ISOLATION, ANTIBIOTIC SUSCEPTIBILITY AND MOLECULAR CHARACTERIZATION OF RESISTANCE GENES IN *PSEUDOMONAS* ISOLATES FROM SELECTED HOSPITALS IN MOMBASA COUNTY, KENYA.

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in Infectious Diseases in Medical Bacteriology of Kenyatta University

SEPTEMBER 2018.

DECLARATION

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

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DEDICATION

I dedicate this study to my lovely family, without whom, this work would not have been possible. Their great love, patience and lots of encouragement have been the source of my inspiration to pull along this far. I sincerely love and cherish my family. Thanks a million and for your relentless support, you are of your own kind.

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ABBREVIATIONS AND ACRONYMS

CLSI	Clinical Laboratory Standards Institute
CPGH	Coast Province General Hospital
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GIM	German imipenemase
Gyr	Gyrase
HAI	Hospital Acquired Infections
ICU	Intensive Care Unit
IMP	Imipenemase
MBL	Metallo-beta-lactamase
MDR	Multidrug resistance
Mex	Multi drug efflux
MIC	Minimum inhibitory concentration
Opr	Outer membrane porin
PCR	Polymerase Chain Reaction
P-Value	Probability Value
SIM	Seoul Imipenemase
SPM	São Paulo Metallo-beta-lactamase
Spp	Specie
SPSS	Statistical Package for the Social Science
TBE	Tris-Borate Ethylenediaminetetraacetic acid
VIM	Verona imipenemase

ABSTRACT

Pseudomonas species are common causes of nosocomial infections. Globally nosocomial infections are among the most important problems complicating health care provision since they are caused by bacteria exhibiting resistance to many antibiotics. Their susceptibility to antibiotics is restricted to merely few drugs, and the occurrence of resistance by initially susceptible strains during therapy occurs at a relatively high frequency. The rate of transmission of *Pseudomonas* infection increases from 5% in non-hospitalized patients to 20% in hospitalized patients within 72 hours after hospital admission. These infections may be contributed by indwelling catheters, surgical or burn wounds as well as abscess in patients who have been admitted for over 48 hours in the hospital. This study was conducted in two selected hospitals in Mombasa County, coastal region of Kenya. A cross sectional study design was adopted and 192 clinical samples from each study hospital were randomly collected and analyzed. Samples were collected from indwelling catheters, swabs from burn wounds, surgical wounds and abscess as well as tubing's from patients who had stayed for over 48 hours in the hospital wards or ICU. Specimens were inoculated in MacConkey and Blood agar, and later sub cultured in blood agar for purity. Species identification and antimicrobial susceptibility was analyzed by the Vitek® system. *Pseudomonas* positive isolates were screened for the presence of Metallo-beta lactamase and fluoroquinolones resistance conferring genes by conventional PCR. Out of the 384 samples, collected 50 were positive for *Pseudomonas species.* The type of specimens collected included 226 (58.9%) pus swabs, 124 (32.3%) catheter tips, 29 (7.6%) endotracheal tube tips and 5 (1.3%) central venous catheter tips. The predominant isolate identified was *Pseudomonas aeruginosa* 41 (82%), followed by *Pseudomonas fluorescence* 3 (6%), *Pseudomonas stutzeri* 3 (6%), *Pseudomonas luteola* 2 (4%), and *Pseudomonas oleovorans* 1 (2%). The relative distribution of the *Pseudomonas* species amongst the two study facilities was not statistically significant ($p= 0.955$). *Pseudomonas* isolates were detected in all the specimen types except central venous catheter tips. Pus swabs recorded the highest *Pseudomonas* burden n=30 (60%) followed by catheter tips n=11 (22%) and finally endotracheal tubes tips n=9 (18%). Antibiotic susceptibility against 26 drugs used was varying. This study observed that (96%) of *Pseudomonas* isolates were resistant to Ampicillin while Colistin was the most effective antibiotic. Metallo-betalactamase (MBL) and fluoroquinolone resistance conferring genes were analyzed from twenty (20) *Pseudomonas* isolates using PCR. VIM was the most prevalent MBL gene observed followed by SPM, SIM, IMP, and GIM. Fluoroquinolone resistant genes *ParC* and *MexR* were detected in all the isolates screened in this study. This study is of great importance to the county, as it will outline the suitable antibiogram for the treatment of *Pseudomonas* infections and serves as a baseline to evaluate the trends in antibiotic resistant genes in the region.

CHAPTER ONE: INTRODUCTION

Background Information

Pseudomonas species are naturally resistant to many antibiotics, thus their susceptibility to the available antibiotics is limited to only a few drugs. Resistance against initially susceptible strains during therapy occurs at a relatively high frequency (Mohmid *et al.,* 2013; Motayo *et al.,* 2013). *Pseudomonas* species comprise of the major agents of nosocomial and community acquired infections. Members of this group are widely distributed in hospital environments and are difficult to eradicate. They usually need minimal nutritional requirements for their growth and treatment of *Pseudomonas* infections is usually difficult which leads to high mortality rates (Gomes *et al.,* 2011). Resistance to antibiotics by these pathogens is mainly because of the diffusion barrier of the bacterial outer membrane, mutations in the target molecules such as, GyrA and/or ParC, as well as antimicrobial inactivating enzymes. In several hospital settings, this natural resistance is more complicated by mutations facilitated via chromosomes and the presence of resistant genes from plasmids and transposons (Belodu *et al.,* 2012).

Nosocomial or hospital acquired infections (HAI) are defined as infections that develop 72 hours after admission to the hospital for reasons other than the primary infection at admission (Ghane & Azimi, 2014; Picão *et al.,* 2008). They are also defined as localized or systemic conditions that results from adverse reactions to the presence of an infectious agent(s) or its toxin(s) and that were present 48 hours or more after hospital admission (Cardoso *et al.,* 2015). Bacteria mostly cause these infections with higher resistance to majority of the available antibiotics thus; they are more dangerous than the other bacterial infections. More importantly they lead to a

worldwide rise in mortality and morbidity as well as increased health care costs (Altayyar *et al.,* 2016). Risk factors determining nosocomial infections depends upon the environment in which care is delivered, the susceptibility and condition of the patient, and the lack of awareness of such prevailing infections among staff and health care providers (Yallew *et al.,*2017). Based on results from most studies, the infection rate and the etiological agent of nosocomial infections varies between different hospitals and geographical locations (Ghane & Azimi, 2014).

There is often ineffective treatment to acute and chronic *Pseudomonas* infection, alongside biofilm formation where *Pseudomonas* species shows limited susceptibility to antibiotics and disinfectants. The remarkable plasticity of the *Pseudomonas* genome is a result of its members being assumed to be able to attain nearly all known antimicrobial resistance mechanisms. Therefore, infections with *Pseudomonas* are frequently associated with multidrug resistance with limited treatment options (Livermore, 2002). Their antibiotic resistance coupled with the lowered immunity in the hospitalized patients undoubtedly affects patient's management including available treatment options.

Pseudomonas species are Gram-negative motile rods, from the family *Pseudomodaceae*. Their existence in the apparent deficiency of nutrients is due to their genetic adaptability, which translates into improved metabolic activity with the capability to adapt and inhabit varied ecological niches. They are able to utilize diverse organic compounds as a source of energy and carbon. Some species of *Pseudomonas* can survive under weak ionic concentrations as well as extreme temperatures (Ndi & Barton, 2012; Belodu *et al.,* 2012).

Infections resulting from antibiotic resistant bacteria are a healthcare problem globally and more so in the developing countries. Studies that show detection,

antibiotic susceptibility and molecular characterization of *Pseudomonas* species in Mombasa county hospitals are scanty. The aim of this study was to define the prevalence, antibiotic susceptibility and molecular characterization of antibiotic resistance conferring genes in this bacterium at the Mombasa hospital and Coast Province General hospital in patient samples 48 hours after hospital admission.

1.2 Statement of the Problem

Pseudomonas species are major etiological agents of nosocomial infections, which translate into longer hospital stays, increased healthcare costs and mortality. They are widely distributed in the hospital environment and can cause a variety of diseases especially among the hospitalized immunocompromised patients. They commonly cause wound infections, surgical sites infections, urinary tract infections, pneumonia and bacteremia in patients undergoing invasive procedures such as catheterization and mechanical ventilation. Ideally, healthcare institutions are required to conduct routine assessments for nosocomial infection and resistance patterns in an effort to mitigate future occurrences. However, studies of this nature in the region are scanty.

Pseudomonas spp are known to be resistant to most antimicrobials alongside methicillin resistant *Staphylococcus aureus*, *Enterococcus, Escherichia coli, Klebsiela pneumoniae, and Acinetobacter baumanii* due to different mechanisms working against antibiotics. Antimicrobials that are now variably ineffective due to increasing resistance include cephalosporins, macrolides, aminoglycosides and fluoroquinolones. Thus, to control infections caused by *Pseudomonas*, it is important to determine the distribution, phenotypic characteristics and genetic elements associated with antibiotic resistance of these organisms. This study aims to determine the prevalence of *Pseudomonas* species, generate suitable antibiogram and determine genetic

determinants of antibiotic resistance in *Pseudomonas* isolates from hospitalized patients in two facilities in Mombasa County, Kenyan.

1.3. Justification

The World Health Organization (WHO) has identified *Pseudomonas* species as a "priority 1 pathogen" (critical) due to the threat that it poses to human health. This is largely due to its resistance to multiple antibiotics. This increased trend in antibiotic resistance worldwide poses a challenge in the choice of an ideal antibiotic for therapy against these bacteria. Determination of an ideal antibiotic for the treatment of this pathogen may greatly aid in effective patient management.

1.4. Research Questions

1. What is the prevalence of *Pseudomonas* spp in the study area?
2. What are the antibiotic susceptibility patterns of the isolated *Pseudomonas* spp?
3. What are the antibiotic resistance conferring genes in the *Pseudomonas* spp isolates in the study area?

1.5. Hypothesis

1.5.1. Null hypothesis

Pseudomonas spp do not contain drug resistant genes.

1.6 Objectives

1.6.1 General Objective

To determine the occurrence, antibiotic susceptibility patterns and resistance conferring genes in *Pseudomonas* species from selected Mombasa County hospitals.

1.6.2 Specific Objectives

1. To determine the prevalence of *Pseudomonas* spp isolated in the study hospitals.
2. To determine the antibiotic susceptibility patterns of *Pseudomonas* spp in the study area.
3. To determine the antibiotic resistance conferring genes in *Pseudomonas* isolates in the study area.

1.7 Study Output

This study aims at providing important information, which will be used by the healthcare providers, clinicians, medical practioneres, infection control committees, drug firms and scientists to combat infections due to *Pseudomonas* spp. This information will be shared with the ministry of health with the aim of improving adherence to antibiotic prescription guidelines and infection control policy.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Pseudomonas* species

The genus *Pseudomonas* is comprised of species of health, economic and ecological importance. Members of this genus exhibit a great deal of metabolic diversity, and are also versatile with the capability of adapting and colonizing a variety of ecological niches (Health Protection Agency, 2013). The genus forms part of the family *Pseudomonadaceae* which consist of over 140 species, out of which more than 25 species are associated with human infections (Ali, 2013). *Pseudomonas* comprise of a diverse group of aerobic gram-negative bacilli isolated from plants, water, soil and animals including humans. Species identified to cause human diseases in this genus are frequently associated with opportunistic infections and comprise species like *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas oleovorans, Pseudomonas luteola* and *Pseudomonas stutzeri* (Chong, 2009).

Pseudomonas spp from clinical sources grow well on solid media such as MacConkey agar, blood agar, and chocolate agar. Colony characteristics on these media may be virtually colorless, cream, or off-white. Yellow or blue green pigmentation is usually a common characteristic of these bacteria, though some species produce fluorescent colonies under ultraviolet light or fluorescent microscope. Most species in this genus are oxidase positive, (with an exception of *Pseudomonas luteola*) catalase positive, citrate utilization test positive but negative to indole test, methyl red test, and voges-proskaurer test. Some species in this genus show beta-hemolysis on blood agar secretes a fluorescent yellow-green (pyoverdin) under iron-limiting environments,

while others like *Pseudomonas fluorescens*, secretes thioquinolobactin. *Pseudomonas aeruginosa* isolates secretes pyoverdine and produce siderophores, such as pyocyanin (Health Protection Agency, 2013; Nordberg *et al.,* 2013).

Pseudomonas species are therefore well adapted to their environment such that they can tolerate various physical environments including extreme low (4°C) and high (43°C) temperature conditions, antimicrobial agents and weak ionic concentrations. Growth specifically at 43°C distinguishes *Pseudomonas aeruginosa* from the other *Pseudomonas* species and this characteristic contributes to its pathogenicity (Ali, 2013).

Almost 70% of *Pseudomonas* infections occur as nosocomial infections and colonization increases significantly in hospitalized patients (Tuon *et al.,* 2012). Members of the genus *Pseudomonas* are also associated with opportunistic infections, which include systemic infections, bacteremia, gastrointestinal, skin, soft tissues, urinary tract and respiratory system in immunocompromised patients. Vehicle or contact transmission is the main route of infection where the pathogen is introduced and re-introduced into the hospital settings by patients transferred from other facilities, wards, ICU, or from the theatre. It may also be introduced by visitors on their shoes, ornamental flowers, as well as healthcare workers during cleaning, ward rounds and during sample collection or even during invasive procedures like catheterization. Some *Pseudomonas* species can be found in moist hospital settings such as disinfectants, ointments, eye drops, mops, foods, sinks, taps, cosmetics as well as hospital equipments (e.g. respirators) and contact lens solutions forming biofilms (Raj, *et al.,* 2015; Shenoy *et al.,* 2002). Sterilization of all patient-care items is not necessary, therefore health-care policies must identify, primarily on the basis of the

items' intended use, whether cleaning, disinfection, or sterilization is indicated (Rutala, *et al.,* 2008)

2.1.1 *Pseudomonas aeruginosa*

P.aeruginosa is an opportunistic bacterium that infects patients especially the immunocompromised. Infections caused by this pathogenic bacterium are always life threatening with difficulties in treatment due to its intrinsic higher resistance to many antimicrobials (Anil & Raza, 2013). Pathogenicity by this bacterium is favored by a number of virulence factors which include secretion of enzymes such as alkaline protease, elastase, and gelatinase, production of toxins such as exotoxin A,S and T, hemolysin, and the possession of flagella, pilli, lipopolysaccharide and pyocyanin on their cell wall (Parija, 2012). *Pseudomonas aeruginosa* biofilms enhances its ability to adhere and survive in medical equipments, environmental surfaces, and airways in patients with prolonged respiratory infections. Pyocyanin produced by this bacterium catalyzes the production of superoxide and hydrogen peroxide that causes tissue damage leading to inflammation. *Pseudomonas aeruginosa* resides in biofilms encased by anaerobic condition thereby protecting this pathogen from being recognized by the host immune system and the action of antibiotics (Jácome *et al.,*2012).

Although classified as an aerobic bacterium, *Pseudomonas aeruginosa* can multiply in environments of limited or total oxygen depletion where it can attain anaerobic growth with nitrate as a terminal electron acceptor or ferment arginine by substrate-level phosphorylation (Filiatrault *et al.,* 2006). *Pseudomonas aeruginosa* is usually recognized by its pearlescent appearance, production of grape-like odor in culture media, while its definitive identification includes production of fluorescein,

pyocyanin and, as well as its capability to grow at extreme temperatures of 43 °C (Ghane & Azimi, 2014).

2.1.2 *Pseudomonas fluorescens*

This bacterium occasionally causes human infection but in some cases, it has been associated with peritonitis in peritoneal dialysis patients, blood transfusion related septicemia and catheter related bacteremia. Most outbreaks of *Pseudomonas fluorescence* infections have been connected with immunocomprised patients (Odjadjare *et al.,* 2012; Wong *et al.,* 2011; Hsueh *et al.,* 1998).

Pseudomonas fluorescens is a Gram-negative, rod shaped, obligate aerobe and a causative agent of nosocomial infections or hospital acquired infections (Trivedi, 2015). It does not ferment lactose though some strains in this group are capable of utilizing nitrate instead of oxygen during cellular respiration as a final electron acceptor. This bacterium grows well at low temperatures such as 4°C, where blood and blood products, disinfectants, normal saline, distilled water and other fluids provide suitable atmosphere for bacteria proliferation in hospital settings. (Trivedi, 2015;Wong *et al.,*2011). It also produces heat-stable proteases and lipases that catalyze the spoilage of milk by causing bitterness, breakdown of casein and ropiness due to production of slime and coagulation of proteins (Samaržija *et al.,* 2012). It secretes a soluble fluorescent siderophore pigment, pyoverdine (Da Silva & De Almeida, 2006).

2.1.3 *Pseudomonas stutzeri*

Pseudomonas stutzeri rarely cause infections, however infections in hospitalized patients is associated with contamination or colonization or in patients with

predisposing factors such as invasive procedures like catheterization or immunocompromised persons (Hummeida *et al.,* 2010). The presumable absence of virulence factors makes this bacteria to be relatively low virulent though its pathogenicity include bacteremia and local infections as well as pneumonia, conjunctivitis, arthritis, brain abscess, endocarditis and meningitis (ECHC, 2015; Hummeida *et al.,* 2010).

Pseudomonas stutzeri is a Gram-negative, rod-shaped, aerobic bacterium. This bacterium moves by means of a single polar flagellum, it's a non-lactose fermenter, oxidase positive and mostly a saprophyte found in soil, water, and hospital environments as an opportunistic pathogen although human infections are rare, (*Final Screening Assessment Report for Pseudomonas stutzeri ATCC 17587*, 2015; Bisharat *et al.,* 2012). *Pseudomonas stutzeri* consist of several strains among which the medically important ones include *Pseudomonas stutzeri*, *Pseudomonas medocina* and CDC group Vb-3. *Pseudomonas stutzeri* is the most commonly encountered strain in clinical isolates and is frequently isolated from wounds respiratory tract, blood and urine.

2.1.4 *Pseudomonas luteola*

P. luteola is a nosocomial pathogen and rarely causes clinical infections, mostly isolated from ulcer, meningitis, endocarditis, septicemia and peritonitis, usually in association with surgical operations or the use of catheters or prostheses, (Bayhan *et al.,* 2015; Kilic *et al.,* 2008).

Pseudomonas luteola is a Gram-negative bacterium, rod-shaped, aerobic and non-spore forming measuring (0.8-2.5 μm). It is motile with single or multiple polar flagella, does not ferment lactose and grows on MacConkey agar at optimum

temperatures of 30°C. *P. luteola* is oxidase negative, nitrate utilization negative and gives a distinctive yellow to orange pigment on MacConkey agar after 48 hours incubation, colonies are typically rough or wrinkled (Chihab *et al.,*2004).

2.1.5 *Pseudomonas oleovorans*

Pseudomonas oleovorans has been classified under the *Pseudomonas* group based on the analysis of 16s rRNA. It is a Gram-negative motile rod strictly aerobic and grows well on blood agar and MacConkey agar at optimum temperatures of 35°C. It is isolated from water, soil and clinical samples such as CSF, urine, sputum and is sometimes associated with pneumonia and meningitis though rarely causes humans infections (Gautam *et al.,* 2015; Faccone *et al.,* 2014).

2.1.6 *Pseudomonas putida.*

Pseudomonas putida has the ability to metabolize a wide variety of compounds, which makes members in this group capable of surviving in moist and inanimate ecological surfaces thus can serve as a reservoir thereby leading to nosocomial infections (Liu *et al.,* 2014). Being an opportunistic pathogen with low-virulence, *P.putida* is a primarily causative of nosocomial infections in patients with indwelling catheters, immunocompromised and those with medical devices. This pathogen has also been implicated in outbreaks of bacteremia due to transfusion with contaminated fluids, blood and blood products (Liu *et al.,* 2014).

P. putida is an aerobic, fluorescent, non-spore forming bacteria, oxidase positive. This bacterium possess a single or multiple flagella and can be found in moist surroundings, such as soil, water, and grows well in temperatures of 25°C to 30°C and

not at 40°C. It can be distinguished from the other species by its inability to liquefy gelatin (HPA, 2013).

2.2 Antimicrobials used to treat *Pseudomonas* infections

Antimicrobials are agents that destroy or prevent the growth of microorganisms, classified according to the type of microbes they act against, their antimicrobial properties or function. Antibiotics are formulations derived from living microbes that act against other microbes and they don't include synthetic antimicrobial substances, semisynthetic or those from animals and plants origin that are used to treat a variety of microbial infections (Abdallah, 2011). Antibiotics are the most commonly used drugs to treat bacterial infections but they are however frequently misused by doctors, medical practitioners and health care workers through inappropriate prescribing protocol and treating of infections other than those caused by bacteria (Fair & Tor, 2014).

Antibiotics can be classified according to their activities, such as their action on the target microbe and their effect on the target organism or chemical structure. Based on the degree of susceptibility to antimicrobials by most bacterial species, antibiotics can be classified as broad spectrum, intermediate or narrow spectrum. Broad spectrum antibiotics are active to a wider range of microorganisms including both gram positive and gran negative bacteria, while narrow spectrum antibiotic activity is limited to particular species of microorganisms (Etebu & Arikekpar, 2016).

The effect of antibiotics to the target organism can lead to an end point of either inactivation or killing of the microbe. Bactericidal drugs usually kill the target organism while bacteriostatic antibiotics inhibit or delay growth and multiplication of the target bacteria whereas some antibiotics can behave as both bacteriostatic as well

as bactericidal (Frederick, 2015). Most antibiotics possess diverse modes of action due to their structural nature and the degree of affinity to certain target molecules within the bacterial cells. Antibiotics can inhibit the functions of bacterial cell membrane, synthesis of cell wall, proteins, nucleic acid and inhibition of other bacterial metabolic processes (Etebu & Arikekpar, 2016; Frederick, 2015).

Most frequently used antibiotic classes for the treatment of infections caused by *Pseudomonas* species include beta-lactams, aminoglycosides, fluoroquinolones and polymyxins (Meletis & Bagkeri, 2013). Majority of *Pseudomonas* species displays natural resistance to a number of beta-lactam antibiotics mostly penicillin, but other species are sensitive to ticarcillin, piperacillin, imipenem, and aminoglycosides (Chong, 2009). Resistance to antibiotics by bacteria is an important medical problem across the world and most bacteria like the *Pseudomonas* can resist at least three classes of antibiotics (MDR) (Ventola, 2015), all the available antibiotic classes (pan resistance), or can resist all the antibiotic classes available but susceptible to one or two classes (extended antibiotic resistance) (Meletis & Bagkeri, 2013).

The widespread and imprudent use of antibiotics has resulted in the occurrence of resistance by bacteria against antibiotics posing serious risk to global public health. These resistant bacteria requires a renewed effort to seek effective antibacterial agents (Bonk, 2015). Resistance to antibiotics is known to be the major existing problem worldwide facing humans and actions to inhibit and contain the rise of antibiotic resistant bacteria need a great understanding of the antibiotic resistant genes as well as their distribution. (Khudaier & Salih, 2014; Adel & Sabiha, 2010). Resistance of *Pseudomonas* spp against certain classes of antibiotics increases owing to diverse trends in different parts of the world and the selection of a proper empiric antibiotic against this bacteria has been a challenge (Adel & Sabiha, 2010).

Consequently, several nosocomial infections caused by *Pseudomonas* spp are becoming more difficult to treat owing to the ability of these bacteria to attain certain mechanisms of adapting and overcoming the actions of antibiotics in their surroundings (Lister *et al.,* 2009). *Pseudomonas* spp resistance can often occur against different antibiotic classes during treatment and may also show additional resistance after ineffective treatment mostly via modification of a porin (Brown & Izundu, 2004). *Pseudomonas* species possess intrinsic resistance to majority of antibiotic classes which may be caused by modifications in the bacterial outer membrane, production of extended-spectrum beta-lactamase or efflux pumps, which also confers several levels of resistance to broad spectrum cephalosporins, such as Cefotaxime, ceftazidime, and azotreonam (Golshani *et al.,* 2012).

2.3 Mechanisms of antibiotic resistance in *Pseudomonas* species

Resistance to antibiotics by *Pseudomonas* species has been known as a major medical problem facing the entire human population. To counter this menace, it requires a great understanding of the antibiotic susceptibility patterns, antibiotic resistant genes and dissemination (Adel & Sabiha, 2010). The genus *Pseudomonas* usually displays intrinsic or acquired resistance to various classes of antibiotics via a variety of mechanisms.

Some species harbours inducible chromosomally encoded beta-lactamase, biofilm formation, mutational genes, plasmids, transposons which are responsible for resistance to majority of antibiotics (Luczkiewicz *et al.,* 2015).

2.3.1 Restricted uptake and efflux pump

Bacterial cytoplasmic membrane forms a protective barrier for the passage of hydrophilic compounds, where entry of these compounds is through carrier dependent transport mechanisms or outer membrane channels (Kugelberg *et al.,*2005). Gram-negative bacteria have an outer membrane barrier that prevents large hydrophilic molecules to pass through it. The outer membrane contains proteins called porins that forms channels which allows the passage of many hydrophilic substances including antibiotics. A reduction in the amount of the outer membrane porin protein OprD results in the decreased drug influx into the cell allowing the bacteria to develop resistance (Dumas *et al.,* 2006). Certain antibiotics such as colistin and aminoglycosides are capable of interacting with bacterial lipopolysaccharides thereby changing the permeability of the bacterial cell membrane in order to pass through, whereas some antibiotic diffuses through certain porin channels example beta-lactams and quinolones (Meletis & Bagkeri, 2013).

Pseudomonas species produces mainly specific porins, which allows only certain molecules to pass. However, most bacteria expresses lots of general porins which allows nearly several hydrophilic molecule to pass through and relatively few specific ones with binding sites for certain molecules (Meletis & Bagkeri, 2013; Lister *et al.*, 2009). To increase the removal of antibiotics from the intracellular compartment or the membrane space in Gram-negative bacteria, some bacteria contain membrane proteins that function as exporters called efflux pumps, for certain antimicrobial agents. These pumps expel the drug from the cell at a higher rate, meaning that the drug concentrations are never sufficiently high to elicit an antibacterial effect (Bockstael & Aerschot, 2009)

The expression of several efflux pumps by the genus *Pseudomonas* allows drugs as well as other substances to be expelled out of the bacterial cell (Chroma & Kolar, 2010). Polyselective efflux is the most common type of efflux pump found in Gram-negative bacteria, which belongs to the resistant nodulation division (RND) superfamily and plays a key role in multidrug resistance (MDR) bacterial phenotype. This type of pump ejects a variety of antibiotics and structurally unrelated molecules such as dyes and bile salts, but also detergents and biocides that are frequently used in medical practice (Kourtesi *et al.,* 2013). Therefore, their first two components are named multidrug efflux (Mex) along with a letter e.g. MexA and MexB. The outer membrane porin is called *Opr* along with a letter e.g. *Opr*M. Most antibiotics except polymyxins are pumped out by these efflux systems (Lister *et al.,* 2009).

2.3.2 Drug inactivating enzymes

Bacteria produce enzymes such as topoisomerase, gyrase, Beta-lactamases, aminoglycoside-modifying enzymes that irreversibly modify and inactivate antibiotics. The fluoroquinolones class of antibiotics acts on the bacterial topoisomerase II/ IV (ParC and ParE) and DNA gyrase (gyrA and gyrB) that are responsible for bacterial DNA synthesis and repair. Each of these two enzymes contains subunits that bears antibiotic resistant mutations in the fluoroquinolones resistant determining region of ParC or gyrA thus resistance is due to mutations in these regions which are common in *Pseudomonas* (Poole *et al.* 2011).

Beta-lactam antibiotics are characterized by the presence of a beta-lactam ring in their structure that is essential for their function. The beta-lactam ring helps in the inactivation of a set of transpeptidase enzymes necessary for the catalyzation of the final cross-linking reactions during peptidoglycan cell wall synthesis in bacteria.

Bacterial isolates that confer resistance to beta-lactam antibiotics are primarily due to the hydrolysis of the antibiotic by beta-lactamase enzymes (Van Hoek *et al.,* 2011). Beta-lactamases are serine protease enzymes that cleave the betalactam ring by opening the amide bond. The corresponding gene may be carried on either chromosomes or plasmids. Some chelating agents such as EDTA are capable of inhibiting the activities of metallo-beta-lactamases. However, they are not affected by clinical beta-lactamase inhibitors like clavulanic acid and normally retain their susceptibility against monobactams (Chroma & Kolar, 2010). The most commonly detected metallo-beta-lactam in *Pseudomonas* include the Imipenemase (IMP) and Verona imipenemase (VIM) (Dötsch *et al.,* 2009).

Ambler classified beta-lactams based on the amino-acid sequence where four classes designated A, B, C, and D were recognized (Aghamiri, Amirmozafari, Fallah Mehrabadi, Fouladtan, & Samadi Kafil, 2014). Classes A, C, and D gathered evolutionarily distinct groups of serine enzymes, and class B the zinc-dependent or "EDTA-inhibited" enzymes. Ambler classified Metallo-beta lactamase enzymes under Group B and sub-divided it into three classes namely BI, BII, and BIII. The subclass BI was further divided according to their molecular structures into four categories namely imipenemase (IMP), Verona imipenemase (VIM), German imipenemase (GIM) and São Paulo Metallo-beta-lactamase (SPM) types (Aghamiri *et al.,* 2014). The increase in the spread of several antimicrobial resistant determinants is because of acquisition or insertion of mobile genetic cassettes GIM, SPM, VIM, and IMP on integrons, which are associated with bacterial transposons and plasmids. Drug resistance in *Pseudomonas* spp can be caused by acquirement of resistance genes like those encoding beta-lactamase or aminoglycoside modifying enzymes via horizontal gene transfer or chromosomal gene mutation of the target fluoroquinolones

particularly ciprofloxacin (Chander & Raza, 2013). The production of metallo-beta-lactamase (GIM, VIM, SPM and IMP), by *Pseudomonas* leads to antibiotic resistance (Bonomo & Szabo, 2006).

Bush proposed a functional classification scheme of beta-lactamases by defining four groups based on their substrate and inhibitor profiles (Bush & Jacoby, 2010). The first group comprised of beta-lactamases that are not well inhibited by clavulanic acid such as cephalosporinases. The second group is that which do active site-directed beta-lactamase inhibitors such as broad-spectrum beta-lactamases, penicillinases, and cephalosporinases generally inhibit. The third group consists of metallo-beta-lactamases which are poorly inhibited by almost all beta-lactam-containing molecules and that they hydrolyze penicillins, cephalosporins, and carbapenems. The fourth group is penicillinases that are not well inhibited by clavulanic acid. Subgroups were also defined according to rates of hydrolysis of carbenicillin or cloxacillin (oxacillin) by the second group penicillinases (Van Hoek *et al.,* 2011). Resistance to aminoglycosides is often as a result of inactivation of enzymes acetyltransferase, phosphotransferase and adenyltransferase by bacteria, due to the acquisition of plasmid genes that encodes for aminoglycoside modifying enzymes. These genes also helps in the dissemination of antibiotic resistance to most bacteria (Shaw *et al.,* 1993).

2.3.3 Mutation of drug targets

Hypermutable strains of *Pseudomonas aeruginosa* are usually observed in prolonged or chronic infections including infections of the lungs in patients with cystic fibrosis (Poole *et al.,* 2011). Intrinsic resistance in bacteria is the inherent or passive resistance to antimicrobials normally due to consequences of general adaptive processes that are not directly linked to specific or a certain class of antimicrobials. Acquired or active

resistance is because of the development of a counterattack mechanism by bacterial specific evolutionary pressure. This pressure is normally against a specific or a class of antimicrobials resulting to previous sensitive bacteria to certain antimicrobials becoming resistant (Bockstael & Aerschot, 2009).

Various antibiotics are capable of overcoming the inherent defenses of *Pseudomonas* and are active against most species in this genus. However, these drugs are prone to being compromised by mutational resistance expressed by these bacteria. Examples include mutations in genes that's encode DNA gyrase A and topoisomerases II and IV, depression of the chromosomal AmpC beta-lactamase, up-regulation of MexAB-OprM and efflux pump regulatory genes (Neda Gorgania *et al.,* 2010; Lister *et al.,* 2009; Livermore, 2002)

The principal target sites for the fluoroquinolones in *Pseudomonas* include the DNA gyrase. This is where observed mutations normally occur within the gyrA that leads to higher levels of resistance among *Pseudomonas* strains with mutations in the fluoroquinolones resistance determining region gyrA and ParC gene. (Agnello & Wong-beringer, 2012; Kugelberg *et al.,* 2005; Hocquet *et al.,* 2003).

The regulatory gene mexAB-oprM efflux system contributes to the natural resistance of *Pseudomonas* to a wide range of antibiotics including fluoroquinolones, beta - lactams, and beta-lactamase inhibitors, whereas MexXY-oprM contributes to aminoglycoside resistance. High expression of MexAB-oprM and MexXY-oprM may confer high levels of resistance to clinical strains as a result of mutations occurring mainly in their regulatory genes MexR and MexZ (Neda Gorgania *et al.,* 2010; Nemat-Gorgani, 2009).

The genus *Pseudomonas* expresses a chromosomal AmpC betalactamase in a low level but the levels increases in the presence of antibiotics. With constant depression

of AmpC, it results to the production of higher amounts of betalactamase due to spontaneous mutations on the regulatory genes. Stimulation of AmpC is attributed to three genes ampD, ampR, ampG that are related to peptidoglycan recycling. Inactivation of ampD results to cytoplasmic build up and over expression of AmpC (Neda Gorgania *et al.,* 2010).

2.3.4 Biofilm Formation

Biofilm is an organization of bacterial cell communities enclosed in an extracellular polysaccharide matrix that forms a slippery solid coat around the bacterial community. It is usually attached to an inert or living surface that normally protects the bacterial community against hostile environments, such as in the human lungs. These bacterial communities cause many persistent and chronic infections, such as *Pseudomonas* infection in immunocompromised patients (Neidig *et al.,* 2013).

Biofilms can manipulate the level of inflammatory response in a host by modulating its chemical appearance and altering its cell-to-cell activity pathways through quorum sensing. Acyl-homo serine lactone (AHL) is an example of a quorum sensing molecule found in *Pseudomonas aeruginosa* (Stinson, 2010). Bacterial cells located deep inside biofilms exist in a starved or slow growing state due to nutrients limitations. This results into a state of dormancy by the bacterial cells leading to antibiotic resistance. In some instance, biofilm matrix may slow down or retard the rate of antibiotic penetration into bacterial cells leading to expression of genes within the biofilms that mediate resistance (Taraszkiewicz *et al.,* 2013).

Though some antibiotics are more effective at penetrating and destroying cells in biofilms, biofilms have inherent resistance to antibiotics through multiple mechanisms. These include, failure of the antibiotics to fully diffuse through the

biofilm, changes in the osmotic environment causing an osmotic stress response, which changes the degree of permeability into *Pseudomonas* cell envelope and porins. This effectively stops majority of antibiotics from gaining entrance in to the bacterial cells and some cells in the biofilm may adopt a distinct phenotype due to environmental factors which protect them from the action of antimicrobial agents (Sachan *et al.,* 2015).

2.4 Current approaches in combating antimicrobial resistance

New therapeutic approaches alternative to antimicrobials have been proposed for the treatment of pathogenic microbes. Some of these approaches include the use of antivirulence medicines, which do not directly kill bacteria but deprive them of their virulent factors for easy elimination by the host immune system. The use of non-antibiotics like efflux pump inhibitors that will act by reducing or reversing antibiotic resistance to drugs which the target microbe has developed intrinsic resistance against it. In addition, the use of vaccines to induce immunity against pathogenic microbes and the use of genetically engineered bacteriophages to reverse a pathogen drug resistance thereby restoring bacterial sensitivity to antibiotics. Finally host pathogen interactions where the host releases cationic antimicrobial peptides at the infected sites which have multiple antimicrobial activities (Nordberg *et al.,* 2013)

Interventions which have been intended to decrease antibiotic resistance rates in hospitals, and which are now drawing considerable interest to most researchers is antibiotic cycling or rotation. Cycling is the programmed rotation of one class of antibiotics with one or more different classes displaying similar spectra of action, in a repeated order in that cycle. A class or a specific antibiotic is withdrawn from use for a defined period then reintroduced later in an effort to limit resistance to the cycled drug. In so doing antibiotics that had greater susceptibility against pathogenic

microbes turn out to be effective in treating bacterial infections (Kollef, 2006). A qualitative meta-analysis has argued that cycling could be beneficial for preserving drug susceptibility in *Pseudomonas aeruginosa*. However there are no current published theoretical models of antibiotic cycling in hospital settings and the greater community suggests that cycling will actually facilitate the spread of resistant strains (Brown & Nathwani, 2005). In a study conducted by (Viechtbauer, Bonhoeffer, Abel, & Kouyos, 2014), it was found that cycling could only prevent the evolution of multiple resistance but not in cases where multiple resistance is wide spread.

Antibiotic mixing is another approach in combating antibiotic resistance. Antibiotic mixing involves random allocation of appropriate antibiotics to patients in a manner with no correlations across patients or time in the used drugs (Beardmore *et al.,*2016). Antimicrobial stewardship programs have proven in some instances to optimize antimicrobial therapy, reduce or stabilize resistance to antimicrobials. Studies have demonstrated the potentiality of these programs in restricting the occurrence and spread of resistance. They have further maintained a relationship between the usage of antimicrobials and the occurrence of resistance (Lee *et al.,*2013).

Modification of target drugs to make them more effective against drug resistant bacteria is also being considered. A study conducted by (Barrios *et al.,* 2014) on doripenem, a betalactam carbapenems which has four membered beta lactamics in one ring connected to a second ring in structure. Based on the five membered thiazolidinic ring, they found out that this structural arrangement offers protection to beta lactamase producing bacteria as well as a wide spectrum of activity and chemical stability. Another study (Lee *et al.,* 2013) showed that the polymixin remained the most consistently effective agents against multidrug-resistant *Pseudomonas*. The study demonstrated that drugs with higher molecular weight such as colistin were able

to infiltrate by active transport into the outer cell membrane of gram-negative bacteria.

2.5 Methods used to determine antibiotic susceptibility

Antibiotic susceptibility testing methods are useful in the prediction of therapeutic outcomes, drug discovery and even epidemiological studies. Testing methods are divided based on the principle of each system and initially involves culturing a sample to obtain a pure isolate and testing to determine which antimicrobial agents inhibit the growth or kill the pathogen (Balouiri *et al.,* 2016; Doern, 2011).

Traditional phenotypic methods currently used to determine antimicrobial resistance profiles such as disk diffusion, broth microdilution remain critical in guiding appropriate treatment options. However, techniques such as these are unable to determine the actual molecular mechanisms of resistance, and are especially lacking in situations where the observed phenotype is a result of the interaction of multiple gene products with overlapping activities (Hanson & Hanson, 2016). Drug susceptibility patterns significantly reduces duration and cost of hospitalization, morbidity and mortality rate when the susceptibility outcomes are provided timely. Routine or automated susceptibility assays can be used to provide quick result (Ataee *et al.,* 2012)

2.5.1 Agar or disk diffusion antibiotic sensitivity testing

Disk diffusion methods measure bacterial growth on agar plates containing paper disks that are commercially prepared and impregnated with a standard concentration of an antimicrobial agent. The paper disks are placed onto the surface of the agar medium onto which a bacterium has been inoculated at a standardized concentration

of cells, then the plate is incubated at 35ºC to 37ºC overnight (Driscoll *et al.,* 2012). Diffusion of the antimicrobial agent from the paper disks into the agar, establishes a gradient with more of the target antibiotic compound in the agar closer to the disk and less further from the disk where the target isolate will grow on the agar to a point at which the drug is concentrated enough to impede growth. Diffusion through the agar is based on the molecular size of the antimicrobial agent, factors that may be present in the agar, and agar concentration. The inhibitory zone diameter is influenced by the diffusion rate of the target antibiotic through the agar, a function of the molecular sizes and hydrophilicities of the compounds (Giani *et al.,* 2012). The inhibition zone diameter is measured manually or using automated systems in millimeters, then compared with standardized (EUCAST) or (CLSI) interpretive criteria to designate the isolate as sensitive, intermediate or resistant to the drug (Turnidge & Paterson, 2007). Interpretive criteria are based on the relationship between minimum inhibitory concentration (MIC) and zone diameter size, which is analyzed against the pharmacokinetics of the antimicrobial agent in normal dosing regimes. The final *in vitro* criteria are obtained following studies of clinical efficacy and response outcomes (Doern, 2011). The disk diffusion method is cost-effective, easy to perform on small or large numbers of isolates, and does not require expensive equipment (Driscoll *et al.,* 2012).

Kirby Bauer disk diffusion method has been useful to clinical and research areas (Doern, 2011) and has been extensively used to characterize antibiotic resistance in environmental isolates. European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Committee for Clinical Laboratory Standards Guidelines (CLSI) have standardized protocols for disk diffusion methods (Ataee *et al.,* 2012).

2.5.2 Broth Dilution

Broth dilution is a method used to estimate the minimum inhibitory concentration on bacterial isolates (Balouiri *et al.,* 2016). This method is based on serial dilutions of an antimicrobial agent in tubes, or microtitre plates, to which a standard concentration of the test bacterium is added, then incubated at prescribed times. The optical density of the broth is then measured which will show the lowermost possible concentration of each antimicrobial that inhibits observable growth of the target organisms. A term designated as the MIC the lowest concentration of antibiotic that completely inhibits the growth of the bacterium (Hilpert *et al.,* 2015). The ranges in concentrations tested vary with the antibiotic and with the identity of the isolate being tested but should encompass the concentration used to define the organism as resistant and the ranges of expected MICs for reference quality control organisms. If the contents of the tubes are cultured onto agar plates then the plate with no growth represents the tube with the minimal bactericidal concentration (MBC) of the antimicrobial (Hilpert *et al.,* 2015).

2.5.3 Agar Dilution Assays

Agar or disk dilution method is like the broth dilution technique in that a serially diluted antimicrobial agent is added to the agar medium which is then inoculated with the test organism in a standardized concentration. Agar plates are prepared with antimicrobial concentrations and must be used within a week of preparation. This method requires more media preparation than the disk diffusion method and is more likely to be used by laboratories with a high sample throughput. Disadvantages of the method include difficulty in verifying the purity of the applied bacterium, and the endpoints being not always easy to read. Both the broth dilution and the agar dilution method may be labour intensive if automated equipment is not used (Jiang, 2011).

2.5.4 Concentration Gradient Method (E-Test)

E-test antibiotic susceptibility assay is based on agar diffusion techniques where a rectangular plastic test strip containing an immobilized antimicrobial agent is used as an antibiotic disk. This plastic tests strip is applied on the surface of the agar plate inoculated with the test organism. The drug diffuses from the strip to the inoculated agar plate causing an elliptical zone of inhibition which intercepts the strip at a point where the MIC is taken using a calibrated scale on the strip (Simpson *et al.,* 1995). This test is rapid and easy to use however, it is limited to the antibiotic range supplied by the manufacturer and is an expensive test to use for screening (Sader *et al.,* 2006).

2.5.5 Calibrated Dichotomous Sensitivity test (CDS)

CDS method is a disk diffusion method which use the agar dilution method as a gold standard in correlating between sizes of inhibition zone and quantitative MIC, (Doern, 2011). An antibiotic is calibrated by plotting the zone sizes recorded from a large number of strains of a bacterial species against the log MIC of each antibiotic. It is referred to as a dichotomous test because it does not recognize an intermediate category. It divides susceptibilities into sensitive and resistant categories. This method is conservative, selecting the lower end of the range of break point MICs. In the interpretation of results, a uniform zone size with an annular radius (measured from the edge of the disk to the edge of the zone of no growth) of 6 mm (18 mm diameter) indicates a susceptible organism. It is the point of diffusion on the sigmoid curve that enables the greatest discrimination. A zone size of less than 6 mm indicates a resistant organism. There are exceptions to this standard interpretation and these are listed in the CDS manual seventh edition, 2013. Thus, an annular zone size of 6 mm will

correspond to a set MIC, in mg/L, for a particular antibiotic. The CDS method is said to increase the specificity of the test by using the dichotomous cut-off values. In some cases, a few marginally sensitive strains may be called resistant and therefore the CDS method may be less sensitive for some bacterial strains compared to other methods. Factors critical to the accuracy of the CDS method include inoculum concentration and preparation, media, disk potency, incubation temperature and incubation atmosphere.

The inoculum is made by stabbing a colony with a straight wire and emulsifying in sterile normal saline to provide a concentration of 10CFU/mL to result in a confluent and uniform growth (Doern, 2011). This facilitates the visualization, on the plates, of the production of enzymes that inactivate antimicrobial agents. The CDS method uses Sensitest agar (Oxoid). The disk potency is designed to promote the uniform cut-off zone between resistant and susceptible results. The CDS manual provides details of testing for a number of bacteria (Doern, 2011).

2.5.6 Automated Antimicrobial Susceptibility Testing Systems.

Several automated and semiautomated systems for antimicrobial susceptibility testing and identification of clinically relevant bacteria have been widely used. Most of these methods are commercially available. All these methods are based on phenotypic identification of bacteria. These methods are cost effective for clinical laboratories with a high throughput of clinical specimens, provide convenient laboratory information systems interface, and reduce the turnaround time compared to conventional tests (Fritsche *et al.,* 2007). They also provide the physicians with convenient susceptibility profiles that help and guide on antimicrobial therapy. When testing certain organism antimicrobial combinations limitations on the accuracy of the

assessment of MIC values by these systems are known. Each of these systems comes with well-known inherent strengths and performance specifications (Sader *et al.,* 2006). Example of these methods include VITEK 2 System (bioMerieux), Micro Scan WalkAway System (Dade Behring) and Phoenix Automated Microbiology System (BD Diagnostic Systems),

2.5.6.1 VITEK 2 System (bioMerieux)

Vitek 2 system is an automated method used for bacterial identification and susceptibility testing available from BioMerieux. It utilizes a growth based photometric technology where the bacterium utilizes a substrate resulting in a colour and density change that is detected by phototransistor detectors. The system uses calorimetric reagent cards, which after inoculation with the suspected bacteria are incubated in the Vitek 2 where species identification and antimicrobial susceptibility are automatically interpreted. The Vitek 2 AST-GN83 and AST-N222 card for Gram-negative bacilli contains wells that test for antimicrobial agents at different concentrations and result in an MIC value. The following antimicrobials are on the AST-GN83 and AST-N222 card, Amikacin, Ampicillin, Amoxicillin/clavulanic acid ,Ampicillin/sulbactam, Ceftriaxone, Cefazolin, Cefuroxime, Cefuroxime axetil, Cephoxitin, Ceftazidime, Ciprofloxacin, Cefepime, Cefotaxime, Colistin, Gentamicin, Imipenem, Meropenem, Minocycline, Nitrofurantoin, Piperacillin/tazobactam, Perfloxacin, Piperacillin, Ticarcillin, Ticarcillin/clavulanic acid, Tobramycin, Trimethoprim/sulphamethoxazole (Pincus, 2010; Hummeida *et al.,* 2010).

2.5.6.2 MicroScan WalkAway system

MicroScan WalkAway system is an automated bacterial identification and antimicrobial susceptibility testing method, which is based on photometric and fluometric technology.

2.5.6.3 DNA Microarray

The use of DNA microarrays is another promising technology for the identification of antimicrobial resistance determinants in any number of species. Due to their inability to determine whether resistance determinants are expressed or gene products are functional, DNA microarrays an electrochemically interrogated platform containing oligonucleotide probes that targets antimicrobial resistant genes. They are not intended to replace standard phenotypic testing, but provide a powerful platform for broad base screening, which have been applied successfully in broad spectrum detection of antibiotic conferring genes (Scaria *et al.,* 2008, Cassone *et al.,* 2006).

Microarrays can trail elements focused against various antibiotic classes that are commonly found grouped in mobile gene elements. This is because they permit concurrent identification of multiple genes in a single assay. The fast spreading of such assemblages makes treatment options unsuccessful and difficult. It also complicates epidemiological investigations aimed at clarifying the spread of antibiotic resistance genes together with the core genetic structures that leads to the occurrence of multidrug resistant phenotypes (Leski *et al.,* 2013).

2.5.6.4 Phoenix Automated Antimicrobial Susceptibility Testing System

The Phoenix^{TM} Automated Microbiology System (BD Diagnostics, Sparks, MD, USA) is a fully automated system for the rapid detection and susceptibility testing of

bacteria. It is the first system to incorporate automated nephelometry in identification and antimicrobial susceptibility a step that has traditionally been the most time consuming, manual step associated with isolate preparation. It is designed for the rapid bacterial identification at the species level and determination of antimicrobial susceptibility testing (AST) of clinically significant human bacterial pathogens (Giani *et al.,* 2012). Performance of this system has been previously evaluated using the CLSI breakpoints but not the EUCAST ones (Menozzi *et al.,* 2006). This system has demonstrated accuracy in identification of isolates with resistance mechanisms. These isolates include extended-spectrum beta-lactamases (ESBL), acquired AmpC beta-lactamases, certain carbapenemases in gram-negatives, PBP2a in *Staphylococcus aureus* and Vancomycin resistance determinants in *Enterococcus* (Giani *et al.,* 2012). To inoculate a panel using this method, a ‘heavy’ suspension of the isolate to be tested is made in Phoenix identification broth. This method has the capability of processing a starting McFarland of 0.20 to 4.0 to the appropriate testing McFarland. Both the identification (ID) broth and the corresponding antibiotic sensitivity test (AST) broth are then placed in a Phoenix AP rack and loaded onto the system for preparation. The estimated rack processing time is 5-7 minutes and each Phoenix AP is capable of processing 200 ID/AST sets in less than 4.5 hours. This System is equipped with software suitable for interpretation of AST results using EUCAST breakpoints (Giani *et al.,* 2012).

2.5.6.5 Polymerase Chain Reaction (PCR)

PCR is a molecular technique that amplifies target genes. It offers rapid and sensitive methods to identify the presence of resistant genes in bacteria, viruses and is crucial in the elucidation of genetic elements responsible for antimicrobial resistance. This

method also enables characterization of target organism and is useful in investigating of non culturable or slow growing organisms (Vaez *et al.,* 2015; Joshi & Deshpande, 2010; Cockerill, 1999).

2.5.6.5.1 Real time Polymerase Chain Reaction

Real time Polymerase chain reaction is a technique used to monitor in real time the progress of a PCR reaction while quantifying relatively small amounts of PCR products such as DNA, cDNA or RNA. This method is based on the detection of a fluorescence produced by a reporter molecule, which increases, as the reaction proceeds, due to the accumulation of PCR products with each cycle of amplification. The fluorescent reporter molecules in this method include dyes that bind to the double stranded DNA or sequence specific probes. Based on the detection molecules this method can be categorized as specific or non-specific. In specific method, they use oligonucleotide probes labeled with both a reporter fluorescent dye and a quencher dye. In the non-specific method the DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction (Paiva *et al.,* 2010). This method has the additional cycle of a reverse transcription that leads to formation of a DNA molecule from an RNA molecule. The advantage of this method is that it can start with a small amount of nucleic acid and quantify the end product accurately. It also saves time, resources and there is no need for the post PCR processing. In addition, it facilitates monitoring of the reaction as it progresses and have high sensitivity with more specificity (Wong & Medrano, 2005).

2.5.6.5.2 Multiplex Polymerase Chain Reaction

Multiplex Polymerase Chain Reaction is a variant of PCR that enables simultaneous amplification or detection of more than one template (many targets of interest) in one reaction in a mixture by addition of more than one set or pair of oligonucleotide primers. Two or more products in parallel are amplified in a single reaction tube. This method is widely used in genotyping applications and DNA testing in research, forensic, and diagnostic laboratories for pathogen detection or genetically modified organisms. It is also used for qualitative and semi-quantitative gene expression analysis using cDNA as a starting template (Kalvatchev, Draganov, & Kalvatchev, 2017). Multiplex assays can be tedious and time consuming due to long optimization procedures because different multiplex PCRs may require unique reaction conditions (Shuber *et al.,* 1995). The simultaneous amplification of several DNA fragments within one reaction helps to reduce the number of reactions needed to test a sample for different targets. This is very important as it saves time, money and makes this method useful especially when large number of sample have to be screened (Sint *et al.,* 2012).

2.5.6.5.3 Nested Polymerase Chain Reaction

Nested polymerase chain reaction is a modified conventional PCR. This method is proposed to minimize binding to products that are not specific due to the amplification of unexpected primer binding sites. It reduces product contamination due to the amplification of unintended primer binding sites or mispriming. This method utilizes a set of two primers in two successive runs. The first set binds to sequences outside the target DNA as in standard PCR, but may also bind to other areas of the template. The second set binds to sequences in the target DNA that are

within the portion amplified by the first set (nested primers). Thus, the second set of primers will bind and amplify target DNA within the products of the first reaction. The primary advantage of nested PCR is that if the first primers bind and amplifies an unwanted DNA sequence, it is unlikely that the second set will also bind within the unwanted region. This method is useful for PCR with high background or those with rare templates and its highly sensitive and specific (Hernández-Rodríguez & Ramirez, 2012).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Area

This study was conducted in two selected Mombasa County hospitals namely The Mombasa hospital and The Coast Province General Hospital. These hospitals are the major referral hospitals in the region one being a private (The Mombasa hospital) hospital and the other a government hospital (The Coast Province General Hospital). They provide both in and outpatient services with operating theaters and intensive care. Mombasa hospital has a bed capacity of 114 beds while Coast General Hospital is the premier public health and reference hospital in the entire coast region with a bed capacity of 700 beds.

3.2 Study Design

A cross sectional study design utilizing a systematic random sampling technique was adopted. All members of the study population had an equal and independent chance of being selected.

3.3 Study Population

Admitted patients who had stayed for over 48 hours with catheters; tubing's, surgical wounds, burn wounds and whose consent was sought and given were eligible. Those patients who did not meet these criteria were excluded from this study.

3.4 Sample size Determination

The sample size was determined by the Fishers formula (Fisher *et al.,* 2000). The study assumed a *Pseudomonas* prevalence rate of 50%.

$$n = \frac{Z^2 P (1-P)}{d^2}$$

Where: n= required sample size.

Z= Confidence level at 95% (standard value or deviation of 1.96) from the tailed normal table.

P= Expected Prevalence rate 50% (0.5)

d= precision if 5% ($d = 0.05$)

$$n = \frac{1.96^2 \text{ x } 0.5\ (1-0.5)}{0.05^2} = \frac{3.8416 \text{ x } 0.25}{0.0025} = 384.16 = 384.16$$

=384 samples

3.5 Sample Collection

A total of 384 samples were collected, which included central venous catheter tips, endotracheal tubes, catheter tips and pus swabs. One sample was collected from each patient who had attained the inclusion criteria. Swabs were collected by cleansing the wound or abscess with sterile saline to irrigate any purulent debris. The swab was moistened with sterile saline and using a “zig-zag” motion whilst simultaneously rotating the swab between the fingers, the whole wound surface was sampled. The specimen was placed straight into the transport medium (Level, 2015; Supplies & Company, 2014).

Before removing the catheter, the skin around insertion site was cleansed with 70% alcohol to reduce contaminating skin flora and remove any residual antimicrobial

ointment. The area was then allowed to dry. Aseptically the catheter was removed and clipped, 2 inches of the distal tip of the catheter was directly cut with a sterile scissor into a sterile container (Health *et al.,* 2016).

A systematic random sampling was employed where every third patient was selected and sample taken (Martínez-Mesa *et al.,* 2016; SMART, 2012). Each sample was preserved in a separate Cary Blair transport media to maintain the organism's viability and transported to the laboratory. A laboratory technologist from each study area served as a research assistant aiding in taking patients consent using the questionnaire and assisting in specimen collection.

3.6 Laboratory Analysis

3.6.1 Bacterial Identification and Susceptibility Testing

3.6.1.1 Culture

For the purpose of standardization, all samples collected were analyzed at one facility. Upon receipt, samples were immediately inoculated into both differential and enriched media (MacConkey agar and blood agar plates). They were then incubated aerobically at 37ºC for 12-48 hours before colonial morphologies were interpreted. Preliminary identification of bacteria was based on colony characteristics of the organisms including beta hemolysis on blood agar, non-lactose fermentation and pigment production (greenish yellow and bluish green pigments) on MacConkey agar. The isolates that showed these characteristics were then sub cultured onto blood agar to obtain a pure culture.

3.6.1.2 Gram Staining and Microscopy

Gram staining was performed on colonies from subcultures for the identification of their gram reaction. Specimens were smeared onto clean grease free glass slides air-dried, heat fixed and Gram stained. The stained slides were examined microscopically under oil immersion lens for bacterial morphology. The isolates were identified as gram-negative if they did not retain the purple stain and were counter-stained pink by safranin.

3.6.1.3 Species identification and Antimicrobial Susceptibility

Biochemical tests were performed to test the enzymatic activities of the organisms. These included oxidase test, citrate utilization test and indole test. Oxidase test was performed by taking a colony of the isolate then smeared onto the Oxidase paper disc moistened in distilled water a purple colour indicated the presence Oxidase enzyme. Citrate utilization was performed by inoculation of a colony of the isolate on the slant of the Simmons citrate agar and incubated at 35°C to 37°C for up to 7 days. A sky blue or royal blue colour indicated citrate utilization positive. Indole test was performed by inoculation of the test organism into tryptophan or peptone broth and incubates at 37°C for 24 – 48 hours, and then 0.5 mL Kovacs reagent was added with gently agitation while observing the upper layer for colour change. A cherry red ring at the top indicates a positive test results.

All gram-negative rods, Oxidase positive, citrate utilization test positive and indole negative isolates were further confirmed by the Vitek system (bioMerieux Vitek) for species identification and antibiotic susceptibility. Some isolates with a characteristic yellow pigmentation on MacConkey agar and oxidase negative were also included because some *Pseudomonas* spp are oxidase negative. Suspension of the isolates were

prepared, and then adjusted between 0.5 to 0.63 McFarland standards according to manufacturer's instruction for Gram-negative bacteria. Gram-negative identification cards AST-GN83 and AST-N22 were used for Gram-negative *Pseudomonas* organisms. VITEK®2 cards were inoculated following manufacturers instruction then, the isolates ID were introduced into the VITEK®2 system to allow it to choose the correct interpretive criteria. The resulting MIC was translated into clinical categories of susceptible, intermediate, or resistant following the Clinical and Laboratory Standards Institute (CLSI) recommendations (Schreckenberger & Binnicker, 2011) .

Antimicrobial agents used were obtained from respective bioMerieux suppliers. 26 antibiotics were used in this study. Quality control was done using commercially available sensitive *Pseudomonas species* standard organisms ATCC 27853 as positive controls. *Pseudomonas* isolates were identified as susceptible, intermediate or resistant according to the 2007 National Committee for Clinical Laboratory Standards Guidelines (CLSI). All the isolates that showed intermediate reaction to antimicrobial agents were considered as resistant.

Based on the clinical breakpoints, a set which uses minimum inhibitory concentration (MIC) measurements defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Turnidge & Paterson, 2007). An isolate classified as susceptible will be inhibited by the MIC at the site of infection, resistant if it will not be inhibited by the achievable concentration. Intermediate are isolates that have displayed a relative resistance by exhibiting growth at the MIC of an antimicrobial but are in fact susceptible to a concentration above the MIC (Heil & Kristie, 2016). For isolates classified as intermediate, the therapeutic effect is uncertain (Giani *et al.,* 2012).

3.6.2 DNA Extraction

DNA was obtained from twenty (20) randomly selected *Pseudomonas* positive isolates using the Qiagen DNA extraction kit (Qiagen, Germany), according to manufacturer's instructions. Bacterial colonies were taken from culture plate with an inoculation loop and suspended in 180 µl of buffer ATL (supplied in the QIAamp DNA Mini Kit) by vigorous stirring. The mixture was then centrifuged for 7 minutes at 400-x g with brake. The supernatant was discarded and the bacterial cells suspended with Hank’s balanced salt solution, in a total volume of 200 µL. Bacterial cells were lysed by pipetting 20 µL of Qiagen Proteinase K into the bottom of a 1.5 mL micro centrifuge tube, and then 200 µL of bacterial cell suspension added together with 200 µL of buffer AL. The mixture was vortexed for 15 seconds then incubated in a 56˚C water bath for 10 minutes. The mixture was then briefly centrifuged to remove any droplets that may have formed at the top.

To facilitate DNA adsorption to the QIAamp Column, 200 µL of 100% ethanol was added to the mixture, then vortexed for 15 seconds. The entire mixture was then briefly centrifuged and added to a QIAamp spin column, which was centrifuged at 13,200 rpm for 1 minute at room temperature. To remove residual contaminants, the spin column was removed and placed in a clean labeled collection tube, where 500 µL of buffer AW1 was added then centrifuged at 10,000 rpm for 1 minute at room temperature. The spin column was again removed and placed in another clean-labeled collection tube where 500 µL of buffer AW2 was added and centrifuged at 13,200 rpm for 3 minutes at room temperature. DNA was Eluted by removing the spin column and placed in a clean, 1.5 mL micro centrifuge tube. The QIAamp Mini spin column was carefully opened and the 60 µL of buffer AE added. The mixture was

incubated at room temperature for 5 minutes then centrifuge at 6,000-x g (8,000 rpm) for 1 minute. The QIAamp mini spin column was then discarded and the micro-centrifuge tube containing the eluted DNA stored at -20˚C or -80°C freezer indefinitely (Technologies, 2012).

3.6.3 Identification of antimicrobial resistant genes.

Out of the *Pseudomonas* isolates obtained in this study twenty (20) were screened for the presence of MBL and fluoroquinolones resistance encoding genes using the polymerase chain reaction (PCR). The presence of metallo-beta-lactamase (MBL) drug resistant genes namely IMP-1(Imipenemase), SIM (Seoul imipenemase), VIM-1 (Verona imipenemase), SPM-1 (São Paulo metallo-β-lactamase), and GIM (German imipenemase) was analyzed using respective primers, in a multiplex PCR using published primers (Ellington, Kistler, Livermore, & Woodford, 2007). Two genes involved in fluoroquinolone drug efflux namely ParC and MexR were amplified in a second PCR using another set of published primers (Neda Gorgania *et al.,* 2010). The primers and PCR conditions used in this study are listed in. Table 3.1

3.6.4. Multiplex PCR

The first conventional multiplex PCR assay was carried out using five sets of primers to detect five families of MBL genes. The second involved two sets of primers to detect two fluoroquinolone resistance genes in single reaction. This was done because the amplicon sizes of the seven genes chosen would not allow their detection in a single multiplex reaction. The MBL genes surveyed included, IMP, VIM, GIM, SPM and SIM. Fluoroquinolone resistance determining region of ParC gene was determined using primers parC-1 and parC-2 to amplify a 267bp region. For the

MexR regulatory gene, mexR-1 and mexR-2 primers were used to amplify the whole 503bp region of the gene.

Amplification reactions for MBL were prepared in a total volume of 25µl per tube. This was comprised of 2µl genomic DNA, 14 pmol of primers (This was a multiplex of 5 pairs of primers working at a concentration of 10uM. 1ul of each primer (F/R) were added to this master mix), 10X buffer, 2.5µM of each deoxyribonucleotide triphosphate (Invitrogen), 3 mM MgCl2 and 0.5U Taq DNA polymerase (Invitrogen). In each round of amplification, sterile water was used as a negative control. The cycling conditions were 94°C for 5 minutes to activate Taq, followed by 35 cycles of denaturation or unzipping at 94°C for 30 seconds, annealing at 52ºC for 40 seconds and extension at 72ºC for 50seconds followed by a final extension for 5minutes at 72°C.

Amplification reactions for Fluoroquinolone resistance-determining genes ParC and MexR genes were prepared in a total volume of 50µl per tube. This comprised of 4µl genomic DNA, 10 pmol of primers (This was a multiplex of 2 pairs of primers working at a concentration of 10uM. 1ul of each primer (F/R) were added to this master mix), 10X buffer, 0.2 mM of each deoxyribonucleotide triphosphate (Invitrogen), 2.5 mM MgCl2 and 0.5U Taq DNA polymerase (Invitrogen). In each round of amplification, sterile water was used as a negative control. The cycling conditions were 95°C for 15 minutes activating Taq, followed by 35 cycles of denaturation or unzipping at 95°C for 45 seconds, annealing at 51ºC for 45 seconds and extension at 71ºC for 1 minute and a final extension at 71°C for 7 minutes.

PCR products were mixed with blue-green dye (Promega) and subjected to electrophoresis on a 2% agarose gel in 1X TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA) in which 10ug/ml Ethidium bromide (1ul/100ml agarose)

had been added. O'GeneRuler Ultra Low Range DNA Ladder and Sigma's Direct Load™ 1kb Ladder were used as markers to indicate the size of the amplicons. This was run under a constant voltage of 70V for 1 hour. To determine the presence of the amplified gene products, the gel was visualized in a U/V trans-illuminator and the image documented.

Table 3.1: Primers and PCR conditions used in the study.

Target	Primer Name	Sequence of Primer (5'-3')	Product Size(bp)	Reaction Conditions
MBL	**Imp-F 50**	GGA ATA GAG TGG CTTAAY TCTC	188	Multiplex PCR for the first 5 pairs of primers cycling condition was 94°C for 5min followed by 36 cycles of 94°C for 30 sec, 52ºC 40 sec extension 72ºC for 50sec and finally 72°C for 5min.
	Imp-R 50	CCA AAC YAC TAS GTT ATC T		
	Vim-F 50	GAT GGT GTT TGG TCG CAT A	373	
	Vim-R 50	CGA ATG CGC AGC ACC AG		
	Gim-F 50	TCG ACA CAC CTT GGT CTG AA	476	
	Gim-R 50	AAC TTCCAA CTT TGC CAT GC		
	Spm-F 50	AAA ATCTGG GTA CGC AAA CG	270	
	Spm-R 50	ACA TTA TCC GCTGGA ACA GG		
	Sim-F 50	TAC AAG GGA TTC GGCATC G	570	
	Sim-F 50	TAA TGG CCT GTT CCC ATG TG		
Fluoroqui-nolones	**parC-1**	CATCGTCTACGCC ATGAG	267	Multiplex PCR of the last 2 pairs of primers cycling condition was 95°C for 15min, followed by 35 cycles of 95°C for 45 sec, 51^{0}C for 45 sec and 71^{0}C for 1min. Finally 71^{0}C for 7min
	parC-2	AGCAGCACCTCGG AATAG		
	mexR-1	CTGGATCAACCAC ATTTACA	503	
	mexR-2	CTTCGAAAAGAAT GTTCTTAAA		

3.7 Data Presentation and Analysis

Data was presented using tables, graphs and pie charts. Data was entered into a Microsoft® Excel spread sheet, exported and analyzed using the statistical package for the social science (SPSS) package version 13.0 (IBM, Chicago). Chi square was used for testing the significance of the findings and data correlation between the two study facilities.

3.8 Ethical Considerations

Data was secured and minimal identification information was collected. Safeguarding confidentiality, it was reported using identification codes in all research materials and was not shared with any third party. Before any data collection, oral and written consent was used to seek the authority of the participants. Ethical Approval was sought from the Kenyatta University Ethical Review Committee and approval from the study hospitals management before study commencement.

CHAPTER FOUR

4.0 RESULTS

4.1 Types of samples collected

A total of 192 samples were collected from each of the study facilities from consenting patients who had stayed in the hospital for over 48 hours. The specimen type's collected during the study period included 226(58.854%) pus swabs, 124(32.292%) catheter tips, 29(7.552%) endotracheal tube tips and 5(1.302%) central venous catheter tips. (Figure 4.1).

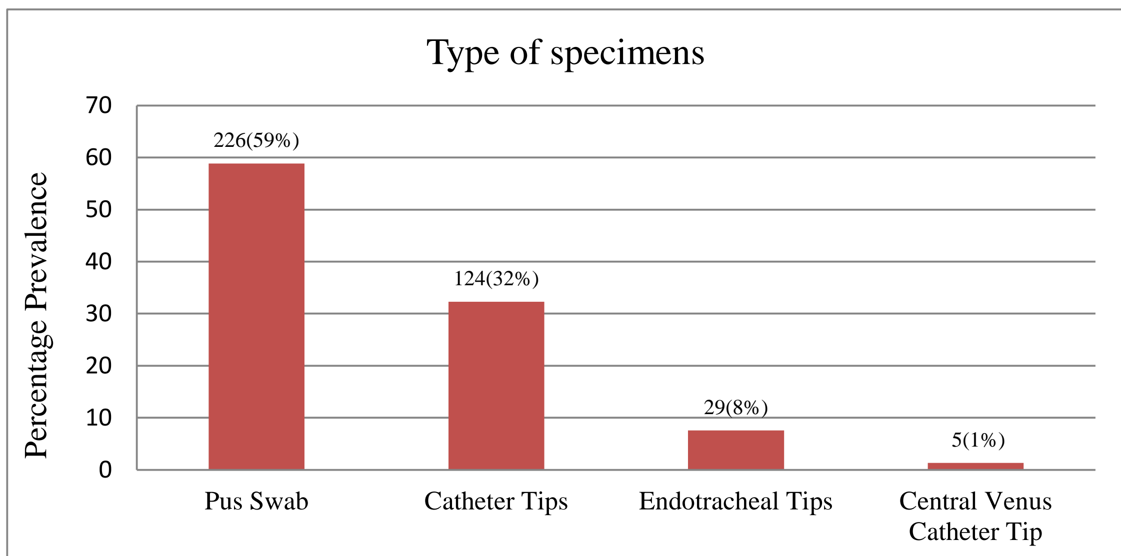

Figure 4.1: Type of specimens collected in the study.

As shown in Figure 4.1, pus swab was the most commonly collected sample (n=226) followed by catheter tube tips (n=124), endotracheal tube tips (n=29) while central venous catheter tube tips were the least collected (n=5) among the total number of samples collected from the two study sites. More pus swabs (n=120) were collected from Coast General Hospital while Mombasa hospital was leading in central venous catheter tube tips samples (n=5) collected. Mombasa hospital collected the highest

number of catheter tips (n=68) while Coast General Hospital was leading in endotracheal tube samples (n=16). There was no significant difference in the type of samples collected during the entire study period between the two hospitals (p= 0.056).

4.2 Prevalence of *Pseudomonas* species in the study hospitals

Out of the 384 samples collected 50(13%) were positive for *Pseudomonas* species while 334(87%) were negative. The overall prevalence of *Pseudomonas* species in Mombasa County was 13%. Of the *Pseudomonas* positive samples, 23(46%) were isolated from Mombasa Hospital while 27(54%) were isolated from Coast General Hospital. Overall, the prevalence of *Pseudomonas* at Mombasa Hospital was 12% (23/192) while at Coast General Hospital it was 14.1% (27/192) (Figure 4.2). However the difference in prevalence of the *Pseudomonas* species between the two study hospitals was not statistically significant (p = 0.544).

4.2.1 *Pseudomonas* species isolated

The predominant isolate from this study was *Pseudomonas aeruginosa* 41(82%), followed by *Pseudomonas fluorescence* 3(6%), *Pseudomonas stutzeri* 3(6%), *Pseudomonas luteola* 2(4%), and *Pseudomonas oleovorans* 1(2%) (Figure 4.3).

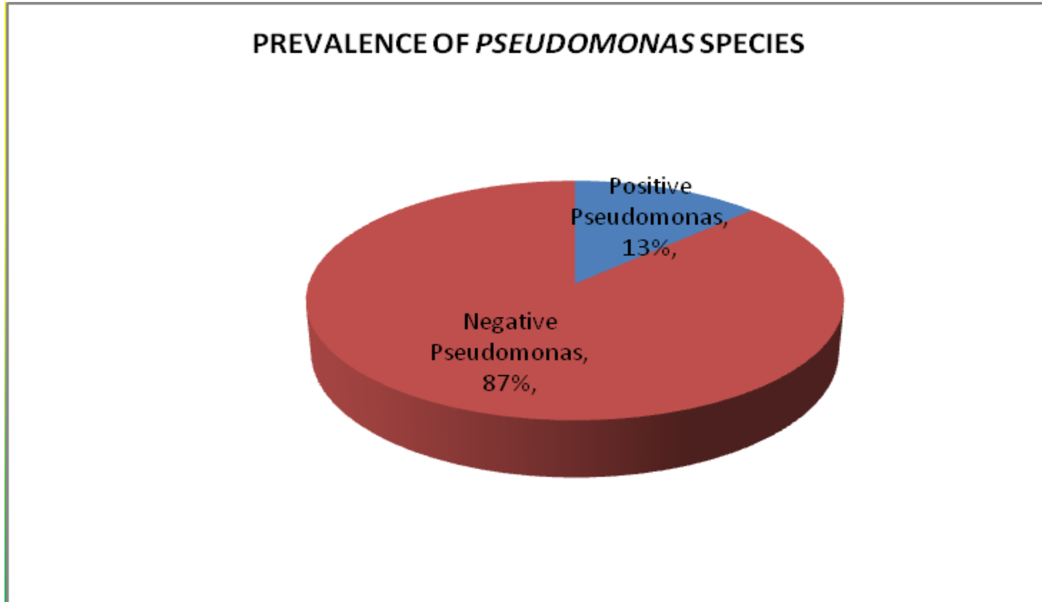

Figure 4.2: Prevalence of *Pseudomonas* in the study area.

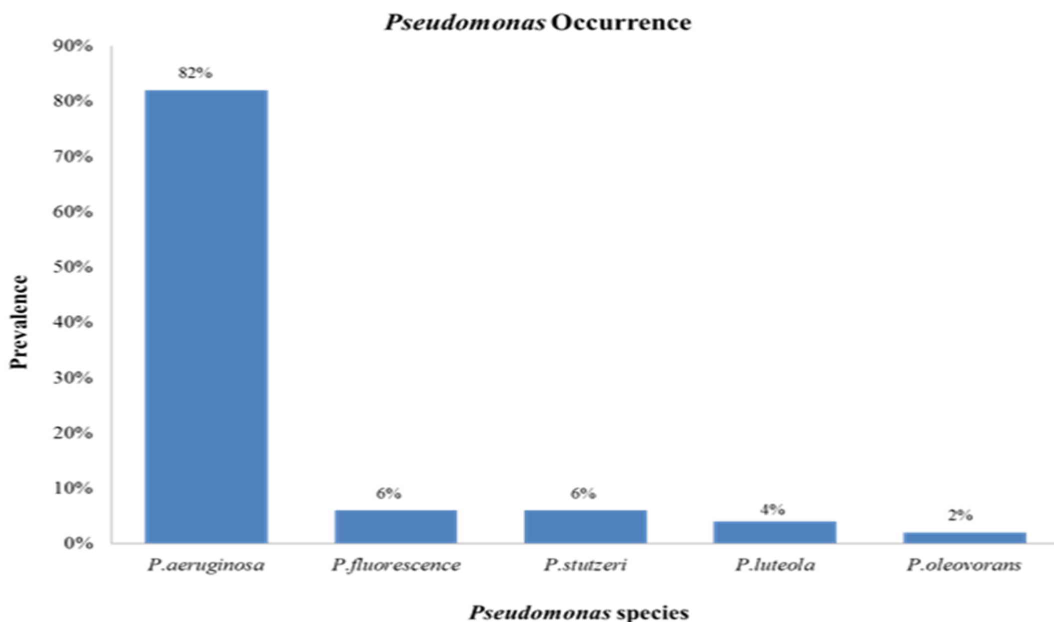

Figure 4.3: *Pseudomonas* species isolated from the study facilities.

4.2.2 Distribution of *Pseudomonas* species in various clinical samples

Isolation of *Pseudomonas* species was highest from pus swab specimens 30(60%) followed by catheter tips 11(22%) and finally endotracheal tubes tips 9(18%). There was no *Pseudomonas* isolation reported from central venous catheter tips. The number of *Pseudomonas* isolates from pus swab specimens was higher in Coast Province

General Hospital (66.7%) as compared to Mombasa Hospital (33.3%). Catheter tip specimens collected at Mombasa Hospital had the highest *Pseudomonas* burden (72.7%) as compared to Coast Province General Hospital (27.3%). The percentage prevalence of *Pseudomonas* isolates in endotracheal tip specimens was low at 18% from the two study hospitals as compared to the other specimens. Mombasa Hospital had 55.6% of the *Pseudomonas* isolates while Coast Province Hospital had 44.4%. There was no *Pseudomonas* isolated from central venous catheter tips in this study as shown in Table 4.1.

Table 4.1: Distribution of *Pseudomonas* isolates among clinical specimens

Specimen Type	CPGH (%)	Msa Hospital (%)	All Hospitals (%)
Pus Swab	20(66.7)	10(33.3)	30(60)
Catheter Tip	3(27.3)	8(72.7)	11(22)
Endotracheal Tip	4(44.4)	5(55.6)	9(18)
CVC	0	0	0

4.3 Antibiotic susceptibility patterns in *Pseudomonas* species

Antibiotic susceptibility of 50 *Pseudomonas* isolates against 26 antibiotics used in this study indicated that, in general *Pseudomonas* species showed a higher resistance to most of the antibiotics. The highest resistance recorded was against Ampicillin (96%) while the most sensitive antibiotic was Colistin (88%). *Pseudomonas* species demonstrated a higher resistance against first, second and third generation cephalosporin family of antibiotics used in this study. The highest resistance observed was with Cefazolin 88%, cefuroxime 84%, Cephoxitin 82%, Cefotaxime 80% and

Ceftriaxone 78%. The isolates showed minimal resistance 32% against fourth generation cephalosporins, cefepime (Table 4.2).

The percentage *Pseudomonas* species resistance to beta lactam inhibitor antibiotic was higher in this study. Out of all the isolates, 88%, showed resistance `to ampicillin sulbactam, 84%, to amoxicillin clavulanic acid, 72%, to ticarcillin clavulanic acid and 60% to piperacillin tazobactam. Resistance to Ampicillin was recorded the highest (96 %) compared to all the other antibiotics used in this study. The 86%, of the isolates showed resistance against nitrofurantoin, trimethoprim/sulfamethaxazole 78%, minocycline 68% and tobramycin 52% (Table 4.2).

Susceptibility to carbapenems was observed in most of the *Pseudomonas* isolates, 80% were sensitive to Meropenem while 78% to imipenem. Higher susceptibility was also observed with polymixin E (colistin, 88 %). Sensitivity also varied among other antibiotics such as aminoglycosides, (Gentamicin 86%, Amikacin 84%), and Fluoroquinolones (ciprofloxacin 66%, Perfloxacin 64%) in this study (Table 4.2).

P.fluorescence and *P.luteola* showed the highest sensitivity to most of the antibiotics used in this study as compared to the other *Pseudomonas* species. These two species did not show 100% resistance against ampicillin as compared to the other species (Table 4.2).

Table 4.2: Antibiotic resistance among *Pseudomonas* isolates

Drugs to which isolates were resistant	% resistance of *Pseudomonas* species					
	P.aeruginosa	*P.fluorescence*	*P.oleovorans*	*P.luteola*	*P.stutzeri*	**Total**
	N=41 (%)	N=3 (%)	N=1 (%)	N=2 (%)	N=3 (%)	N=50 (%)
Colistin	(15)	0	0	0	0	(12)
Gentamicin	(17)	0	0	0	0	(14)
Amikacin	(20)	0	0	0	0	(16)
Meropenem	(24)	0	0	0	0	(20)
Imipenem	(27)	0	0	0	0	(22)
Ceftazidime	(29)	0	0	0	0	(24)
Piperacillin	(37)	0	0	0	0	(30)
Cefepime	(39)	0	0	0	0	(32)
Ciprofloxacin	(32)	0	(100)	(100)	(33)	(34)
Perfloxacin	(44)	0	0	0	0	(36)
Tobramycin	(63)	0	0	0	0	(48)
Piperacillin/tazobactam	(71)	0	(100)	0	0	(60)
Minocycline	(78)	0	0	(100)	0	(68)
Ticarcillin	(88)	0	0	0	0	(72)
Ticarcillin/clavulanic acid	(88)	0	0	0	0	(72)
Ceftriaxone	(85)	0	(100)	0	(100)	(78)
Trim/sulphamethoxazole	(90)	0	(100)	(50)	0	(78)
Cefotaxime	(98)	0	0	0	0	(80)
Cefuroxime axetil	(90)	0	(100)	0	(100)	(82)
Cephoxitin	(90)	0	(100)	0	(100)	(82)
Cefuroxime	(90)	(33)	(100)	0	(100)	(84)
Amoxicillin/clavulanic acid	(93)	(67)	(100)	0	(33)	(84)
Nitrofurantoin	(95)	0	(100)	0	(100)	(86)
Ampicillin/sulbactam	(93)	(67)	(100)	0	(100)	(88)
Cefazolin	(95)	(33)	(100)	0	(100)	(88)
Ampicillin	(100)	(67)	(100)	(50)	(100)	(96)

4.3.1 Antibiotic susceptibility of *Pseudomonas aeruginosa* isolates

P. aeruginosa isolates showed 100% resistance against Ampicillin in both the two study facilities. More of *Pseudomonas aeruginosa* isolates that showed 100% resistance against the rest of the antibiotics used was from Mombasa hospital. This was observed against 35% of the antibiotics used. In CPGH, apart from ampicillin there was no 100% *Pseudomonas aeruginosa* resistance observed against the remaining antibiotics. However, were observed higher resistance against Cefazolin, cefotaxime, nitrofurantoin (95%), amoxicillin/clavulanic acid, trimethoprim/sulphamethoxazole (86%), cefuroxime, cefuroxime axetil, Cephoxitin (82%), ticarcillin, ticarcillin/clavulanic acid (77%) and ceftriaxone (73%). In Mombasa hospital, higher resistance above 50% in *P. aeruginosa* isolates was observed against 65% of the antibiotics used in this study (Table 4.3).

Table 4.3: Antibiotics susceptibility in *Pseudomonas aeruginosa* isolates

ANTIBIOTICS	Frequency of *Pseudomonas aeruginosa* isolates susceptibility			
	CPGH		Msa Hospital	
	Sensitive n (%)	Resistant n(%)	Sensitive n (%)	Resistant n (%)
Amikacin	20(91)	2(9.1)	13(68)	6(32)
Amoxicillin/clavulanic acid	3(14)	19(86)	0	19(100)
Ampicillin	0	22(100)	0	19(100)
Ampicillin/sulbactam	2(9.1)	20(91)	1(5)	18(95)
Cefazolin	1(4.5)	21(95)	1(5)	18(95)
Cefepime	13(59)	9(41)	12(63)	7(37)
Cefotaxime	1(4.5)	21(95)	0	19(100)
Ceftazidime	17(77)	5(23)	12(63)	7(37)
Ceftriaxone	6(27)	16(73)	0	19(100)
Cefuroxime	4(18)	18(82)	0	19(100)
Cefuroxime axetil	4(18)	18(82)	0	19(100)
Cephoxitin	4(18)	18(82)	0	19(100)
Ciprofloxacin	16(73)	6(27)	12(63)	7(37)
Colistin	19(86)	3(14)	16(84)	3(16)
Gentamicin	19(86)	3(14)	15(79)	4(21)
Imipenem	19(86)	3(14)	11(58)	8(42)
Meropenem	19(86)	3(14)	12(63)	7(37)
Minocycline	8(36)	14(64)	1(5)	18(95)
Nitrofurantoin	1(4.5)	21(95)	1(5)	18(95)
Perfloxacin	14(64)	8(36)	9(47)	10(53)
Piperacillin	12(55)	10(45)	14(74)	5(26)
Piperacillin/tazobactam	11(50)	11(50)	1(5)	18(95)
Ticarcillin	5(23)	17(77)	0	19(100)
Ticarcillin/clavulanic acid	5(23)	17(77)	0	19(100)
Tobramycin	13(59)	9(41)	2(11)	17(89)
Trimethoprim /sulphamethoxazole	3(14)	19(86)	1(5)	18(95)

4.3.2 Antibiotic susceptibility of *Pseudomonas fluorescence* isolates

Susceptibility profiles of the three (3) *P. fluorescence* isolates in the two study hospitals were varying. Mombasa hospital had isolated only one *Pseudomonas fluorescence* that showed 100% susceptibility to 96% of the antibiotics used in this

study except ciprofloxacin. Coast Province General Hospital (CPGH) had isolated two *Pseudomonas fluorescence*, which were susceptible to majority of the antibiotics used. In (CPGH) *Pseudomonas,* isolates showed 100% resistance against three antibiotics only (amoxicillin/clavulanic acid, ampicillin and ampicillin/sulbactam). In (CPGH) 50% of the isolates were susceptible to cefazolin and cefuroxime while the other half showed resistance to the same antibiotics, (Table 4.4)

4.3.3 Antibiotic susceptibility of *Pseudomonas luteola* isolates

Two *Pseudomonas luteola* isolates were obtained in this study with each study hospital having one isolate. The isolates showed (100%) susceptibility to majority of the antibiotics used. In CPGH, the isolate showed resistance against ampicillin, ciprofloxacin and minocycline. In Mombasa hospital, the isolate also showed (100%) resistance against ciprofloxacin, minocycline and Trimethoprim /sulphamethoxazole. In general, the isolates showed higher susceptibility to majority of the antibiotics 88.5% used in this study. (Table 4.5)

Table 4.4: Antibiotics susceptibility in *Pseudomonas fluorescence* isolates

ANTIBIOTICS	Frequency of *Pseudomonas fluorescence* isolates susceptibility			
	CPGH		Msa Hospital	
	Sensitive n (%)	Resistance n (%)	Sensitive n (%)	Resistance n (%)
Amikacin	2(100)	0	1(100)	0
Amoxicillin/clavulanic acid	0	2(100)	1(100)	0
Ampicillin	0	2(100)	1(100)	0
Ampicillin/sulbactam	0	2(100)	1(100)	0
Cefazolin	1(50)	1(50)	1(100)	0
Cefepime	2(100)	0	1(100)	0
Cefotaxime	2(100)	0	1(100)	0
Ceftazidime	2(100)	0	1(100)	0
Ceftriaxone	2(100)	0	1(100)	0
Cefuroxime	1(50)	1(50)	1(100)	0
Cefuroxime axetil	2(100)	0	1(100)	0
Cephoxitin	2(100)	0	1(100)	0
Ciprofloxacin	2(100)	0	0	1(100)
Colistin	2(100)	0	1(100)	0
Gentamicin	2(100)	0	1(100)	0
Imipenem	2(100)	0	1(100)	0
Meropenem	2(100)	0	1(100)	0
Minocycline	2(100)	0	1(100)	0
Nitrofurantoin	2(100)	0	1(100)	0
Perfloxacin	2(100)	0	1(100)	0
Piperacillin	2(100)	0	1(100)	0
Piperacillin/tazobactam	2(100)	0	1(100)	0
Ticarcillin	2(100)	0	1(100)	0
Ticarcillin/clavulanic acid	2(100)	0	1(100)	0
Tobramycin	2(100)	0	1(100)	0
Trimethoprim/sulphamethoxazole	2(100)	0	1(100)	0

Table 4.5: Antibiotics susceptibility in *Pseudomonas luteola* isolates

ANTIBIOTICS	Frequency of *Pseudomonas luteola* isolates susceptibility			
	CPGH		Msa Hospital	
	Sensitive n (%)	Resistance n (%)	Sensitive n (%)	Resistance n (%)
Amikacin	1(100)	0	1(100)	0
Amoxicillin/clavulanic acid	1(100)	0	1(100)	0
Ampicillin	0	1(100)	1(100)	0
Ampicillin/sulbactam	1(100)	0	1(100)	0
Cefazolin	1(100)	0	1(100)	0
Cefepime	1(100)	0	1(100)	0
Cefotaxime	1(100)	0	1(100)	0
Ceftazidime	1(100)	0	1(100)	0
Ceftriaxone	1(100)	0	1(100)	0
Cefuroxime	1(100)	0	1(100)	0
Cefuroxime axetil	1(100)	0	1(100)	0
Cephoxitin	1(100)	0	1(100)	0
Ciprofloxacin	0	1(100)	0	1(100)
Colistin	1(100)	0	1(100)	0
Gentamicin	1(100)	0	1(100)	0
Imipenem	1(100)	0	1(100)	0
Meropenem	1(100)	0	1(100)	0
Minocycline	0	1(100)	0	1(100)
Nitrofurantoin	1(100)	0	1(100)	0
Perfloxacin	1(100)	0	1(100)	0
Piperacillin	1(100)	0	1(100)	0
Piperacillin/tazobactam	1(100)	0	1(100)	0
Ticarcillin	1(100)	0	1(100)	0
Ticarcillin/clavulanic acid	1(100)	0	1(100)	0
Tobramycin	1(100)	0	1(100)	0
Trimethoprim /sulphamethoxazole	1(100)	0	0	1(100)

4.3.4 Antibiotic susceptibility of *Pseudomonas stutzeri* isolates

Three *Pseudomonas stutzeri* isolates were obtained in this study. CPGH isolated two *Pseudomonas stutzeri* while Mombasa Hospital had only one isolate. In CPGH, the isolates showed (100%) resistance against ampicillin, ampicillin sulbactam, Cefazolin, ceftriaxone, cefuroxime, cefuroxime axetil, cefoxitin, and nitrofurantoin. In Mombasa, hospital the isolate showed (100%) resistance against amoxicillin/clavulanic acid, ampicillin, ampicillin/sulbactam, Cefazolin, ceftriaxone, cefuroxime, cefuroxime/axetil, cefoxitin, ciprofloxacin and nitrofurantoin. *Pseudomonas stutzeri* isolates showed (100%) susceptibility to Majority of the antibiotics used in this study. *P. stutzeri* showed 100% susceptibility to 69% of the antibiotics in CPGH while in Msa hospital; only 62% of the antibiotics were susceptible. (Table 4.6)

4.3.5 Antibiotic susceptibility of *Pseudomonas oleovorans* isolates

There was no *P.oleovorans* isolated at CPGH. Msa hospital had a single isolate, which showed 100% resistance against 46% of the antibiotics used in this study. (Table 4.7)

Table 4.6: Antibiotics susceptibility patterns in *Pseudomonas stutzeri* isolates

ANTIBIOTICS	Frequency of *Pseudomonas stutzeri* isolates susceptibility			
	CPGH		Msa Hospital	
	Sensitive n (%)	Resistance n (%)	Sensitive n (%)	Resistance n (%)
Amikacin	2(100)	0	1(100)	0
Amoxicillin/clavulanic acid	2(100)	0	0	1(100)
Ampicillin	0	2(100)	0	1(100)
Ampicillin/sulbactam	0	2(100)	0	1(100)
Cefazolin	0	2(100)	0	1(100)
Cefepime	2(100)	0	1(100)	0
Cefotaxime	2(100)	0	1(100)	0
Ceftazidime	2(100)	0	1(100)	0
Ceftriaxone	0	2(100)	0	1(100)
Cefuroxime	0	2(100)	0	1(100)
Cefuroxime axetil	0	2(100)	0	1(100)
Cephoxitin	0	2(100)	0	1(100)
Ciprofloxacin	2(100)	0	0	1(100)
Colistin	2(100)	0	1(100)	0
Gentamicin	2(100)	0	1(100)	0
Imipenem	2(100)	0	1(100)	0
Meropenem	2(100)	0	1(100)	0
Minocycline	2(100)	0	1(100)	0
Nitrofurantoin	0	2(100)	0	1(100)
Perfloxacin	2(100)	0	1(100)	0
Piperacillin	2(100)	0	1(100)	0
Piperacillin/tazobactam	2(100)	0	1(100)	0
Ticarcillin	2(100)	0	1(100)	0
Ticarcillin/clavulanic acid	2(100)	0	1(100)	0
Tobramycin	2(100)	0	1(100)	0
Trimethoprim /sulphamethoxazole	2(100)	0	1(100)	0

Table 4.7: Antibiotics susceptibility patterns in *Pseudomonas oleovorans*

ANTIBIOTICS	Frequency of *Pseudomonas oleovorans* isolates susceptibility			
	CPGH		Msa Hospital	
	Sensitive (%)	Resistance (%)	Sensitive (%)	Resistance (%)
Amikacin	0	0	1(100)	0
Amoxicillin/clavulanic acid	0	0	0	1(100)
Ampicillin	0	0	0	1(100)
Ampicillin/sulbactam	0	0	0	1(100)
Cefazolin	0	0	0	1(100)
Cefepime	0	0	1(100)	0
Cefotaxime	0	0	1(100)	0
Ceftazidime	0	0	1(100)	0
Ceftriaxone	0	0	0	1(100)
Cefuroxime	0	0	0	1(100)
Cefuroxime axetil	0	0	0	1(100)
Cephoxitin	0	0	0	1(100)
Ciprofloxacin	0	0	0	1(100)
Colistin	0	0	1(100)	0
Gentamicin	0	0	1(100)	0
Imipenem	0	0	1(100)	0
Meropenem	0	0	1(100)	0
Minocycline	0	0	1(100)	0
Nitrofurantoin	0	0	0	1(100)
Perfloxacin	0	0	1(100)	0
Piperacillin	0	0	1(100)	0
Piperacillin/tazobactam	0	0	0	1(100)
Ticarcillin	0	0	1(100)	0
Ticarcillin/clavulanic acid	0	0	1(100)	0
Tobramycin	0	0	1(100)	0
Trimethoprim/sulphamethoxazole	0	0	0	1(100)

4.4 Molecular characterization of antibiotic resistance genes in *Pseudomonas* species

DNA was extracted from twenty (20) randomly selected *Pseudomonas* positive isolates. Two multiplex PCR assays were then conducted to amplify the metallo-beta-lactamase (MBL) and fluoroquinolone resistance conferring genes.

4.4.1 Metallo-betalactamase genes

All the five MBL genes screened were detected in the *Pseudomonas* isolates tested in this study. Out of the twenty antibiotic resistant *Pseudomonas* isolates screened, MBL genes were detected from eight of the isolates (40%). Some of the isolates had more than one gene each. VIM was the predominant MBL gene detected from six *Pseudomonas* isolates (75%). Others were SIM and SPM, which were detected in two different isolates and another isolate having both the SIM and SPM genes. IMP genes were detected in two of the isolates and GIM in one isolate. In general, one isolate had three MBL genes, five isolates had two MBL genes each while the remaining two isolates had one MBL gene each (Figure 4.4)

4.4.2 Fluoroquinolone resistance genes

Two fluoroquinolone resistance-conferring genes were screened in this study. All the twenty *Pseudomonas* isolates tested positive for the presence of the two-fluoroquinolone resistance conferring genes (MexR and ParC) (Figure 4.5)

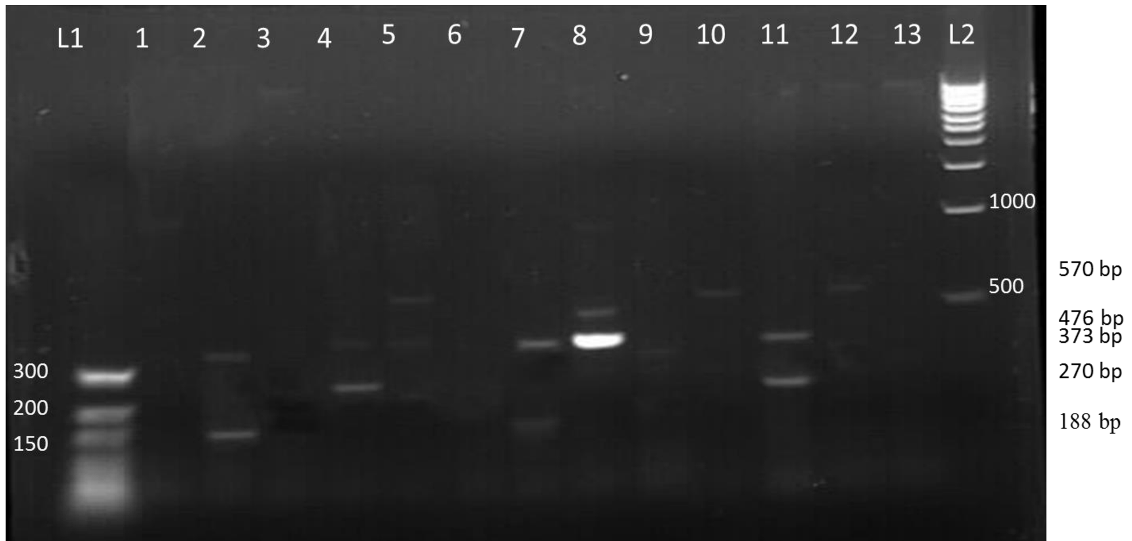

Figure 4.4: Gel image of PCR amplification of the 5 Metallo-betalactamase genes from 13 of the 20 isolates. Lanes 1-13 represent corresponding isolates and are numbered 1-13 while L1 and L2 are O'GeneRuler Ultra Low Range DNA Ladder and Sigma's Direct Load™ 1kb Ladder respectively. Lanes 2,4,5,7,8 and 11 were positive for VIM. Lanes 5,10 and 12 positive for SIM. Lanes 2 and 7 positive for IMP. Lanes 4 and 11 positive for SPM. Lane 8 positive for GIM.

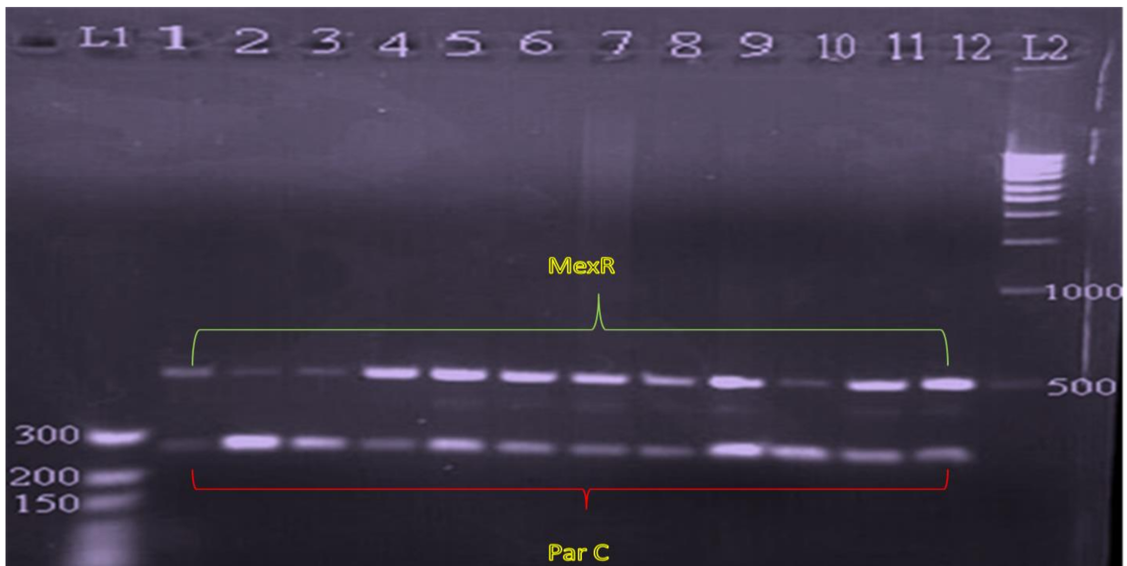

Figure 4.5: Gel image of PCR amplification of ParC and MexR genes from 12 of the 20 isolates. Lanes 1-12 represent corresponding isolates and are numbered 1-12 while L1 and L2 are O'GeneRuler Ultra Low Range DNA Ladder and Sigma's Direct Load™ 1kb Ladder respectively.

To assess the correlation between the phenotypic and molecular resistance, results from the molecular analysis were compared with the phenotypic antibiotic susceptibility profiles obtained in this study. The phenotypic antibiotic resistant results of most *Pseudomonas* isolates were comparable with the molecular findings obtained. Most *Pseudomonas* isolates showed phenotypic resistance of greater than 50% against most of the antibiotic used in this study (Table 4.2). On the other hand, some 34% of *Pseudomonas* isolates showed phenotypic resistance against fluoroquinolone ciprofloxacin and 36% against Perfloxacin, while 100% of the *Pseudomonas* isolates had the fluoroquinolone resistant conferring genes MexR and ParC (Table 4.2) (Figure 4.5).

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMENDATIONS

5.1 DISCUSSION

5.1.1 Prevalence

This study aimed at determining the prevalence, antibiotic susceptibility and molecular antibiotic resistance gene patterns of *Pseudomonas* isolates obtained from 384 different clinical samples. There was a variation in the type and number of samples collected between the two facilities under study. CPGH collected the highest number of pus swab (n=120) and endotracheal tube tips (n=16) specimens while Mombasa hospital was leading in catheter tube tips (n=68) and central venous catheter tube tips (n=5). In general, pus swab was the leading specimen collected during the entire study period (59%). The higher number of catheter tips in Mombasa hospital indicates that, most of the patients admitted were catheterized to facilitate direct drainage of the urinary bladder for diagnostic or therapeutic purposes.

The prevalence of *Pseudomonas* species observed in this study was 13%. The prevalence observed in this study was in line to that obtained in a study conducted in two study areas, Shaheed Beheshti Medical University Centre for Infectious Diseases Research and Loghman Hakim Hospital in Tehran Iran where they observed a prevalence rate of 13.2%. (Aminizadeh & Kashi, 2011).

This study detected only five *Pseudomonas* species out of the 50 positive *Pseudomonas* isolates from the two hospitals. The predominant species was *Pseudomonas aeruginosa* (82%), followed by *Pseudomonas stutzeri* (6%), *Pseudomonas fluorescence* (6%), *Pseudomonas luteola* (4%), and *Pseudomonas oleovorans* (2%). These results showed similarities with the study conducted by Singh *et al.,* 2015 at the Dr. Rajendra Prasad Government Medical College in Kangra

Tanda, India. In their study they found that the predominant isolates were *Pseudomonas aeruginosa* 337(84.2%), *Pseudomonas stutzeri* 28(7%), *Pseudomonas fluorescence* 17(4.2%), *Pseudomonas putida* 5(1.2%), *Pseudomonas pickettii* 5 (1.2%). The smaller variations in prevalence between the two studies might have been due to the different sample sizes used as well as the study design.

Among the 50 *Pseudomonas* isolates obtained in this study, 30 (60%) were from pus swabs, 11 (22 %) from catheter tube tips and 9 (18%) from endotracheal tube tips. There was no *Pseudomonas* isolated from central venous catheter tips. Pus swab samples were collected from different sites including surgical wounds, burn wounds as well as abscesses. In a study conducted in India by Singh *et al.,* 2015, similar results were observed. The higher *Pseudomonas* burden in pus swab might have been because of cross infection between healthcare workers and the patients during routine procedure.

There was observed in our study a higher rate of isolation of *Pseudomonas* from Mombasa hospital among catheter tips and endotracheal tips compared to CPGH. The study found out that 72.7% of catheter tips at Mombasa hospital had *Pseudomonas* compared to 27.7% at CPGH while 55.6% of endotracheal tips at Mombasa hospital harbored the bacteria compared to 44.4% at CPGH. Although there was no association between the site and the type of *Pseudomonas* specie isolated, it is important to note that most of the specimens yielded significant bacterial growth and were thus deemed to indicate infection. However, two previous studies done in Nigeria had associated specific micro-organisms with particular specimen types (Pondei, Fente, & Oladapo, 2013) but more studies are required to clarify this observation.

In this study, there was no *Pseudomonas* isolate from central venous catheter samples detected. This might have been contributed by the smaller sample size in central venous catheters collected during the study period. Only five central venous catheters were collected and all of them were from Mombasa hospital. This finding may also be contributed by the sterile conditions observed during the procedure. This study provided evidence of high prevalence of *Pseudomonas aeruginosa*, in the county and the existence of other *Pseudomonas* species.

5.1.2 Antibiotic Susceptibility

The drug-resistance pattern of *Pseudomonas* isolates obtained in this study indicates that the antibiotics that are the first line of therapy according to CLSI 2015 are still sensitive, showing higher susceptibility to these drugs, i.e., aminoglycosides such as gentamicin (86%), amikacin (84%) and ceftazidime (76%) and cefepime (68%) with an exception of piperacillin-tazobactam (40%). Determining antibiotic resistance pattern of antibacterial agents may assist in appropriate drug selection.

The susceptibility pattern of *Pseudomonas* species in this study showed *P.aeruginosa* to be the most resistant isolate against majority of the antibiotic tested compared to the other *Pseudomonas* species. This finding though not unexpected might be due to the smaller proportion of the other *Pseudomonas* species isolated in this study.

In this study majority of the isolates showed higher susceptibility to imipenem and meropenem. Similar findings were obtained in studies conducted at the University of Prishtina, Kosovo and Kahramanmaraş, Turkey where the isolates showed low resistance to imipenem and Meropenem (Deniz Yilmaz *et al.,* 2016, Shenoy *et al.,* 2002). However, other studies from India showed a much higher resistance to

imipenem (66.6%) (Sivanmaliappan & Sevanan, 2011). This pattern of resistance indicates a probable overuse of broad-spectrum antibiotics like carbapenems.

In the current study the percentage *Pseudomonas aeruginosa* resistance to ampicillin was (100%) which is similar to the data in a study conducted in India (Sivanmaliappan & Sevanan, 2011). Comparable rates were also reported at the Lagos University Teaching Hospital, Nigeria in a study conducted by Akingbade *et al.,* 2012 *P.fluorescence* showed antibiotic susceptibility to ceftazidime, piperacillin/tazobactam, gentamicin, tobramycin and colistin (Table 4.4). Similar findings were observed in a study conducted in India by (Trivedi, 2015) where the bacteria was sensitive to the five antibiotics used. In the same study, the bacteria showed resistance to ticarcillin/clavulanic acid and trimethoprim sulphamethoxazole, which were in disagreement with the results obtained in this study where *P.fluorescence* showed 100% susceptibility to ticarcillin/clavulanic acid, and trimethoprim sulphamethoxazole (Table 4.4). These differences in the resistance rates are probably related to differences in antibiotic use in different settings and selective pressure.

Pseudomonas luteola showed resistance to ciprofloxacin where similar findings were obtained in a study conducted by Yousefi *et al.,* (2014). *P.luteola* showed susceptibility to colistin and piperacillin/tazobactam (Table 4.5). This results were in disagreement with the findings obtained in two studies conducted in Turkey (Bayhan *et al.*, 2015; Senel & Children, 2014). In the same studies conducted in Turkey (Bayhan *et al.,* 2015; Senel & Children, 2014) *P.luteola* showed susceptibility to amikacin, gentamicin and meropenem which were in line with the findings obtained in this study.

Previous studies conducted by Bisharat *et al.,* (2012) showed *Pseudomonas stutzeri* susceptibility to gentamicin (99%), Ofloxacin (99%), amikacin (98%), imipenem (98%), ciprofloxacin (97%), meropenem (97%), ceftazidime (95%), piperacillin (93%), polymixin-B (92%), trimethoprim-sulfamethoxazole (91%), piperacillin/tazobactam (91%), cefepime (71%),ceftriaxone (60%), amoxicillin-clavulanic acid (50%), cefotaxime (50%), nitrofurantoin (27%), cefuroxime (14%) and Cephoxitin (12.5%). In this study, *P.stutzeri* showed 100% resistance to ampicillin, ampicillin-sulbactam, Cefazolin, cefuroxime, ceftriaxone, cefuroxime axetil, Cephoxitin and nitrofurantoin (Table 4.6). This finding could be due to the small sample size of this bacterial isolate in this study

P. oleovorans was isolated only in Mombasa hospital. This isolate showed resistance to amoxicillin/clavulanic acid, ampicillin, ampicillin/sulbactam, cefazolin, cefuroxime, cefuroxime axetil, Cephoxitin, ciprofloxacin, nitrofurantoin, piperacillin/tazobactam and trimethoprim/sulphamethoxazole among the antibiotics used in this study (Table 4.7). In the study findings by Gautam *et al.,* (2015), *Pseudomonas oleovorans* exhibited susceptibility to ceftazidime, amikacin, piperacillin/tazobactam and colistin. The isolates also showed resistance to ciprofloxacin and levofloxacin.

The current study observed variability in the antibiotic resistant patterns between the two-study hospitals. More of resistant isolates were detected at The Mombasa Hospital than in CPGH. This finding may be due to a lack of regional antibiogram data and also may be due to overuse of antibiotics by patients at Mombasa Hospital than CPGH.

5.1.3 Molecular characterization of antibiotic resistance genes

A major clinical problem regarding *Pseudomonas* infection is due to their possession of metallo-beta-lactamase (MBL) genes. As a result, this study sought to investigate the distribution of these genes. All the five MBL genes screened in this study were detected in the *Pseudomonas* isolates. This suggests that majority of the *Pseudomonas* isolates were resistant to carbapenems antibiotics, a finding that was corroborated by the phenotypic antibiotic susceptibility results. VIM was the most commonly identified MBL gene in the current study (Figure 4.4). This finding is in agreement with that of a previous study at the Aga khan Hospital, Nairobi which identified VIM in all the isolates studied (Muthoni, 2014). These findings are also in agreement with the findings observed in a study among several hospitals in Zeheden Iran which showed that VIM was the most predominant MBL gene detected Ghamgosha *et al.,* (2015).

The identification in this study of the MBL genes represents the first documentation of their presence in *Pseudomonas* isolates in the country. However, this is not unexpected phenomenon bearing in mind that bacterium harboring some or all these genes have been detected in other species locally and elsewhere respectively. Factors such as horizontal gene transfer across species, increases in international travel and medical tourism that involves patient transfer between countries raises the possibility of rapid dissemination of these MBL genes in the country.

The two-fluoroquinolone resistance conferring genes were detected in the current study albeit at a higher frequency than the MBL genes. All the isolates tested in this study showed the presence of the two fluoroquinolones resistance-conferring genes under investigation. P*arC* has been detected previously in *E.coli* and *Shigella* isolates from patients with diarrhea from Machakos District Hospital (Juma *et al.,* 2016). With

the possibility of horizontal gene transfer, the presence of these genes among the *Pseudomonas* isolates is not unexpected. The presence of M*exR* gene in the country has not been previously documented. The detection of this gene in the current study may be attributed to the rapid international travel and medical tourism.

In a study conducted by (Savov & Kardjeva, 2014) similar findings as in this study were obtained where all the *Pseudomonas* isolates demonstrated higher frequency of fluoroquinolones resistance conferring genes. These study findings however are in disagreement with those obtained in a study conducted in California,USA where M*exR* was the most predominant fluoroquinolone resistance conferring gene detected (Neda Gorgania *et al.,* 2010). An important observation was also made between the phenotypic results and the genotypic results. While phenotypic results indicate a 34-36 % resistance to the fluoroquinolones tested, the genotypic results indicate a 100% resistance. Similar nonconcordance findings were observed in a different study conducted (Binyamin, Pastukh, On, Paritsky, & Peretz, 2017). This finding is not unexpected. It is generally known that genotype is not always predictive of phenotype due to factors including poor gene expression, non-functional expressed gene products, presence of other genes or via other mechanisms that are not known (Taitt *et al.,* 2017).

5.2 CONCLUSION

Pseudomonas species were found prevalent in the two Mombasa County hospitals. A prevalence of 13% was obtained where *Pseudomonas aeruginosa* isolates showed highest incidence followed by *Pseudomonas fluorescence Pseudomonas stutzeri, Pseudomonas luteola* and *Pseudomonas oleovorans*. Apart from *Pseudomonas*

aeruginosa, there are other *Pseudomonas* species isolated from clinical samples that cause infections.

Pseudomonas isolates exhibited high to moderate levels of resistance against different classes of antibiotics. Colistin, Gentamicin, Amikacin, Meropenem, imipenem, ceftazidime, Piperacillin and Cefepime were suitable against *Pseudomonas infections*. The susceptibility data from this report may be worth consideration while implementing empiric treatment strategies for *Pseudomonas* infections. At the same time, Updated knowledge of antimicrobial susceptibility profiles of clinical isolates will not only assist in designing the most appropriate dose-regimen and treatment schedule against *Pseudomonas* infections but also help in curbing the alarmingly expanding menace of drug resistance

Our study showed the presence of MBL and fluoroquinolones resistance conferring genes in *Pseudomonas* isolates for the first time in Mombasa County. This underscores the necessity of screening all antibiotic-resistant *Pseudomonas* isolates for MBL production as well as fluoroquinolones resistance conferring genes and advocating for the implementation of infection control programs to prevent spread of such organisms. Early and accurate detection of antibiotic resistant genes may control the spread of multi drug resistant pathogens in the future.

5.3 RECOMMENDATIONS

Clinicians and other healthcare workers need to follow the appropriate safe medical procedures and give attention to the immunocompromised patients for the prevention and control of hospital-acquired infections. Regular surveillance in infection control should be conducted in hospital settings to monitor the occurrence of pathogenic microbes.

Strict health policies need to be implemented to regulate the purchase and prescription of antibiotic use. There should be a continuous monitoring and reporting of antibiotic resistance.

The use of molecular techniques aids in antibiotic resistant genes detection in regional laboratories that provide the appropriate diagnosis and identification of outbreaks by antibiotic resistant genes producing pathogens. Thus, regular surveillance of antibiotic resistant genes producing *Pseudomonas* isolates along with judicious use of antibiotics may prevent the spread of drug resistance

5.4 RECOMMENDATIONS FOR FUTURE RESEARCH

1. A larger sample size should be used to determine the possible presence of other disease causing *Pseudomonas* species in the region.

2. Studies should be carried to determine the susceptibility of other antipseudomonal drugs that were not included in this study like monobactams e.g. aztreonam

3. Further studies should be conducted to determine whether the MBL and fluoroquinolone resistance-conferring genes are transferable or not.

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APENDICES

Appendix i: Ethical approval letter from Kenyatta University Ethical Review Committee

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Email: chairman.kuerc@ku.ac.ke
secretary.kuerc@ku.ac.ke
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P. O. Box 43844 - 00100 Nairobi
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Our Ref: KU/R/COMM/51/557

Date: 16th October, 2015

Issa Suleiman Mwinyikombo
Kenyatta University,
P.O Box 43844,
Nairobi.

Dear Suleiman,

RE APPLICATION NUMBER PKU/398/I 367- "DETECTION AND MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANCE IN PSEUDOMONAS ISOLATES IN HOSPITAL IN MOMBASA, KENYA".- VERSION 2

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic, "Detection and molecular characterization of antibiotic resistance in pseudomonas isolates in hospital in Mombasa, Kenya" - Version 2 dated 16th October, 2015.

2. APPLICANT

Issa Suleiman Mwinyikombo

3. STUDY SITE

Mombasa County, Kenya

4. DECISION

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines AND APPROVED that the research may proceed for a period of ONE year from 16th October, 2015.

5. ADVICE/CONDITIONS

i. Progress reports are submitted to the KU-ERC every six months and a full report is submitted at the end of the study.
ii. Serious and unexpected adverse events related to the conduct of the study are reported to this board immediately they occur.
iii. Notify the Kenyatta University Ethics Committee of any amendments to the protocol.
iv. Submit an electronic copy of the protocol to KUERC.

When replying, kindly quote the application number above.

If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.

for: PROF. NICHOLAS K. GIKONYO
CHAIRMAN ETHICS REVIEW COMMITTEE

I Issa Suleiman accept the advice given and will fulfill the conditions therein.

Signature.................................. Dated this day of 16th October 2015.

cc. Vice-Chancellor

Appendix ii: Ethical approval letter from CPGH Ethical Review Committee

MOMBASA COUNTY GOVERNMENT

Telegrams: "MEDICAL", Mombasa
Phone: Mombasa 2314202/5, 2222148, 2225845
Fax: 2220161 E-mail: chiefadmin@cpgh.co.ke
Address all correspondence to the Chief Admin.
When replying, please quote Ref. No. & date.

COAST PROVINCE GENERAL HOSPITAL
P.O. BOX 90231
MOMBASA

Ref. No. ERC-CGH/VOL.I **Date: 24th MAY, 2016**

Issa Suleiman Mwinyikombo
P.O. Box 96275
MOMBASA.

RE: DETECTION AND MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANCE IN PSEUDOMONAS ISOLATES IN SELECTED HOSPITALS IN MOMBASA COUNTY OF KENYA

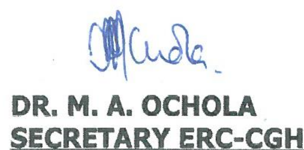
Reference is made to your letter dated 15th December, 2016. The Ethics Review Committee acknowledges receipt of your protocol.

This is to inform you that the Ethics Review Committee reviewed the document submitted and is satisfied that the issues raised at the meeting of Ethics Review Committee on 20th April, 2016 have been adequately addressed.

The study is granted approval for implementation effective from the date of this letter. Please note that authorization to conduct this study will automatically expire on the 20th April, 2017. If you plan to continue with data collection and analysis beyond this date, please submit an application for continuing approval to the ethical Review Committee-Coast General Hospital in appropriate time.

Any unanticipated problem resulting from the implementation of this protocol should be brought to the attention of the ERC-CGH. You are also required to submit any changes to this protocol to the ERC- CGH.

The ERC-CGH looks forward to receiving a summary of the research findings upon completion of the study to be part of the data base to be consulted when processing related researches to minimize duplication.



DR. M. A. OCHOLA
SECRETARY ERC-CGH