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

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Abstract

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

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

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is responsible for 10–15% of the nosocomial infections worldwide. Often these infections are hard to treat due to the natural resistance of the species, as well as to its remarkable ability of acquiring further mechanisms of resistance to multiple groups of antimicrobial agents (Strateva and Yordanov, 2009). The global emergence of *P. aeruginosa* resistance to multiple antibiotics including Beta-lactams represents an extraordinary threat to public health (Toval et *al.*, 2015). *P. aeruginosa* develops resistance by various mechanisms like multi-drug resistance efflux pumps, outer-membrane permeability, biofilm formation, production of β-lactamases and aminoglycoside modifying enzymes (Mahmoud et *al.*, 2013). In this context, infections by *P. aeruginosa* harboring acquired mechanisms of resistance, such as production of MBLs and ESBLs have high clinical impact. These "newer Beta-lactamases" are capable of hydrolyzing a wide range of Beta-lactam antibiotics, notably the extended-spectrum penicillins and the third and fourth generation cephalosporins, which include the carbapenems (Oberoi et *al*., 2013; Wadekar et *al.*, 2013). Carbapenems are the drug of choice for treatment of serious infections due to ESBL-producing organisms including P. aeruginosa but yet, carbapenem-resistant isolates have been reported (Ejikeugwu et *al.*, 2012). MBL-producing *P. aeruginosa* isolates are responsible for several nosocomial outbreaks from different parts of the World, illustrating the need for proper infection control practices (Pitout et *al.*, 2008). Those strains are particularly problematic leading to increased mortality, longer hospital stays and higher hospital costs over and above the values associated with susceptible strains of this pathogen (Hong et *al.*, 2015). Its current impact on antibacterial therapy failure encouraged us to find out the Beta-lactamases mediated resistance rate and current antibiotic options for effective treatment of this opportunistic organism (Ansari et *al.*, 2016). This study aimed to address the molecular characterization of Beta-lactams resistance in *P. aeruginosa* isolated from clinical sources in different wards at The Nairobi hospital in Kenya.

2. Materials and Methods

2.1 Collection of strains

P. aeruginosa used in this study were clinical isolates obtained from in-and out-patients at The Nairobi Hospital, Nairobi-Kenya. A total of 185 non-duplicate consecutive isolates were collected from the Pathology Department of the hospital between May 2017 and April 2018 and were identified from clinical Samples as *Pseudomonas aeruginosa* by use of Vitek 2 Compact System and stored at -20°C in a Brain Heart Infusion (BHI) Medium (Himedia, India) with glycerol (30%) according to standard techniques.

2.2 Antimicrobial Susceptibility patterns (David H., BioMérieux, USA*)*
Isolates on MacConkey (Oxoid, UK) were subcultured on Nutrient Agar plate (Oxoid, UK) and incubated at 35°C overnight. Inoculums were prepared from a pure culture, according to good laboratory practices. The 0.5McFarland bacterial suspension was diluted with $1.5X10^7$/ml in 0.45% saline and measured with DensiCheck. Aseptically, 3.0 ml of sterile saline (0.45% to 0.5% NaCl, pH 4.5 to 7.0) were transferred into a clear plastic (polystyrene) test tube (12 mm x 75 mm). A sterile swab was used to transfer a sufficient number of morphologically similar colonies to the saline tube. In a second tube containing 3.0 ml of saline, 145 µl of the suspension was transferred for AST-GN cards. (BioMérieux, USA) Then this tube was placed in the cassette with a susceptibility card. All the cards were automatically filled, sealed, and loaded into the Vitek 2 compact for instrument and reading. The MICs of each antimicrobial agent (piperacillin/tazobactam PTZ 110µg, cefotaxime CTX 30 µg, ceftazidime CAZ 30 µg, cefepime FEP 30 µg, meropenem MER 10 µg, aztreonam AZM 30 µg, gentamycin CN 10 µg, amikacin 30 µg and ciprofloxacin 5 µg) tested against *Pseudomonas aeruginosa* were generated by the Vitek 2 Compact according to MIC interpretation Standard in line with CLSI recommendations. In the Same time, the resistance to meropenem was interpreted by the expert system as a mechanism of Carbapenemase production for *P. aeruginosa*. The results were finally generated by the Expert Advanced System (EAS) of the Vitek 2 Compact for MBL phenotype. Alternative test methods such as targeted PCR amplification were required to confirm MBL-producing the isolates.

2.3 Extraction of total DNA
The total DNA content from ≤5X106 bacterial cells was extracted using Quick-DNA Mini-Prep plus Kit (Zymo Research, USA) via mechanical method according to kit's instructions. The eluted DNA was stored at ≤ -20°C for future use.

2.4 PCR assays for the detection of Metallo-Beta-Lactamases (MBL) encoding genes
Four pairs of specific primers introduced by Shibata et *al.* (2003) with one pair primer experienced by Mushi et *al.* (2014) (See Table 1) were used in this study, which specifically detected vim1, vim2, imp1, imp2, and ndm1 genes. The mixture for amplification of these genes consisted of Taq 2x Master Mix (BioLabs, UK) 12.5 µl followed by Forward-primer (10µmol/µl) 0.5 µl and Reverse-primer (10µmol/µl) 0.5 µl then Nuclease free water 9µl and lastly the DNA template 2.5 µl, in final volume of 25 µl. The PCR conditions were 5 minutes at 94ºC and 35 cycles of 1 min at 94ºC for the denaturation of the template, 45 seconds at an annealing temperature (A.T) of 51ºC and 1 min 30 seconds at 72ºC extension before a final extension at 72ºC (Sepehriseresht et *al.*, 2012).

2.5 Agarose gel electrophoresis
Horizontal electrophoresis system containing 2% agarose (AMRESCO, USA) gel in the TAE Buffer was used. Voltage was set at 110V for 35 minutes. E-Z vision, in gel solution staining (AMRESCO, USA) was performed for 20 minutes with 100 bp DNA Ladder (BioLabs, UK) to determine molecular weight. Additionally, the PCR products were visualised by gel imaging instrument.

2.6 Statistical analysis
The results were expressed using descriptive analyses (mean, standard deviation, frequency, and proportion). The data was analysed with the Statistical Software SPSS version 21.0 for windows. Chi-square or Fisher's exact test was used where applicable. The data presentation by tables, graphs or figures was done using Excel Software version 2013 (Microsoft Corporation, USA). This investigation was approved by Bioethics and Research Committee of The Nairobi Hospital.

Table 1. Primers sequences used for detection of MBL genes (Shibata et *al.*, 2003; Mushi et *al.*, 2014)

Genes	Oligonucleotides sequences	A.T (°C)	Product size	Reference
vim1	F: AGT GGT GAG TAT CCG ACA G R: ATG AAA GTG CGT GGA GAC	55	261	Shibata et *al.*(2003)
vim2	F: ATG TTC AAA CTT TTG AGT AAG R: CTA CTC AAC GAC TGA GCG	51	801	Shibata et *al.*(2003)
imp1	F: ACC GCA GCA GAG TCT TTG CC R: ACA ACC AGT TTT GCC TTA CC	51	587	Shibata et *al.* (2003)
imp2	F: GTT TTA TGT GTA TGC TTC C R: AGC CTG TTC CCA TGT AC	51	678	Shibata et *al.* (2003)
ndm1	F:GGT TTG GCG ATC TGG TTT TC R: CGG AAT GGC TCA TCA CGA TC	52	621	Mushi et *al.* (2014)

3. Results

Out of 185 clinical isolates, the high prevalence of the bacterium isolates was seen among the male gender than female (61.1% and 38.9%, respectively), and the high rate of isolates were found among the age groups above 45 years (64.3%) with the mean age of patients of 56.1±21.6 years old, ranging from 4 to 100 years (Table 2). This table 2 demonstrates also that out of 185 subjects who showed the growth of *P. aeruginosa* isolates, 137 (74.1 %) were inpatients and 48 (25.9%) were outpatients.

Table 2. Distribution of demographic characteristics for patients with *P. aeruginosa* infections

Age/Year	Number of isolates	%
Age group <15	4	2.2
Age group 15-30	23	12.4
Age group 31-45	39	21.1
Age group 46-60	37	20.0
Age group 61-75	39	21.1
Age group 76-90	38	20.5
Age group >90	5	2.7
Sex	Frequency (N=185)	%
Female	72	38.9
Male	113	61.1
Type of patient	Frequency (N=185)	%
Inpatient	137	74.1
Outpatient	48	25.9

Table 3. Distribution of *P. aeruginosa* isolates by type of clinical samples

Type of specimen	Number of isolates	Proportion (%)
Pus and wound swab	73	39.5
Sputum	48	25.9
Body fluid	9	4.9
Urine	35	18.9
Blood	3	1.6
Catheter tip	11	5.9
Tissue or Biopsy	6	3.2
Total	185	100.0

In the present study, the specimens showing the highest isolation of *P. aeruginosa* were from pus and wound swab (39.5%), sputum and respiratory aspirates (25.9%), and urine (18.9%), followed by the lowest isolation of *P. aeruginosa* such as catheters tips (5.9%), body fluid (4.9%), biopsy (3.2%) and blood (1.6%) (Table 3).

Table 4. Antimicrobial susceptibility profile for *Pseudomonas aeruginosa* isolates

Antibiotics tested	Sensitive (%)	Intermediate (%)	Resistant (%)
Piperacillin/tazobactam (PTZ)	102 (55.1)	14 (7.6)	69 (37.3)
Cefepime (FEP)	125 (67.6)	8 (4.3)	52 (28.1)
Ceftazidime (CAZ)	126 (68.1)	7 (7.6)	52 (28.1)
Cefotaxime (CTX)	0 (0)	2 (3.8)	183 (98.9)
Meropenem (MER)	122 (65.9)	5 (2.7)	58 (31.4)
Gentamycin (CN)	125 (67.6)	1 (0.5)	59 (31.9)
Amikacin (AK)	126 (68.1)	0 (0)	59 (31.9)
Ciprofloxacin (CIP)	126 (68.1)	1 (0.5)	58 (31.4)
Aztreonam (AZM)	70 (37.8)	3 (1.6)	40 (21.6)

The resistant rates for β-lactam antibiotics including cefepime, ceftazidime, meropenem, piperacillin/ tazobactam, and aztreonam were respectively 28.1%, 28.1%, 31.4%, 37.3%, 21.6%. The resistant rates for non-β-lactam antibiotics including amikacin, gentamycin, and ciprofloxacin were 31.9%, 31.9% and 31.4% (Table 4).

Of the One Hundred and Eighty Five (185) *P. aeruginosa* isolates, forty two (22.7%) positively expressed MBL Carbapenemases enzymes. The antimicrobial susceptibility patterns of MBL producing *P. aeruginosa* showed that all the MBL isolates were resistant to the antibiotics tested. Chi-square (χ^2) or two-tailed Fisher's exact test was used where applicable. The difference in resistance levels between MBL and non-MBL producing isolates for all the antimicrobial drugs (fluoroquinolones, aminoglycosides, and Beta-lactams) tested were statistically significant, ($p<0.05$, $P<0.001$) (Table 5).

In this study, the highest numbers of cases of MBL-producing *P. aeruginosa* were isolated from Intensive Care Unit (ICU) (45.2%) and High Dependency Unit (HDU) (28.6%) with predominant distribution in pus swab (38.1%) and sputum (33.3%). The isolates with resistant pattern to meropenem and MBL production were subjected to PCR for MBL genes BlaVIM-1, BlaVIM-2, BlaIMP-1, BlaIMP-2 and BlaNDM-1.

Table 5. Antibiotic Resistance for MBL and Non-MBL *P. aeruginosa* isolates

Variable	MBL(=42)	Non-MBL(=143)	Significance level
Antibiotics tested	Resistant (%)	Resistant (%)	P-Value
Piperacillin/tazobactam (PTZ)	42 (100)	27 (18.9)	P<0.001
Cefepime (FEP)	42 (100)	10 (7.0)	P<0.001
Ceftazidime (CAZ)	42 (100)	10 (7.0)	P<0.001
Meropenem (MER)	42 (100)	16 (11.2)	P<0.001
Gentamycin (CN)	42 (100)	17 (11.9)	P<0.001
Amikacin (AK)	42 (100)	17 (11.9)	P<0.001
Ciprofloxacin (CIP)	42 (100)	16 (11.2)	P<0.001
Aztreonam (AZM)	42 (100)	19 (20.7)	P<0.001

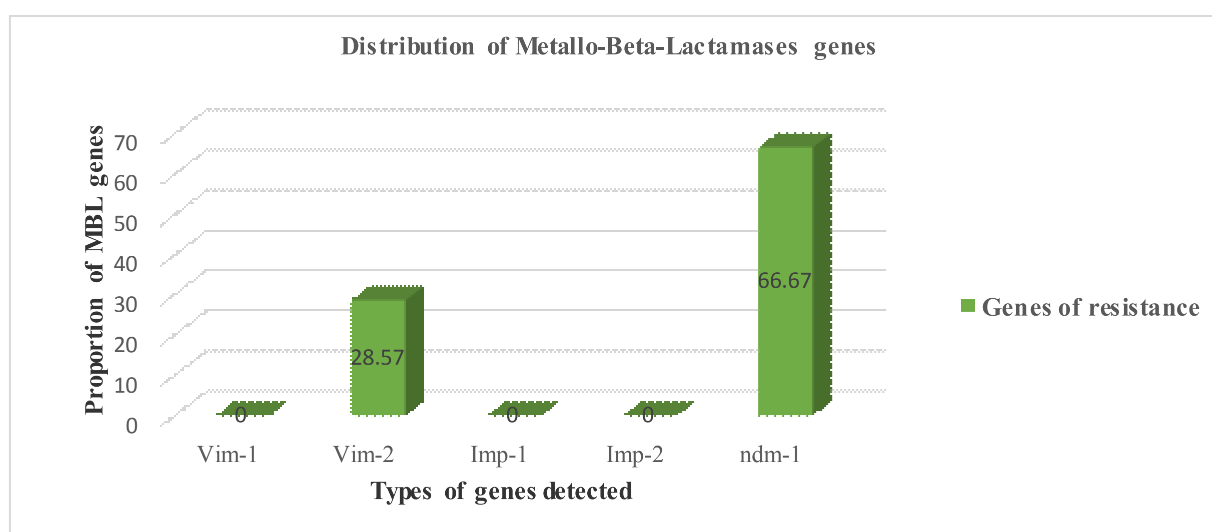

Figure 3. Distribution of Metallo-Beta-Lactamases genes among the *P. aeruginosa* isolates

The PCR assays performed for metallo-beta-lactamases were positive in twelve MBL isolates (28.57%) for BlaVIM-2 and twenty eight MBL isolates (66.67%) for BlaNDM-1. None of the MBL *P. aeruginosa* isolates carried BlaVIM-1, Bla IMP-1 and BlaIMP-2. From the 42 MBL isolates, three (7.14%) isolates had both BlaNDM-1 and BlaVIM-2 genes (Figure 1, 2, 3).

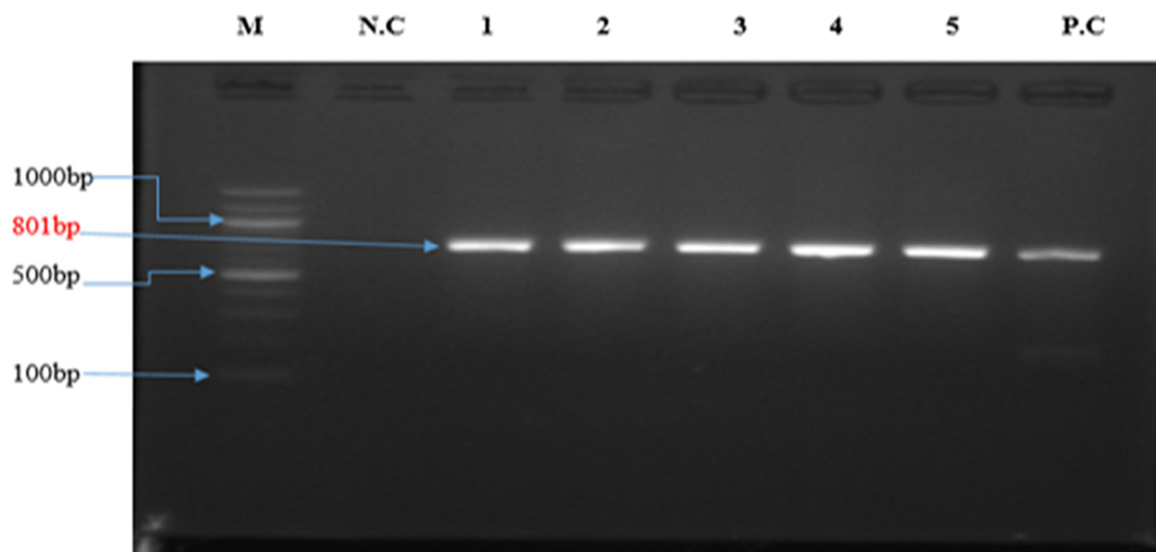

Figure 1. Agarose gel electrophoresis of BlaVIM-2 PCR products
Lanes 1, 2, 3, 4 and 5 show isolates positive for BlaVIM-2. M shows 100 bp DNA Ladder. Lane P.C shows the positive control. Lane N.C is the Negative Control.

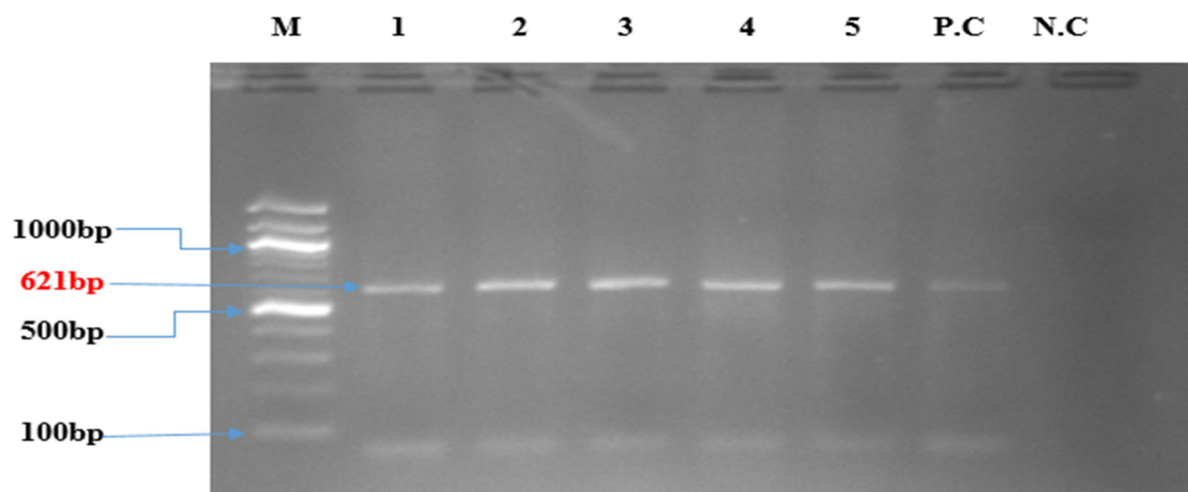

Figure 2. Agarose gel electrophoresis of BlaNDM-1 PCR products.
Lanes 1, 2, 3, 4 and 5 show isolates positive for BlaNDM -1. M shows 100 bp DNA Ladder. Lane P.C shows the positive control. Lane N.C is the Negative Control.

4. Discussion

This is one of the few studies that has evaluated the magnitude of Metallo-Beta-Lactamases producing isolates and characterized the antimicrobial resistance among *Pseudomonas aeruginosa* isolates obtained from clinical sources in Kenya. In the present study, a total of 185 isolates from clinical samples were identified as *Pseudomonas aeruginosa* from May 2017 to April 2018. According to gender and age group distribution, our findings agree with the study of Mahmoud et *al.* (2013) and Kiana et *al.* (2016) where *P. aeruginosa* infections were common in males than female cases and their mean age were 44.6±25 and 49.39 ± 16 years old, respectively (Mahmoud et *al*., 2013; Shirani et *al.*, 2016). These results differ with the study of Zafer et al. in which the highest number of cases were found in males with a low mean age of 24.5 years with the highest number of cases in younger patients (54%)

Same observation has been made by Al-Zaida (2016) in which results of the studies showed higher occurrence of the bacterium in male (55.6%) and younger patients (38.9%) (Zafer et *al*., 2014; Al-Zaida et *al.*, 2016). The preponderance of males can be explained by greater number of cases from Critical Care Nursing ward (Intensive Care Unit) which should have more admissions of male patients in this hospital. This particular age group should be explained by the immunocompromised condition due to lifestyle disease among the old age

groups (>45 years old). In our study *P. aeruginosa* isolates are more prevalent in inpatients (74.1 %) as compared to the outpatients (25.9%). A similar finding was observed by others authors (Sandhya and Swathi, 2014). These results are importantly expected because *Pseudomonas aeruginosa* is most frequently associated with hospital-acquired infection compared to the community-acquired infection of this organism reported in different studies (Zafer et *al.*, 2014; Sandya and Swathi, 2014; Farajzadeh et *al.*, 2014; Jayarani et *al.*, 2014).

The specimens showing the highest isolation of *P. aeruginosa* were from pus and wound swab (39.5%), followed by the sputum (25.9%), and urine (18.9%). This is also comparable with studies of many researchers in which the highest number of *P. aeruginosa* strains were isolated from pus and wound swab followed by urine and sputum (Mahmoud et *al.*,2013; Zafer et *al.*, 2014; Mohanam and Thangam, 2017; Wassef et *al.*, 2015). These findings should be explained by the long hospital stay of the patient in ICU in which is found the application of medical devices, which prompt the selection of nosocomial pathogens. *P. aeruginosa*, as nosocomial strain, is well known to be the leading cause of cystic fibrosis among lower respiratory tract infections, and surgical wounds infections. *P.aeruginosa* from patients demonstrated resistance to piperacillin/tazobactam, gentamycin, amikacin, ciprofloxacin, meropenem, cefepime, ceftazidime and aztreonam in the following order respectively: 37.1%, 31.9%, 31.9%, 31.4%, 31.4%, 28.1%, 28.1%, and 21.6%. Similar results have been found by Bangera et *al.* (2016) where 38.0% of the isolates showed resistance to gentamicin followed by ceftazidime (31.69%) and meropenem (33.03%) (Bangera et *al.*, 2016). This is in contrast with the study of Radan et *al.* (2016) in which the resistance rate to ciprofloxacin, imipenem, meropenem, amikacin, ceftazidime, and cefepime was higher than 90%, while the resistance rates to piperacillin/tazobactam and aztreonam were 70.7% and 86%, respectively (Radan et *al.*, 2016). This difference can be attributed to the production of different factors through the resistance genes and mutational processes. Resistance of *P. aeruginosa* to antibiotics is the result of the production of enzymes that inactivate and degrade antibiotics, reducing the membrane permeability and the multidrug resistance efflux system (Mahmoud et *al.*, 2013; Wassef et *al.*, 2016). Our study revealed good activity of cefepime (67.6%), and Ceftazidime (68.1%). In contrast, cephalosporins tested in a study conducted in Egypt, showed that 90% of the isolates were resistant to cefepime (91%) and Ceftazidime (98%) (Mahmoud et *al.*, 213). This difference may be due to the absence of Extended Spectrum βeta-Lactamases (ESBLs) production in our *P. aeruginosa* isolates used β–lactamase mediated resistance against cephalosporins.

Out of 185 strains of *P. aeruginosa* 31.5 % were meropenem resistant. This prevalence of carbapenem resistance is comparable with the study of Ghasemian et *al.* (2018) in which the prevalence of Carbapenem-Resistant *P.* aeruginosa (CRPA) was 37.7% among the isolates (Ghasemian et *al.*, 2018). In most countries, the reported Carbapenem-resistance of *P. aeruginosa* (CRPA) ratio ranged from 10 to 50%. Many CRPA strains have been identified and their prevalence is increasing gradually (Hong et *al.*, 2015; Meradji et *al.*, 2016, Dogonchi et *al.*, 2018). This discrepancy can be explained by both carbapenem use and the application of medical devices, which prompt the selection of resistant organisms. Both factors are related to patient characteristics, clinical practice policies and antibiotic prescription patterns of health care facilities in each hospital.

The rate of MBL producing *P. aeruginosa* (22.7 %) was not alarming and still awareness compared to the study of others researchers in which MBL producing *P. aeruginosa* were 14% in a tertiary hospital in Kenya and 17 % in Egypt (Pitout et *al.*, 2008, Abaza et *al.*, 2017). Different studies have reported higher rates of MBL production among *P. aeruginosa* isolates (E.g.: 84.5% in Brazil and 68.6% in Nepal) (Acharya et *al.*, 2017; Kalluf et *al.*, 2017). This difference can be attributed to the continuous development of Multi-drug resistant strains in different parts of the world, also a considerable geographic difference in the prevalence of MβL in different countries.

Our study showed also that all MBL producing-*Pseudomonas aeruginosa* were resistant to the antibiotics tested. These results were similar with the results of others researchers in which MBL-producing *P. aeruginosa* isolates were resistant to the examined antibiotics (Sandhya and Swathi, 2014; Liew et *al.*, 2018). This high level of resistance (100%) to all classes of antibacterial agents tested can be probably explained by the co-production of MBL and others non-investigated mechanisms of the resistant strains in our study. Despite these facts, the difference in resistance levels between MBL and non-MBL producing isolates for all the antimicrobial drugs (Beta-lactams, fluoroquinolones and aminoglycosides) tested were statistically significant, ($p<0.05$, $P<0.001$). Our findings showed that the highest rate of isolation of MBL producers was found from critical Care Nursing ward, ICU (45.2%) and HDU (28.6%) with predominant distribution in pus swab and sputum. Similar results have been observed by Subramaniyan et *al.* (Subramaniyan et *al.*, 2018). This finding should be explained by the increase of incidence of infection associated with the use of invasive devices for the diagnostic purpose or medical applications as well as inadequate sterilization and immunocomprised condition due to lifestyle disease.

Among carbapenemases, Metallo-Beta-Lactamases (MBL) and mostly Verona integron-mediated (VIM) and New Dehli MBL (NDM) have played the crucial role in carbapenem-resistance *Pseudomonas aeruginosa* emergence. Our study showed that twenty eight (66.67%), and twelve (28.57%) of the isolates had blaNDM-1

and blaVIM-2 genes in their PCR results, respectively. The PCR assays performed for metallo-beta-lactamases were negative in all MBL isolates for BlaVIM-1, BlaIMP-1 and BlaIMP-2. Different findings have been observed by the study of Pitout et *al.* (2008) in which fifty seven *Pseudomonas aeruginosa* were screened for MBL producers for VIM-type and IMP-type genes through the PCR method; and showed that all the MBL isolates (100%) carried blaVIM-2 genes from a tertiary care Centre in Kenya (Pitout et *al*., 2008). Moyo et *al.* demonstrated that all carbapenem-resistant isolates from a tertiary care hospital in Dar-es-Salaam (Tanzania) were phenotypically positive for MBL production. PCR followed by sequencing showed that the isolates harbored blaVIM-2 (Moyo et *al*., 2015). To our knowledge, this is the first study done on molecular characterization of blaNDM-1-producing *P. aeruginosa* in Kenya. Few others studies and cases reports have shown the presence of VIM-2 and NDM-1 from the African continent including South Africa, Ghana, Nigeria, Tunisia, Algeria and Egypt (Manenzhe et *al.*, 2015, Sekyere et *al.*, 2016). VIM-2 and NDM-1 are increasingly reported worldwide. Similar studies have recently reported the emergence of the *Pseudomonas aeruginosa* carrying the blaNDM-1 which exhibited resistance to carbapenems for the first time in Iraq, blaVIM-2 in Spain, and blaNDM-1 and VIM-2 genes in Malaysia (Bellés et *al*., 2018; Ismail et *al*., 2018; Liew et *al*., 2018). The negative PCR results for VIM-1- and IMP-types can be explained by a considerable geographic difference in the distribution of Metallo-βeta-Lactamases genes in different countries.

The authors did not investigate the clonality of the isolates and the sequence of the genes and also did not use primers to target all known MBL genes. Despite these limitations, the study has provided the distribution of the common MBLs genes and the magnitude of the problem in The Nairobi Hospital.

5. Conclusion and Recommendation

The high level of resistance to antibiotics of MBL-producing *P. aeruginosa* is present in this hospital with a prevalence of 22.7 % that suggests the spread of resistance among the population of nosocomial strains. To overcome the problem of emergence and the spread of multidrug resistant *P. aeruginosa* a combined interaction and cooperation between the microbiologists, clinicians and the infection control team is required in order to develop infection control strategies that can limit the dissemination of resistant strains. We also recommend further studies on epidemiology of MBL-producing *P. aeruginosa*, others mechanisms of antimicrobial resistance in *P. aeruginosa* isolates, proper antibiotic stewardship, and rapid diagnostic methods for proper detection of Beta-Lactamases-producing isolates.

6. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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