Molecular Characterisation and Antimicrobial Susceptibility Pattern of *Pseudomonas* Species Isolated from Infected Wounds of Patients in Insurgency-Stricken Maiduguri, Borno State

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Abstract: Multidrug resistant *Pseudomonas aeruginosa* has been known to cause infections among hospitalised patients leading to significant morbidity and mortality. Carbapenems are usually deployed for treating these infections. This work focused on molecular characterisation and antimicrobial susceptibility pattern of wound infections due to *Pseudomonas* species from four major hospitals within Maiduguri, Nigeria. Four hundred and twenty non-duplicate wound specimens were collected from surgical units of four hospitals within Maiduguri metropolis between February 2020 and October 2020. The samples were analysed for *Pseudomonas* species and molecularly identified using standard methods. *Pseudomonas* species constituted 72 (17.1%) of the isolates, with *P. aeruginosa* being responsible for 68 (94.4%); the remaining were *P. putida* 2(2.8%) and *P. fluorescens* 2(2.8%). The highest prevalence of 36.8% was in the 21-40-year age group, with 66% being males. ($P<0.05$). Resistance was documented at 100%, 83.6%, 35.3% 29.4%, 27.9% and 4.4% for ceftazidime, aztreonam, meropenem, gentamicin, ciprofloxacin and piperacillin-tazobactam, respectively. Genotypic characterisation of the *P. aeruginosa* isolates was positive for bla_{VIM}, bla_{IMP}. An alarmingly high level of carbapenem resistance was discovered among *Pseudomonas* species from our study population, with the presence of VIM and IMP. This finding will serve as a guide for empiric therapy of infected surgical wounds among our patients. To effectively tackle the menace of antimicrobial resistance, strict adherence to antimicrobial stewardship guidelines, infection prevention and control, and the need for improved surveillance; employing the One Health approach need to be universally adopted.

Key word: Carbapenemases, insurgency, molecular characterisation, *Pseudomonas* species, wound infection

INTRODUCTION

Antimicrobial resistance, particularly in Gram-negative bacteria, challenges the ability to treat common infections and is one of the greatest threats to global public health systems (Breijyeh *et al*., 2020). Resistance is more worrisome in resource-limited countries, especially in sub-Saharan Africa, where infections are common and last-resort antimicrobial agents are scarce and/or unaffordable (Tompkins *et al*., 2021). Carbapenems play a significant role as "last resort" antibiotics in the treatment of infections caused by extended spectrum of β-lactamase (ESBL)-producing Enterobacteriaceae and other multidrug-resistant Gram-negative bacteria. Widespread use of carbapenems in the treatment of multidrug resistant infections has led the emergence of carbapenem-resistance, posing a serious source of healthcare concern (Garbati *et al.*, 2016; WHO, 2018). Globally, concurrent resistance to colistin and carbapenem by Enterobacteriaceae has been increasingly

reported in isolates from animals and humans with increasing frequency (Du *et al*., 2016; Yao *et al*., 2016; Lomonaco *et al*., 2018). Concurrent resistance determinants are usually located on conjugative plasmids and can thereby be co-transferred, setting the stage for pandrug resistance (PDR) (Long *et al*., 2019). Carbapenem resistant Enterobacteriaceae (CRE) comprise both carbapenemase-producing (CP-CRE) and non-carbapenemase producing CRE (non-CP-CRE) strains. The CPE produce carbapenemases to hydrolyze carbapenems, non-CP-CRE have β-lactamase (ESBL and AmpC) activities combined with structural mutations of the outer membrane protein and drug efflux pumps (Logan and Weinstein, 2017). Carbapenem-resistant *Acinetobacter baumannii* (CRAB) and CR *Pseudomonas aeruginosa* (CRPA) are among the top tier of the World Health Organization (WHO) list of antibiotic-resistant "priority pathogens" that pose the greatest threat to human health (Tacconelli *et al*., 2018). Infections with these resistant bacteria are a matter of national and international concern as they are an emerging cause of Hospital Acquired Infections (HAIs) that pose a significant threat to public health and responsible for hospital outbreaks worldwide (WHO, 2014).

Moreover, they are associated with high rates of morbidity and mortality, especially in patients with serious underlying disorders or patients admitted to the intensive care unit (ICU) (MacVane, 2017). Although, carbapenems were introduced to treat infections caused by bacteria resistant to penicillins, cephalosporins, and fluoroquinolones, the reliability of these antibiotics has been reduced due to the development of resistance. Multiple mechanisms of resistance to carbapenems have been identified including overexpression of efflux pumps, porin mutations, and enzymatic inactivation (Codjoe and Donkor, 2017).

Pseudomonas aeruginosa is one of the major opportunistic and nosocomial pathogens associated with many severe and often fatal infections, especially in immunocompromised patients or those with underlying diseases (Poole, 2011). Multidrug resistant *P. aeruginosa* isolates have been detected in hospitals worldwide and associated with increased mortality and costs due to prolonged hospitalization, need of surgery, and prolonged treatment with antibiotics (Woodford *et al*., 2011). Increasing resistance in *P. aeruginosa* isolates complicates the selection of adequate empirical therapy in such situations. Carbapenems are potent broad spectrum β-lactam antibiotics, and one of the few remaining agents that have reliable activity against *P. aeruginosa* (Poole, 2011; Partridge, 2012). However, increased prevalence of resistance to carbapenems among these organisms has been noted (Woodford *et al*., 2011). Resistance against carbapenems by *P. aeruginosa* may occur through different mechanisms including: loss of the outer membrane porin OprD protein, reduced levels of drug accumulation due to efflux-pumps over-expression, and increased production of AmpC β-lactamases (Poole, 2011).

The exact prevalence in AMR among enteric bacteria of public health importance in Nigeria is unknown. However, ample reports of multidrug resistance in enteric bacteria exist (Nsofor *et al*., 2013; Olowe *et al*., 2008). Several factors have been reported by various investigators to contribute to the current state of evolutionary trend towards drug resistance in microbes of medical importance. Drug misuse and abuse by patients and individuals have encouraged the spread of these resistance genes in the microbial population (Tenover, 2001). In addition, the widespread use of low-cost, broad-spectrum antibiotics over-the-counter without prescription in Nigeria has led to the proliferation of self-medication practices leading to the development of drug resistance (Ventola, 2015). The lack of routine drug sensitivity testing and surveillance in resource-limited countries equally contributes to irrational use of antibiotics, thus adding to the menace of

antimicrobial resistance (Okeke, 2006). The increasing use of antimicrobial agents in livestock also contributes significantly towards development and spread of resistant pathogens in the environment. (Mattew *et al*., 2007, Oloso *et al*., 2018). This multi-centre study was undertaken to detect the prevalence of metallo-beta-lactamases (*bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{OXA}$) in carbapenem-resistant isolates of *P. aeruginosa* among patients attending four major healthcare institutions in Maiduguri, Borno State, Nigeria.

MATERIALS AND METHODS

Study design: This cross-sectional study was carried out to determine the antimicrobial resistance patterns and molecular characteristics of isolates of *P. aeruginosa* using polymerase chain reaction (PCR). To isolate *P. aeruginosa* from wounds and to assess antibiotic resistance, as well as the trends and patterns in sensitivity profiles, samples were collected from patients irrespective of their age after obtaining an informed consent.

Study setting and population: This study was carried out in four healthcare institutions - University of Maiduguri Teaching Hospital (UMTH), State Specialist Hospital (SSH), Umaru Shehu Ultra-Modern Hospital (UMH), and Mamman Shuwa Memorial Hospital (MSMH), all situated within the Maiduguri metropolis, Borno State, Northeast Nigeria. The UMTH is the largest healthcare institution in the region, while the remaining are secondary healthcare institutions that provide sub-specialty clinical and laboratory services to the populations within Borno and neighboring states and countries through shared boundaries with Chad, Cameroon and Niger Republics.

Sample Size: The sample size was calculated using a prior prevalence of 51.5% (Zubairu and Iregbu, 2018) and Fisher's statistical formula:

$n= Z^2pq/d^2$

n= the desired sample size

Z= the standard normal deviation usually 1.96 which corresponds to 95% confidence level

p= 51.5(0.515) (Zubairu and Iregbu, 2018)

q= 1.0-p (1.0-0.515) = 0.485

d= degree of accuracy usually set at 0.05 (precision)

$n= (1.96)^2 \times 0.515 \times 0.485/(0.05)^2 = 384$

To take care of an estimated attrition rate of 10%, the sample size was increased to 420.

Sampling technique: All the clinical samples were collected consecutively from four different hospitals (UMTH, SSH, MSMH, USUH) in Maiduguri, Nigeria.

Sample collection: A total of 420 samples were collected from patients admitted for the care of various types of wounds across the four hospitals listed. Single use sterile cotton swab sticks were used to collect the samples. The sterile swab sticks were moistened in normal saline and rolled over the wounds for at least 3 – 5 seconds using the Levine technique (Levine *et al*., 1976) in order to obtain a good sample. Swab sticks were returned aseptically into their containers and samples were transported immediately in an ice box to the University of Maiduguri Microbiology laboratory for processing within one hour of collection.

Isolation, identification and biochemical characterisation of Pseudomonas aeruginosa: In the laboratory, respective non-duplicate swab samples from hospital clinical specimens were cultured first on a selective agar, Cetrimide agar and MacConkey agar. Then, suspected colonies were sub-cultured on blood agar and on Muller-Hinton agar (MHA) to observe for haemolysis and pigmentation. All the inoculated plates were incubated at 37°C for 18-24 hours and growth was evaluated on these media. Isolates were identified on the basis of standard bacteriological methods - morphology, colonial characteristics, haemolysis, as well as pigment production (Mahmoud *et al*., 2013). Further

identification was done by Gram reaction, odour in cultures, and biochemical tests such Analytical Profile Index (API). The data was then analysed by the manufacturer's software and positive results with ≥89% probabilities were confirmed as *Pseudomonas* spp.

Antibiotic susceptibility testing of bacterial isolates: A disc diffusion technique, which is a reliable and accurate technique, was employed for this study. A disc of blotting paper was impregnated with a known volume and appropriate concentration of an antimicrobial agent. This was placed on a plate of sensitivity testing agar uniformly inoculated with the test organism (*Pseudomonas aureginosa*). The antimicrobial diffuses from the disc into the medium, and the growth of the test organism was inhibited at a distance from the disc that is related (among other factors) to the sensitivity of the organism. Strains sensitive to the antimicrobials are inhibited at a distance from the disc whereas resistant strains have smaller or no zones of inhibition (Collee *et al.,* 1996; CLSI, 2020). Antimicrobial susceptibility was performed on Mueller-Hinton agar by standard disc diffusion method recommended by Clinical and Laboratory Standards Institute (CLSI). This was done by soaking a sterile swab in 0.5 MacFarland solution and then carefully swabbing the entire surface of Mueller Hinton agar plates. The antibiotic discs were placed on the surface of the inoculated plates and gently pressed. Susceptibility testing of the *P. aeruginosa* isolates was done using the following anti-pseudomonas antibiotic discs - meropenem (10 µg), ceftazidime (30 µg), gentamicin (10 µg), ciprofloxacin (10 µg), aztreonam (30 µg) and piperacillin-tazobactam (100 µg). All antibiotics were procured from Oxoid limited (Oxoid, UK). The diameter zone of inhibition was measured, recorded and interpreted based on the CLSI criteria for standard zone sizes of inhibition to define sensitivity or resistance to the antimicrobials. The plates were incubated at 37°C for 18-24 hours. The diameter zone of inhibition was measured in millimeters and isolates were scored as sensitive or resistant by comparing with values recommended on CLSI standard (CLSI, 2020).

Phenotypic detection of carbapenemase production: Screening and phenotypic detection of carbapenemase production for resistance was determined by disc diffusion method on Mueller-Hinton agar using commercially available single discs of meropenem and imipenem-cilastatin (Oxoid, England, UK) (CLSI, 2022). All isolates with diameter zone of inhibition less than the cut-off values for imipenem-cilastatin (<23 mm) and meropenem (<25 mm) are classified as carbapenem-resistant and were tested for phenotypic production of carbapenemases (EUCAST, 2013).

DNA extraction by boiling: The harvested cells were transferred into 1000 µL of sterile water and Vortexed until it is completely dissolved. The mixture was then centrifuged for 5 minutes at 10,000rpm. The supernatant was discarded, and 1000µl of sterile water was added. The mixture was then vortexed and centrifuged and the supernatant was decanted. 200 µl of sterile water was added and vortexed until thoroughly mixed. The mixture was then boiled for 10 minutes at 100^{o}C and then cooled immediately on ice and vortexed again. This was centrifuged for 5 minutes at 10,000 rpm. The supernatant was transferred into fresh Eppendorf tubes and the pellets were discarded.

Polymerase chain reaction (Multiplex): The PCR reaction was carried out using the Solis Biodyne 5× HOT FIREPol Blend Master mix. The PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1× concentration containing 1× Blend Master mix buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 25 pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture (Table 1).

Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) for an initial denaturation of 95°C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 1 minute at 56°C and 58°C and 1 minute 30 Seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80 V for 1 hour 30 minutes (Table 2). After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100 bp DNA ladder was used as DNA molecular weight standard (Lee *et al.*, 2012).

RESULTS

A total of 420 clinical specimens were received from the surgical units of UMTH, SSH, USUH, and MSMH, Borno State between February 2020 and October 2020. The total of 72 *Pseudomonas* species were isolated, out of which sixty-eight (68) isolates were *Pseudomonas aeruginosa*, giving a prevalence of 94.44% among the *Pseudomonas* species; and the others were *P. putida* 2(2.8%) and *P. fluorescens* 2(2.8%). Table 3 below shows the age distribution of the studied population. The highest prevalence of 36.8% was in the 21-40-year age group, followed by 25.0% among those aged 0-20 years. The lowest prevalence of 1.3% was recorded in those aged between 81-100 years. Those aged 40 years and younger had more chances of wound infection with carbapenem-resistant *P. aeruginosa* (P<0.05). Majority (60%) of the patients were males and harboured about two-thirds (66.2%) of the isolates.

Table 1:16SrRNA identification of *Pseudomonas aeruginosa* isolates

No sample	Primer Name	Sequence (5^1-3^1)	Base pair (bp)
30	PA-GS-F	5^1GACGGGTGAGTAATGCCTA3^1	618
	PA-GS-R	3^1CACTGGTGTTCCTTCCTATA5^1	

Table 2: Carbapenemase primers and their sequences

S/N	Target gene	Sequence (5^1-3^1)	Annealing Temperature (°C)	Target Amplicon	References
1	VIM – F	TTTGGTCGCATATCGCAACG	56	500bp	Hujer *et al*., 2006
	VIM- R	CCATTCAGCCAGATCGGGCAT			
2	IMP- F	GTTTATGTTCATACATCG	56	440bp	Hujer *et al*., 2006
	IMP-R	GGTTTAACAAAACAACCAC			
3	Bla OXA-F	TAATGCTTTGATCGGCCTTG	56	552bp	Kobs *et al*., 2016
	Bla OXA-R	TGGATTGCACTTCATCTTGG			

Table 3: Age and gender distribution of the studied population

Age (mth/yrs)	No. of Samples	No. Positive samples (%)	X^2	P-value
0-20	139	17 (25.0)	10.57	P<0.05
21-40	155	25 (36.8)		
41-60	87	15 (22.0)		
61-80	20	10 (14.7)		
81-100	9	1 (1.3)		
Total	420	68 (100)		
Gender	No. of Patients	No. of Positive patients (%)	X^2	P-value
Male	252	45(66)	0.91	P>0.05
Female	168	23(33)		
Total	420	68(100.0)		

Key: mnth = months; yrs = years; X^2 = Chi squared

Table 4: Prevalence of *Pseudomonas aeruginosa* isolated from infected wounds in selected hospitals

Sample Area	No. of Samples	No. of Positive isolates (%)	Prevalence (%)	X^2	P-value
UMTH	120	25 (36.8)	6.0	4.67	P>0.05
SSH	210	35 (51.5)	8.3		
USUH	40	5 (7.3)	1.1		
MSMH	50	3 (4.4)	1.0		
Total	420	68	16.4		

Keys: UMTH: University of Maiduguri of Teaching Hospital; SSH: State Specialist Hospital; USUH: Umaru Shehu Ultra-Modern Hospital; MSMH: Mamman Shuwa Memorial Hospital

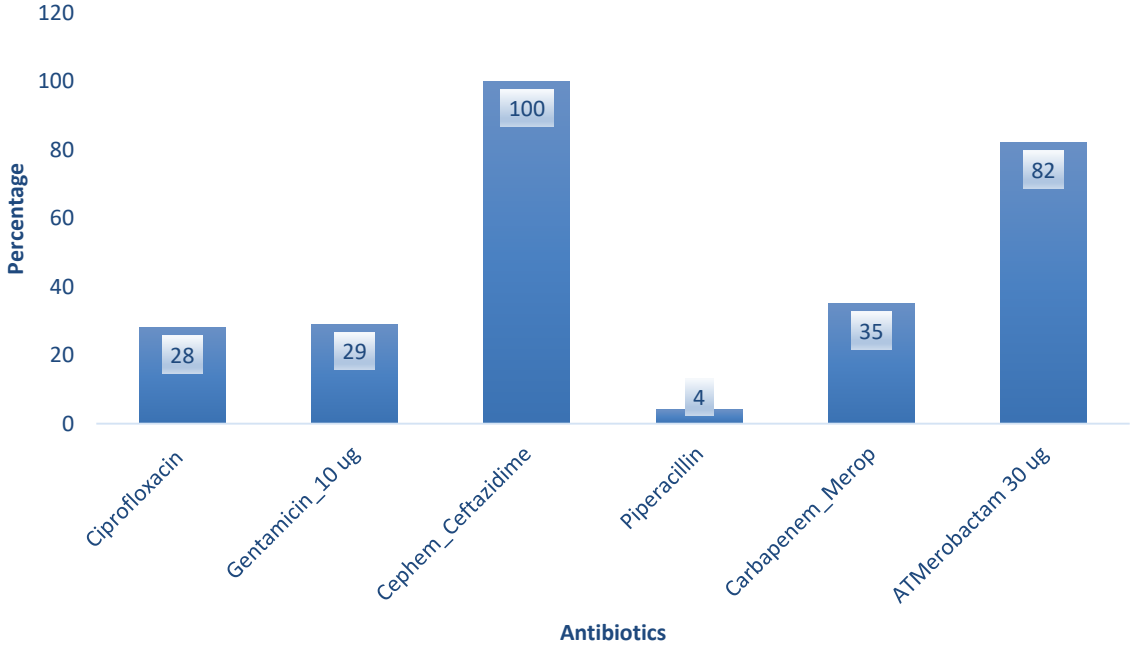

Figure 1: Antibiotic resistance profile of *Pseudomonas aeruginosa* from wound infections

The number of isolates of *P. aeruginosa* in infected wounds from the selected hospitals is presented in Table 4. Majority of the *P. aeruginosa* were recovered from SSH with a prevalence of 8.3%, followed by UMTH (6%), USUH (1.1%) and MSMH (1.0%). The antibiotic resistance profile of the studied isolates is presented in Figure 1, the highest resistance was reported against ceftazidime 68 (100%), followed by aztreonam 56 (83.6%). Different antibiotics had varying degrees of resistance. Meropenem 24 (35.3%), Gentamicin 20 (29.4%) and Ciprofloxacin 19 (27.9%); while piperacillin-tazobactam 3 (4.4%) had the lowest resistance. Isolates were considered multi-resistant (MDR) if they showed resistance to three or more of the tested antibiotics, and this was observed in 30 of the 68 (16.8%) isolates of *P. aeruginosa* recovered from the infected wounds as shown in Table 5. In this study about 14 (46.7%) of the MDR *P. aeruginosa* isolates were recovered from samples from SSH, followed by UMTH 9 (30.0%) and USUH 7 (23.3%). No MDR *P. aeruginosa* isolates were recovered from samples from MSMH. Agarose gel electrophoresis (1.5%) showing the typical amplicon of the gene 16S rRNA product of *P. aeruginosa* isolate. The amplification of DNA appears as a ladder-like pattern. Where, Lane (M) DNA marker (100 bp), Lane (1-30) represent positive isolates (Figure 2 and Figure 3).

Table 5: Multi-drug resistance pattern (MDR) of *Pseudomonas aureginosa* isolates in the study area

Study Area	Frequency	Percentage Positive (%)
UMTH	9	30.0
SSH	14	46.7
USUH	7	23.3
Total	30	100.0

Key: UMTH = University of Maiduguri Teaching Hospital, SSH = State Specialist Hospital, USUH = Umaru Shehu Ultramodern Hospital

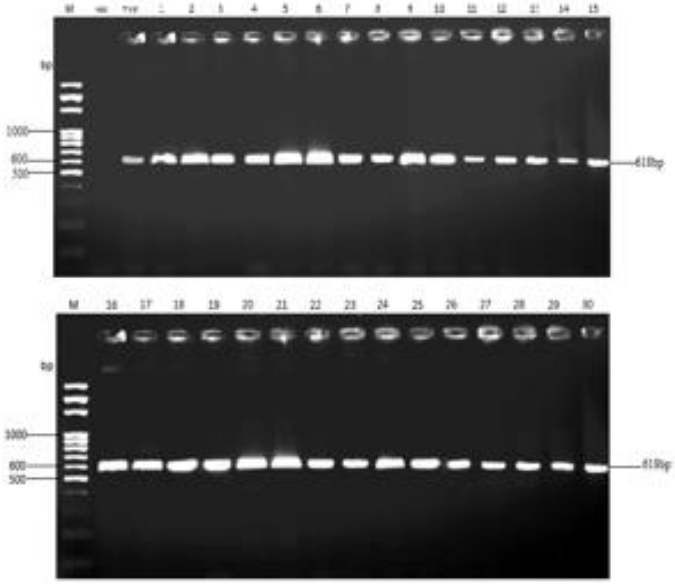

Figure 2: 16SrRNA identification of *Pseudomonas aeruginosa* isolates from wound infections

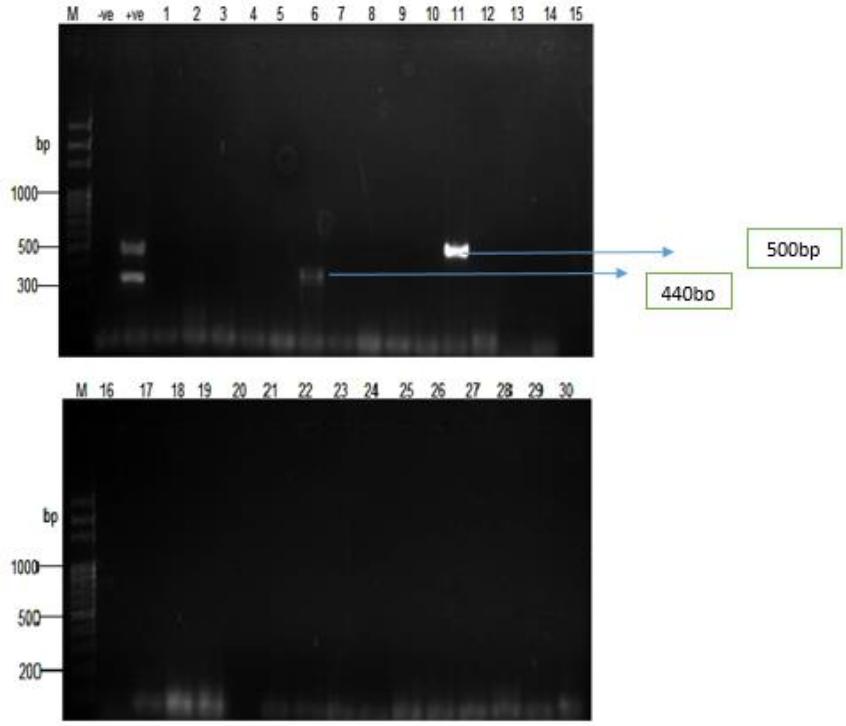

Figure 3: A representative agarose gel electrophoresis image (1.5%). The amplification of DNA appears as a ladder-like pattern. *P. aeruginosa* positive for bla_{VIM}, bla_{IMP} while bla_{OXA} is negative

DISCUSSION

The advent of carbapenems in the 1980s heralded a new treatment option for serious bacterial infections. Carbapenems have broad spectrum of activity and are stable to hydrolysis by most of the beta-lactamases, including the extended-spectrum beta-lactamases (ESBL) and the AmpC beta-lactamases (Shanthi *et al*., 2012). Carbapenem–resistant *P. aeruginosa* has become an important infectious agent all over the world challenging the current diagnostic and therapeutic approaches. It is important to identify carbapenemase genes transmitted on mobile genetic elements which can lead to the spread of resistance of *P. aeruginosa* to carbapenems, which are the main treatment options for infections caused by this highly resistant bacteria.

Pseudomonas species were isolated from 72 (17.1%) out of 420 samples of swabs from patients with infected wounds across four government hospitals in Maiduguri, Borno State, Nigeria. *Pseudomonas aeruginosa* constituted 68 (94.44%) of the isolates, while 4(5.6%) were other Pseudomonas species. This is similar to that by Ezeafor *et al*. (2020) who reported a *P. aeruginosa* prevalence rate of 18.3% of the total bacteria pathogens isolated in their study and also 13.6% of all clinical specimens and 14.6% of all environmental specimens. A similar study carried out in Zaria, North Central Nigeria by Olayinka *et al*. (2009) revealed a prevalence rate of 10.4% while a study in Egypt by Mahmoud *et al*. (2013) revealed a prevalence rate of 19.5%. In addition, a study by Yusuf *et al*. (2014) in North West Nigeria recovered 83 isolates of *P. aeruginosa* from various clinical samples with a prevalence of 13.1%. This difference could also be attributed to the differences in the study population and the larger number of specimens in their studies.

Almost 62% of the *P. aeruginosa* isolated in this study were from individuals aged 40 years or younger, with the majority in the 21–40-year ager group ($p<0.05$). This age group are the most productive in the community which agrees with results by Ranjan *et al.* (2010). The males predominated in this study as reported by Baba *et al*. (2018) who reported that *P. aeruginosa* was isolated more in males than females.

In this study, antimicrobial susceptibility pattern of *P. aeruginosa* shows highest sensitivity to piperacillin-tazobactam followed by ciprofloxacin, meropenem, gentamicin and aztreonam; with 100% resistance to ceftazidime, which is commonly employed as empiric therapy in nosocomial infections. Similar results of ceftazidime resistance were also observed by Trivedi *et al.,* (2012); Shrivastava *et al.* (2014).

Bacteria resistant to carbapenem have emerged and spread in the healthcare settings worldwide (Garbati and Al Godhair 2013, Dortet *et al*., 2014; Duin and Doi, 2017; Logan and Weinstein, 2017). This constitutes immediate threat to public health with attendant morbidity and mortality and thus require urgent and aggressive action (CDC, 2013). Resistance to meropenem in this study was however higher than 15.2% and 10.2% reported earlier in Lagos and Maiduguri, southwest and northeast Nigeria, respectively (Oduyebo *et al*., 2015; Mohammed *et al*., 2015).

Carbapenemase genes are one of the most frequent mechanisms reported in carbapenem-resistant *P. aeruginosa* (Villegas *et al*., 2007; Correa *et al*., 2012). The *P. aeruginosa*, resistance to carbapenems is mostly due to impermeability to imipenem-cilastatin, associated with qualitative or quantitative changes of the porin OprD2 (Mesaros *et al*., 2007). Overexpression of the MexXY-OprM porin may lead to decreased susceptibility to meropenem. However, carbapenemases have also been reported in *P. aeruginosa*. These are mostly MBLs (VIM, IMP) (Cornaglia *et al*., 2011). In general, for clinical samples from all countries where data were available, blaVIM is the most prevalent MBL, followed by blaIMP/blaNDM. In this study 3.3% of the *P. aeruginosa* isolates harbour bla_{VIM} and bla_{IMP}, while no bla_{OXA} was

detected. A similar study carried out in Asia reported *bla*$_{IMP}$ and *bla*$_{VIM}$ being more prevalent; with *bla*$_{IMP}$ found mainly in Japan, Korea, China, Taiwan, and Iran (Fang *et al*., 2008; Franco *et al*., 2010; Peymani *et al*., 2011).

CONCLUSION

Pseudomonas species were found to be prevalent in wound cultures from all the four major hospitals in Maiduguri, Nigeria. The susceptibility pattern from this report may be worth considering while implementing empiric treatment strategies for *Pseudomonas* infections in our locality. This study also revealed the presence of VIM and IMP in isolates of *P. aeruginosa*, the genes conferring resistance to carbapenems in the locality. Strict adherence to antimicrobial stewardship guidelines, infection prevention and control, and the need for improved surveillance on antimicrobial resistance on clinical specimens, animals and the environment; employing the One Health approach in order to mitigate against the spread of infections due to multidrug resistant organisms.

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