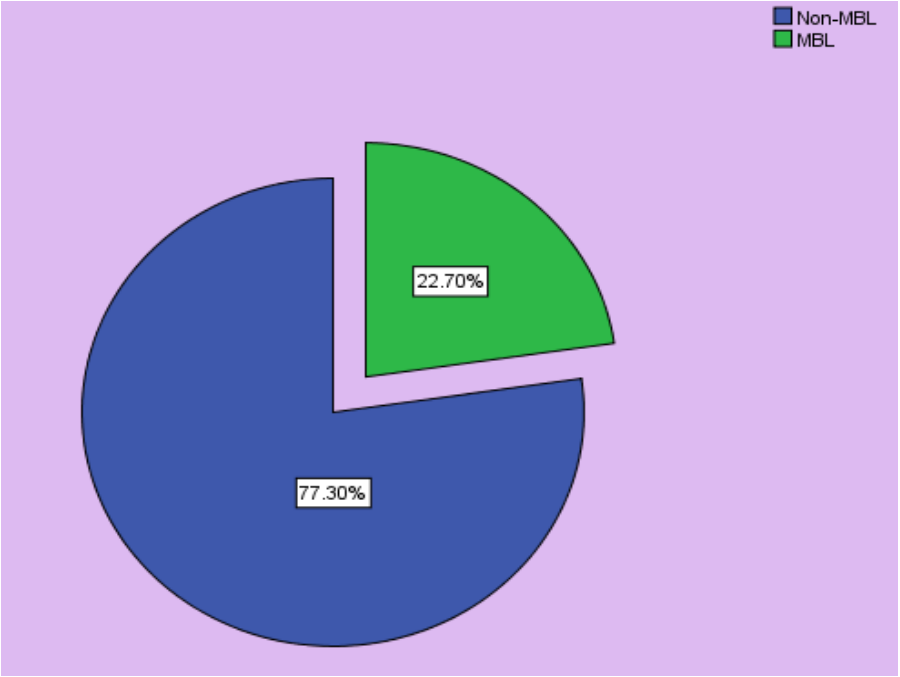

Figure 4.2: Metallo-Beta-Lactamases profile among the isolates

Key: %: percentage, **Non-MBL:** Metallo-β-lactamases negative isolates (77.3 %), **MBL:** Metallo-β-lactamases positive isolates (22.7 %)

The antimicrobial susceptibility profile of MBL producers showed that all the MBL isolates were resistant to the 6 classes of antibiotics tested (β-lactam/β-lactam β-lactamase inhibitor combinations, monobactams, cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones).

Overall the prevalence of MBL producing *P. aeruginosa* was 22.7 % as compared to non-MBL isolates (77.3 %). The MBL producers were resistant to tested antibiotics (piperacillin/tazobactam, aztreonam, ceftazidime, cefepime, meropenem, gentamycin, amikacin and ciprofloxacin). The difference in resistance levels between MBL and non-MBL producing isolates for all the antimicrobial drugs tested were statistically significant ($p \leq 0.05$; $p < 0.001$) (Table 4.4).

Table 4.4: Comparison of resistance patterns of MBL and non-MBL isolates

	MBL N=42 (22.7 %)	Non-MBL N=143 (77.3 %)	
Antimicrobial drugs	**Resistant (%)**	**Resistant (%)**	**P-Value**
PTZ (100/10) µg	42 (100)	27 (18.9)	P<0.001
FEP 30 µg	42 (100)	10 (7.0)	P<0.001
CAZ 30 µg	42 (100)	10 (7.0)	P<0.001
MER 10 µg	42 (100)	16 (11.2)	P<0.001
CN 10 µg	42 (100)	17 (11.9)	P<0.001
AK 30 µg	42 (100)	17 (11.9)	P<0.001
CIP 5 µg	42 (100)	16 (11.2)	P<0.001
AZM 30 µg	42 (100)	32 (22.4)	P<0.001

Key: - **µg**: microgram, **%**: percentage; Resistant: proportion of isolates resistant to each antibiotic in MBL producers and non-MBL producers, **p<0.001**: significance level ($p \leq 0.05$).

PTZ 110 µg-piperacillin/tazobactam, FEP 30 µg-cefepime, CAZ 30 µg-ceftazidime, MER 10 µg-meropenem, CN 10 µg-gentamycin, AK 30 µg -amikacin, CIP 5 µg-ciprofloxacin, AZM 30 µg-aztreonam.

4.5 Distribution of MBL isolates based on demographic characteristics

Based on demographic characteristics of patients with *P. aeruginosa* infections, there was no statistical significance ($p \leq 0.05$; $p=0.200$) in terms of MBL production among the two age groups compared to MBL negative isolates; in which 31 (25.8 %) were MBL producers in patients above 45 years old while 11 (16.9 %) were MBL producers in patients below 45 years old. Out of 42 patients from whom MBL producers were isolated, 32 (28.3 %) were males and 10 (13.9 %) were females which was statistically significant ($p \leq 0.05$; $p=0.035$) when compared to gender-distribution of patients from whom MBL negative strains were isolated (Table 4.5).

Table 4.5: MBL profile among the age groups and gender of patients

Variable	Non-MBL (%)	MBL (%)	P-Value
Age/Year			
Age groups (≤45)	54 (83.1)	11 (16.9)	
Age groups (>45)	89 (74.2)	31 (25.8)	P=0.200
Gender			
Female	62 (86.1)	10 (13.9)	P=0.035
Male	81 (71.7)	32 (28.3)	

Out of 42 patients from whom MBL producers were isolated; 34 (16.7 %) were outpatients and 8 (24.8 %) were inpatients which was not statistically significant ($p \leq 0.05$; p=0.337) when compared to the type-distribution of patients from whom MBL negative strains were isolated (Table 4.6).

Table 4.6: MBL profile among the type of the patient

Type of the patient	Non-MBL	MBL	P-value
Outpatient	40 (83.30)	8 (16.70)	
Inpatient	103 (75.20)	34 (24.80)	P=0.337

Based on the origin and specimen of the patients, the high prevalence of cases of MBL-producing *P. aeruginosa* isolates was obtained from Intensive Care Unit (45.2 %) and High Dependency Unit (28.6 %) with predominant distribution in pus and wound swab (38.1 %), and respiratory secretions (33.3 %). A total of four MBL-producing isolates (9.5%) was recovered from the outpatient department (OPD) (Table 4.7).

Table 4.7: Distribution of MBL isolates by type of specimen and origin of patients

Type of Specimen	**Number of MBL isolates**	**%**
Pus and wound swab	16	38.1
Sputum and aspirates	14	33.3
Urine	7	16.7
Body fluid	2	4.8
Catheter tip	1	2.4
Biopsy	2	4.8
Origin of the patient	**Number of MBL isolates**	**%**
HDU	12	28.6
ICU	19	45.2
OPD	4	9.5
STL	3	7.1
Others	4	9.5
Total	**42**	**100.0**

ICU: Intensive Care Unit (Critical care Nursing Ward); **HDU**: High dependency Unit (Critical Care Nursing Ward) **OPD**: Outpatient Department; **STL**: St Lukes Ward

4.5 Detection of genes encoding-MBL among the isolates

The PCR assays for the detection of Metallo-β-lactamases genes showed the presence of positive MBL isolates for Bla_{VIM-2} (28.57%) and Bla_{NDM-1} (66.67%). From the 42 MBL isolates, three MBL isolates (7.14%) harbored both Bla_{NDM-1} and Bla_{VIM-2} genes (Figure 4.3, Figure 4.4, and Figure 4.5).

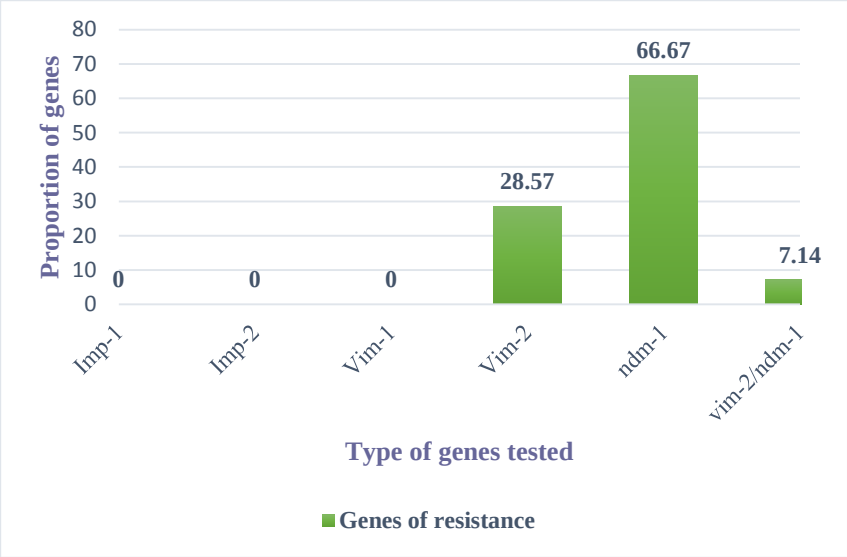

Figure 4.3: Distribution of Metallo-Beta-Lactamases genes among the isolates

Imp: ***bla$_{IMP}$*** genes Vim: ***bla$_{VIM}$*** genes ndm: ***bla$_{NDM}$*** genes

Twelve *bla$_{VIM-2}$* genes (28.57 %) and twenty eight *bla$_{NDM-1}$* genes (66.67 %) were present. Three MBL isolates (7.14 %) harbored both *bla$_{VIM-2}$* and *bla$_{NDM-1}$* genes. None of the *bla$_{IMP-1,}$* *bla$_{IMP-2}$* and *bla$_{VIM-1}$* genes was detected.

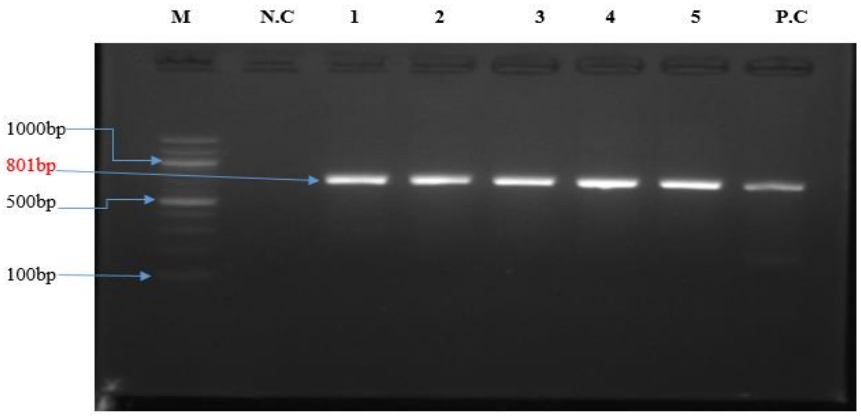

Figure 4.4: Agarose gel electrophoresis of *bla$_{VIM-2}$* genes

Lanes 1, 2, 3, 4 and 5 show isolates positive for *Bla$_{VIM-2}$* (801bp). M shows 100 DNA Ladder; ranging from 100 to 1500 bp. Lane P.C shows the positive control (Well-known sample of *P. aeruginosa* as quality control strain). N.C is the Negative Control (*P. aeruginosa* ATCC 27853 as quality control strain).

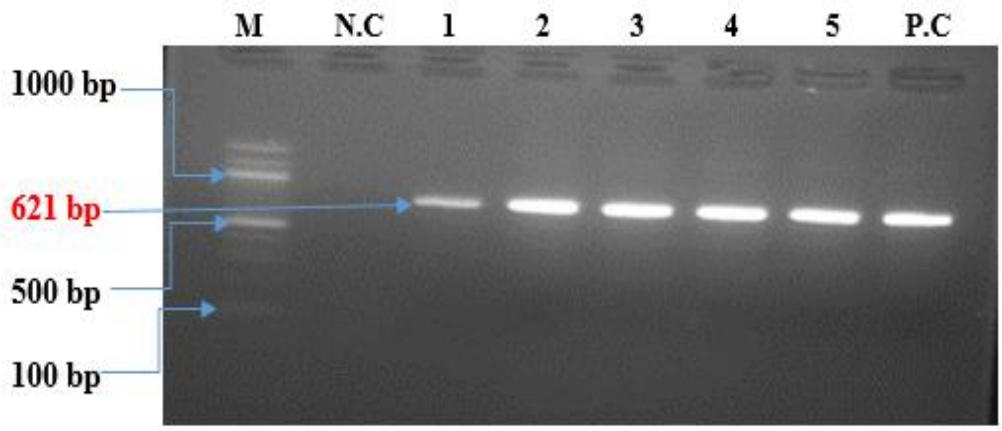

Figure 4.5: Agarose gel electrophoresis of *bla*$_{NDM-1}$ genes

Lanes 1, 2, 3, 4 and 5 show isolates positive for *Bla*$_{NDM-1}$ (621bp). M shows 100 bp DNA Ladder; ranging from 100 to 1500 bp. Lane P.C shows the positive control (*Klebsiella pneumoniae* ATCC BAA-2472 as quality control strain). N.C is the Negative Control (*P. aeruginosa* ATCC 27853).

CHAPTER FIVE

DISCUSSION

This is one of the few studies that has evaluated patients' demographic characteristics, antimicrobial susceptibility profiles and molecular characterization of β-lactams resistance in *P. aeruginosa* isolates obtained from clinical sources in Kenya.

5.1 Demographic characteristics of patients with pseudomonal infections

A total of 185 *P. aeruginosa* isolates were collected from clinical specimens during May 2017 to April 2018. Based on gender and age group distribution, *P. aeruginosa* isolates were more prevalent in males (61.1 %) than females (38.9 %) with a high mean age of 56.1±21.6 years old. In Kenya, a recent study conducted at Tigoni Hospital showed that Wounds from females were more likely to be colonized by *P. aeruginosa* at 67 % compared to those of males at 33 % (Gachuki *et al.*, 2019). These findings are comparable with those from different studies conducted by Mahmoud *et al*. (2013) and Shirani *et al.* (2016) which showed that *P. aeruginosa* infections were common in males than females respectively from patients hospitalized with secondary immunodeficiency in Iran and at a University Hospital in Egypt.

The Egyptian study by Zafer *et al.* (2014) reported the highest rate of *P. aeruginosa* isolated from hospitalized patients in males with a low mean age of 24.5 years and highest number of cases in younger patients (54 %). In Iraq, a study conducted by Al-Zaida (2016) showed a high occurrence of the bacterium within the male population (55.6 %) and younger patients (38.9 %). The preponderance of males could be explained by greater number of cases from hospital wards including critical care nursing ward which had more admissions of male patients during the time of study. Futher research is necessary to clarify the gender differences in the prevalence of healthcare-associated infections caused by *P. aeruginosa.* These age groups (>45 years old) could be probably due to decreasing immunity in the older patients due to a hiher underlying disease prevalence in this population (Wang *et al.*, 2019).

The study reported the highest rate of isolation of *P. aeruginosa* in indoor patients (74.1 %) than outdoor patients (25.9 %). The predominance of *P. aeruginosa* isolates in inpatients (83.7 %) than in outpatients (16.3 %) has been reported from a tertiary care hospital in India (Sandhya Rani and Swathi, 2014). These findings were expected because *P. aeruginosa* is most commonly associated with hospital-acquired infections compared to the community-acquired infections (Farajzadeh *et al.,* 2014; Jayarani *et al.*, 2014).

5.2 Antimicrobial Susceptibility profiles of *P. aeruginosa* isolates

P. aeruginosa isolates demonstrated different rates of resistance to antibiotic tested in piperacillin/tazobactam (37.3 %), gentamycin (31.9 %), amikacin (31.9 %), ciprofloxacin (31.4 %), meropenem (31.4 %), cefepime (28.1 %), ceftazidime (28.1 %) and aztreonam (40 %). In kenya, the resistance towards CAZ, FEP, CIP, and MER was 37 % from selected hospitals in Mombasa County while the rates of resistance to AK and PTZ were 32 % and 95 % respectively (Mwinyikombo, 2018). In the current study conducted at Tigoni Hospital in kenya, the antimicrobial resistance towards CAZ, FEP, MER, CN and AK were 64 %, 52 %, 40 %, 45 %, and 40 % respectively while PTZ and CIP were the most effective antimicrobial agents with a resistance prevalence rate of 20 % and 25 % in *P. aeruginosa* isolates from wound infections of outpatient population respectively (Gachuki *et al.,* 2019). In South Africa, the resistance to ATZ, MER, CAZ, PTZ , and AK was higher than 75 % in the isolates from a private hospital in Durban with exception to CIP (29 %) (Adjei *et al.,* 2018). In India, a different study reported similar results for some antibiotics where the resistant rates of the isolates from wound infections were reported as 38.0 % for CN, 31.69 % for CAZ, and 33.03 % for MER (Bangera *et al.*, 2016). In a different study conducted also in India, the resistance rates to AK, CIP, CAZ, FEP, and MER were higher than 90 % while the rates of resistance to PTZ and AZM were higher than 70 % (Radan *et al.*, 2016). The difference in resistance level could be attributed to the factors that cause antibiotic resistance through mutational processes and resistance genes transfer; including over-prescription and misuse of antibiotics, poor infection control in heathcare settings. Resistance of *P. aeruginosa*

to antibiotics could also be due to the production of enzymes that inactivate and degrade antibiotics, reducing the membrane permeability and the multidrug resistance efflux system (Mahmoud *et al.*, 2013; Radan *et al.*, 2016).

The research revealed a susceptibility rate of 67.6 % for FEP, and 68.1 % for CAZ. In kenya, a low susceptibility towards CAZ (36 %) and FEP (48 %) has been recorded in a recent study conducted at Rural Hospital in Outdoor patients with wound infections (Gachuki *et al*., 2019). In Egypt, cephalosporins tested in a different study indicated that 90 % of the isolates were resistant to FEP (91 %) and CAZ (98 %) (Mahmoud *et al.*, 2013). This difference could be due to the absence of ESBLs production in this study which mediate resistance against cephalosporins.

Among *P. aeruginosa* isolates, the resistant rate to meropenem was 31.4 %. In a Kenyan study conducted at Tigoni Hospital, the resistance to meropenem was 40 % in *P. aeruginosa* isolates from wound specimens (Gachuki *et al*., 2019). In the Iranian study of Ghasemian *et al.* (2018), the resistance towards meropenem was 55.5 % among the isolates. In general, the observed Carbapenem-resistance of *P. aeruginosa* (CRPA) ratio varied between 10 and 50 %; and increasing prevalence of CRPA has been reported in different studies (Dogonchi, *et al.*, 2018; Hong *et al.*, 2015; Mehdi *et al*., 2015, Meradji *et al*., 2016). This increase can be linked to a wide use of carbapenems and the application of invasive and aggressive devices, which prompt the selection of resistant organisms. These conditions are associated with the characteristics of patients and clinical practice policies or antibiotic prescription patterns of health care facilities in each hospital. Various risk factors of carbapenems resistance have been reported; such as previous exposure to intensive care units, hospitalization for >48 hours, previous exposure to antibiotics (cephalosporins third generation, carbapenems, fluoroquinolones), exposure to immunosuppressants, previous infection by a multidrug-resistant organism and presence of indwelling devices (Malande, 2018).

5.3 Distribution of β-Lactamases producers among the isolates

The rate of MBL producing *P. aeruginosa* was 22.7 % as compared to the study of other researchers in which MBL producing *P. aeruginosa* were 14 % in a tertiary care hospital in Kenya and 17 % in Egypt (Abaza *et al.*, 2017; Pitout *et al.*, 2008). Alisha *et al.* (2015) and Ameen *et al.* (2015) demonstrated high rates of MBL positive isolates; 48.3 % and 49.5 % among *P. aeruginosa* isolates respectively. Different studies have reported a higher rate of MBL production among *P. aeruginosa* isolates, for example, 72 % in Tehran (Iran), 84.5 % in Brazil and 68.6 % in Nepal (Acharya *et al.*, 2017; Fazeli *et al.*, 2008; Kalluf *et al.*, 2017). The discrepancy between the rate of MBL isolates and other reported results can be associated with the spread of MBL positive isolates in different regions of the world. Local and international travel to different regions and in conjunction with patient transfer between healthcare centers from different countries create the possibility for further spread of MBL strains. This makes a considerable geographic difference in the distribution of MBL enzymes in different countries.

The study showed also that the resistance rate of MBL isolates was 100 % to antibiotics tested. In Kenya, a recent study conducted at Kenyatta National Hospital reported a high resistance rate of MBL isolates from hospitalized patients to AZM (89.7 %), CAZ (82.4 %), MER (100 %), and CN (88.2 %) while the lowest resistance was recorded for AK (46.3 %) (Kilivwa *et al.*, 2018). Other researchers showed that MBL-producing isolates were resistant to the antibiotics tested in different studies (Liew *et al.*, 2018; Moosavian & Mohammad , 2015; Shamaeva *et al.*, 2015). The high prevalence of resistance can be associated with the presence of MBL carbapenemases which mediate resistance to all β-lactams except monobactams; and other non-investigated mechanisms of the resistant strains such as impaired penetration of drug to the target PBP and the presence of efflux pumps in this study. Therefore, these MBL isolates may carry other genes of resistance to other classes of antibiotics including quinolones and aminoglycosides. But these facts are not in line with our findings because the difference in terms of antibiotic resistance between

MBL and non-MBL producers for all tested drugs (β-lactams, fluoroquinolones and aminoglycosides) were statistically significant, ($p<0.001$).

In terms of MBL production among the gender, there was a statistical significant association ($p=0.035$) when MBL producers and non-MBL producers were compared. A different study showed a high occurrence of MBL producers in male patients (>90 %) (Tsakris *et al.*, 2009). There was no statistical significant association ($p=0.200$) between MBL and non-MBL producers among the studied age groups. In Egypt, Zafer *et al.* (2014) reported a predominant distribution of *P. aeruginosa* infections in younger aged group (54 %) with a statistical significance among MBL positive and MBL negative isolates. Indoor patients were more prevalent with no statistical significant association between MBL positive isolates and MBL negative isolates ($p=0.337$). In India, Sandhya Rani & Swathi (2014) showed that *P. aeruginosa* isolates were more prevalent in indoor patients than outdoor patients with wound infections. These findings can be attributed to the duration of the hospital stay (≥5 days) which was directly proportional to a high prevalence of the infection since the rate of isolation of the organism was higher in inpatients than outpatients (Sandhya Rani & Swathi, 2014**).** The risk of infection due to resistant strains could likewise increase over the duration of hospital stay (1.4 % increased risk per week of hospitalisation) (Daneman *et al.*, 2012).

The highest rate of MBL isolates was obtained from ICU (45.2 %) and HDU (28.6 %) with predominant distribution in pus swab (38.1 %) and sputum (33.3 %). In the indian study conducted at a tertiary care hospital, the high level of MBL producers was reported from ICU with predominant distribution of MBL isolates in tracheal aspirates and wound swab samples (Subramaniyan & Jeya, 2018). These findings could be due to prolonged hospitalisation (more than 5 days) of patients with high risk of acquiring nosocomial infections (underlying disease, permanent urinary catheter and respiratory equipment including ventilators). The increase in incidence of infection associated with the use of invasive procedures could prompt the selection of resistant strains and contribute to the high occurrence of this resistant phenotype in high risk groups (those receiving prior antibiotics, exposed to intensive care units,

or suffering from respiratory tract infections) (Daneman *et al.*, 2012). The predominance of pus and sputum specimens showed that wounds and respitory system were the most frequent sites of infection.

Phenotypical investigation of ESBLs did not reveal a positive result. Recent studies conducted in Kenya have reported the existence of ESBLs from hospitals (Gachuki *et al.*, 2019; Kilivwa *et al.*, 2018; Mwinyikombo, 2018). In Egypt, a recent study using molecular methods reported the absence of ESBLs from infecting cystic fibrosis patients with *P. aeruginosa* infections (Courtois et *al.*, 2018). The presence of ESBL-producing *P. aeruginosa* is observed in some African countries such as Senegal, Ivory Coast, Nigeria, and Central African Republic (Cholley *et al.*, 2014; Olowo-Okere *et al.*, 2018). Consequently, the detection of multiple β-lactamases in highly resistant bacteria should be useful for the selection of suitable antibiotic therapy and avoiding treatment failure as well as reducing mortality rates in hospitalized patients (Roya *et al.*, 2014).

5.4 Distribution of Metallo-β-Lactamases genes among the isolates

The worldwide development and dissemination of carbapenem-resistant *P. aeruginosa* strains indicates the spread of genes encoding MBL through horizontal gene transfer (Hong *et al.*, 2015). MBLs and mostly Verona integron-mediated MBL's play a crucial role in the carbapenem-resistance *P. aeruginosa* emergence (Karampatakis *et al.*, 2018). The PCR assays for the detection of MBLs genes showed the presence of positive MBL isolates for bla_{VIM-2} (28.57 %) and bla_{NDM-1} (66.67 %). From the 42 MBL isolates, three MBL isolates (7.14 %) harbored both bla_{NDM-1} and bla_{VIM-2} genes. Different findings have been observed by Pitout *et al.* (2008) in which fifty seven *P. aeruginosa* were screened for MBL producers for bla_{VIM}-type and bla_{IMP}-type genes through the PCR method; and showed that all the MBL isolates (100 %) carried bla_{VIM-2} genes from a tertiary care centre in Kenya. Moyo et *al.* (2015) demonstrated that all carbapenem-resistant isolates from a tertiary care hospital in Dar es Salaam (Tanzania) produce MBL carbapenemases enzymes. PCR followed by sequencing showed that all MBL isolates carried bla_{VIM-2}. To our

knowledge, this could be the first study done on molecular characterisation of *bla*$_{NDM-1}$-producing *P. aeruginosa* in Kenya but the presence of *bla*$_{NDM-1}$ has been observed in other studies done on *Klebsiella pneumoniae* and *Acinetobacter baumanii* isolates from a tertiary Care Centre in Kenya (Poire *et al.*, 2011; Revathi *et al.* , 2013). Few other studies and cases reports have reported the existence of *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ from African contries, such as Nigeria, Egypt, Ghana, Algeria and South Africa (Manenzhe *et al.*, 2015; Mushi *et al.*, 2014; Sekyere *et al.*, 2016**)**.

The presence of *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ genes are increasingly reported to be worldwide. Recent studies have indicated the emergence of *P. aeruginosa* haboring the *bla*$_{NDM-1}$ which showed resistance to carbapenems for the first time in Iraq **,** *bla*$_{VIM-2}$ in Spain**,** and *bla*$_{NDM-1}$ and *bla*$_{VIM-2}$ genes in Malaysia (Belles *et al.*, 2018; Ismail & Mahmoud, 2018; Liew *et al.*, 2018). The reported different PCR results for β-lactamases genes can be explained by a large geographic difference in the distribution of MBL genes in different countries of the World. The spread of MBLs genes between regions is different and their relationship within geographical areas could therefore be evaluated. Therefore, local and international travel to different regions; and patients transfer between healthcare centers could explain the large geographic difference in the distribution of MBL genes between regions.

The Nairobi Hospital is a tertiary care hospital in Kenya where different types of patients who have moved from one hospital to the other both locally and internationally are referred, for management and continuity of treatment. This implies that most of the patients would have been on one form of therapy or the other before getting to this hospital care center, thus the selective pressure of overuse and misuse use of antimicrobial agents cannot be underestimated in the emergence and dissemination of these resistance phenotypes.

The resistance genes could be probably imported from other hospitals around the world. The data generated from this research will help in the therapeutic management of nosocomial infections caused by *P. aeruginosa* species. This should reduce morbid-mortality rate by ensuring that only effective drugs are prescribed and

implemented infection control measures are followed by the medical staff, visitors and patients. It will also contribute to control antibiotic resistance by preventing the spread of *P. aeruginosa* resistant strains in healthcare settings.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. *P.aeruginosa* isolates were more prevalent in males, inpatients and older age groups (> 45 years). The infection due *P. aeruginosa* is a global concern threatening not only patients with immunocompromised conditions in hospitals but also community health members.

2. The resistant rates to non-β-lactams were higher than those of β-lactams with exception to AZM and PTZ. Resistance of *P. aeruginosa* to antibiotics may result in the production of enzymes that inactivate and degrade antibiotics, reducing the membrane permeability and the multidrug resistance efflux system.

3. The prevalence of MBL producing-*P. aeruginosa* was of serious concern as compared to non-MBL isolates, since all MBL isolates were resistant to antibiotics tested. The rate of MBL producing isolates reported in this hospital suggests the existence of resistance emergence among the population of nosocomial bacteria.

4. The highest rate of MBL isolates was obtained from Intensive Care Unit and High Dependency Unit with predominant distribution in pus swab and sputum. The resistance among nosocomial strains is linked to the increase in incidence of infection associated with the use of invasive procedures which could prompt the selection of resistant strains in ICU.

5. There were two predominant MBL genes bla_{NDM-1} and bla_{VIM-2} among *P.aeruginosa* nosocomial isolates circulating in this hospital; but it may also harbor other multiple antimicrobial resistance genes and should have the ability to transfer these to other strains. This facts has a high clinical impact because of limited therapeutic management options for patients infected with MBL strains.

6.3 Recommendations

1. Infection control measures that could prevent the transmission of *P. aeruginosa* infections among hospitalized patients are recommended in hospitals.

2. The study recommends that the correct use of antibiotics including carbapenems which are very effective drugs in treatment of patients as a part of infection control strategies in hospital is required in order to reduce the risk of dissemination of resistant *P. aeruginosa* strains.

3. The early detection of MBL producing *P. aeruginosa* may help in appropriate treatment of infections and prevent further spread of these multidrug resistant strains.

4. The study recommends also a routine surveillance of antibiotic resistance in every clinical microbiology laboratory in order to aid infection control.

5. Finally, more studies on epidemiology of pseudomonal infections, other mechanisms of antibiotic resistance in *P. aeruginosa* isolates, proper antibiotic stewardship, and rapid diagnostic methods for proper identification of β-lactamase producers are recommended in order to develop infection control strategies that could prevent the dissemination of these multi-drug resistant strains.

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APPENDICES

Appendix I: Bacteria Media preparation

MACCONKEY AGAR (Oxoid, UK)

Formula

Gelatin peptone 20.0

Bile salts No: 5.0g

Lactose 10.0g

Neutral red 0.075g

Sodium chloride 5.0g

Peptone mixture 3.0g

Bacteriological agar 12g

Preparation

Suspend 52 g in 1 litre of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50 °C and pour in 15 – 20 ml amounts into Petri dishes.

Use: For selection of enteric or others Gram-Negative bacteria.

C.L.E.D AGAR (Himedia, INDIA)

Formula

Peptic digest of animal tissue: 4.0g

Casein enzyme hydrolysate: 4.0g

Beef extract 3.0g

Lactose 10.0g

L-cystine 0.128g

Agar 15.0g

PH: 7.3±0.2

Preparation

Suspend 36.1 g in 998 millilitres (ml) of distilled water. Add rehydrated contents of 1 vial of Bromo Thymol Blue supplement. (FD091). Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Mix well and pour in 15 – 20 ml amounts into sterile Petri dishes.

Use: For selection of enteric bacteria.

MUELLER HINTON AGAR. (Oxoid, UK)

Formula

Beef infusion 300.0g

Acid hydrolysate of casein 17.5g

Starch 1.5g

Agar 17.0g

Preparation

Dissolve 38 g in 1 litre of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes

Use: For sensitivity testing

NUTRIENT AGAR (Oxoid, UK)

Formula

Lab-Lemco powder 1.0g

Yeast extract: 2.0g

Peptone 5.0g

Sodium chloride 5.0g

Agar 5.0g

Preparation

Dissolve 28g in 1 litre of distilled water. Bring to the boil to dissolve completely. Autoclave at 121°C for 15 minutes.

Use: General purpose media.

BRAIN HEART INFUSION BROTH (Himedia, India)

Formula

HM infusion powder 12.5g

Beef heart infusion powder 5.0g

Sodium chloride 5.0g

Proteose peptone 10.0g

Dextrose (Glucose) 2.0g

Sodium chloride 5.0g

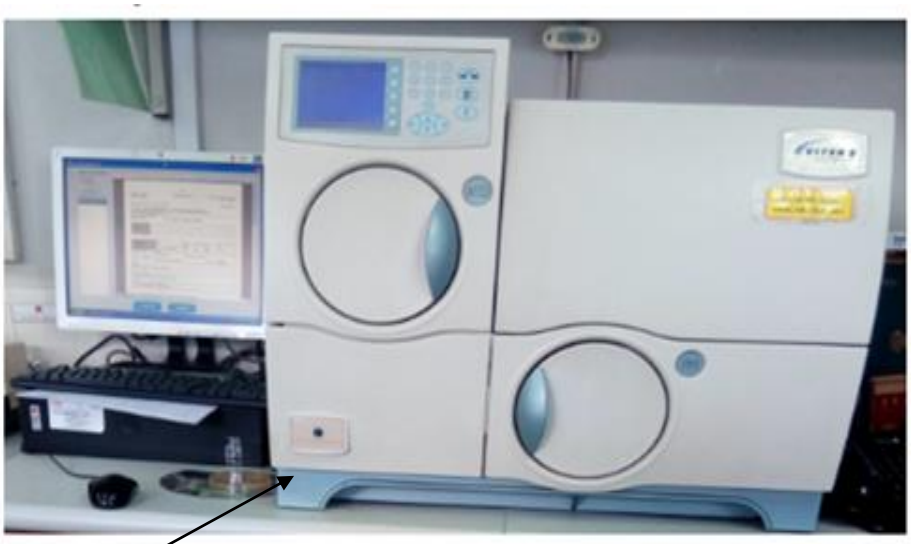
Disodium phosphate 2.5g

Preparation

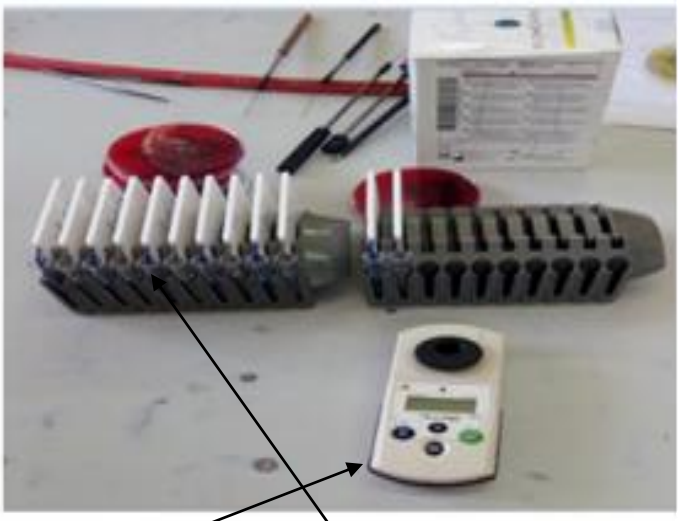
Dissolve 37g in 1 litre of distilled water. Mix and distribute into final containers. Autoclave at 121°C for 15 minutes.

Use: General purpose broth media.

Appendix II: Vitek 2 system and DensiCheck



1. Vitek 2 System machine



2. DensiCheck with calorimetric cards

Appendix III: Substrates on GN Cards for biochemical tests by Vitek 2 system

Well	Test	Mnemonic	Amount/Well
2	Ala-Phe-Pro-ARYLAMIDASE	APPA	0.0384 mg
3	ADONITOL	ADO	0.1875 mg
4	Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018 mg
5	L-ARABITOL	lARL	0.3 mg
7	D-CELLOBIOSE	dCEL	0.3 mg
9	BETA-GALACTOSIDASE	BGAL	0.036 mg
10	H2S PRODUCTION	H2S	0.0024 mg
11	Beta-N-ACETYL-GLUCOSAMINIDASE	BNAG	0.0408 mg
12	GLUTAMYL-ARYLAMIDASE pNA	AGLTp	0.0324 mg
13	D-GLUCOSE	dGLU	0.3 mg
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	0.0228mg
15	FERMENTATION/GLUCOSE	OFF	0.45 mg
17	BETA-GLUCOSIDASE	BGLU	0.036 mg
18	D-MALTOSE	dMAL	0.3mg
19	D-MANNITOL	dMAN	0.1875 mg
20	D-MANNOSE	dMNE	0.3mg
21	BETA-XYLOSIDASE	BXYL	0.0324 mg
22	BETA-Alanine arylamidase pNA	BAlap	0.0174 mg
23	L-Proline ARYLAMIDASE	ProA	0.0234 mg
26	LIPASE	LIP	0.0192 mg
27	PALATINOSE	PLE	0.3 mg
29	Tyrosine ARYLAMIDASE	TyrA	0.0276 mg
31	UREASE	URE	0.15 mg
32	D-SORBITOL	dSOR	0.1875 mg
33	SACCHAROSE/SUCROSE	SAC	0.3 mg
34	D-TAGATOSE	dTAG	0.3 mg
35	D-TREHALOSE	dTRE	0.3 mg
36	CITRATE (SODIUM)	CIT	0.54 mg
37	MALONATE	MNT	0.15 mg
39	5-KETO-D-GLUCONATE	5KG	0.3 mg
40	L-LACTATE alkalinisation	ILATK	0.15 mg

41	ALPHA-GLUCOSIDASE	AGLU	0.036 mg
42	SUCCINATE alkalinisation	SUCT	0.15 mg
43	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	0.0306 mg
44	ALPHA-GALACTOSIDASE	AGAL	0.036 mg
45	PHOSPHATASE	PHOS	0.0504 mg
46	Glycine ARYLAMIDASE	GlyA	0.012 mg
47	ORNITHINE DECARBOXYLASE	ODC	0.3 mg
48	LYSINE DECARBOXYLASE	LDC	0.15 mg
52	DECARBOXYLASE BASE	0DEC	N/A
53	L-HISTIDINE assimilation	lHISa	0.087 mg
56	COUMARATE	CMT	0.126 mg
57	BETA-GLUCORONIDASE	BGUR	0.0378 mg
58	O/129 RESISTANCE (comp.vibrio.)	O129 R	0.0105 mg
59	Glu-Gly-Arg-ARYLAMIDASE	GGAA	0.0576 mg
61	L-MALATE assimilation	IMLTa	0.042 mg
62	ELLMAN	ELLM	0.03 mg
64	L-LACTATE assimilation	lLATa	0.186 mg

Appendix IV: Preparation of molecular reagents

TE Buffer (Tris hydromethyl amino methane-EDTA)

For 100ml TE

10mM EDTA Tris-HCl pH 8.0	1mL 1 M Tris-HCl, pH 8.0
1mM EDTA pH 8.0	2mL 0.5 M EDTA pH 8.0
QS to 100mL with H_2O	

Filter sterilize, store at room temperature

TAE Buffer (Tris-Acetate-EDTA)

Composition of 50X TAE buffer	Working solution 1X TAE buffer
2.0M Tris acetate	40mM Tris acetate
0.05M EDTA	1mM EDTA
PH 8.2-8.4 (at 25^{o}C)	PH 8.2-8.4 (at 25^{o}C)

Preparation of working solution (1X TAE Buffer) and Agarose 2%

Add 20 ml of 50X TAE Buffer into 980 ml of Distilled Water

Put 2g of agarose into 100 mL 1X TAE Buffer (working solution)

Add 5 µl of E-Z VISION into the agarose solution (mixture)

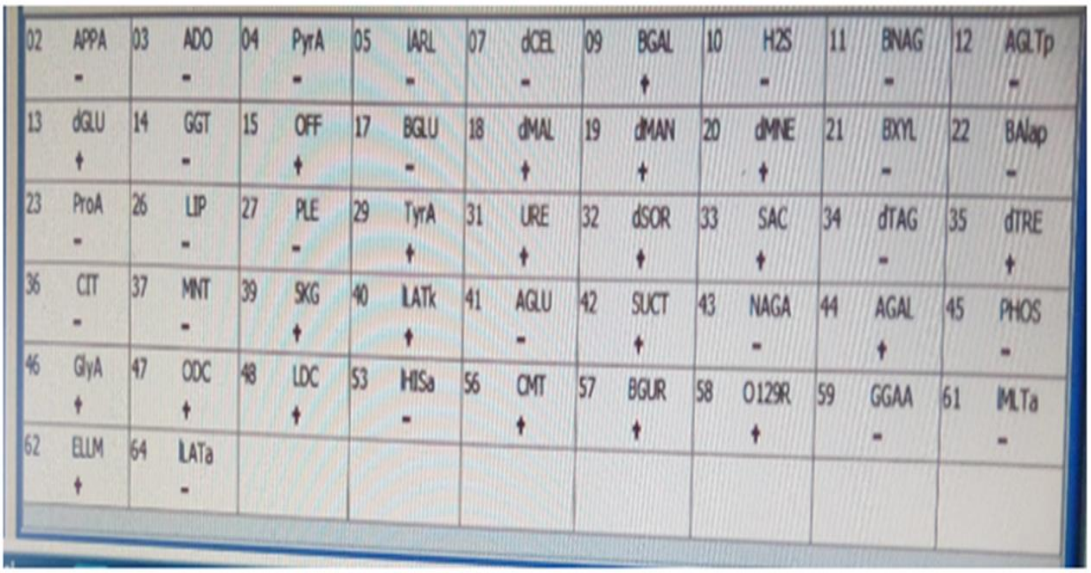
Preparation of DNA Ladder for use (BioLabs, UK)

Prepare 4Xbuffer: 1X Ladder: 1X Loading Dye

40 µl distilled water: 10 µl Ladder: 10 µl Loading Dye

Appendix V: Biochemical substrates of *P. aeruginosa* by Vitek 2 System

(*P. aeruginosa* ATCC 27853)



02	APPA	03	ADO	04	PyrA	05	IARL	07	dCEL	09	BGAL	10	H2S	11	BNAG	12	AGLTp		
	-		-		-		-		-		+		-		-		-		
13	dGLU	14	GGT	15	OFF	17	BGLU	18	dMAL	19	dMAN	20	dMNE	21	BXYL	22	BAlap		
	+		-		+		-		+		+		+		-		-		
23	ProA	26	LIP	27	PLE	29	TyrA	31	URE	32	dSOR	33	SAC	34	dTAG	35	dTRE		
	-		-		-		+		+		+		+		-		+		
36	CIT	37	MNT	39	5KG	40	ILATk	41	AGLU	42	SUCT	43	NAGA	44	AGAL	45	PHOS		
	-		-		+		+		-		+		-		+		-		
46	GlyA	47	ODC	48	LDC	53	IHISa	56	CMT	57	BGUR	58	O129R	59	GGAA	61	IMLTa		
	+		+		+		-		+		+		+		-		-		
62	ELLM	64	ILATa																
	+		-																

Appendix VI: Diameter and MICs breakpoints for *P. aeruginosa* (27th Edition)

Table 2B-1. *Pseudomonas aeruginosa* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)			Interpretive Categories and MIC Breakpoints (µg/mL)			Comments
			S	I	R	S	I	R	
PENICILLINS									
O	Piperacillin	100 µg	≥21	15–20	≤14	≤16	32–64	≥128	**(5) Breakpoints** for piperacillin (alone or with tazobactam) are based on a piperacillin dosage regimen of at least 3 g every 6 h.
β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS									
A	Piperacillin-tazobactam	100/10 µg	≥21	15–20	≤14	≤16/4	32/4–64/4	≥128/4	**(6) Breakpoints** for piperacillin (alone or with tazobactam) are based on a piperacillin dosage regimen of at least 3 g every 6 h.
B	Ceftolozane-tazobactam	30/10 µg	≥21	17–20	≤16	≤4/4	8/4	≥16/4	**(7) Breakpoints are based on a dosage regimen of 1.5 g every 8 h.**
O	Ticarcillin-clavulanate	75/10 µg	≥24	16–23	≤15	≤16/2	32/2–64/2	≥128/2	**(8) Breakpoints** for ticarcillin (alone or with clavulanate) are based on a ticarcillin dosage regimen of at least 3 g every 6 h.
CEPHEMS (PARENTERAL) (Including cephalosporins I, II, III, and IV. Please refer to Glossary I.)									
A	Ceftazidime	30 µg	≥18	15–17	≤14	≤8	16	≥32	**(9) Breakpoints** are based on a dosage regimen of 1 g every 6 h or 2 g every 8 h.
B	Cefepime	30 µg	≥18	15–17	≤14	≤8	16	≥32	**(10) Breakpoints** are based on a dosage regimen of 1 g every 8 h or 2 g every 12 h.
MONOBACTAMS									
B	Aztreonam	30 µg	≥22	16–21	≤15	≤8	16	≥32	**(11) Breakpoints** are based on a dosage regimen of 1 g every 6 h or 2 g every 8 h.
CARBAPENEMS									
B	Doripenem	10 µg	≥19	16–18	≤15	≤2	4	≥8	**(12) Breakpoints** for doripenem are based on a dosage regimen of 500 mg every 8 h.
B	Imipenem	10 µg	≥19	16–18	≤15	≤2	4	≥8	**(13) Breakpoints** for imipenem are based on a dosage regimen of 1 g every 8 h or 500 mg every 6 h.
B	Meropenem	10 µg	≥19	16–18	≤15	≤2	4	≥8	**(14) Breakpoints** for meropenem are based on a dosage regimen of 1 g every 8 h.
LIPOPEPTIDES									
O	Colistin	–	–	–	–	≤2	–	**≥4**	**(15) Colistin (methanesulfonate) should generally be administered with a loading dose and at the maximum recommended doses, in combination with other agents.**
O	Polymyxin B	–	–	–	–	≤2	4	≥8	

Table 2B-1. *Pseudomonas aeruginosa* (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)			Interpretive Categories and MIC Breakpoints (µg/mL)			Comments
			S	I	R	S	I	R	
AMINOGLYCOSIDES									
A	Gentamicin	10 µg	≥15	13–14	≤12	≤4	8	≥16	
A	Tobramycin	10 µg	≥15	13–14	≤12	≤4	8	≥16	
B	Amikacin	30 µg	≥17	15–16	≤14	≤16	32	≥64	
O	Netilmicin	30 µg	≥15	13–14	≤12	≤8	16	≥32	
FLUOROQUINOLONES									
B	Ciprofloxacin	5 µg	≥21	16–20	≤15	≤1	2	≥4	
B	Levofloxacin	5 µg	≥17	14–16	≤13	≤2	4	≥8	
O	Norfloxacin	10 µg	≥17	13–16	≤12	≤4	8	≥16	**(16) For testing and reporting of urinary tract isolates only.**
O	Lomefloxacin	10 µg	≥22	19–21	≤18	≤2	4	≥8	**See comment (16).**
O	Ofloxacin	5 µg	≥16	13–15	≤12	≤2	4	≥8	
O	Gatifloxacin	5 µg	≥18	15–17	≤14	≤2	4	≥8	

Abbreviations: ATCC®, American Type Culture Collection; CAMHB, cation-adjusted Mueller-Hinton broth; I, intermediate; MHA, Mueller-Hinton agar; MIC, minimal inhibitory concentration; QC, quality control; R, resistant; S, susceptible.

Appendix VII: Abstract of the published data

Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.8, No.20, 2018

Molecular Characterization of Beta-lactams Resistance in Pseudomonas aeruginosa Isolated from Clinical Sources at the Nairobi Hospital

Armstrong Ndihokubwayo[1*] Caroline W. Ngugi[1] Paul Makau[2] Andrew K. Nyerere[1]
1. Department of Medical Microbiology, School of Biomedical Sciences, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, P.O BOX 62000-00200, Nairobi, Kenya
2. Department of Pathology, The Nairobi Hospital, P.O BOX 30026-00100, Nairobi, Kenya

Abstract
The increase of Beta-lactamases producing organisms can cause major therapeutic failure and poses a significant clinical challenge in healthcare settings. A total of 185 clinical isolates of *Pseudomonas aeruginosa* strains were collected from in-and out-patients at The Nairobi Hospital, 74.1 % were inpatients and 25.9% were outpatients with the high prevalence of this bacterium among the male gender (61.1%) than female(38.9%); and preponderantly comprising the patients above 45 years old (64.3%). The highest numbers of *P. aeruginosa* were isolated from pus swab (39.5%), respiratory secretions (25.9%), and urine (18.9%). The resistance rate of *P. aeruginosa* against carbapenem was 31.5% among the isolates. The prevalence of MBL producing *P. aeruginosa* was 22.7% as compared to non-MBL isolates (77.3%). The MBL isolates were resistant to the examined antibiotics. There were two predominant genes VIM-2 (28.57%) and NDM-1 (66.67%) types among MBL *P. aeruginosa*, and more prevalent genes were isolated from Critical care nursing ward; Intensive Care Unit (45.2%) and High Dependency Unite (28.6%) at The Nairobi Hospital. These findings suggest that the early detection of Metallo-Beta-Lactamases-producing isolates and the cooperation between medical professionals and infection control team may help in appropriate antimicrobial therapy and avoid further spread of these multidrug resistance strains.

Keywords: *Pseudomonas aeruginosa*, Metallo-Beta-Lactamases, Resistance, Beta-lactams.

Appendix VIII: Ethical approval letter

THE NAIROBI HOSPITAL

Our Ref. TNH/ADMIN/CEO/08/12/17

8 December 2017

Mr. Armstrong Ndikokubwayo
Jomo Kenyatta University of
Agriculture & Technology
Nairobi

Dear Mr. Ndihokubwayo,

RE: DETECTION OF GENES ENCODING-EXTENDED SPECTRUM BETA-LACTAMASE AND METALLO-BETA LACTAMASE AMONG BETA-LACTANS RESISTANT PSEUDOMONAS AERUGINOSA ISOLATED FROM CLINICAL SOURCES AT THE NAIROBI HOSPITAL

Reference is made to your request to carry out the above study at The Nairobi Hospital.

We are pleased to advise that approval has been granted.

In line with the Research Projects Policy, you will be required to submit a copy of the final research findings to the Bioethics & Research Committee for records.

Do note that information/data collected and potential findings shall not be in conflict with the Hospital's confidentiality clause which states that "You will not without consent of the Association disclose any of its secrets or other confidential matters to anyone who is not authorized to receive them".

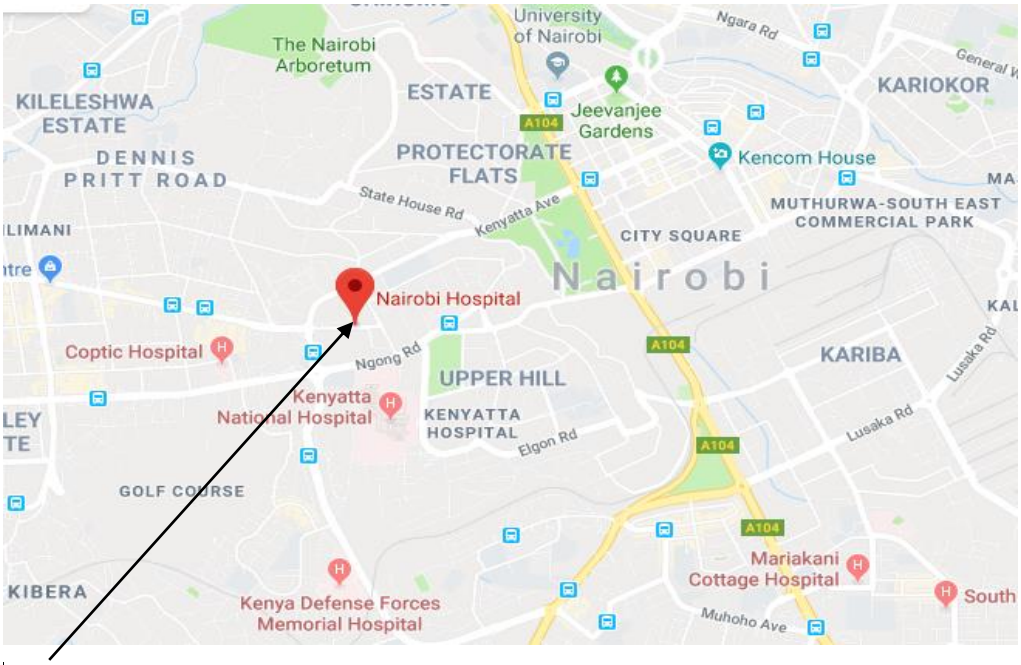
Please note that this approval is valid for the period December 2017 to December 2018, if an extension is required, a fresh application should be done before proceeding with the research.

Yours sincerely,
FOR: THE NAIROBI HOSPITAL

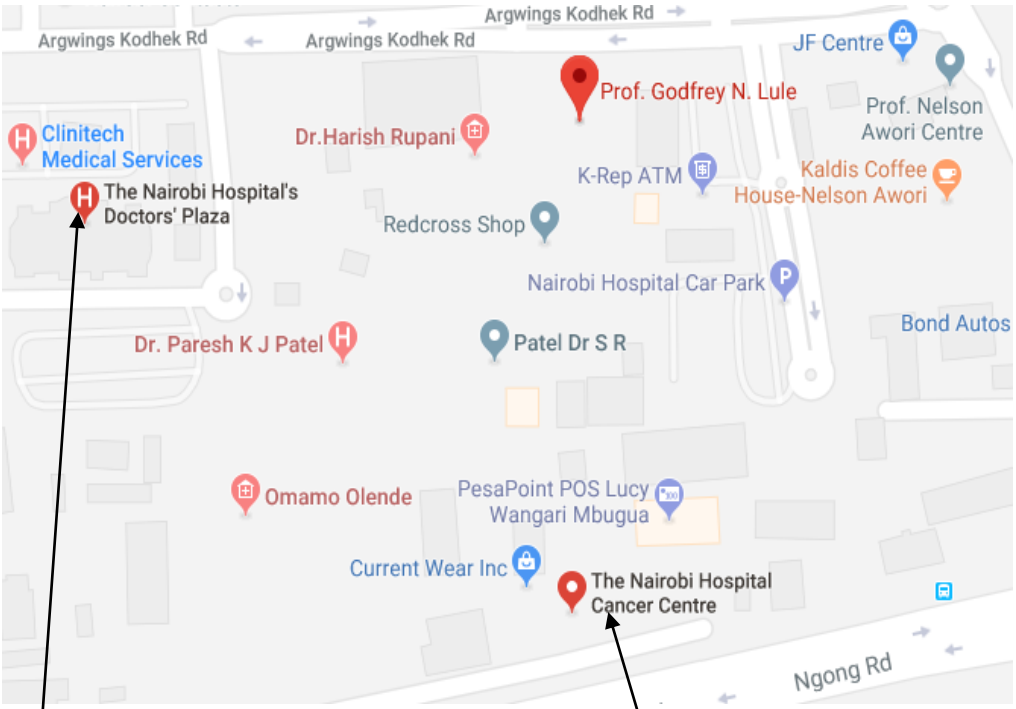
Gordon Otieno Odundo
CHIEF EXECUTIVE OFFICER

c.c. Chairman - Bioethics & Research Committee
AMD/Chief of Pathology

Appendix IX: A map indicating the localization of The Nairobi Hospital



THE NAIROBI HOSPITAL



TNH Doctor's Plaza **TNH Cancer Centre**