ANALYSIS OF ANTIMICROBIAL RESISTANCE PATTERNS, GENETIC BASIS OF RESISTANCE AND PHYLOGENETIC RELATEDNESS IN CLINICAL *PSEUDOMONAS AERUGINOSA* ISOLATES FROM IN-PATIENTS AT KENYATTA NATIONAL HOSPITAL

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Analysis of Antimicrobial Resistance Patterns, Genetic Basis of Resistance and Phylogenetic Relatedness in clinical *Pseudomonas aeruginosa* isolates from in-patients at Kenyatta National Hospital

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DECLARATION

This thesis is my original work and has not been presented for a degree at any other university.

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DEDICATION

Special dedication goes to my late parents Hezrom Mukaya Mujumba and Elika Ngoya Mukaya for nurturing and relentlessly supporting me thereby making me become the person that I am today.

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LIST OF ABBREVIATIONS

CCU - Critical Care Unit

CLSI - Clinical and Laboratory Standards Institute

CS - Cystic Fibrosis

DNA - Deoxyribonucleic acid

ERC - Ethical Review Committee

EsβL - Extended spectrum β-lactamases

ICU - Intensive Care Unit

KEMRI - Kenya Medical Research Institute

KNH - Kenyatta National Hospital

MDR - Multidrug Resistance

PCR - Polymerase Chain Reaction

RFLP - Restriction fragment length polymorphism

UV - Ultra violate light

VCR - Variable cassette region

ABSTRACT

This study sought to determine antimicrobial susceptibility profiles of *Pseudomonas aeruginosa* strains recovered from in-patient population at the Kenyatta National Hospital, the largest referral hospital in Kenya. A total of 188 *P. aeruginosa* strains were obtained from different in-patient wards from August 2015 to January 2016. Minimum inhibitory concentrations (MICs) were conducted on the Vitek 2-Compact (Biomereux company-France). High resistance in *P. aeruginosa* isolates was recorded towards tetracycline (92%) with an MIC of ≥ 128µg/ml followed by cefotaxime (88.8%) and ceftriaxone (86.2%) with MICs of ≥ 64µg/ml. Lowest resistance was recorded towards piperacillin (25%) and amikacin (46.3%). *P. aeruginosa* isolates recovered from the Intensive Care Unit (ICU) recorded the highest resistance proportion of 83% to all the antimicrobial tested while least resistance proportions was observed among strains from the Newborn unit (NBU) ward (38%). On the other hand, majority (92%) of isolates obtained from urine specimen were resistant to any given antimicrobial drug tested while lowest resistance was recorded among isolates obtained from blood (29%).Resistance to CAZ, CIP, CN, and AMK was 82.4%, 80.9%, 88.2% and 78% respectively. A high proportion (86%) of the MBL positive strains were recovered from patients in the CCU, followed by the medical wards (13%), while new born unit was the least affected (1%). From a total of 127 *P. aeruginosa* that were resistant to meropenem, (68%) were positive for bla_{NDM}, a carbapenemase while 64 isolates harbored $bla_{VEB,}$ an ESBL gene. A total of 45 isolates tested positive for both bla_{NDM}, bla_{VEB} and for class 1 integron. A single isolate from tracheal aspirate sample from a 39 years old female admitted in the ICU harbored NDM, VEB, integron class 1 and 3. Plasmid screening revealed 3 types of incompatibility groups, *inc*W, *inc*FIB and *inc*FIB. One *P. aeruginosa* isolate had both *inc*W and *inc*FIB, while another had an *inc*N. Phylogenic cluster analysis using the Gelcompar2® revealed four major clusters based on age, specimen type and wards. The four clusters had a significant genetic similarity of >80% amongst *P. aeruginosa* strains obtained from different wards which is indicative of cross-infection. The high resistance recorded in this study is therefore worrying and may impair our ability to combat severe *P. aeruginosa* infections. Resistance to β-lactams, aminoglycosides and fluoroquinolones further narrows down the available treatment options considering carbapenems are not readily available in Kenya like other developing countries. Even worse, in absence of proper usage of these agents it could partially explain why resistance to carbapenems is on the rise. If left unchecked, this resistance may lead to drastic increase in pan-resistance strains that may cause in turn high mortality in hospital and community settings. The strong evidence of clonal spread in various wards show that this problem is not confined in a specific unit and therefore all relevant bodies should engage to help contain this problem.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Pseudomonas aeruginosa is one of the common cause of nosocomial infections globally (Zavascki *et al*., 2010). The prevalence of this organism is approximately 29% in Europe alone and has been implicated in many cases of blood stream and urinary tract infections (Nathwani *et al*., 2014b).The other serious infections that have been associated with this organism include wound, respiratory, skin and burn infections (Ahmed *et al.,* 2009,). Despite of the health challenge posed by this organism, the prevalence and disease burden in developing countries remains underestimated due poor diagnosis and lack of baseline surveillance.

The intrinsic resistance in *P. aeruginosa* has led to treatment failure leading to prolonged hospitalization which is traumatizing to the patients and their relatives (Vaishali *et al.,* 2015). The more they (patients) stay in the hospital, the more the cost, and, the more the anxiety it causes to the patients/relatives. These multidrug (MDR) *P. aeruginosa* strains in hospital isolates has become a great health concern world over due to the high mortality rates associated with treatment failures (Nathwani *et al.*, 2014b). *P. aeruginosa* is a common cause of severe nosocomial infections, especially in immune-compromised patients.

P. aeruginosa develops resistance to antimicrobial agents with continued use resulting in MDR-strains. Another mechanism of resistance involves the production of β-lactamases and acquisition of plasmid-borne integron through horizontal transfer (Carrattoli, 2009). These resistance genes are acquired from resistant strains of *P. aeruginosa* or from other bacterial species such as *Acinetobacter baumannii, Klebsiella pneumoniae* and *Salmonella* spp. Several studies have attributed antimicrobial resistance in *P. aeruginosa* to the presence of one or more of these genetic elements (Su *et al.,* 2010). Genetic elements such as plasmids, transposons, and integron are means of antimicrobial resistance genes acquisition (Bonomo *et al.*, 2006). Previous research has shown that these strains are more prevalent in the ICU

possibly due to the immune-compromised status of hospitalized patients (Kali *et al.*, 2013). Other risk factor for MDR strains of *P. aeruginosa* colonization in such settings includes concurrent infections, prolonged hospitalization and use of invasive procedures such as catheters and mechanical ventilation. The occurrence of *P. aeruginosa* MDR-strains in ICU settings where patients have a weak immune system is therefore likely to cause even more fatalities and prolonged hospitalization (Aloush *et al*., 2006). The emergence of carbapenem resistance has even made this problem of antimicrobial resistance worse. This is because these drugs are considered the last resort for treatment of serious Gram-negative infections. Of particular importance is the Metallo-beta-lactamases (MBL) such as the bla_{NDM} because of their high resistance capabilities and are harbored in plasmid bearing integrin (Janvier *et al.*, 2013). This therefore means that such strains are intrinsically resistant to a broad range of antimicrobials making resultant infections difficult to treat. Among the MBL variants, bla_{VIM} and bla_{IMP} are the most prevalent in *P. aeruginosa* and have been widely implicated in numerous nosocomial outbreaks (Bereket *et al.,* 2012). Integron harbored in plasmid have been implicated in the spread of resistance genes in the environment. Such spreading is facilitated by use of medical devices such as urinary catheters in the hospital set up as well as person-to-person contamination (Aiken *et al.*, 2011). We therefore set to conduct a survey in Kenyatta national Hospital to help broaden knowledge on antimicrobial resistance patterns and molecular factors associated with *P. aeruginosa* isolates recoverable from in-patients in this medical facility. Since determination of genetic relatedness is essential in examination and monitoring of cross-infection especially in hospital setting, we therefore set to do low resolution fingerprint analysis to determine the genetic relatedness of the recovered strains.

1.2 Problem statement

In Kenya, resistance to cheap and readily available antimicrobials has been on the increase due to irrational use of drugs and ready availability over the counter (Gould *et al.,* 2011). Consequently, MDR.*P. aeruginosa*, as well as other resistant nosocomial pathogens has been associated with severe infections due to indiscriminate use of broad-spectrum antibiotics. According observation data recorded at the KNH (Microbiology laboratory), more than 60% of isolates from patients in critical care

wards show resistance to three or more antibiotics used. Over 30% of the outpatient cases also show resistance to at least three antibiotics (Ngumi, 2006). The increase in antimicrobial resistance has led to drastic increase in financial burden, ever-increasing number of patients and prolonged hospitalization in this referral facility (Elamenya *et al.*, 2015). This menace has also been implicated in severity of infections and death rates from certain infections that could possibly have been avoided by prudent and rational use of the existing and newer antimicrobial agents (Guardabassi *et al*., 2008).

Some of these nosocomial infections are believed to be spreading between wards through person-to-person contamination and use of mechanical devices (Khan *et al*., 2015). Such multidrug infections have been reported as a result of horizontal gene transfer via genetic elements such as class 1 integron. Class 1 integron harbors resistance gene cassettes that can be easily dispersed among other bacteria, resulting in the rapid spread of antibiotic resistance genes (Kiiru *et al*., 2012). This integron has widely been reported in multidrug-resistant P. aeruginosa harbored in resistant plasmids. Widespread dissemination of the class 1 integron and associated gene cassettes in *P. aeruginosa* and other clinically essential pathogens gravely complicate treatments of *P. aeruginosa* infections.

1.3 Study justification

Pseudomonas aeruginosa remains the prevalent cause of nosocomial infections around the globe (Nathwani *et al.*, 2014a). Through various transmission pathways such as human vectors, this organism is able to cause nosocomial infections within hospital wards consequently leading to prolonged hospitalization and increased cost of health-care treatment (Juan *et al.*, 2019). The burden and prevalence of nosocomial infections caused by this organism however, has not been accessed in Kenya through research-based documentation. Treatment of infections in many parts of Kenya does not involve culture and susceptibility testing to determine etiological agent and effective antimicrobial agents. Hence, surveillance of antimicrobial resistance and appropriate, effective measures geared towards curbing the indiscriminate and unregulated use of antibiotics are urgently needed to prevent outbreaks of multidrug-resistant bacteria in KNH.

1.4 Null hypothesis

P. aeruginosa isolates recoverable from clinical samples of in-patients at Kenyatta National Hospital are not resistant to multiple antimicrobial agents and do not carry integrons.

1.5 Study objectives

1.5.1. Broad objective

To determine integron associated with multidrug resistant strains of *P. aeruginosa* recoverable from in-patients in various wards at Kenyatta National Hospital.

1.5.2 Specific objectives

1. To isolate *P. aeruginosa* from specimens obtained from in-patients at Kenyatta National Hospital.
2. To determine the resistance patterns of *P. aeruginosa* to the various antimicrobials that are heavily used against Gram-Negative bacteria.
3. To determine genetic basis of resistance to various antimicrobial classes.
4. To determine phylogenetic relatedness among isolates obtained from different wards and from different specimen

CHAPTER TWO

LITERATURE REVIEW

2.1 Pseudomonas *aeruginosa infections and challenges in combating antimicrobial resistance*

P. aeruginosa is a common bacterium that can cause disease in animals, including humans. It is found in soil, water, skin flora, and most man-made environment throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, and has, thus, colonized many natural and artificial environments (Vaishali *et al.*, 2015). It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonization occurs in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal (Owens *et al.*, 2008). Because this organism normally thrives on moist surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is implicated in hot-tub rash. *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections (Partridge, 2009).This organism is an opportunistic pathogen that causes severe infections in immunocompromised individuals. It is the most common cause of infections in burn injuries and of the outer ear (*otitis externa*) and is the most frequent colonizer of medical devices (e.g., catheters). *P. aeruginosa* can in rare circumstances cause community-acquired pneumonia (Lu *et al*., 2012), as well as ventilator-associated pneumonia, being one of the most common agents isolated in several studies (Parker *et al.*, 2008). Pyocyanin is a virulent factor in this bacterium and has been reported to cause death by oxidative stress. However, research indicates salicylic acid can inhibit pyocyanin production (Queipo-Ortuno *et al.*, 2008).

Intrinsic and acquired resistance makes treatment of *P. aeruginosa* infections problematic. For severe infections, a combined dosage of a β-lactam such as ceftazidime, aminoglycoside such as gentamicin and fluoroquinolone such as ciprofloxacin may be administered (Rossolini *et al.*, 2005). Combined resistance to

these classes of antimicrobial therefore should be a serious concern if successful treatment is to be achieved. In intensive care unit where patients may be in coma, antimicrobial administration via oral route is very difficult, therefore, injectable aminoglycosides such as gentamicin and amikacin are often relied on. If these injectable drugs are ineffective in treating infections in such patients, yet other effective antimicrobials administrable via oral route cannot be utilized, such patients are likely to die from infections (Tamma *et al.*, 2012).Carbapems are effective in most Gram negative bacteria and are often regarded as last resort option for treating serious infections (Cisneros-Farrar *et al.*, 2007). Although this antimicrobial class is not readily available in developing countries due to high cost, current data in the region show that resistance is on the rise (Pitout *et al.*, 2008). A study conducted in Agha Khan hospital between 2006 and 2007 reported 57 *P. aeruginosa* isolates from urine, blood, wounds and respiratory tract specimens that were highly resistant to imipenem and meropenem. All these isolates were positive for bla_{VIM} carriage by polymerase chain reaction screening. This study also reported a pan resistant cluster that was associated with nosocomial outbreak in intensive care unit. Carbapenem resistance is often accompanied with resistance to other antimicrobial agents. In fact, it has been reported that carbapenem are less effective on *P. aeruginosa* isolates that are resistant to some cephalosporin antimicrobials such as ceftazidime (Kanj *et al.*, 2012). Furthermore, lack of proper diagnosis of infections caused by *P. aeruginosa* in most of health facilities in most of developing countries make early intervention and proper treatment difficult. Many health facilities lack proper equipment and skill to perform diagnosis and antimicrobial sensitivity testing and therefore empirical treatment is widely used (Connolly *et al.*, 2004).

2.2 Pseudomonas aeruginosa *pathogenesis and virulence factors*

P.aeruginosa is able to cause a wide range of infections through the expression of virulence factors such as flagella, pilli and exopolysaccharide alginate (Kaye *et al.*, 2015). Single polar flagella is used as an adhesion, key for motility of the organism and also important in bacterial chemotaxis (Toutain *et al.*, 2007). Pili (type 4) is a key component in formation of biofilms and aggregation of bacterium in target host tissues which essentially enable respiratory pathogenesis. Formation of the biofilms protects

this bacterium from host immune defense systems and antibiotics which may lead to treatment failure (Gellatly *et al.,* 2013).

Type 3 secretion system (T3SS) enables injection of effector proteins (ExoY, ExoS, ExoT and ExoU) in infected host cells and this process is important in acute invasive infections caused by *P. aeruginosa* (Gellatly *et al.*, 2013*)*. Auto inducer molecules produced by this bacterium like many Gram negative microbial, enable this organism to adapt to environmental changes, including those that occur in host and this enables survival (Hassett *et al.*, 2010). Protease degrades epithelial junctions and immunoglobulin play an important role in ocular infections and sepsis caused by *P. aeruginosa* (Gellatly *et al.*, 2013). Other virulence factors include lipopolysaccharide (lipid A and O polysaccharide) which convey antibiotic interactions, inflammatory response and antigenicity.

2.3 Identification and diagnosis

P. aeruginosa is an obligate aerobe and is usually recognized by the greenish-blue pyocyanin pigment it produces (except about 4% of its strains) (Hassani *et al*., 2012). It also produces fluorescein so that colonies fluoresce green in ultraviolet (UV) light. *P. aeruginosa* produces large, flat, hemolytic colonies on blood agar. All *P. aeruginosa* strains are strongly oxidase positive (Odumosu *et al.*, 2013).

2.4 *Treatment* of Pseudomonas aeruginosa *infections*

Presumptive treatment of *Pseudomonas aeruginosa* infections involves the use of combination therapy while awaiting susceptibility results (Kanj *et al.*, 2012). This therapy usually involves the use of antibiotics with anti-Pseudomonal activity such as Ticarcillin and piperacillin and third and fourth generation cephalosporins such as ceftazidime and cefepime. Other drugs include aminoglycosides for example amikacin, gentamicin, aztreonam, oxazolidinones, and carbapenems such as imipenem and meropenem. Fluoroquinolones includes ciprofloxacin and levofloxacin, colistin, and polymixin B. (WHO, 2008). It is recommended that patients with severe *Pseudomonas* multidrug resistance (MDR) infections should be treated with a combination therapy consisting of an anti-Pseudomonal β-lactam such as Meropenem (carbapenems), an

aminoglycoside for example amikacin or fluoroquinolones such as ciprofloxacin to provide adequate therapy cover and improve patient outcomes (Bassetti *et al*., 2018). Colistin administered intravenously has been used for bacteremia, urinary tract infections, surgical sites infections, abdominal, skin and central nervous system infections (Sanchez *et al.*, 2011).

2.5 *Antibiotic resistance* in Pseudomonas aeruginosa

P. aeruginosa is naturally resistant to a broad range of antibiotics and may demonstrate additional resistance after unsuccessful treatment, in particular, through modification of a porin (Delcour, 2009). Resultant infections are better treated using informative antimicrobial test as opposed to empirical treatment. If antibiotics are started empirically, then every effort should be made to obtain cultures, and the choice of antibiotic used should be reviewed when the culture and sensitivity results are available.

Large amounts of non-rational use of antibiotics in human therapy have resulted in the selection of pathogenic bacteria resistant to multiple drugs (De Bruycker *et al*., 2013). Multidrug resistance in bacteria may be generated by one of two mechanisms. First, these bacteria may accumulate multiple genes, each coding for resistance to a single drug, within a single cell. This accumulation occurs typically on resistance (R) plasmids or transposons, of genes, with each coding for resistance to a specific agent (Bennett, 2008). Second, multidrug resistance may also occur by the increased expression of genes that code for multidrug efflux pumps, extruding a wide range of drugs (Westfall *et al*., 2006).

The emergence of ‘pan-resistant’ gram-negative strains, notably those belonging to *P. aeruginosa* and *Acinetobacter baumannii*, occurred more recently after most major pharmaceutical companies stopped the development of new antibacterial agents (Falagas *et al.*, 2007). Hence, there are almost no agents that could be used against these strains, in which an outer membrane barrier of low permeability and an array of efficient multidrug efflux pumps are combined with multitudes of specific resistance mechanisms.

Resistance to aminoglycosides for example gentamicin and amikacin involves the MexXY-OprM efflux pump as well as the aminoglycoside modifying enzymes (AMEs) (Islam *et al*., 2004). Resistance to fluoroquinolones for example, ciprofloxacin involves mutations in target genes (*gyrA*, *gyrB*, *parC*, and *parE*), aminoglycoside modifying enzymes or to drug efflux systems (MexAB-OprM, MexCD-OprJ, MexAB-OprM, MexXY-OprM, OqxAB and Qep). (Hocquet *et al.*, 2007).

2.6 *Integron*

Integron are gene capture system inform of resistance gene cassettes. Cassettes carried by integron usually encode multiple resistance mechanisms, such as, resistance to beta-lactams ($bla_{\text{VIM-1)}}$, Aminoglycosides (*aacA4*allele) and other antimicrobial agents (Jeong *et al.*, 2009). Integrons are linked to chromosomes, plasmids and transposons. The integron has three important core elements: The *intI* gene which encodes an integrase (*IntI*) required for site specific recombination; *attI* which is recognized by integrase; and integron which is associated promoter (Pc) and is needed for transcription and expression of gene cassettes within the integrin (Hall, 2012). Gene cassettes are genetic elements that encode antibiotic resistance genes, and consist of a specific- site recombination recognized by integrase that is called *attC* (or 59-base elements). The class 1 integron remains the most common integron found in members of the family Enterobacteriaceae such as *Enterobacter* spp, *K. pneumoniae, E. coli* and *Proteus* spp, as well as other clinically significant Gram-negative bacteria such as *P. aeruginosa* and *Acinetobacter baumannii* (Weldhagen *et al.*, 2004). Detection of class 2 and class 3 integron among these nosocomial pathogens is not widely reported (Poirel *et al.*, 2002).

2.7 *Plasmids*

P. aeruginosa is well known for harboring numerous copies of plasmids, some of which are conjugative with resistance genes that are responsible for multiple drug resistance. The relationship between plasmid profiles and multiple drug resistance patterns suggests that plasmids may play a significant role in the multidrug resistance of *P. aeruginosa* strains because multiple antibiotic resistance genes, as well as

virulence genes, have often been found clustered together on a single plasmid (Toleman *et al.*, 2006). Plasmid-mediated horizontal gene transfer has been implicated in *P. aeruginosa* resistance to β-lactams, carbapenem and aminoglycosides.

Antimicrobial resistance plasmids often contain many resistance genes; they are maintained stably in the host strains of bacteria and are transferred very efficiently to neighboring drug-susceptible cells (White *et al.*, 2000). This is a conjugative factor in bacteria cell that promotes resistance to antibiotics.

Most drug resistant genes are active when expressed from plasmids; many such genes are often present on a single resistance plasmid so that multidrug resistance can be transferred to a susceptible bacterium in a single conjugation event. When the resistance plasmids were first reported in Japan in the 1950s, many of them already contained resistance genes for aminoglycosides, tetracycline, chloramphenicol, and sulfonamides. The sequence of early-generation R plasmids indicates resistance genes are components of transposons, which can deliver the genes to any piece of DNA. Tn*21* is a particularly remarkable example of abundant, complex, multiple composite transposons, and it contains mercury resistance genes ('Gene expression; posttranscriptional modifications (2C-01 - 2C-09)', 2004).

Recent studies have unveiled that the integron structures are associated with a downstream IS*C*R element which contains a putative transposase gene (Owens *et al.*, 2008). It apparently functions in an unusual, open-ended transposition event and recruit various resistance genes and delivers them close to the integron structure, resulting in the assembly of yet more resistance genes.

2.8 *Clonal spread* of Pseudomonas aeruginosa

Pseudomonas aeruginosa is able to survive on inanimate surfaces, medical equipment, including catheters, causing cross-infections in hospitals and clinics. Intrinsic resistant of this bacterium to disinfectants enable this organism to survive on various hospital surfaces and can be spread from one person to another (de Abreu *et al.*, 2014). Health-care providers are the major vectors of transmission within in Health-care setting leading to emergence and spread of nosocomial infections (Albrich *et al.*, 2008).

Although thorough washing of hands should help minimize this problem, most of the health-care providers do not wash hands before touching their patients which in turn facilitate the spread of microbial clones.

Bio-typing is therefore essential in establishing cross-transmission and sources of nosocomial infections.

CHAPTER THREE

MATERIALS AND METHOD

3.1 Study design

A cross-sectional study design was used in this study to help broaden knowledge of the antimicrobial resistance profiles, diversity of selected resistance genes, mobile elements that includes integron and plasmids encountered among *P. aeruginosa* recovered from a large tertiary urban hospital. A judgmental/purposive sampling method was used to collect clinical samples that includes; blood, urine, respiratory aspirates and pus.

3.2 Study site

The current study was conducted at Kenyatta National Hospital (KNH), the largest referral hospital in East Africa and the Sub-Sahara region located in Nairobi, the Kenyan Capital. The hospital was founded in 1901 as a Native Civil hospital with a bed capacity of 40 which has since grown to 1800. KNH referral hospital was ideal for this study due to a diverse population the facility attracts across the East Africa region. This hospital gives priority to patients with critical medical conditions such as chest infection, severe head injury, sepsis, diabetes complications, cardiac complications, burns, autoimmune-related diseases, kidney complications among others. As such, antibiotic use among hospitalized patients is higher than what is expected in smaller hospitals.

3.3 Study population

This study ideally recruited from in-patient population whose medical record strongly suggested bacterial etiological agent in respiratory, urinary, sepsis and wound infections. Patients of all ages who met the inclusion criteria and consented were recruited in this study.

3.4 Sample size

Fisher *et al.* (2005) method was used to calculate the sample size in this study.

$N = Z^2 P (l-P)/d^2$

Where N = Minimal sample size:

Z = Standard normal deviation corresponding to 95% confidence interval (=1.96);

P = Estimated prevalence of *Pseudomonas aeruginosa* in hospital setting, which in this case is 0.02 (Pitout *et al.,* 2008)

d = degree of precision (5%)

Therefore, $N = 1.96^2 X 0.02 (1-0.02)/0.02^2$ which gives us a minimum of 246 samples.

Therefore, a total of 1,106 clinical samples were collected in this study and they include; Tracheal aspirates (608), urine (155), blood (22), pus (321).

3.5 Inclusion

A medical Doctor or a nurse was used to identify patients with key infections that are associated with *P. aeruginosa* that includes urinary tract infections, sepsis, respiratory infections and wound infections. However, only patients who met the following conditions were included;

I. Must be conversant with either English or Swahili language since the study was done in a metropolis city.
II. Must willingly agree to participate in this research study.
III. Must have been hospitalized for a minimum of 3 days (in-patients).

3.6 exclusion criteria

The following category of patients were excluded from this study

I. In patients who did not meet all the inclusion criteria.
II. Patients in coma and whose relatives did not consent
III. Assenting child patients whose guardians did not give consent

3.7 Specimen collection

A clinician as indicated in section 3.7.1/2/3/4 collected specimen that included pus swab, wounds, blood, urine and aspirates.

3.7.1 Blood (septicemia)

The clinician at KNH collected blood aseptically; a tincture of iodine was used to clean thoroughly the site of collection. The cap of the culture bottle was removed to allow cleansing the top of the bottle using an ethanol-ether swab (McCall *et al.,* 2008). A Sterile syringe (5ml/10 ml.) and a 21-gauge needle was used to withdraw a 5ml blood sample from adult patients. In case of neonates, a 25-gauge needle with a 2ml syringe was used to draw at least 1ml blood sample. The needle was then replaced with a sterile one, and then inserted into the rubber liner of the bottle cap to dispense blood into culture bottle. The top of the culture bottle was then wiped and a protective cap replaced. The blood was then gently mixed with the tryptone soya diphasic medium. The container was then labeled and incubated at 37^{o}cimmediately for 7 days (Al-Charrakh *et al.,* 2016).

3.7.2 Pus from wounds, abscesses, burns, and sinuses (skin infection)

A pus specimen was aseptically obtained from ruptured or incised abscesses and transferred into a leak-proof sterile container. In case the pus was not discharged, a sterile cotton wool swab was used to collect a sample from the infected site. The swab was then immersed in a container of Amies transport medium, Oxoid Ltd. The specimen was then delivered to the laboratory immediately for processing. In case of delay, then the specimen was kept at 4^{o}c.

3.7.3 Urine (urinary infection)

Midstream urine specimen was collected in the morning in a bijou bottle. For indisposed patients, (CCU) collection of urine involved the use of a fresh urine bag connected to a freshly inserted catheter, where 20 ml. of urine were then transferred to a sterile bijou bottle(Vigil *et al.,* 2016). Proper labeling was done and then the specimen transported to the laboratory immediately. Where a delay of more than 1 hour was anticipated, 10 g/l boric acid was added.

3.7.4 Effusions (inflammation of respective areas)

The Tracheal fluids were aspirated by a clinician and dispensed aseptically into a sterile screw-capped bottle and labeled before delivering immediately to the laboratory. In a case where a delay was anticipated, then the specimen was kept at between 4^{o}c to 10^{o}c.

3.8 Sample processing and bacterial culture

Tracheal aspirates were first mixed for 30 seconds on a vortex machine before culture. Bacterial culture was done on blood and MacConkey and Mueller Hinton agar plates and incubated at 37°c for 24–48 h. Urine samples were additionally cultured on CLED and blood agar and incubated overnight at 37^{o}c for 24-48 hrs. Inoculation was done using a wire loop sterilized by heating with a Bunsen burner flame then allowed to cool before picking the specimen. Sterilization was repeated before using the same wire loop to do striking on the plate (Sanders *et al.,* 2012).

Blood for culture was brought to the laboratory in Tryptone soya diphasic medium. Incubation was done at 37^{o}C in a Bactec machine (Becton Dickinson & Company USA, 9050 Series) for up to 2 weeks (examination was done daily for the first 7 days, then twice a week for up to four weeks). Observation for colonies on the agar slope checked with the help of hand lens, and signs of bacterial growth in the broth. Resultant bacterial colonies were subjected to gram stain reaction and a series of biochemical tests for identification of presumed *P. aeruginosa* species.

3.9 Gram stain

A loop full of the test organism was emulsified in a drop of normal saline on a glass slide using a sterile wire loop. The smear was then fixed by passing the slide over a heat flame 3-4 times. After the smear had cooled, primary staining was done by flooding the smear with crystal violet (Mahasneh *et al.*, 2006). The stain was removed after 1 minute by gently washing off with running water. The smear was then flooded with lugol' iodine for 1 minute and then washed off. Decolorization to remove the unbound crystal violet was done by dipping the slide in 95% ethanol for 30 seconds. A counter stain safranin was lastly flooded on the smear and then washed off after 1 minute. Then slides were observed on a microscope using x100 magnifying lens (oil emulsion). Gram negative rods were considered candidates for *P. aeruginosa* and further identification tests were done by bio typing.

3.10 Biochemical test

Gram-negative rods suspected to be *P. aeruginosa* isolates were subjected to biochemical testing as detailed below.

3.10.1 Oxidase test

Oxidase test was used to test presence of Oxidase enzyme which catalyzes oxidation of cytochrome C in the presumed *P. aeruginosa* isolates. A fresh Oxidase test strip was placed on a clean glass slide and then moistened with distilled water. Using a sterile loop, the test colony was smeared on the moistened surface of the Oxidase strip. Presence of a deep-purple hue within 5-10 seconds was considered positive candidate for *P. aeruginosa* (Al-Charrakh *et al.*, 2016)**.**

3.10.2 Motility indole lysine test (MIL)

This test was used to test organism ability to decarboxylate and deaminate lysine and motility. The test was done by stabbing the MIL semi-solid medium contained in a test tube with a straight wire loop containing test inoculums. Incubation was done at 37^{o}c for 24 hours. A characteristic purple band and butt was considered positive test for lysine decarboxylation. A narrow purple with a yellow butt was considered a negative

test. A deep red band with yellow butt was considered positive test for lysine deamination (Hemraj *et al.,* 2013). A purple band with yellow butt was considered a negative test. Indole test was done by adding 3 drops of kovac's reagent. Reaction of the added kovac's reagent with indole was characterized by formation of a red ring on top which was also considered a positive test. Lysine decarboxylation and deamination negative test, motility positive and indole negative tests were considered possible *P. aeruginosa*.

3.10.3 Triple sugar iron test

This test was used to test organism ability to ferment glucose and lactose to produce acid and gas and also sulfur reduction to form hydrogen sulfide (H_2S) (Lehman *et al*., 2014). The test was done by stabbing the butt and streaking the slant of the TSI medium using a loop containing the test colony. Incubation was then done at 37^oc for 24 hours. Glucose fermentation was characterized by acid production which turned butt yellow while the slant remained yellow. Production of hydrogen sulfide was characterized by black precipitation of the medium. Lactose fermentation was characterized by acid production evidenced by cracking of the medium leading to yellow butt and slant. Red coloration on both butt and slant was considered negative test for glucose and lactose fermentation. Organisms that can ferment lactose continue to produce acidic byproducts and the media remains yellow (Lehman, 2014). Negative test for glucose and sucrose fermentation, H_2S production and acid production was considered positive for *P. aeruginosa.*

3.10.4 Simmon's Citrate Agar

This medium was used to test the ability of the test organism to use citrate as a sole carbon source. Organisms capable of utilizing citrate as a carbon source hydrolyzed citrate enzyme into oxaloacetic acid and acetic acid. The oxaloacetic acid was then hydrolyzed into pyruvic acid and CO_2. Carbon dioxide production leads to alkaline pH formation which in turn converts the medium from green to blue which was indicative of positive test (Lehman, 2014). *Pseudomonas aeruginosa* is able to utilize citrate as carbon and hence positive for this test.

3.10.5 Urease test

Urease medium was used to test the ability of an organism to produce urease. The urease produced hydrolyzed urea to ammonia and carbon dioxide. Urease medium contains pH buffers, urea and minute nutrients and also phenol red which is a pH indicator. In acid environment, phenol red changed to yellow and fuchsia in alkaline environment and the medium turned red which is indicative of a positive test (Hemraj *et al.*, 2013). Organisms that were positive for this test were ruled out as possible *P. aeruginosa*.

3.11 Antimicrobial susceptibility testing

A pure colony was used to make a 0.5 McFarland solution using normal saline. The solution was spread on a Mueller Hinton plate to make a confluent growth after which antimicrobial disc were dispensed. MIC was done using Vitek 2 technology. Quality control of the test was done using *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 (Bamidele *et al.*, 2013). Antimicrobial sensitivity tests were performed for 13 antimicrobial agents: ceftriaxone (CRO, 30µg), ceftazidime (CAZ, 30µg), cefotaxime (CTX, 30µg), carbenicillin (CAR, 100µg), piperacillin (PRL, 100µg), aztreonam, (ATM, 30µg) levofloxacin (LEV, 5µg), ciprofloxacin (CIP, 5µg), gentamicin (CN, 10µg), amikacin (AK, 30µg), tetracycline (TET, 30µg), meropenem (MEM,10µg), piperacillin/tazobactam (TZP, 100/10µg) and trimethoprim (W, 25µg).

3.12 DNA extraction for PCR screening

Genomic DNA extraction was carried out as described previously (Queipo-Ortuno *et al.*, 2008) with slight modifications. Briefly, the *P. aeruginosa* isolates were inoculated into 2 ml of Trypticase Soy broth (Difco, Detroit, MI) and incubated overnight at 37°c. The bacterial cells were harvested by centrifugation at 8,000 rpm for 5 min, and the supernatant was obtained using sterile Pasteur pipette. The pellet was re-suspended in 500µl of Tris EDTA (TE) buffer. The cells were then lysed by boiling for 10 min in a water bath, cooled on ice, and centrifuged at 14,000 rpm for 5 min to remove any cell debris before storage at -20°C.

3.13: Screening for resistance markers

Aliquots of 2µL of the template DNA were used in polymerase chain reaction (PCR) for detection of integron class 1, 2&3, plasmids and Metallo β-lactamases (MBL). Amplification of target MBLs including *bla*$_{GES}$, *bla*$_{VEB}$, *bla*$_{NDM}$, *bla*$_{SPM}$, *bla*$_{GIM}$ and *bla*$_{KPC}$ was done using published primers (Table 3.1). Specific primers for integron class 1, 2 and 3 were used are indicated in table 1 below. Polymerase chain reaction products for amplification using thermos cycler were prepared by adding 2 µL of the DNA extract in 25 µL of master mix that contained forward and reverse primers 1µL each, 15µL of PCR water, Taq polymerase, dNTPs, Q solution, Magnesium chloride and PCR buffer (Watson, 2012). Plasmid typing was done using published primers (Table 3.2). Amplification of test genes was done using a thermal cycler under the following conditions; initial denaturation at 95 °C for 2 min, annealing at 40 - 62 °C (depending on the primer) from 1 min, extension at 65 °C for 8 min and a single final extension step at 65 °C for 8 min for 30 cycles (Carattoli *et al.*, 2006).

Table 3.1: Amplification primers for Metallo β-lactamases and integron

Primer Name	5' -3' Sequence	Base pairs	Annealing temperature (°C)	Reference
VEB-F	CGACTTCCATTTCCCGATGC			
			56	(Magiorakos *et al*., 2012)
VEB-R	TGTTGGGGTTGCCCAATTTT	371		
NDM-F	ACTTGGCCTTGCTGTCCTT			
		621		(Monicah 1999)
NDM-R	CATTAGCCGCTGCATTGAT		56	
SPM-F	AAAATCTGGGTACGCAAACG			
		271	52	(Ellington *et al*. 2007)
SPM-R	ACATTATCCGCTGGAACAGG			
PER-F	ATGAATGTCATTATAAAAGC			
		933	50	(Carraccio *et al* 1991)
PER-R	AATTTGGGCTTAGGGCAGAA			
GES-F	ATGCGCTTCATTCACGCAC			
		863	56	(Monicah 1999)
GES-R	CTATTTGTCCGTGCTCAGGA			
GIM-F	TCGACACACCTTGGTCTGAA			
		477	52	(Ellington *et al*., 2007)
GIM-R	AACTTCCAACTT TGCCATGC			
IntM1_U	ACGAGCGCAAGGTTTCGGT			
		441	60	(Acharya *et al*., 2017)
IntM1_D	GAAAGGTCTGGTCATACATG			
INT_1U				
	GTTCGGTCAAGGTTCTG	923	60	(Acharya *et al*., 2017)
INT2-L	CACGGATATGCGACAAAAAGGT			
		789	50	(Jellinger *et al*., 1979)
INT2-R	GTAGCAAACGAGTGACGAAATG			
Int3-F	AAATGACAAACCTGACTG			
		922	60	[15]
Int3-R	CGAATGCCCCAACAACTC			
ERICR	ATGTAAGCTCCTGGGGATC			
		Variable	50	(Mohapatra *et al*., 2007)
ERIC2	AAGTAAGTGACTGGGGTGAGCG			

Table 3.1 These primers were used to screen for more resistance genes that confer carbapenems resistance in *Pseudomonas aeruginosa*. We also tested for carriage of class 1, 2 and 3 integron (*intI*) where most of these resistance genes are harbored in resistance cassettes. The low resolution enteric repetitive intergenic consensus (ERIC) method was used to check possible proliferation of these strains within and between Hospital wards.

Table 3.2: Amplification primers incompatibility group plasmids

	Sequence (5'-3')	Primers	Annealing temperature (°C)	Product
MultiPlex -1	GGAGCGATGGATTACTTCAGTAC	HI1 – F	60	471 bp
	TGCCGTTTCACCTCGTGAGTA	HI1 – R		
	TTTCTCCTGAGTCACCTGTTAACAC	HI2- F	60	644 bp
	GGCTCACTACCGTTGTCATCCT	HI2 – R		
	CGAAAGCCGGACGGCAGAA	I1 – F	60	139 bp
	CGTCGTTCCGCCAAGTTCGT	I1 – R		
MultiPlex -2	AACCTTAGAGGCTATTTAAGTTGCTGAT	X – F	60	376 bp
	GAGAGTCAATTTTTATCTCATGTTTTAGC	X –R		
	GGATGAAAACTATCAGCATCTGAAG	L/M – F	60	785 bp
	CTGCAGGGGCGATTCTTTAGG	L/M – R		
	GTCTAACGAGCTTACCGAAG	N – F	60	559 bp
	GTTTCAACTCTGCCAAGTTC	N – R		
Multiplex -3	CCATGCTGGTTCTAGAGAAGGTG	FIA – F	60	462 bp
	GTATATCCTTACTGGCTTCCGCAG	FIA – R		
	GGAGTTCTGACACACGATTTTCTG	FIB – F	60	702 bp
	CTCCCGTCGCTTCAGGGCATT	FIB – R		
	CCTAAGAACAACAAAGCCCCCG	W – F	60	242 bp
	GGTGCGCGGCATAGAACCGT	W – R		
Multiplex –4	AATTCAAACAACACTGTGCAGCCTG	Y – F	60	765 bp
	GCGAGAATGGACGATTACAAAACTTT	Y – R		
	CTATGGCCCTGCAAACGCGCCAGAAA	P – F	60	534 bp
	TCACGCGCCAGGGCGCAGCC	P- R		
	GTGAACTGGCAGATGAGGAAGG	FIC – F	60	262 bp
	TTCTCCTCGTCGCCAAACTAGAT	FIC – R		
Multiplex -5	GAGAACCAAAGACAAAGACCTGGA	A/C – F	60	465 bp
	ACGACAAACCTGAATTGCCTCCTT	A/C – R		
	TTGGCCTGTTTGTGCCTAAACCAT	T – F	60	750 bp

Table 3.2: Five (5) multiplex primers were used to detect presence of 18 incompatibility plasmids (*inc*plasmids). These plasmids carry integrons or chromosomal mediated antimicrobial resistance genes and are most common among members of the family enterobacteriaceae and *P. aeruginosa.*

3.14 DNA fingerprinting of recovered bacterial isolates

Fingerprint analysis using the GTG^{5}method was used to determine the genetic relatedness of *bla*$_{NDM}$ and *bla*$_{VEB}$ positive *P. aeruginosa* isolates recovered from different wards. Polymerase chain reaction products for amplification using thermo cycler were prepared by adding2 µl of the DNA extract in 25µL of master mix that contained 2µLof GTG^{5}primer, 15µL of PCR water, Taq polymerase, dNTPs, Q solution, Magnesium chloride and PCR buffer. The PCR amplicons were separated by running on 1% agarose with ethidium bromide gel for 1 hour. Banding patterns were visualized under ultraviolet light using a Gelmax® imager. Cluster analysis was done using Gelcompar®2 software version 6.6. Cluster analysis was done using the dice method based on banding pattern with arithmetic mean UPGMA. Isolates that had a correlation of ≥ 80% were considered genetically related (Lister *et al.,* 2009b).

3.15 Ethical approval

Study approval was obtained from KEMRI Scientific Ethical Review Committee (SERU) and Kenyatta National Hospital Ethical Committee before commencing the study. Recruitment was done on consenting from in-patient wards such as Intensive Care Unit (CCU), Renal Unit Ward (RU), Burns Unit (BU), Newborn Unit (NBU) and Medical Wards at Kenyatta National Hospital (general admission wards, maternity wards, oncology wards, theatres, accident and emergency, cardiology unit, Infectious Respiratory Disease Unit and Orthopedic) between August 2015 and January 2016.

CHAPTER FOUR

RESULTS

4.1 *Isolation*

The prevalence of *P. aeruginosa* study from a total of 1106 clinical specimens analyzed was 17% where multiple-drug resistant 188 *P. aeruginosa* isolates were recovered. Colonies with a characteristic green color, spread and serrated edge with a grape smell on Mueller Hinton agar were presumed to be *P. aeruginosa* and were confirmed by Gram stain reaction and biochemical tests. One hundred and eighty-eight non-duplicate *P. aeruginosa* strains were isolated during a six-month period (August 2015 - January 2016). Of these, 103 were from ICU, 2 from Renal Unit, 4 from Burns Unit, 2 from Newborn Unit, and 77 from Medical Wards. A total of 153 isolates were obtained from patients aged 50 years and below, and 62% of 188 isolates were obtained from males while the rest (38%), were from females. Out of the 188 *P. aeruginosa* isolates recovered in this study, 103 (55%) were from tracheal aspirates samples, 55 (29%) from pus swabs, 26 (14%) from urine samples and 4 (2%) from blood samples (Table 4.1).

Table 4.1: *Pseudomonas aeruginosa* isolates from in-patients at KNH

		***P.aeruginosa* n (%) isolates**				
	n	**ICU**	**R.U**	**B.U**	**N.B.U**	**M.W**
All	188	103(55)	2(1)	4(2)	2(1)	77(41)
0-12 yrs	15	2(13)	0(0)	0(0)	2(13)	11(87)
13-17 yrs	29	7(24)	1(3)	1(3)	0(0)	20(69)
18-50 yrs	90	53(59)	1(1)	2(2)	0(0)	34(38)
>50 yrs	54	41(76)	0(0)	1(2)	0(0)	12(22)
Male	130	69(53)	0(0)	3(2)	2(2)	56(43)
Female	58	34(59)	2(3)	1(2)	0(0)	21(36)
Blood	4	1(25)	0(0)	0(0)	2(50)	1(25)
Urine	26	17(65)	2(8)	0(0)	0(0)	7(27)
T.apirates	103	63(61)	0(0)	0(0)	0(0)	40(39)
Pus	*55*	22(40)	0(0)	4(7)	0(0)	29(53)

Table 4.1: *n*-number, CCU-critical care unit, R.U- Renal unit, B.U burn unit, NBU- new borne unit, M.W- medical wards. A total of 188 multi-drug resistant *P. aeruginosa* isolates were isolated from patients admitted at Kenyatta national Hospital. Most of these isolates were recovered from tracheal aspirates of CCU patients.

4.2 *Antimicrobial susceptibility test*

4.2.1 Antimicrobial susceptibility test based on disc diffusion method

All the 188 *P. aeruginosa* isolates recovered in this study were multiple drug resistant (MDR) strains (≥3 antimicrobial class). High resistances were recorded towards piperacillin-tazobactam (96%) while ciprofloxacin (34%) was the least resisted antimicrobial. All the isolates were resistant to one or more of extended cephalosporin (CAZ, CTX and CRO) with a percentage resistance of 63%, 82% and 79% respectively

(Figure 4.1). A high resistance to carbapenems (meropenem, 54%) and aztreonam (54%) was also observed in this study.

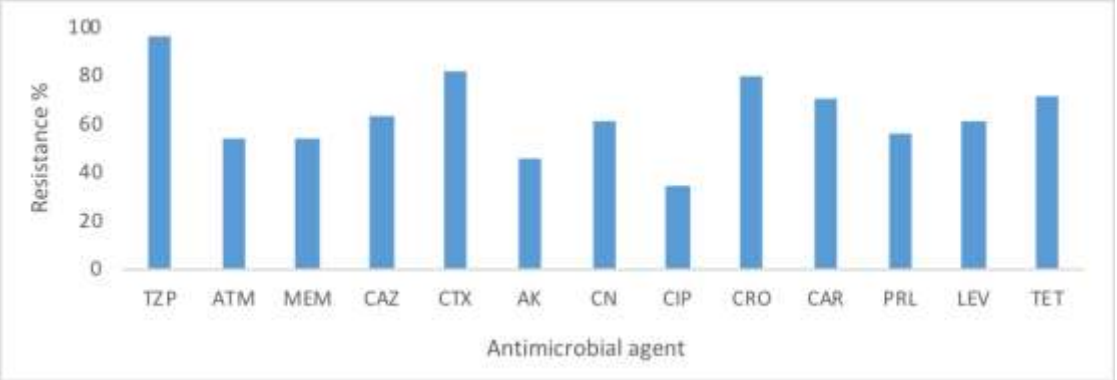

Figure 4.1: Resistance profiles of P. aeruginosa based on disc diffusion method

Key: MEM: meropenem, **ATM**: aztreonam, **CAZ**: ceftazidime, **CTX**: cefotaxime, **AK**: amikacin, **CN**: gentamycin, **CIP**: ciprofloxacin, **CRO**: ceftriaxone, **CAR**: carbenicillin, **PRL**: piperacillin, **LEV**: levofloxacin, **TET**: tetracycline, **TZP**: piperacillin/tazobactam. These 13 antimicrobial agents were tested against *Pseudomonas aeruginosa* isolates where piperacilllin, cefotaxime and ceftriaxone were the most resisted agents.

4.2.2 Antimicrobial susceptibility testing based on the Minimum inhibitory concentration

More than 132 (70%) *Pseudomonas aeruginosa* isolates were resistant to cephalosporins including ceftazidime, cefotaxime, and/or ceftriaxone with an MIC value of ≥ 32 µg/ml. High resistance to aztreonam (67.4%) was also recorded in this study. Resistance towards meropenem was 67.6% with an MIC value of ≥ 8µg/ml (Table 4.2). Most antimicrobial resistance was recorded towards tetracycline (92%) with MIC value of ≥ 8 µg/ml. *P. aeruginosa* isolates recovered from urine samples were the most resistant to tested antimicrobials with an overall resistance of (72%) recorded. High resistance in *P. aeruginosa* isolates from tracheal aspirates (76%) was also recorded while blood isolates were the least resistant.

The MIC test also revealed relatively high resistance to ciprofloxacin (52%) compared to the disc diffusion method where resistance was 34.3%. Isolates obtained from the

critical care unit (CCU) were the most resistant to any given antimicrobial. Isolates recovered from burns unit also had high resistances of (58%) followed by medical wards (43%) while those obtained from newborn unit (38%) were least resistant.

Table 4.2: MIC of P. aeruginosa isolates obtained from various sample types from different wards.

Drug	Resistance breakpoint* (µg/ml)	% resistance	Mode MIC	MIC_{50} (µg/ml)	MIC90 (µg/ml)
MEM	≥ 8	67	128	16	128
ATM	≥ 32	67	128	64	128
CAZ	≥ 32	70	64	64	64
CTX	≥ 32	88	64	64	64
AK	≥ 64	46	128	32	128
CN	≥ 16	67	128	64	128
CIP	≥ 4	52	128	32	128
CRO	≥ 32	86	64	64	64
CAR	≥ 64	57	64	64	64
PRL	≥128	25	64	64	128
LEV	≥ 8	73	128	64	128
TET	≥ 8	92	128	64	128
TZP	≥ 128	50	128	64	128

Key: MIC: Minimum inhibitory concentration, **MEM**: meropenem, **ATM**: aztreonam, **CAZ**: ceftazidime, **CTX**: cefotaxime, **AK**: amikacin, **CN**: gentamycin, **CIP**: ciprofloxacin, **CRO**: ceftriaxone, **CAR**: carbenicillin, **PRL**: piperacillin, **LEV**: levofloxacin, **TET**: tetracycline, **TZP**: piperacillin/tazobactam.

4.3 PCR analysis of Metallo-β-lactamases, Integron and Plasmid typing carriage in *P. aeruginosa* isolates

Amplified PCR products were obtained respectively for bla_{VEB} and bla_{NDM} using consensus primers. PCR experiments with primers specific for $bla_{TEM,}$ bla_{SHV}, bla_{GES}, bla_{PER}, bla_{KPC}, $bla_{GIM,}$ bla_{SPM}were negative. Integron class I, II and III were screened in all *P. aeruginosa* isolates positive for bla_{NDM} and/or bla_{VEB}. From a total of 127 *P. aeruginosa* isolates that were resistant to meropenem, only 68 were positive for

bla$_{NDM,}$ a gene encoding carbapenem resistance while 64 harbored *bla*$_{VEB,}$ a gene encoding an ESBL gene. A total of 45 isolates screened positive for both of *bla*$_{NDM}$, *bla*$_{VEB}$ and for class 1 integron (Plate 4.1). A single isolate from tracheal aspirate sample from a 39-year-old female admitted in the ICU harbored *bla*$_{NDM}$, *bla*$_{VEB,}$ integron class 1 and 3. Chi-square analysis found significant association in antimicrobial resistance to the drugs tested except for CTX and TET with carriage of integron (Table 4.3). Plasmid screening revealed 3 types of incompatibility groups. One *P. aeruginosa* isolate had both W-Plasmid and a FIB-Plasmid, while another isolate had an N-Plasmid. The nucleotide sequences of the *bla*$_{NDM}$ and the *bla*$_{VEB}$ reported in this paper have been submitted to the EMBL/GenBank nucleotide sequence database under accession numbers KX857136 (https://www.ncbi.nlm.nih.gov/nuccore/KX857136) and KX857137 (https://www.ncbi.nlm.nih.gov/nuccore/KX857137), respectively.

Table 4.3: Integron carriage association to antimicrobial resistance

Antimicrobial	Integron positive (n=48) n(%)			Integron negative (n=140) n(%)			X^2 P-value	significance
	R	I	S	R	I	S		
MEM	44 (91.6)	2 (4.2)	2 (4.2)	83 (59.2)	0 (0)	57 (40.7)	0.00001	S*
ATM	44(91.6)	2 (4.2)	2 (4.2)	82 (58.6)	0 (0)	58 (41.4)	0.0001	S*
CAZ	40 (83.3)	2 (4.2)	6 (12.5)	91 (65)	0 (0)	49 (35%)	0.004955	S*
CTX	47 (97.9)	0 (0)	1 (2)	119 (85)	10 (7.1)	11 (7.9)	0.141657	NS*
AK	40 (83.3)	0 (0)	8 (16.6)	41 (23.9)	5 (3.6)	94 (67.1)	0.00001	S*
CN	42 (87.5)	0 (0)	6 (12.5)	85 (60.7)	2 (1.4)	51 (36.4)	0.006661	S*
CIP	42 (87.5)	2 (4.2)	4 (8.3)	57 (40.7)	1 (0.7)	82 (58.6)	0.00001	S*
CRO	46 (95.8)	2 (4.2)	0 (0)	116(82.9)	1 (0.7)	23 (16.4)	0.028865	S*
CAR	44 (91.6)	2 4.2)	2 4.2)	63 (45)	45 (32.1)	32 (22.9)	0.00001	S*
PRL	38 (79.1)	8 (16.6)	2 4.2)	9 (6.4)	58 (41.4)	73 (52.1)	0.00001	S*
LEV	42 (87.5)	2 4.2)	4 (8.3)	96 (68.6)	1 (0.7)	43 (30.7)	.003063	S*
TET	48 (100)	0 (0)	0 (0)	124(88.6)	0 (0)	16 (11.4)	0 .137591	NS*
TZP	44 (91.6)	4 (8.3)	0 (0)	50 (35.7)	36 (25.7)	54 (38.6)	0.00001	S*

Table 4.3: R=Resistant, I=Intermediate, S=Susceptible, S*=significant, NS*=Non-significant. The test was considered significant at $P < 0.05$. Chi-square test (X^2) was used to test association between integron carriage to antimicrobial resistances recorded. .

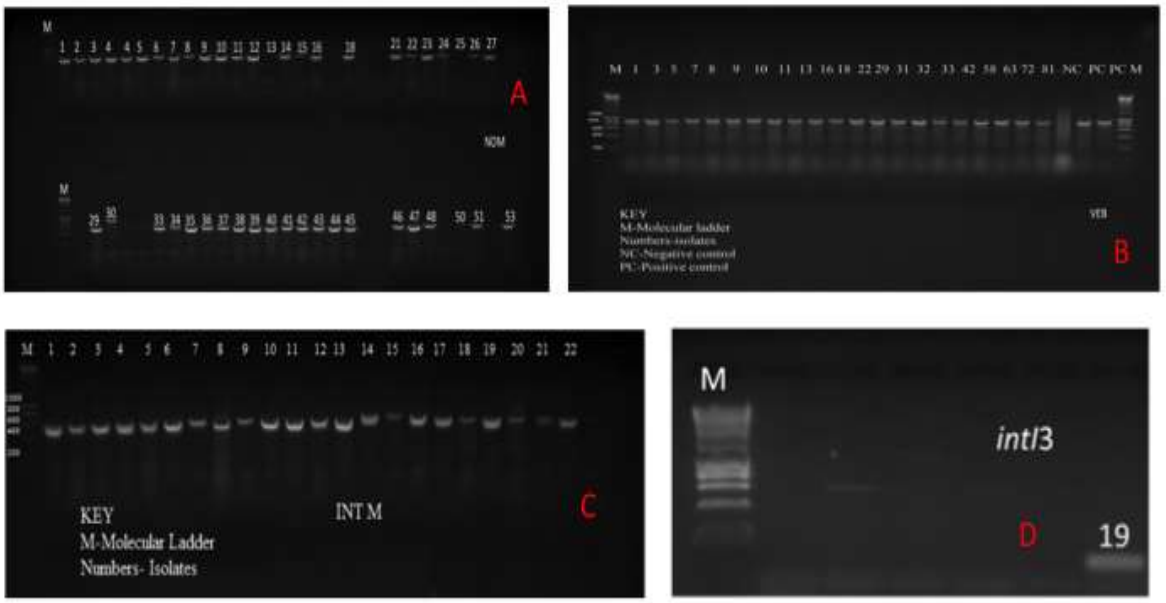

Plate 4.1: Gel images of detected carbapenemases and integrin in *Pseudomonas aeruginosa*

A: This show a gel image of *bla*$_{NDM}$ detected in *Pseudomonas aeruginosa* isolates that were resistant to meropenem.

B: This show a gel image of *bla*$_{VEB}$ detected in *Pseudomonas aeruginosa* isolates that were resistant to meropenem.

C: This figure shows a gel image of class I integron (*intI*1) detected in *Pseudomonas aeruginosa*. Most of these isolates were resistance to ceftazidime and ceftriaxone.

D: Only a single *Pseudomonas aeruginosa*isolate carried class 3 integron (*intI*3) and was also positive for carriage of *intI*1.

4.4 *DNA fingerprinting of recovered bacterial isolates*

Cluster analysis revealed four major clusters based on banding patterns with >80% similarity (Figure 4.2). Isolates in the first cluster (C-1) harbored bla_{NDM} and bla_{VEB} with exception of a single strain that harbored only bla_{NDM}. Five of these isolates were recovered from CCU, while 2 were from the medical ward. This cluster also contained a sub-cluster (a) with 2 isolates from male CCU ward that harbored bla_{NDM} and bla_{VEB} with a 94% similarity. Another sub-cluster (b) showed 4 *P. aeruginosa* isolates obtained from patients in medical and CCU ward which had a significant genetic similarity of 96%. The second cluster revealed 8 isolates with a similarity matrix of 96%. All the isolates in the second cluster (C-2) were obtained from CCU patients in urine and tracheal aspirate samples. Cluster 3 (C-3) uncovered a sub-cluster (d) with isolates obtained in CCU and medical ward that had a 96% similarity. With exception of a single strain that carried bla_{NDM}, all other isolates in this sub-cluster harbored class 1 integron, bla_{NDM} and $bla_{VEB.}$ Another sub-cluster (e) had 5 *P. aeruginosa* isolates obtained from CCU ward with a homogeneous resistance pattern and a significant similarity of 96%. A single isolate in this sub-cluster harbored *inc*W and *inc*FIB in addition to the other resistance genetic elements. Cluster 4 (C-4) on the other hand uncovered 3 homogenous isolates all of which were recovered from the CCU. The 3 isolates had a similarity matrix of >90%, all carrying class 1 integron, bla_{NDM} and $bla_{VEB.}$

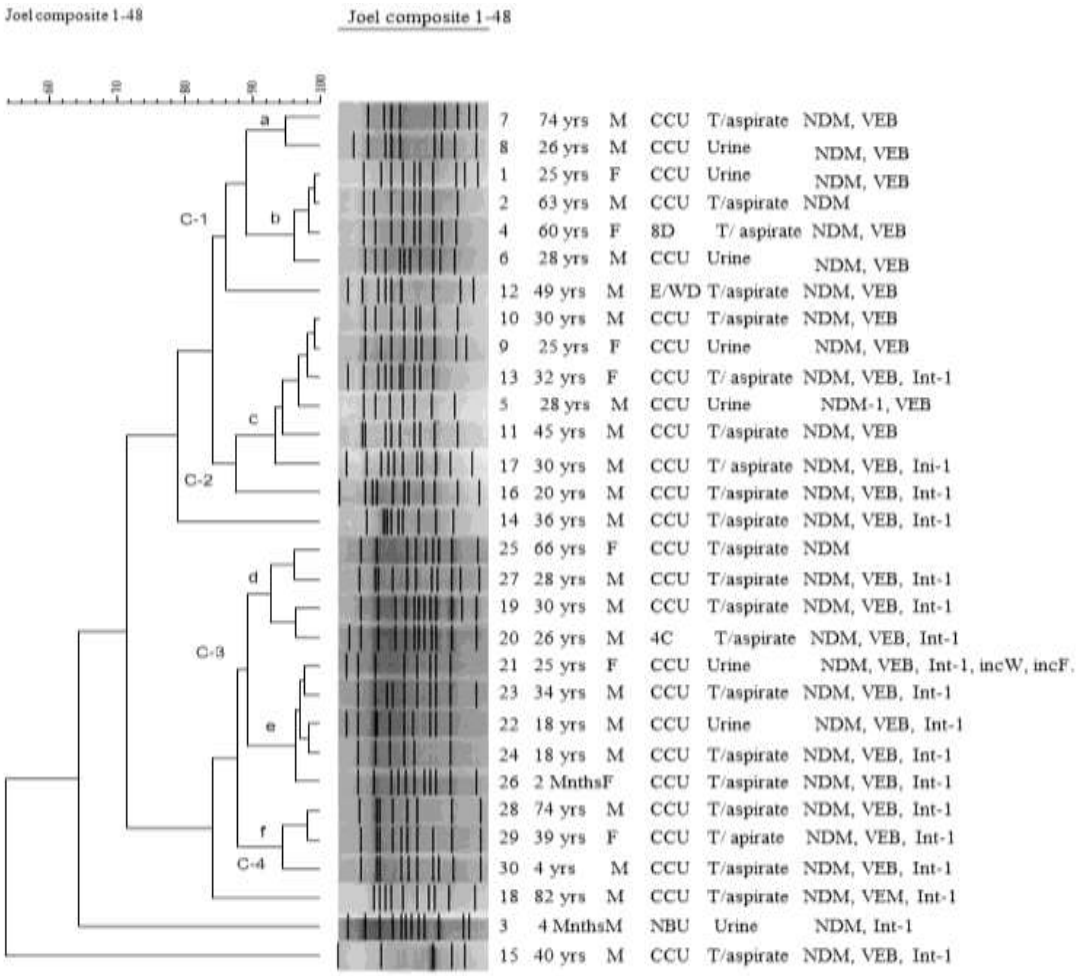

Figure 4.2: Fingerprint analysis for *P. aeruginosa* isolates recovered from various wards of Kenyatta hospital

Key: **C**- cluster, **F**- female, **M**- male, CCU- critical care unit, NBU- new-born unit, 8D,4C& E/WD- medical wards, T/aspirate- tracheal aspirate, NDM- New Delhi Metallo-beta lactamases, VEB- Verona extended beta lactamases, Int-1- class 1 integron, *inc*- incompatibility group. Phylogeny analysis showed high similarity of isolates in many cluster which is highly indicative of clonal proliferation of these *Pseudomonas aeruginosa* strains.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The current study recorded *P. aeruginosa* prevalence of 17% at Kenyatta National Hospital. This findings show an increase of 7% in nosocomial infections associated with *P.aeruginosa* compared to 10% documented earlier in Kenyatta national Hospital (Muthoni, 2012). *P. aeruginosa* is an opportunistic pathogen but is heavily implicated in nosocomial infections amongst immunocompromised patients. In the wake of widespread antibiotic resistance in both environmental and hospital settings, control of this pathogen has been difficult. Of major concern are *P. aeruginosa* strains with inherent resistance to multiple antimicrobial agents that are heavily used in inpatient wards. Although clinical *P. aeruginosa* are generally sensitive to ceftazidime, amikacin and ciprofloxacin, our findings were different. We recorded high levels of resistance to cephalosporins, aminoglycosides, and fluoroquinolones. Ciprofloxacin has been widely used in Kenya in combined therapy with ceftazidime among CCU patients, which potentially increases the risk of resistance build-up due to selective pressure. However, resistance to ciprofloxacin (53.2%) was found to be relatively lower compared to that of levofloxacin (73.7%) probably suggesting overuse leading to resistance build up.

Carbapenemases *P.aeruginosa* producers are normally susceptible to monobactams such as Aztreonam (Lister*et al.*,2007), however, this study revealed high resistance to this antimicrobial agent (67.4%). Although we only screened for MBLs in our isolates, previous studies have associated aztreonam resistance in carbapenemases producers to carriage of Extended Spectrum β-lactamases (ESBL) and AmpCs such as CMY-1 and CMY-2 (Lister *et al.*, 2009a).The high level of resistance observed for aminoglycosides, β-lactams and fluoroquinolones suggest additional resistance mechanisms in addition to the bla_{NDM} and bla_{VEB} detected in our isolates. Resistance to β-lactams antibiotics has also been associated with the production of Extended spectrum β-lactamases enzymes (ESBLS) that are mostly plasmid-encoded (Bush *et al.*,2010). Therefore, such genes can easily spread to susceptible strains and in turn

impact negatively on our ability to combat serious life-threatening infections in multiple wards. The combined resistance to β-lactams, fluoroquinolones and aminoglycosides which are widely used as anti-Pseudomonal agents makes treatment problematic especially in developing countries where carbapenem are not readily available or affordable. Resistance to carbapenem further narrows the treatment of MDR *P. aeruginosa* infections.

Although 127 out of 188 *P. aeruginosa* isolates were resistant to meropenem, only 62 (48.8%) were positive for bla_{NDM} and/or $bla_{VEB.}$ None of the isolates tested positive for bla_{VIM} genes that have been reported in this species in previous studies in Kenya. A study conducted in Kenya reported bla_{VIM-2} from all imipenem resistance *P.aeruginosa* isolates obtained from urine and blood samples (Pitout *et al.,* 2008). However, the current study revealed higher resistance to carbapenem compared to an earlier study that reported a prevalence of 53%. Our findings also differ from the Agha Khan study where resistance to ceftazidime, ciprofloxacin, gentamicin and amikacin was 100%. In both studies however, most of the multidrug resistance strains of *P. aeruginosa* were obtained from the CCU (Pitout *et al.,* 2008). This observation is in line with previous studies that have shown that CCU are hotspots of MDR strains (Acharya *et al.,* 2017). MDR *P. aeruginosa* organisms are highly adaptable to antimicrobial agents' selective pressure (Threlfall *et al.,* 2000). This selective pressure is even higher in hospital settings such as the CCU due to stronger antimicrobial therapy including injectable drugs such as amikacin and ceftriaxone. Other risk factors for colonization by MDR *P. aeruginosa* in these settings include concurrent diseases, previous exposure to anti-*Pseudomonas* antibiotics and mechanical ventilation. The current study found high levels of resistance to meropenem and amikacin in this ward, both of which are widely used in such settings. Our results are consistent with findings of a study conducted in a tertiary hospital in Nepal (Acharya *et al.*, 2017) where most of MBLs-producing *P. aeruginosa* were from CCU.

To date, only a handful of studies in Africa have reported bla_{VEB} positive *P. aeruginosa*, and this include studies in Egypt and South Africa (Khajuria *et al.,* 2013). The prevalence of VEB among carbapenem resistant isolates in the current study was however lower compared to the later studies. In the East Africa region, *bla*NDM-1 has

been reported in carbapenem resistant *Acinetobacter baumannii* clinical isolates (Revathi *et al.*, 2013). In other parts of the world, *bla*$_{\text{NDM-1}}$ positive *P. aeruginosa* has been reported in Serbia, France, India, Italy and Singapore (Carattoli *et al.*, 2013). In another study, *bla*$_{\text{VEB-1}}$-like genes were present as a gene cassette on class 1 integron in *P. aeruginosa* from Thailand (Girlich *et al.*, 2002). This, therefore, means that these resistance mechanisms may be spreading across continents. This is most likely being fuelled by international travels.

To the best of our knowledge, this is the first report of co-carriage of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{VEB-1}}$ genes in single isolate in Kenya. Isolates that harbored these two determinants were also resistant to the third-generation cephalosporin, aminoglycosides, fluoroquinolones and also aztreonam. Although we did not confirm the cassette content of the integron detected, high MDR phenotype and carriage of MBLs genes was associated with carriage of an integron. Previous studies have implicated these kinds of resistance to carriage of *bla*$_{\text{VEB}}$ in *P. aeruginosa*. Co-carriage of *bla*$_{\text{NDM}}$ and *bla*$_{\text{VEB}}$ in *P. aeruginosa* therefore means that only a few anti-Pseudomonal agents are effective to such strains. The high resistance recorded in both studies particularly against gentamicin and amikacin pose a serious health-care problem in the country especially in the CCU where injectable aminoglycosides are more important for coma and other patients who may be unable to swallow other drugs taken orally.

In the current study, 36 MDR isolates were found to carry class 1 integron. The higher prevalence of class-1 integron compared to class 2 and 3 is consistent with findings of previous studies conducted in south Nigeria which reported 57.4% incidence rate. Integron class 1 has also been reported in *P. aeruginosa* encoding *bla*$_{\text{GES-2}}$ extended β-lactamases in a study conducted in South Africa (Poirel *et al.*, 2002). We also recorded significant statistical association between carriage ofclass-1 integron and combined resistance to β-lactams, aminoglycosides, and fluoroquinolones. Our findings are in line with a study conducted by Odumosu, *et al.*, 2013, where resistance to carbenicillin (80.6%), ceftriaxone (87.1%) and tetracycline (100%) was significantly associated with carriage of class-1 integron in *P. aeruginosa* isolates. Other studies have also implicated resistance to these antimicrobial agents to genes carried by integrons (Pazhani *et al.*, 2011). Resistance to extended spectrum β-lactams and carbapenem is mostly plasmid-

borne bearing integron (Poirel *et al.*, 2002). Genetic elements carried by these integron are very stable and can easily spread to other strains and bacterial species leading to more resistance (Tenover, 2006). Plasmids have also been reported to transfer resistance agents to other bacterial strains or species via horizontal gene transfer. Plasmid-mediated horizontal gene transfer has been implicated in *P. aeruginosa* resistance to β-lactams, carbapenem and aminoglycosides (Walsh, 2008). Our study however was not able identify resistance cassettes of the detected integrons and whether they were borne in mobile plasmids. We detected two MDR-strains that harbored both *inc*FIB and *inc*W plasmids and a single isolate that carried *inc*N. In the absence of whole genome sequencing or conjugation experiments, it was however not possible to determine what resistances are conferred by these plasmids. To the best of our knowledge, this is the first report of these types of plasmids in *P. aeruginosa* isolates in Africa. These plasmids have been implicated in resistance to ampicillin, streptomycin, gentamicin, amikacin, trimethoprim, nalidixic acid and chloramphenicol in *Escherichia coli* (Sader *et al.*, 2001).The *Inc*N plasmid has been reported to harbor β-lactamase and carbapenemases genes such as $bla_{\text{CTX-M}}$, bla_{IMP}, bla_{NDM}, and bla_{KPC} in *Klebsiella pneumoniae* and *Escherichia coli* isolates (Humphrey *et al.*, 2012). Integron class 1 and 2 borne in plasmid *inc*FIB has been reported in clinical isolates of *Escherichia coli* that were highly resistant to sulfonamide and streptomycin (Odetoyin *et al.*, 2018).

The phylogenetic clustering of the isolates collected in a 6-months period in this study revealed a significant similarity of >80%. Significant genetic similarity was observed in isolates obtained from diverse in-patient population from different wards in Kenyatta National Hospital. There is therefore a strong indication that some of these strains are spreading in various wards possibly through contaminated devices or human contamination. The distinct clustering observed in this study may indicate cross-infection and transmission within different wards. The low resolution cluster analysis PCR may indicate clonal proliferations in such settings but future studies based on SNP analysis of whole genome data may shed better light on this assumption. It was also noted that most of the genetically related strains were from intensive care unit from tracheal swab specimen which possibly could be the source origin. The strong evidence of genetic relatedness of MDR strains established in this study may reflect a

dysfunctional or lack of proper antimicrobial resistance monitoring, prevention and control policy in this health facility.

5.2 Conclusion

From the research findings documented in this study, we conclusively deduce the following;

1. The high prevalence of *P. aeruginosa* documented in this study indicates that a majority of in-patients in most wards in Kenyatta National Hospital are at risk of multiple severe infections previously associated with this bacterium.
2. There is a great risk of treatment failure of infections that may emanate from this bacterium as noted by high level of resistances to multiple antimicrobial agents.
3. The high level of *P. aeruginosa* recovery and antimicrobial resistance in critical care unit (CCU) is a major indication that this section is hotspot for MDR-strains hence a great risk for in-patients.
4. The high similarity matrix of isolates within and across wards at KNH strongly suggests clonal spread of MDR-strains that perhaps leads to nosocomial infections in this health facility.

5.3 Recommendations

1. Proper diagnosis and antimicrobial susceptibility testing should be conducted for infections prior to treatment. This will consequently reduce over reliance on empirical treatment which is nonspecific, improving treatment and possibly preventing antimicrobial resistance build up.
2. Measures should be put in place to prevent cross-infection between wards which in return will help in reduction of nosocomial infections in Kenyatta National Hospital.

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APPENDICES

Appendix I: Gel electrophoresis

Procedure for Preparation of 1% agarose for electrophoresis;

1.0 g of agarose powder will be weighed and added to 99 ml of distilled autoclaved H_2O in a 400 ml conical flask, then add 1ml of 50X TAE buffer.

The mixture will be swirled and heat in a microwave for 1.5 minutes before allowing the mixture to cool then add 5 microlitre of Ethidium bromide. The warm agarose solution will then be poured into a mold that has combs then allowed to set by leaving it on the bench at room temperature for a while.

Loading of samples on the agarose gel

The gel will be placed in an electrophoresis tank that has TAE buffer up to level. The samples will be mixed with the loading dye on a paper film and loaded onto the wells (the dye contains glycerol that aids in visibility and has a density to assist in the migration).

The electric current will be connected for migration to take place (at 80volts for 1 hour). The results will be examined under UV illumination (the two dyes have different molecular weights i.e., leading dye-bromophenol blue has low molecular weight). Good bands should always be between the two dyes. Primer dimmers always go beyond bromophenol blue. Gels can last up to one week when stored at 4 degrees centigrade and will be covered with Parafilm or aluminum foil. Photos will be taken immediately after running the gel, to avoid the bands diffusing.

Appendix II: Information and Consent form

ANALYSIS OF INTEGRON AND ASSOCIATED GENE CASSETTES IN CLINICAL ISOLATES OF MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA*FROM KENYATTA NATIONAL HOSPITAL

INTRODUCTION: My name is KILIVWA J S MUKAYA, a Master of Science degree (Molecular Medicine) student at Jomo Kenyatta University of Agriculture and Technology (ITROMID campus). We are undertaking a study on integron and associated gene cassettes in clinical isolates of multidrug-resistant *P. aeruginosa* from KNH

We would wish to recruit you to participate in this study, and we are seeking your consent.

PURPOSE OF STUDY: To analyze integron and associated gene cassettes in clinical isolates of multidrug-resistant *P. aeruginosa* from KNH.

PROCEDURE: If you agree to participate in this study, you will be required to answer a few questions in the questionnaire provided and give a specimen for culture and sensitivity

ANY RISKS OF STUDY? No additional specimen will be obtained from you apart from the specimen collected by a clinician for routine laboratory investigation (urine, pus swab, blood or aspirates).

ARE THERE BENEFITS OF TAKING PART IN THE STUDY? There are direct benefits to the study subjects; no payments will be made for the testing and results of the study will (upon consent from subjects) be dispatched to the attending physician for further management and other relevant authorities, who will take measures to formulate guidelines to the hospital based on the results, and take the necessary course(s) of action.

WHAT ABOUT CONFIDENTIALITY? All the information obtained will be strictly confidential and data password protected and only accessed by the principal

investigator, subjects/participants in the study will be kept anonymous, being identified only by specific numbers assigned by the principal investigator and results obtained will be made available to the health care givers only with consent from the subjects.

WHAT ARE THE COSTS? There will be no costs for the participants in this study.

SUMMARY OF YOUR RIGHTS AS A PARTICIPANT IN A RESEARCH STUDY

This study is voluntary, subjects will be free to withdraw from the study at any point and will not be penalized in any way, and subjects will not also be waiving any of their legal rights by signing this informed consent document.

CONTACT INFORMATION

The following persons will be available for contact in the event of any research related questions, comments or complaints:

Principal Investigator: Kilivwa J S Mukaya

Tel. number 0722215825

Email: mkilivwa@gmail.com

Ethical Review committee:

Postal address 54840-00200, Nairobi

Tel. number 254-202726781

1. **Adult consent**

I have read the Consent Form and conditions of this project. I have had all my questions answered. I at this moment acknowledge the above and give my voluntary consent.

Before we involve you in the study, we kindly request you to append your signature below in the consent form.

I…… have read and understood the purpose and benefits of the study and I at this moment agree to participate in the study.

Participant's Name.....

Study Case Number.....

Address.....

Telephone Number.....

Signature.....Date.....

Child assent form

I at this moment acknowledge the above and give my voluntary consent. All details regarding this research study have been explained to me and in presence of my guardian and I have understood.

I…… have read and understood the purpose and benefits of the study and I at this moment agree to participate in the study.

Child participant's Name.....

Study Case Number.....

I…… have read and understood the purpose and benefits of the study and I at this moment agree to

participate in the study. By signing this assent form I acknowledge my permission to allow my child to participate in this research study.

Guardian’s Name.....

Address.....

Telephone Number.....

Signature.....Date.....

Appendix III: Questionnaire

ANALYSIS OF INTEGRON AND ASSOCIATED GENE CASSETTES IN CLINICAL ISOLATES OF MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA* FROM KENYATTA NATIONAL HOSPITAL

1. Study Case Number……..

2. Sex……

-Male……..

-Female……..

3. Age (Years)…….

4. Residence.....

5. Occupation.....

6. Clinical diagnosis……..

4. Duration hospitalized…….

5 Whether on any antibiotics (if the answer is yes, for how long) …….

6. Type of specimen……

Appendix IV: Maelezo na kudhibitisha

ANALYSIS OF INTEGRON AND ASSOCIATED GENE CASSETTES IN CLINICAL ISOLATES OF MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA* FROM KENYATTA NATIONAL HOSPITAL

Utangulizi: Jina langu ni KILIVWA J S MUKAYA mwanafunzi wamasomo ya uthibiti katika chuo cha Jomo Kenyatta University of Agriculture and Technology (ITROMID campus). Tunafanya utafiti kuchunguza chanzo cha viini sugu kwa madawa (MDR *P. aeruginosa)* katika Hospitali Kuu ya Kenyatta.

Tungependa uwe mshiriki katika utafiti huu na tunaomba uthibitishe kushiriki kwa hiari.

Kiini cha utafiti: Kuchunguza chembechembe zinazo sababisha viini sugu (MDR *P. aeruginosa*) kwa madawa yanayo tumika kutibu

Mwelekeo: ukikubali kushiriki utahitajika kujibu maswali kadhaa kwa mwelekezo utakayo pewa na kisha baadaye kutolewa sampuli kama damu, mkojo usaa na kadhalika, ili kufanyiwa uchunguzi katika maabara ya hospitali kuu ya Kenyatta.

Jeemadhara? Hakuna sampuli ya ziada ambayo itatolewa kwako ila tuu ile itakayotolewa na muhudumu wa afya kwa uchunguzi wa kawaida.

Kuna faida gani kushiriki katika utafiti huu? Kuna manufaa ya moja kwa moja kwa mshiriki; hatahitajika kulipia gharama ya maabara na uchunguzi ukishafanywa, matokeo kwa idhini ya mshiriki yatapewa daktari ili kuanzisha ama kuendeleza matibabu.

Siriyamshiriki? Mtafiti mkuu atahakikisha kwamba matokeo na kushiriki kwa kila atakaye jitolea yanadhibitiwa na siri kuhifadhiwa. Washiriki watatambuliwa tuu kwa nambari maalumu na walasi kwa majina yao. Matokeo yanaweza tuu kutolewa kwa daktari kwa idhini kutoka kwa mshiriki.

Kuna gharama kwa mshiriki? Hakuna gharama yoyote kwamshiriki.

MUKTASARI WA HAKI YA MSHIRIKI

Utafiti huu ni huru kwa mshiriki na hakuna kushurutishwa kwa yeyote. Mshiriki ako huru kujiondoa kutoka kwa utafiti huu katika kiwango chochote na hatagharamika kwa njia yeyote ile. Mshiriki hatakuwa akiasi haki yake kwa kutia sahihi kushiriki utafiti huu.

ANWANI YA KUWASILIANA

Watu hawa wanaweza kupatikana kwa anwani ambayo imepeanwa kwa habari na maswali kuhusiana na utafiti huu:

Principal investigator:

- **Kilivwa J S Mukaya.**

  Nambari ya simu 0722-215 825.

Barua pepe:mkilivwa@gmail.com

- **Ethical Research Committee**

  Anwani ya posta 54840-00200, Nairobi

Nambari ya simu 254-202726781

Idhini ya kushiriki

Nimesoma na kuelewa maelezo kwa kushiriki utafiti huu na kujibiwa maswali yote na nimekubali kwa hiari bila kushurutishwa.

Kabla ya kukushirikisha kwa utafiti huu, tungependa kukusihi kwa hiari utie sahihi yako hapa chini kama utakavyo elekezwa.

Mimi……nimesoma na kuelewa lengo na manufaa ya utafiti huu na ninathibitisha kushiriki utafiti huu.

Jina la mshiriki……

Nambari ya usajili……...

Anwani…….

Nambari ya simu……....

Sahihi……Tarehe……

Appendix V: Maswali elekezi

ANALYSIS OF INTEGRON AND ASSOCIATED GENE CASSETTES IN CLINICAL ISOLATES OF MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA* FROM KENYATTA NATIONAL HOSPITAL

1. Nambari ya usajili …….

2. Jinsia

    -Kiume…….

    -Kike…….

3. Miaka…….

4. Makaazi…….

5. Kazi…….

6. Mudawa kulazwa hospitalini……

7. Kama mshiriki ameanzishwa madawa (kama jawabu ni ndio, ni kwa muda gani? Nani madawa gani?) ……

8. Ni sampuli aina gani imechukuliwa (damu, mkojo, usaa na kadhalika) ……

Role of researchers

Each of the five research assistants was assigned specific wards to work with clinicians during specimen collection. They also ensured that specimen containers are available. One laboratory technologist worked with the principal investigator (PI) at KNH Microbiology Laboratory during specimen processing and analysis. The other two worked with the PI at CMR laboratory to carry out molecular analysis. The Quality Officer ensured that all processes involved in this research work were carried out as per the Standard Operating Procedures and quality upheld. Porters are tasked with transportation of specimen to the laboratory immediately they were collected.

For microbiology analysis (culture, identification and antimicrobial sensitivity testing), Muller Hinton culture medium was procured. 4 kits of Identification Cards (ID) and 4 kits of Antimicrobial Sensitivity Test cards (AST) for Vitek machine were bought for identification and sensitivity testing respectively. 300 disposable Petri dishes were bought.

For molecular analysis, agarose gel for plasmid and genomic DNA extraction were bought as well as the other requirement for the analysis at this stage. For PCR amplification and sequencing analysis, specific Primers were bought from the manufacturer. Sequencing were done at International Livestock Research Institute at a fee. (Kshs.100 per sample).

Appendix VI: Published manuscript 1

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Antimicrobial Resistance Profile and Genetic Profiling of *Pseudomonas aeruginosa* Strains Obtained from Different Inpatient Wards at Kenyatta National Hospital

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**Correspondence author: Kilivwa J S Mukaya*

Abstract: *This study sought to determine the antimicrobial susceptibility profiles of Pseudomonas aeruginosa isolates from inpatients populations at the Kenyatta National Hospital. A total of 188 P. aeruginosa strains were obtained from different inpatient wards from August 2015 to January 2016. Minimum inhibitory concentrations (MICs) were conducted on the Vitek 2-Compact (Biomereux company-France). P. aeruginosa ATCC 27853 and E. coli ATCC 25922 were used as reference strains for drug resistance testing and interpretation done based on the CLSI 2017 guideline.*

High resistance was recorded towards Tetracycline (92%) with an MIC of ≥ 128µg/ml followed by Cefotaxime (88.8%) and Ceftriaxone (86.2%) with MICs of ≥ 64µg/ml. Lowest resistance was recorded towards Piperacillin (25%) and Amikacin (46.3%). Pseudomonas aeruginosa isolates recovered from the Critical care unit (CCU) recorded the highest resistance of 83% to all the antimicrobial tested while least resistance was observed in strains from the Newborn unit (NBU) ward (38%). On the other hand, isolates obtained from urine (92%) sample were the most resistant while lowest resistance was recorded from blood samples (29%). PCR screening revealed 68 Metallo β-lactamase (MBL) positive strains amongst 127 isolates that were Meropenem resistance. Resistance to Aztreonam amongst the 68 MβL positive producers was 89.7%. Resistance to CAZ, CIP, CN, and AMK was 82.4%, 80.9%, 88.2% and 78% respectively. At least 52(76.5%) of these MβL positive isolates were recovered from patients in the Critical Care Unit. Among the total 188 recovered P. aeruginosa, 48 (25.5%) carried class-1 integron with a single strain among them also harbouring a class-3 integron. Carriage of integron among the 64 bla_{VEB} positive isolates was 70.3%. Among the 68 isolates that were positive for bla_{NDM}, 47 (69.1%) carried class-1 integron. Overall, 45 (23.9%) among the 188 P. aeruginosa isolates were positive for a co-carriage of bla_{NDM}, bla_{VEB} and class I integron. Plasmid screening revealed 3 types of incompatibility groups. One P. aeruginosa isolate had both incW and incFIB, while another isolate had an incN. Phylogenic cluster analysis using the Gelcompar2® revealed four major clusters based on age, specimen type and wards. The four clusters had a significant genetic similarity of >80% amongst P. aeruginosa strains obtained from different wards which is indicative of cross-infection.

Keywords: *New Delhi Metallo-beta-lactamase; MβL – Metallo βeta lactamase; VEB-type beta-lactamases; Pseudomonas aeruginosa, Kenya*

Date of Submission: 20-07-2018 Date of acceptance: 04-08-2018

I. Introduction

Pseudomonas aeruginosa is a common flora of the skin, gut and also ambiguous in the environment. This organism however has a significant clinical importance as one of the major cause Health-care associated infections (HAI) and has been implicated in severe opportunistic infections in immunocompromised individuals. High antimicrobial resistance has globally been reported in clinical *P. aeruginosa* strains raising alarm due to associated high mortality rates ranging between 18% and 61% due to treatment failure [1]. Infections caused by this organism range from bacteremia, respiratory, urinary tract, skin and burn wound infections with a positive culture from blood, urine and tracheal aspirate specimens of infected patients [2]. Nosocomial infections caused by *P. aeruginosa* is approximated to be 8% in United States alone, however, prevalence in developing countries like Kenya remain largely underestimated. In the 2014 WHO report, Africa was identified as one of the regions that lack an established antimicrobial surveillance system. This underestimation is partially attributed to the complex HAI diagnosis and inadequate surveillance due to limited resources.

Antimicrobial resistance *in Pseudomonas aeruginosa* has partially been attributed to over-use and miss-use of antimicrobial agents resulting in the emergence of multiple drug resistance (MDR) strains. Another

mechanism of resistance involves production of β-lactamases and acquisition of plasmid-borne integron through horizontal gene transfer (HGT). Carriage of multiple integron containing long arrays of resistance cassettes consequently withstand high antibiotic selection pressure and are therefore likely to spread faster in hospitals, and in particular, in intensive care units. Class 1 integron implicated in resistance to important anti-Pseudomonal drugs such as third generation Cephalosporin, fluoroquinolones and advanced classes of Aminoglycosides such as Amikacin have been reported in *P. aeruginosa* [3-5]. Plasmid-borne integron contains a pool of gene cassettes therefore are more common among multiple drug resistance (MDR)-strains of *P. aeruginosa*.

Previous studies has shown that MDR strains of *P. aeruginosa* are predominant in the Intensive Care Units possibly due to the immune-compromised status of hospitalized patients and associated high volumes of antimicrobials used in such settings[6]. Other risk factor for colonization in such settings includes concurrent infections, prolonged hospitalization and use of invasive procedures such as catheters and mechanical ventilation [7]. The spread of plasmid borne integron has partially been attributed to usage of medical devices such as indwelling urinary catheters as well as person-to-person contamination. The emergence of Carbapenemase capable of hydrolyzing carbapems has aggravated the antimicrobial resistance problem in this burgs. This is because such drugs are considered the last resort for treatment of serious Gram-negative infections. Of particular importance are the Metallo-beta-lactamase (MβL) such as the bla_{NDM} that confer high resistance to Carbapenem and are harbored in plasmid bearing integron. These MβL borne in integron are therefore have a higher potential to spread to other susceptible bacteria. Among the MβL variants, bla_{VIM} and bla_{IMP} are the most prevalent in *P. aeruginosa* and have been widely implicated in numerous nosocomial outbreaks. Other MBL of clinical and epidemiological importance includes the bla_{SPM}, bla_{NDM} and bla_{VEB}.

Despite of the enormous threat associated with *P. aeruginosa* burgs, data on prevalence of Carbapenemase producers in the Africa continent remains scarce. In Kenya, only bla_{VIM-2} has been reported in *Pseudomonas aeruginosa* isolates from a tertiary hospital [4]. To the best of our knowledge, there is no data on other Carbapenemases and mobile genetic elements in multidrug resistance isolates of *Pseudomonas aeruginosa* in the country. We therefore set this cross-sectional study to determine the antimicrobial resistance profile and carriage of Carbapenemases, plasmids and integron in *P. aeruginosa* recoverable from different in-patient ward at Kenyatta National hospital. In order to access possible cross-infection within the hospital, we also sought to determine the genetic relatedness of recovered burgs using low resolution fingerprinting (ERIC-PCR).

II. Methods

Recruitment of patients and sample collection

In this hospital-based cross-sectional study, recruitment of the hospitalized participants and sample collection was done between August 2015 and January 2016. Upon participant consenting, specimens (pus swab, blood, urine, aspirates) were collected by clinicians in their respective wards/units using previously published method [8]. Clinical isolates were obtained from patients admitted in the Critical Care Unit (103), Renal Unit Ward (1), Burns Unit (2), Newborn Unit (4) and Medical Wards (77) (admission wards, maternity wards, oncology wards, theatres, accident and emergency, cardiology unit, Infectious Respiratory Disease and Orthopedic Units). Approximately 1ml of blood was collected into EDTA-coated vacationers while midstream urine was collected in sterile containers for analysis. Wound specimen were obtained from aspirated pus from ruptured or incised abscesses and transferred into a leak-proof sterile container.

Culture and susceptibility testing

Standard blood culture was done as previously published [8]. Aspirates were first homogenized by vortexing for 1 minute before culture on MacConkey agar and Blood agar [8]. Samples from urine were cultured on CLED and blood agar [8]. Standard colony and biochemical tests were used for identification of *Pseudomonas aeruginosa* [8]. Since *Pseudomonas aeruginosa* infections are rarely multi-clonal, a single colony from each successful culture was analyzed. Antimicrobial susceptibility testing disc diffusion method on Mueller-Hinton was performed for 12 antimicrobial agents; Ceftriaxone (CRO, 30µg), Ceftazidime (CAZ, 30µg), Cefotaxime (CTX, 30µg), Carbenicillin (CAR, 100µg), Piperacillin (PRL, 100µg), Aztreonam, (ATM, 30µg), Levofloxacin (LEV, 5µg), Ciprofloxacin (CIP, 5µg), Gentamicin (CN, 10µg), Amikacin (AK, 30µg), Tetracycline (TET, 30µg), Piperacillin/tazobactam (100/10µg) and Meropenem (MEM,10µg). Minimum inhibitory concentrations (MICs) were conducted on the Vitek 2-Compact (Biomereux Company-France). *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as reference strains. Interpretation of the zones was done using the CLSI guidelines.

2.2. PCR Amplification

Bacterial DNA extraction was done using the Chelex method as previously described [9]. PCR amplification of the DNA was done using selected consensus primers for detection of Carbapenemases frequently reported in *P. aeruginosa* that includes *bla*PER, *bla*GES, *bla*NDM, *bla*GIM, *bla*SPM and *bla*VEB (table 1). The PCR amplification program consisted of an initial denaturation (94 °C, 5 minutes) followed by 35

cycles of denaturation (94 °C, 60 seconds), annealing temperature depending on the primer and a single final extension for 5 minutes at 72 °C. Replicon typing of plasmid was done using 5 multiplex and 3 simplex PCR assays as previously described by Carattoli [16] (table 2). Separation of the amplified DNA was done suing 1.5% agarose gel and visualization of the bands done on UV Gelmax imager.

Table1. Amplification primers for Metallo βeta-lactamases and integron

Primer Name	5'-3' Sequence	Base pairs	Annealing temperature (°C)	Reference
VEB-F VEB-R	CGACTTCCATTTCCCGATGC TGTTGGGGTTGCCCAATTTT	371	56	[9]
NDM-F NDM-R	ACTTGGCCTTGCTGTCCTT CATTAGCCGCTGCATTGAT	621	56	[9]
SPM-F SPM-R	AAAATCTGGGTACGCAAACG ACATTATCCGCTGGAACAGG	271	52	[11]
PER-F PER-R	ATGAATGTCATTATAAAAGC AATTTGGGCTTAGGGCAGAA	933	50	[11]
GES-F GES-R	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGGA	863	56	[9]
GIM-F GIM-R	TCGACACACCTTGGTCTGAA AACTTCCAACTT TGCCATGC	477	52	[11]
5_CS 3_CS	GGCATACAAGCAGCAAGC AAGCAGACTTGACCTGAT	Variable	52	[11]
IntM1_U IntM1_D	ACGAGCGCAAGGTTTCGGT GAAAGGTCTGGTCATACATG	441	60	[11]
INT_1U INT_1D	GTTCGGTCAAGGTTCTG GCCAACTTTCAGCACATG	923	60	[11]
INT2-L INT2-R	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	789	50	[11]
Int3-F Int3-R	AAATGACAAACCTGACTG CGAATGCCCCAACAACTC	922	60	[11]
ERIC R ERIC2	ATGTAAGCTCCTGGGGATC AAGTAAGTGACTGGGGTGAGCG	variable	50	[12]

Table 2: Amplification primers incompatibility group plasmids

	Sequence (5'-3')	Primers	Annealing temperature (°C)	Product
Multiplex -1	GGAGCGATGGATTACTTCAGTAC	HI1 – F	60	471 bp
	TGCCGTTTCACCTCGTGAGTA	HI1 – R		
	TTTCTCCTGAGTCACCTGTTAACAC	HI2- F	60	644 bp
	GGCTCACTACCGTTGTCATCCT	HI2 – R		
	CGAAAGCCGGACGGCAGAA	I1 – F	60	139 bp
	CGTCGTTCCGCCAAGTTCGT	I1 – R		
Multiplex -2	AACCTTAGAGGCTATTTAAGTTGCTGAT	X – F	60	376 bp
	GAGAGTCAATTTTTATCTCATGTTTTAGC	X –R		
	GGATGAAAACTATCAGCATCTGAAG	L/M – F	60	785 bp
	CTGCAGGGGCGATTCTTTAGG	L/M – R		
	GTCTAACGAGCTTACCGAAG	N – F	60	559 bp
	GTTTCAACTCTGCCAAGTTC	N – R		
Multiplex -3	CCATGCTGGTTCTAGAGAAGGTG	FIA – F	60	462 bp
	GTATATCCTTACTGGCTTCCGCAG	FIA – R		
	GGAGTTCTGACACACGATTTTCTG	FIB – F	60	702 bp
	CTCCCGTCGCTTCAGGGCATT	FIB – R		
	CCTAAGAACAACAAAGCCCCCG	W – F	60	242 bp
	GGTGCGCGGCATAGAACCGT	W – R		
Multiplex -4	AATTCAAACAACACTGTGCAGCCTG	Y – F	60	765 bp
	GCGAGAATGGACGATTACAAAACTTT	Y – R		
	CTATGGCCCTGCAAACGCGCCAGAAA	P – F	60	534 bp
	TCACGCGCCAGGGCGCAGCC	P- R		
	GTGAACTGGCAGATGAGGAAGG	FIC – F	60	262 bp
	TTCTCCTCGTCGCCAAACTAGAT	FIC – R		
Multiplex -5	GAGAACCAAAGACAAAGACCTGGA	A/C – F	60	465 bp
	ACGACAAACCTGAATTGCCTCCTT	A/C – R		
	TTGGCCTGTTTGTGCCTAAACCAT	T – F	60	750 bp

CGTTGATTACACTTAGCTTTGGAC	T – R		
CTGTCGTAAGCTGATGGC	FII – F	60	270 bp
CTCTGCCACAAACTTCAGC	FII – R		
TGATCGTTTAAGGAATTTTG	FrepB - F	60	270 bp
GAAGATCAGTCACACCATCC	FrepB - R		
GCGGTCCGGAAAGCCAGAAAAC	K/B – F	60	160 bp
TCTTTCACGAGCCCGCCAAA	K – R		
GCGGTCCGGAAAGCCAGAAAAC	B/O – F	60	159 bp
TCTGCGTTCCGCCAAGTTCGA	B/O – R		

DNA fingerprinting of recovered bacterial isolates

Enteric repetitive intergenic consensus (ERIC-PCR) using published primers (table 1) was used to determine the genetic relatedness of bla_{NDM} and bla_{VEB} Positive *Pseudomonas aeruginosa* isolates recovered from different wards. The PCR products were separated by running on 1% agarose with ethidium bromide gel for 1 hour. Banding patterns were visualized under ultraviolet light using a Gelmax® imager. Cluster analysis was done using Gelcompar®2 software version 6.6. Cluster analysis was done using the dice method based on banding pattern with arithmetic mean UPGMA. Isolates that had a correlation of ≥ 80% were considered genetically related [18].

Ethical consideration

All specimens were collected and processed in accordance with ethical Clearance approved by The National Ethics Committee number: SERU 3048 and Institutional Ethical Committee of Kenyatta National Hospital, reference number: UP44/02/2010.

III. Results

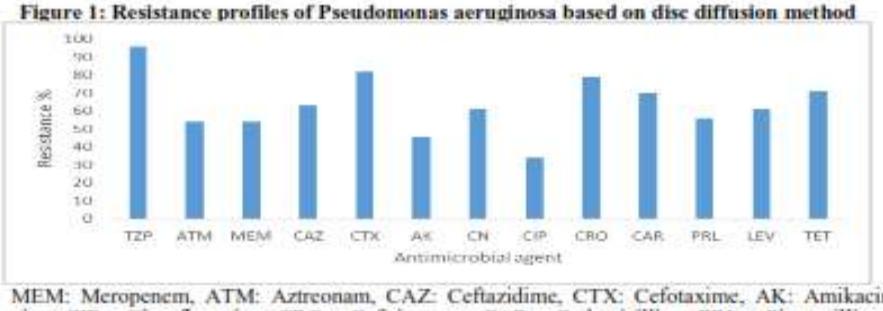
Bacterial Isolates

One hundred and eighty-eight non-duplicate clinical *P. aeruginosa* strains were isolated during a six-month period (August 2015 - January 2016) from CCU (103), Renal Unit Ward (1), Burns Unit (4), Newborn Unit (2), and Medical Wards (77). A total of 153 isolates were obtained from patients aged 50 years and below and 62% of 188 isolates were obtained from males while the rest (38%), were from females. A total of 103 tracheal aspirates, 55 pus swabs, 26 urine samples and 4 blood samples yielded clinical isolates of MDR *P. aeruginosa* respectively.

Antimicrobial susceptibility test based on disc diffusion method

All the 188 *Pseudomonas aeruginosa* isolates recovered in this study were multiple drug resistance strains (≥3 antimicrobial class). High resistance was recorded towards Piperacillin-tazobactam (96%) while Ciprofloxacin (34%) was the least resisted antimicrobial. All the isolates were resistance to one or more of extended cephalosporin (CAZ, CTX and CRO) with a percentage resistance of 63.1%, 82% and 79.7% respectively (figure 1). A high resistance to Carbapenemases (Meropenem, 54%) and Aztreonam (54%) was also revealed in this study.

Figure 1: Resistance profiles of Pseudomonas aeruginosa based on disc diffusion method



MEM: Meropenem, ATM: Aztreonam, CAZ: Ceftazidime, CTX: Cefotaxime, AK: Amikacin, CN: Gentamycin, CIP: Ciprofloxacin, CRO: Ceftriaxone, CAR: Carbenicillin, PRL: Piperacillin, LEV: Levofloxacin, TET: Tetracycline, TZP: Piperacillin/tazobactam

Antimicrobial susceptibility testing based on the Minimum inhibitory concentration

More than 70% of *P. aeruginosa* were resistant to β-lactams tested including Ceftazidime, Cefotaxime, and ceftriaxone with an MIC value of ≥ 32 µg/ml. High resistance to Aztreonam (67.4%) was recorded in this study. Resistance to Meropenem was 67.6% with an MIC value of ≥ 8µg/ml (table 3). However, contradictory to

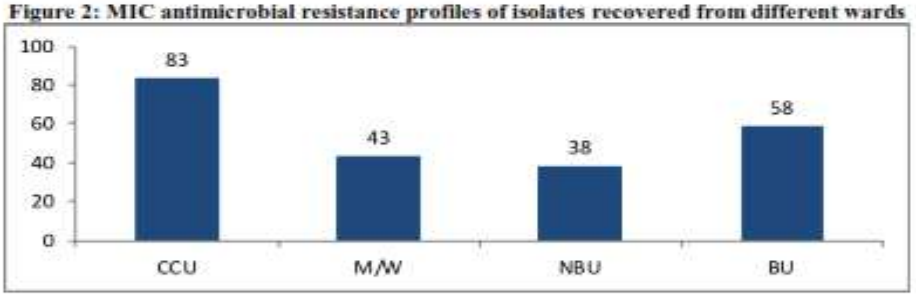
disc diffusion results, most resistance towards Tetracycline was recorded (92%) with MIC value of ≥ 8 µg/ml. The MIC test also revealed relatively high resistance to Ciprofloxacin (52.7) compared to the disc diffusion method where resistance was 34.3%. Piperacillin was the most effective antimicrobial, where only 47 (25.1%) out of the 188 *P. aeruginosa* isolates recovered been resistance. *P. aeruginosa* isolates obtained from the critical care unit (CCU) were the most resistant to overall antimicrobial (83%) used which includes. Isolates recovered burn unit also revealed a high resistance of (58%) followed by medical wards (43%) while those obtained from newborn unit (38%) were least resistance (figure 2). *Pseudomonas aeruginosa* isolates recovered from urine samples were the most resistant to tested antimicrobial with an overall resistance of (72.2%) been recorded. High resistance in *P. aeruginosa* isolates from Tracheal aspirates (76%) was also recorded while blood isolates were the least resistance (figure 3).

Table 3: MIC of *P. aeruginosa* isolates obtained from various sample types from different wards.

Drug	Resistance breakpoint* (µg/ml)	% resistance	Mode MIC	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
MEM	≥ 8	67.6	128	16	128
ATM	≥ 32	67.4	128	64	128
CAZ	≥ 32	70	64	64	64
CTX	≥ 32	88.8	64	64	64
AK	≥ 64	46.3	128	32	128
CN	≥ 16	67.9	128	64	128
CIP	≥ 4	52.7	128	32	128
CRO	≥ 32	86.2	64	64	64
CAR	≥ 64	57.4	64	64	64
PRL	≥128	25.	64	64	128
LEV	≥ 8	73.9	128	64	128
TET	≥ 8	92	128	64	128
TZP	≥ 128	50.5	128	64	128

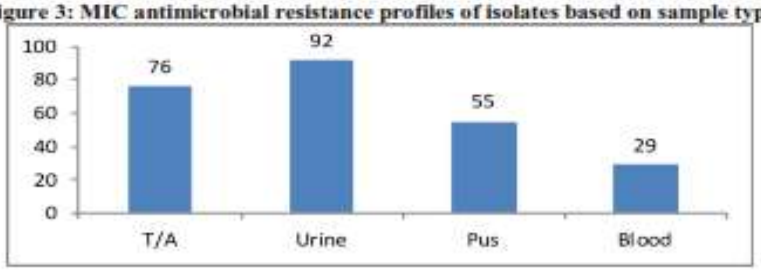
MIC: Minimum inhibitory concentration, MEM: Meropenem, ATM: Aztreonam, CAZ: Ceftazidime, CTX: Cefotaxime, AK: Amikacin, CN: Gentamycin, CIP: Ciprofloxacin, CRO: Ceftriaxone, CAR: Carbenicillin, PRL: Piperacillin, LEV: Levofloxacin, TET: Tetracycline, TZP: Piperacillin/tazobactam.

Figure 2: MIC antimicrobial resistance profiles of isolates recovered from different wards



CCU: Critical care unit, B/U: Burn unit, NBU: New born unit, R/U: Renal unit

Figure 3: MIC antimicrobial resistance profiles of isolates based on sample type



T/A: Tracheal aspirate

PCR analysis of Metallo-β-lactamase, Integron and Plasmid typing carriage in *P. aeruginosa* isolates

Amplified PCR products were obtained respectively for bla_{VEB} and bla_{NDM} using consensus primers PCR experiments with primers specific for bla_{TEM} bla_{SHV}, bla_{GES}, bla_{PER}, bla_{KPC}, bla_{GIM} bla_{SPM} were negative. Integron class I, II and III were screened in all *P. aeruginosa* isolates positive for bla_{NDM} and/or bla_{VEB}. The prevalence of bla_{NDM} carriage was the highest at 51.9% while carriage of bla_{VEB} was 49.6%. Fifty-two (76.5%) of these isolates were obtained from patients in Critical Care Unit among the 188 *P. aeruginosa*, 48 (25.5%) of these isolates carried class-1 integron with a single strain among them also harbouring class-3 integron. Carriage of class 1integron among the 64 bla_{VEB} positive isolates was 70.3%. Amongst the 68 isolates that were positive for bla_{NDM}, 47 (69.1%) carried class-1 integron. Overall, 45 (23.9%) among the 188 *P. aeruginosa* isolates were positive for a co-carriage of bla_{NDM}, bla_{VEB} and class I integron. Chi-square analysis found significant association in antimicrobial resistance to the drugs tested except for CTX and TET with carriage of integron (table 4). Plasmid screening revealed 3 types of incompatibility groups. One *P. aeruginosa* isolate had both W-Plasmid and a FIB-Plasmid, while another isolate had an N-Plasmid. The nucleotide sequences of the bla_{NDM} and the bla_{VEB} reported in this paper have been submitted to the EMβL/GenBank nucleotide sequence database under accession numbers KX857136 (https://www.ncbi.nlm.nih.gov/ nuccore/KX857136) and KX857137 (https://www.ncbi.nlm.nih.gov/nuccore/KX857137), respectively.

Table 4: Integron carriage association to antimicrobial resistance

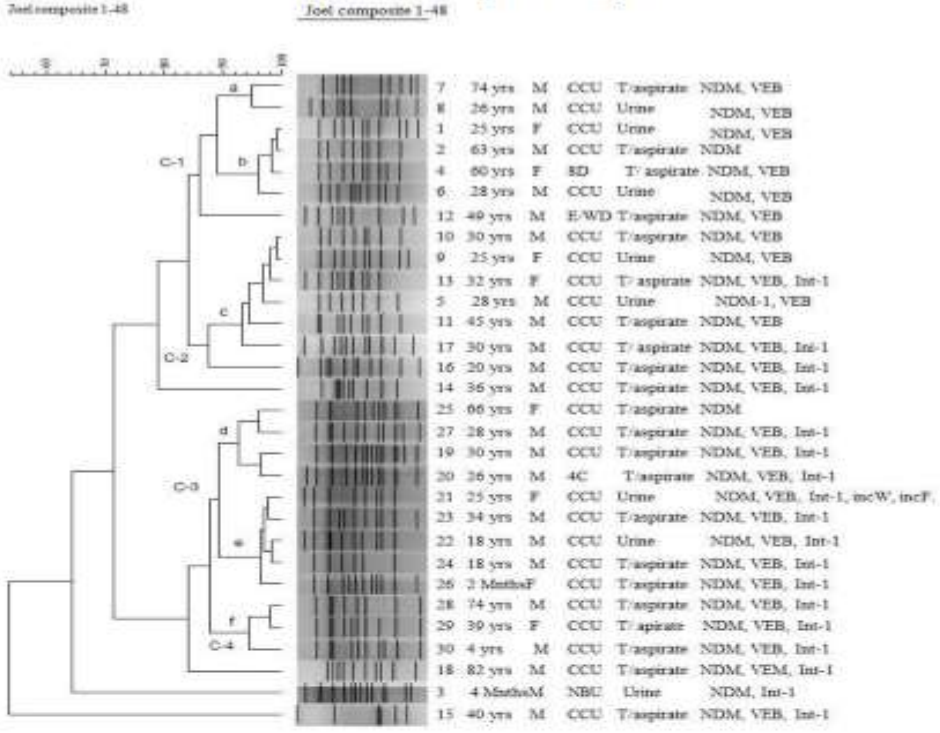
Antimicrobial	Integron positive (N=48) No.(%)			Integron negative (N=140) No.(%)			Chi-square P-value	Test significance
	R	I	S	R	I	S		
MEM	44 (91.6)	2 (4.2)	2 (4.2)	83 (59.2)	0 (0)	57 (40.7)	0.00001	S*
ATM	44(91.6)	2 (4.2)	2 (4.2)	82 (58.6)	0 (0)	58 (41.4)	0.0001	S*
CAZ	40 (83.3)	2 (4.2)	6 (12.5)	91 (65)	0 (0)	49 (35%)	0.004955	S*
CTX	47 (97.9)	0 (0)	1 (2)	119 (85)	10 (7.1)	11 (7.9)	0.141657	NS*
AK	40 (83.3)	0 (0)	8 (16.6)	41 (23.9)	5 (3.6)	94 (67.1)	0.00001	S*
CN	42 (87.5)	0 (0)	6 (12.5)	85 (60.7)	2 (1.4)	51 (36.4)	0.006661	S*
CIP	42 (87.5)	2 (4.2)	4 (8.3)	57 (40.7)	1 (0.7)	82 (58.6)	0.00001	S*
CRO	46 (95.8)	2 (4.2)	0 (0)	116(82.9)	1 (0.7)	23 (16.4)	0.028865	S*
CAR	44 (91.6)	2 4.2)	2 4.2)	63 (45)	45 (32.1)	32 (22.9)	0.00001	S*
PRL	38 (79.1)	8 (16.6)	2 4.2)	9 (6.4)	58 (41.4)	73 (52.1)	0.00001	S*
LEV	42 (87.5)	2 4.2)	4 (8.3)	96 (68.6)	1 (0.7)	43 (30.7)	.003063	S*
TET	48 (100)	0 (0)	0 (0)	124(88.6)	0 (0)	16 (11.4)	0 .137591	NS*
TZP	44 (91.6)	4 (8.3)	0 (0)	50 (35.7)	36 (25.7)	54 (38.6)	0.00001	S*

R=Resistant, I=Intermediate, S=Susceptible, S*=significant, NS*=Non-significant. The test was considered significant at $P < 0.05$.

Enterobacterial repetitive intragenic consensus polymerase chain reaction (ERIC-PCR)

Cluster analysis revealed four major clusters based on banding patterns with >80% similarity (figure 3). Isolates in the first cluster (C-1) harbored bla_{NDM} and bla_{VEB} in exception of a single strain that harbored only bla_{NDM}. Five out seven isolates were recovered from critical care unit (CCU) while only 2 were from medical ward. This cluster also revealed a sub-cluster (a) with 2 isolates from male CCU ward that harbored bla_{NDM} and bla_{VEB} with a 94% similarity. Another sub-cluster (b) showed 4 *P. aeruginosa* isolates obtained from patients in medical and critical care unit ward which had a significant genetic similarity of 96%. The second cluster revealed 8 isolates with a similarity matrix of 96%. All the isolates in the second cluster (C-2) were obtained from CCU patients in urine and tracheal aspirate samples. Cluster 3 (C-3) uncovered a sub-cluster (d) with isolates obtained in CCU and medical ward that had a 96% similarity. With exception of a single strain that carried bla_{NDM}, all other isolates in this sub-cluster harbored class 1 integron, bla_{NDM} and bla_{VEB}. Another sub-cluster (e) had 5 *P. aeruginosa* isolates obtained from CCU ward with a homogeneous resistance pattern and a significant similarity of 96%. A single isolate in this sub-cluster harbored *inc*W and *inc*FIB in addition to the other resistance genetic elements. Cluster 4 (C-4) on the other hand uncovered 3 homogenous isolates all of which were recovered from the critical care unit. The 3 isolates had a similarity matrix of >90%, all carrying class 1 integron, bla_{NDM} and bla_{VEB}.

Figure 3: Showing cluster analysis



C- cluster, F- female, M- male, CCU- critical care unit, NBU- new-born unit, 8D,4C & E/WD- medical wards, T/aspirate- tracheal aspirate, NDM- New Delhi Metallo-βeta lactamase, VEB- Verona extended βeta lactamase, Int-1- class 1 integron, *inc*- incompatibility group.

IV. Discussion

Pseudomonas aeruginosa is an opportunistic pathogen which plays a major role in nosocomial infections amongst immunocompromised patients. In the wake of widespread antibiotic resistance strains in both environmental and hospital settings, control of this pathogen has been futile. Of major concern are *P. aeruginosa* strains with inherent resistance to multiple antimicrobial agents that are heavily used in inpatient wards. Although clinical isolates of *P. aeruginosa* are generally sensitive to Ceftazidime, Amikacin and Ciprofloxacin, our findings were contradictory. We recorded high levels of resistance to Cephalosporin's, Aminoglycosides, and fluoroquinolones. Ciprofloxacin has been widely used in Kenya in combined therapy with Ceftazidime among ICU patients, which potentially increases the risk of resistance build-up due to selective pressure. However, resistance to Ciprofloxacin (53.2%) was found to be relatively lower compared to that of Levofloxacin (73.7%).

Carbapenemase *P.aeruginosa* producers are normally susceptible to Monobactam such as Aztreonam[16], however, this study revealed high resistance to this antimicrobial agent (67.4%). Although we only screened for MBLs in our isolates, previous studies have associated Aztreonam resistance in Carbapenemase producers to carriage of Extended Spectrum β-lactamases (ESBL) and AmpCs such as CMY-1 and CMY-2[17]. The high level of resistance observed in Aminoglycosides, B-lactams and fluoroquinolones suggest addition resistance mechanisms in addition to the bla_{NDM} and bla_{VEB} detected in our isolates. Resistance to β-lactam antibiotics has also been associated with the production of Extended spectrum β-lactamase enzymes (ESBLS) that are mostly plasmid-encoded[18]. Therefore, such genes can easily spread to susceptible strains and in turn impact negatively on our ability to combat serious life-threatening infections in multiple wards. The combined resistance to β-lactams, Fluoroquinolones and Aminoglycosides which are widely used as anti-Pseudomonal agents makes

treatment problematic especially in developing countries where Carbapenem are not ready available or affordable. Resistance to Carbapenem further narrows the treatment of MDR-*P. aeruginosa* resultant infections.

Although 127 out of 188 *P. aeruginosa* isolates were resistant to Meropenem, only 62 (48.8%) were positive for bla_{NDM} and/or bla_{VEB}. This findings are contradictory with other studies conducted in Kenya that reported bla_{VIM-2} from all imipenem resistance *P.aeruginosa* isolates obtained from urine and blood samples[22]. However, the current study revealed higher resistance to Carbapenem compared to later study that reported a prevalence of 53%. Our findings also differ from the Agha Khan study where resistance to Ceftazidime, Ciprofloxacin, Gentamicin and Amikacin was 100%. In both studies however, most of the multidrug resistance strains of *P. aeruginosa* were obtained from the critical care unit.

To date, only a handful of studies in Africa have reported bla_{VEB} positive *P. aeruginosa*, and this includes a study in Egypt and South Africa[9,23]. The prevalence of VEB among the Carbapenem resistance isolates in the current study was however lower compared to the later studies[24-26]. In the East Africa region, *bla*NDM-1 has been reported in Carbapenem-resistant *Acinetobacter baumannii* clinical isolates [19]. In other parts of the world, NDM-1 positive *P. aeruginosa* has been reported in Serbia, France, India, Italy and Singapore [26,28-30]. In another study, bla_{VEB-1} -like genes were present as a gene cassette on class 1 integron in *P. aeruginosa* from Thailand [25]. This, therefore, means that these resistance mechanisms may be spreading across continents. This is most likely been fuelled by international travels.

To the best of our knowledge, this is the first report of co-carriage of bla_{NDM-1} and bla_{VEB-1} genes in clinical isolate of *P. aeruginosa* in Kenya. Isolates that harbored these 2 resistance determinants were also resistant to the third-generation cephalosporin, Aminoglycosides, Fluoroquinolones and also Aztreonam. Although we did not confirm the content of the integron detected, high MDR phenotype and carriage of MβL genes was associated with carriage of an integron. Previous studies have implicated resistance antimicrobial resistance to carriage of bla_{VEB} in Pseudomonas aeruginosa isolates. The Co-carriage of bla_{NDM} and bla_{VEB} in *P aeruginosa* therefore means only a few anti-Pseudomonal agents are effective to such strains. The high resistance recorded in both studies particularly in Gentamicin and Amikacin in the ICU pose a serious health-care problem in the country. This is injectable antimicrobials are amongst the few available treatment options for patients in coma where oral treatment is not feasible.

Notably, recovery of multiple-drug resistance *P. aeruginosa* strains was predominant in the critical care unit. This observation is in line with previous studies that have shown that Critical Care Units (CCU) are a hotspots of MDR strains[20]. Multiple-drug resistance *Pseudomonas aeruginosa* organisms are highly adaptable to antimicrobial agents' selective pressure [3]. This selective pressure is even higher in hospital settings such as the ICU due to stronger antimicrobial therapy including injectable drugs such Amikacin and ceftriaxone. Other risk factors of colonization by MDR *P aeruginosa* in this settings included concurrent disease, previous exposure anti-*Pseudomonas* antibiotics and use of mechanical ventilation. The current study found high levels of resistance to Meropenem and Amikacin in this ward, both of which are widely used in such settings. Our results are consistent with findings of a study conducted in a tertiary hospital in Nepal[20] where most of MβL-producing *P. aeruginosa* were from ICU.

In the current study, 36 multidrug-resistant *P. aeruginosa* isolates were found to carry of class 1 integron. We also identified a single strain that harbored both class 1 and 3 integron. The high detection of class-1 integron opposed to class 2 and 3 is consistent with findings of previous studies conducted in south Nigeria which reported 57.4% incidence rate[9] Integron class 1 has also been reported in *P. aeruginosa* encoding bla_{OXA-2} extended β-lactamases in a study conducted in South Africa [21]. We also recorded significant statistical association of class-1 integron with high resistance recorded in β-lactams, aminoglycosides, and fluoroquinolones. Our findings are in line with a study conducted by Odumosu *et al* 2013 where resistance to Carbenicillin (80.6%), Ceftriaxone (87.1%) and Tetracycline (100%) was significantly associated with carriage of class-1 integron in *P. aeruginosa* isolates. Other studies have also implicated resistance to these antimicrobial agents to genes carried by integron [31]. Resistance to extended spectrum β-lactams and Carbapenem is mostly plasmid-borne bearing integron [22]. Genetic elements carried by these integron are very stable and can easily spread to other strains and bacterial species leading more resistance[15].

Plasmid have also been reported to transfer resistance agents to other bacterial strains or species via horizontal gene transfer. Plasmid-mediated horizontal gene transfer has been implicated in *P. aeruginosa* resistance to β-lactams, Carbapenem and Aminoglycosides[10]. We detected 2 multidrug *P. aeruginosa* that harbored both *inc*FIB and *inc*W and a single isolate that carried *inc*N. It was however not clear the kind of resistance conferred by these incompatibility plasmids groups. To the best of our knowledge, this is the first report of these types of plasmids in *Pseudomonas aeruginosa* isolates in Africa. These plasmids have been implicated with resistance to Ampicillin, Streptomycin, Gentamicin, Amikacin, Trimethoprim, Nalidixic Acid and Chloramphenicol in *Escherichia coli*. Previous studies have demonstrated transferability of such resistance genes harbored in plasmids to *P. aeruginosa* isolates [23].

The genotype cluster created from *P. aeruginosa* isolates collected in 5-months period revealed a significant similarity of >80%. Significant genetic similarity was observed in isolates obtained from diverse inpatient population from different wards in Kenyatta National Hospital. The distinct temporal association observed is therefore a strong evidence of persistent spread of MDR clones among patients in different wards. This finds may also reflect a lack or dysfunctional Antimicrobial resistance monitoring, prevention and control unit in this health facility.

In conclusion, reliable surveillance and control initiative programs should be initiated to prevent the spread of antimicrobial resistance in our medical facilities. Consequently, this will impact on early detection of strains with unique resistance and possible spread control initiatives enactment. Early detection also means appropriate treatment regime which is important in the prevention of antimicrobial resistance build up.

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Appendix VII: Published manuscript 2

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Research Article

Prevalence of Multidrug-Resistant *Pseudomonas aeruginosa* at Kenyatta National Hospital

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Abstract: This cross-sectional study was designed to determine the prevalence of multidrug resistance *P. aeruginosa* in Kenyatta National Hospital. Recruitment of patients and bacterial isolation was done in the period between August 2015 to January 2016. Aspirates, blood, urine and pus swab samples were obtained from patients in the critical care unit, new-born unit, renal unit and medical wards. A total of 188 non-duplicate *P. Aeruginosa* isolates were recovered. Antimicrobial susceptibility testing on 13 drugs was done using Kirby technique. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used for quality control of all susceptibility testing. Our findings revealed that all the 188 isolates were multidrug resistant. Piperacillin-tazobactam (96%) was the most resisted antimicrobial while Ciprofloxacin (65.7%) was the most susceptible. High resistance to Carbapenem (Meropenem 54%) and *β*-lactams (CAZ 63.1%, CTX 82%, CRO 79.7%, CAR 70.1% and ATM 54%) was uncovered. Notably, *P. aeruginosa* isolates recovered from Critical Care Unit (73.4%) were the most resistant.

Keywords: America Type Culturecollection (ATCC), Burns Unit (BU), Critical Care Unit (CCU), Kenyatta national hospital (KNH), Multiple-Drug Resistance (MDR), New-born Unit (NBU), *Pseudomonas aeruginosa* (*P. aeruginosa*), Renal Unit Ward (RU)

INTRODUCTION

Kenyatta National Hospital (KNH) is the largest hospital in Kenya with a bed capacity of 1800. This hospital gives priority to serious medical conditions (chest infection, severe head injury, sepsis, diabetes complications, cardiac complications, burns, autoimmune-related diseases, kidney complications among others). Previous independent observations have noted that more than 60% bacterial isolates from clinical specimen analyzed in Microbiology laboratory (KNH) from critical care areas are resistant to at least three antimicrobials. Multiple drug resistance has also been noted among the outpatients with over 35% representation (Microbiology Laboratory Kenyatta National Hospital). Although the actual drive to this observation has not yet been established, extended hospitalization has been suggested as a risk factor for acquisition of multidrug resistance in the hospital. Although it is illegal to buy drugs over the counter without the Doctor's prescription in Kenya, most of the correspondents admitted to previous use of un-prescribed medications. The high prevalence of *P. aeruginosa* MDR strains from urine revealed in the current study may reflect a corresponding heavy use of antimicrobials among these patients (Kiiru *et al*., 2012). The heavy use of antimicrobials has also been attributed as a major cause of resistance brought about by selective pressure to these agents (Gales *et al*., 2003).

The global threat of nosocomial multidrug-resistant *P. aeruginosa* is a growing concern among hospitalized patients. Infections caused by *P. aeruginosa* are severe and often associated with high mortality and morbidity rates. *P. aeruginosa* frequently develops resistance during therapy hence becoming challenging to treat (El Solh and Alhajhusain, 2009). *P. aeruginosa* has been reported to be resistant to structurally unrelated antibiotics attributed to a vast array of chromosomal and plasmid-mediated antibiotic resistance mechanisms (El Solh and Alhajhusain, 2009). Antimicrobial resistance in these strains has also been due to the acquisition of newer resistant genes from other organisms such as *Acinetobacter baumannii, Klebsiella pneumoniae* and *Salmonella* spp (Bonomo and Szabo, 2006).

Previous studies have attributed antimicrobial resistance in *P. aeruginosa* to the presence of one or more of these genetic elements (Su *et al*., 2010). Genetic elements such as plasmids, transposons and integron are means through which resistance genes are

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acquired leading to rising multidrug resistance *P. Aeruginosa* (Szabo *et al*., 2006a). These MDR strains have been implicated with high mortality and morbidity rates resulting from severe nosocomial infections ranging from the bloodstream, wound, urinary tract and respiratory tract infections especially in patients in ICU (Rossolini and Mantengoli, 2005; Varaiya *et al*., 2008).

This cross-sectional study sought to unravel the prevalence of Multidrug-Resistant *P. aeruginosa* at Kenyatta National Hospital.

MATERIALS AND METHODOLOGY

Kenyatta national hospital: The current study was conducted at Kenyatta National Hospital (KNH). This is the biggest referral hospital in East Africa and the sub-Sahara region located in the capital of Kenya, Nairobi. The hospital was founded in 1901 as a Native Civil hospital with a bed capacity of 40 which has since grown to 1800.

Recruitment of patients and sample collection: A cross-sectional study design was used to obtain aspirates, blood, pus swabs and urine samples from In-patients and Out-patients seeking medical attention at Kenyatta National Hospital between August 2015 and January 2016. These samples were obtained from consenting patients (relatives' approval sort for unconscious patients) in Critical Care Unit (CCU), Renal Unit Ward (RU), Burns Unit (BU), New-born Unit (NBU) and Medical Ward.

Clinical samples were obtained from patients using previously published methods (Monicah, 1999). In brief, a 1 mL blood sample was collected into EDTA-coated vacutainers while midstream urine was collected in a urine tube. Wound specimen and aspirates from incised abscesses were transferred into a leak-proof sterile container.

Bacterial isolation and Bio-typing: Blood cultures were done using the previously published methods (Monicah, 1999). Aspirate samples were first homogenized by vortexing before culture on Blood agar and MacConkey agar (Monicah, 1999). Fresh urine samples were cultured on CLED and blood agar (Monicah, 1999). Presumed *P. aeruginosa* on MacConkey and Blood agar were verified by biotyping using published methods.

Antimicrobial susceptibility testing: Kirby-Bauer disc diffusion method was used to perform antimicrobial susceptibility testing for *P. aeruginosa* isolates. Susceptibility testing was done using oxoid Mueller-Hinton agar on Tazobactam and Piperacillin (TZP, 110 µg), Amikacin (AK, 30 µg), Aztreonam, (ATM, 30 µg), Carbenicillin (CAR, 100 µg), Cefotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Ceftriaxone (CRO, 30 µg), Ciprofloxacin (CIP, 5 µg), Gentamicin (CN, 10 µg), Levofloxacin (LEV, 5 µg), Meropenem (MEM, 10 µg), Piperacillin (PRL, 100 µg) and Tetracycline (TET, 30 µg).*P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used for quality control susceptibility testing. The antimicrobial discs' zones were interpreted using CLSI guidelines (27th edition).

Ethical consideration: Ethical Clearance approved by Scientific Ethical Review Unit (SERU), KEMRI reference number 3048 and the Institutional Ethical Committee of Kenyatta National Hospital/University of Nairobi, reference number: UP44/02/2010.

RESULTS

Bacterial isolates: A total of 188 non-duplicate *P. aeruginosa* isolates were obtained from MacConkey and Blood agar cultures. Out of the 188 *P. aeruginosa* isolates,103 were from patients in CCU, 4 in Burns Unit, 77 in Medical Wards, 2 in New-born Unit and 1in the Renal Unit Ward. One hundred and seventeen (117) of these isolates were obtained from male participants while (71) were obtained from females.

Table 1: Antimicrobial resistance patterns of *P. aeruginosa* isolates recovered from various samples types

Antimicrobial agent	Resistance (%)	CCU (103) 73.4% R	M/W (77) 46.8% R	R/U (1) 46% R	NBU (2) 23.1% R	B/U (4) 44.2% R	T/A (103) 69% R	Urine (26) 77.2% R	P/S (55) 41.1% R	Blood (4) 13.5% R
TZP	96	72	22	0	0	2	67	19	9	1
ATM	54	74	25	0	0	2	71	18	12	0
MEM	54	75	24	1	0	1	71	17	13	0
CAZ	63.1	75	39	1	0	3	72	23	23	0
CTX	82.9	89	60	1	2	3	85	25	44	1
AK	46.5	64	23	0	0	0	59	17	11	0
CN	61	73	37	1	0	3	68	23	23	0
CIP	34.3	67	14	0	0	0	61	17	3	0
CRO	79.7	89	56	1	1	3	86	25	38	0
CAR	70.1	71	56	1	1	2	66	16	45	4
PRL	56.7	73	31	0	0	2	66	19	21	0
LEV	61	74	36	0	0	0	73	21	20	0
TET	71.1	84	45	0	2	2	70	21	32	1

TZP = Tazobactam/Piperacillin; MEM = Meropenem; ATM = Aztreonam; CAZ = Ceftazidime; CTX = Cefotaxime; AK = Amikacin; CN = Gentamycin; CIP = Ciprofloxacin; CRO = Ceftriaxone; CAR = Carbenicillin; PRL = Piperacillin; LEV = Levofloxacin; TET = Tetracycline; CCU = Critical Care Unit; B/U = Burn Unit; NBU = New born unit; R/U = Renal Unit; T/A = Tracheal Aspirate; P/S = Pus Swab; M/W = Medical Ward; R = Resistance

Antimicrobial susceptibility test: All of the 188 *P. aeruginosa* isolates were resistant to ≥ 1 drug from ≥3 class of antimicrobial and therefore multidrug resistant. Piperacillin-tazobactam (96%) was the most resisted antimicrobial while Ciprofloxacin was the most effective drug for *P. aeruginosa* isolates (Table 1). High resistance to Meropenem (54%) and β-lactams (CAZ 63.1%, CTX 82%, CRO 79.7%, CAR 70.1% and ATM 54%)) was revealed in this study. *P. aeruginosa* isolates obtained from patients in Critical Care Unit (CCU) were the most resistant as compared to other wards. Isolates obtained from urine samples were also revealed to be the most resistant to the tested antimicrobial.

DISCUSSION

P. aeruginosa has been implicated in severe infections among immune-compromised patients. This organism develops resistance to antimicrobial agents during treatment (El Solh and Alhajhusain, 2009) or through resistance genes acquisition via horizontal transfer from resistant strains or other species (Bonomo and Szabo, 2006). Infections caused by multidrug resistant *P. aeruginosa* have proven problematic to treat and have also been implicated with high mortality rate in hospitalized patients.

In the current study, all the 188 isolates of *P. aeruginosa* were multidrug resistant. High level of resistance to Carbapenem (Meropenem 54%) was revealed in the present study. Our findings are higher compared to results of a survey conducted in Nigeria (Odumosu *et al*., 2013) where Imipenem resistance of 9.6% among *P. aeruginosa* isolates was recorded. Resistance to Amikacin (25.5%), Gentamicin (51.6%), Ceftazidime (22.5%) and Cefotaxime (77.4%) was comparatively low compared to the current study. However, level of resistance to Tetracycline (100%), Ceftriaxone (87.1%) and Carbenicillin (80.6%) was higher compared to the current study which was at 71. %, 79.7% and 70.1%, respectively. In both studies, however, all the *P. aeruginosa* isolates were multidrug resistant. These findings therefore suggest that emergence and spread of MDR strains of *P. aeruginosa* isolates are on the rise. Resistance in these strains has been attributed to chromosomal and plasmid-mediated antimicrobial resistance determinants (El Solh and Alhajhusain, 2009). Integron that carries resistance gene cassettes has also been implicated in multidrug resistance *P. Aeruginosa* (Jeong *et al*., 2009). These integrons have been reported to carry genes that mediate resistance to *β*-lactams, aminoglycosides and other antimicrobial agents (Elbourne and Hall, 2006; Jeong *et al*., 2009)

In the current study, we revealed a high level of Resistance in *P. aeruginosa* isolates recorded in Critical Care Unit (73.4%). Our findings are supported by results of a previous study conducted in the ICU unit in Iran (Vaez *et al*., 2015). In the current study, however, resistance frequency was lower compared to the survey conducted in Iran; Meropenem (100%), Aztreonam (90%), Ceftazidime (90%), Cefotaxime (90%) and Ciprofloxacin (90%). The findings of the current study support findings of previous studies which have reported ICU to be a hotbed for MDR strains (Vaez *et al*., 2015). These resistances are associated with certain medical procedures like the use of catheters and mechanical ventilators (Rodrigues *et al*., 2011). Other risk factors for MDR-*P. aeruginosa* colonization in the ICU includes extended hospitalization and concurrent diseases.

The high level of resistance in Meropenem revealed in this study may suggest inefficacy of the drug in the affected patients. The high Carbapenem resistance poses a serious threat in treatments of serious infections caused by multidrug resistance *P. aeruginosa*. This is because these drugs are regarded the last resort drugs for the treatment of severe infections caused by gram-negative bacteria. Our study, however, revealed a low level of resistance to Ciprofloxacin (34.3%). This drug has been used to treat serious infections caused by *P. aeruginosa* and therefore proves to be still active.

CONCLUSION

The high prevalence of multidrug resistance *P. aeruginosa* uncovered by this study is an indication of continued emergence and spread of resistant strains. This situation therefore requires an urgent need to formulate Multidrug surveillance and control initiatives in hospitals to curb this menace. Clinical studies geared towards identifying risk factors for MDR development and establishing most efficacious antimicrobial regimes should also be encouraged. The hospital should also make it a mandatory undertaking, to conduct environmental surveillance through swabbing surfaces in wards such as sinks, to obtain samples for laboratory analysis. Regular fumigation in precincts and their environs should be embraced. The staff should also be screened on regular basis to avoid chances of clinicians becoming carriers, hence, source of transmission.

CONFLICT OF INTEREST

The author declares no conflict of interest in this study.

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