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MOLECULAR PROFILE OF METALLO-β-LACTAMASE PRODUCING BACTERIAL ISOLATES FROM CLINICAL SAMPLES; SOUTH-SOUTH NIGERIA PERSPECTIVE

One of the major clinical problems regarding β-lactam antibiotics resistance is attributed to metallo-beta-lactamases (MβL), which are a group of enzymes that is a subset of beta- lactamases belonging to group B of the Ambler classification, which causes hydrolysis of carbapenems. The study was conducted to check the prevalence of MβL and its genes (IMP, VIM, and NDM) among Gram-negative isolates. ***Methods.*** *312 clinical samples (urine and wound) were cultured, and antimicrobial susceptibility testing was performed using the conventional disk diffusion method. MβL-phenotypic detection was uncovered by standard bacteriological techniques, MβL genes were amplified using pre-determined conditions set on an AB19700 Applied Biosystem thermal cycler.* ***Results.*** *157 (56.1%) Gram-negative and 123 (43.9%) Gram-positive were isolated. Escherichia coli 32 (11.4%) and Pseudomonas aeruginosa 32 (11.4%) were the most predominant. Providencia stuartii 3 (1.1%), Klebsiella ornitholytica 2 (0.7%), and Stenotrophomonas maltophilia 1 (0.4%) were some of the less predominant isolates. Imipenem and Ertapenem were the most sensitive, while Gentamicin, Amoxicillin-Clavulanate, and Ceftriaxone were the most resistant. Twelve species (7.6%) were identified as MβL producers. The VIM gene (12: 100%) was the predominant gene, followed by the NDM gene (6: 50%) and the IMP gene (2: 16.7%).* ***Conclusions.*** *The detection of blaVIM, blaNDM, and blaIMP genes in South-south Uyo is really worrisome, and proper infectious control measures should be taken in order to prevent outbreaks of MβL-producing Gram-negative bacteria isolated in Uyo, South South Nigeria.*

Keywords: *carbapenem, efflux pump, multiple drug resistance (MDR), Metallo-beta-lactamases (MβL), antibiotic susceptibility.*

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In recent times, there have been reports on carbapenem resistance to various microorganisms, and the management of various infections caused by these harmful pathogenic bacteria has become a challenge [1]. The number of resistant microbial strains, geographic areas affected by drug resistance, and the extent of resistance in clinical isolates continue to escalate therefore contributing to one of the most serious jeopardies to global public health in the 21st century [2, 3]. Multiple drug resistance (MDR) has left few efficient antibiotics to take care of hard to treat life-threatening infections [4] and because of this, carbapenem was discovered to defeat the drug resistance menace [5]. The carbapenems were found to be potent against all MDR Gram-negative bacteria, including extended-spectrum β-lactamases (ESBLs) [6], and less toxic to the host [7]. In the recent past, carbapenems were the last resort antibiotics for the treatment of MDR Gram-negative bacterial infections but its resistance constitutes a major public health problem [8]. This resistance can occur through three possible mechanisms in the family Enterobacteriaceae: efflux pump overactivity, porin loss or mutation, and carbapenemase production [9]. Carbapenemase is an enzyme like β-lactamase and structurally belongs to Ambler classes (A, B, and D) and can hydrolyze a broad range of β-lactams, including carbapenems, cephalosporins, penicillin, and aztreonam [8]. Also, bacterial strains (mostly Enterobacteriaceae) that possess carbapenemase are often resistant to multiple drugs (MDR) [9]. Metallo-β-lactamases (MBLs) are a type of carbapenemases comprising Ambler class B beta-lactamases; these include IMP (imipenemase), VIM (Verona integron-encoded MβL), and NDM (New Delhi MBL), SPM (São Paulo metallo-β-lactamase), GIM (Germany imipenemase), SIM (Seoul imipenemase), KHM, AIM, DIM, SMB, TMB and FIM [10]. These genes which are biological instructors are located on mobile genetic units such as plasmids [11]. Carbapenem resistance caused by MβLs is considered more serious than other resistance mechanisms because MβLs can almost hydrolyze all beta-lactam antibiotics except monobactams [12, 13]. The presence of MβL-producing bacteria in the hospital and non-hospital environment puts the use of carbapenems under threat [14], and Uyo does not record any published report on MβL or the molecular analysis of genes acquired by carbapenem-resistant isolates. Therefore, it is pertinent to determine the incidence of MβLs through phenotypic and genotypic analyses and also detect the gene variants responsible for resistance to carbapenem drugs in Uyo.

Materials and methods. ***Study design and bacterial isolates.*** This descriptive cross-sectional study of minimum 312 samples comprising urine and wound from patients in the General Out-patients Department, Obstetrics and Gynaecology Department, Orthopaedic ward, Medical ward, surgical ward, as well as Burns and Plastic Surgery unit, was carried out in the Medical Microbiology and Parasitology Laboratory of University of Uyo Teaching Hospital, Uyo, Nigeria. This study was approved by the Ethical Committee of the University of Uyo Teaching Hospital, Uyo, Nigeria. Bacterial species were identified using standard laboratory methods, including Gram staining, biochemical method using the Microbact 24E (MB24E) (Oxoid, UK) system, and assessments of growth on Cysteine Lactose Electrolyte Deficient agar (CLED) and Blood agar (BA) plates while the wound swabs were inoculated on blood agar (BA) and MacConkey agar (MA) plates at 37 and 44 °C [15].

Antimicrobial susceptibility tests. Antibiotic susceptibility test of all isolates was performed by Kirby Bauer disc diffusion method recommended by Clinical Laboratory Standard Institute [16] guidelines using the Mueller-Hinton Agar and recommended antibiotics [Amoxicillin/clavulanate (30μg), Ceftazidime (30 μg), Ertapenem (10 μg), Cefepime (30 μg), Imipenem (10 μg), Ceftriaxone, (30 μg), Gentamicin (30 μg), and Ciprofloxacin (5 μg)]. In this study, those isolates that were non-susceptible to at least one agent in three

or more antimicrobial categories were regarded as MDR [17]. Control strains of *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) obtained from the National Research Institute Umudike, Abia State, Nigeria were tested primarily.

MβL screening, confirmation test, and interpretation. The isolates resistant to imipenem and/or meropenem were screened for MβL production using Total Metallo-β-Lactamase confirmation Kit 98016 from ROSCO Diagnostica (ROSCO Diagnostica A/S, Taastrupgaardsvej, Denmark) according to the manufacturer's instructions. Two imipenem discs were placed on agar plates containing the lawn of the test organism. 10 µL of 0.5 M EDTA solution was applied to one of the imipenem discs placed 25 mm apart, and the plate was incubated at 37 °C. After 18—24 hours of incubation, an increase of ≥ 5 mm in the zone diameter of the imipenem+EDTA disc as compared to imipenem+DPA was considered a positive test for the presence of MβL.

Amplification of Metallo-Beta-Lactamase Genes. Amplification was carried out with the following thermal cycling conditions: 5 min at 94 °C and 36 cycles of amplification consisting of 1 min at 94 °C, 1 min at 52—56 °C, and 1 min at 72 °C, with 5 min at 72 °C for the final extension. PCR product bands were analyzed after electrophoresis on a 1.5% agarose gel at 120 V for 20 min in 1X Tris-boric EDTA containing ethidium bromide, and the result was checked under an ultraviolet transilluminator.

Detection of VIM, IMP, and NDM Genes from Carbapenem-Resistant isolates. This was carried out in the Molecular Biology Laboratory of the Department of Medical Laboratory Science, Faculty of Health Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria. Deoxyribonucleic acid (DNA) from twelve carbapenem-resistant isolates and two other isolates, which served as positive and negative control, were extracted using the Zymo Research™ (ZR) Bacterial Mini prep extraction Kit (Inqaba, South Africa). The DNA quantification was done on a Nano-drop-1000 spectrophotometer (SN 1844 ND-1000UV/VIS Spectrophotometer, USA) to check the purity of the extracted DNA. The bacterial cell emulsion was centrifuged, and DNA in the supernatant was directly used as a template for PCR amplification.

Statistical analysis. Statistical analysis was conducted using SPSS, version 22.0 (Chicago, IL, USA). Associations between variables were considered statistically significant at p-values less than or equal to 0.05 ($p \leq 0.05$).

Results. Among 312 samples consisting of wound and urine, 157 (50.3%) yielded growth of Gram-negative bacterial isolates, 123 (39.4%) yielded Gram-positive isolates, and 32 (10.3%) had no significant growth (Fig. 1). *Escherichia coli* 32 (11.4%) and *Pseudomonas aeruginosa* 32 (11.4%) were the most predominant, while *Providencia stuartii* 1 (0.3%), *Klebsiella ornitholytica* 1 (0.3%), and *Stenotrophomonas maltophilia* 1 (0.3%) were the least (Fig. 1).

The resistance profile of Gram-negative organisms is shown in Fig. 2. The isolated Gram-negative organisms were mostly sensitive to imipenem (123; 91.0%), ertapenem (121; 77.6%), Cefepime (111; 71.6%), and Ceftazidime (101; 65.2%). The most resistant antibiotics included Gentamicin (103; 65.6%), Ciprofloxacin (90; 57.3%), Amoxicillin-Clavulanate (99; 63.1%), and Ceftriaxone (77; 49%).

Among the 157 Gram-negative isolates, 24 (15%) were carbapenem-resistant (Fig. 3). Of the 24 carbapenem-resistant Gram-negative isolates, 12 (50%) were MβL-producing Gram-negative isolates (Fig. 4). The prevalence of MβL-producing Gram-negative isolates was 50% among carbapenem-resistant organisms (15.3%) and 7.6% among the total number of Gram-negative isolates.

The zone diameters and difference between the IMPDP and IMP-10 tablet sizes for the 12 MβL-positive cases are shown in Table 1. As shown, if the difference in zone diameter of ≥ 5 mm (IMPDP-IMP10) was observed, the isolate is reported as showing MβL activity.

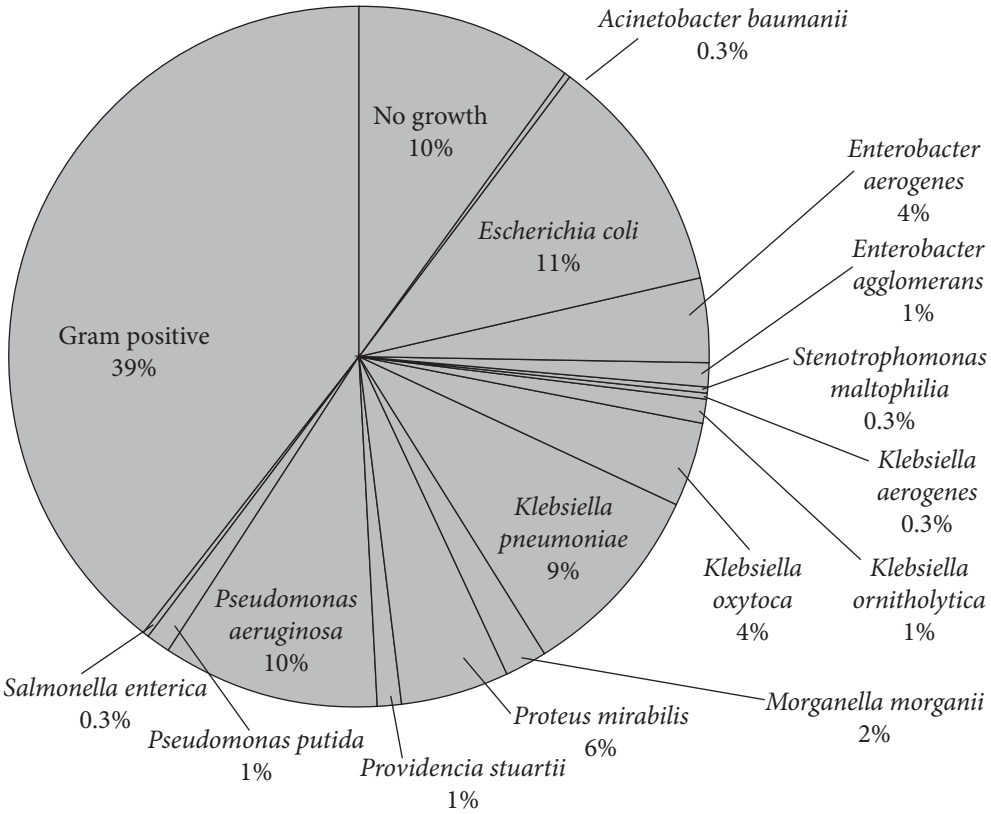

Fig. 1. Distribution of bacterial isolates
Source: Research data (2019)

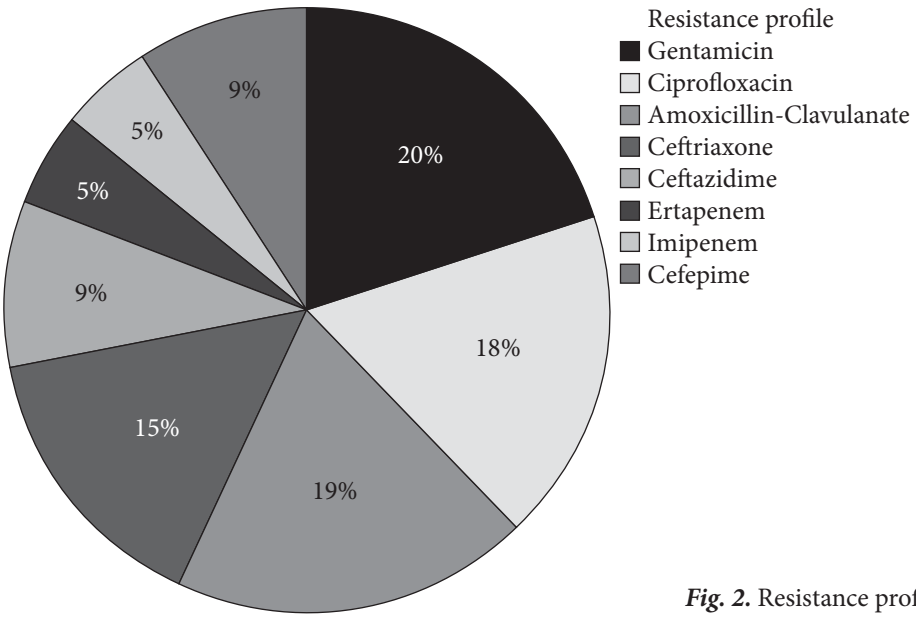

Fig. 2. Resistance profile of Gram-negative isolates

Table 1. **Difference between IMPDP and IMP-10 Diatab zone diameters**

Organism	IMP10	MRP10	1MPDP	IMPE	DPA	MβL Detection
Pseudomonas aeruginosa	10mm	9mm	20mm	21mm	9mm	MβL detected
Escherichia coli	23mm	22mm	20mm	20mm	9mm	No MβL
Morganella morganii	9mm	9mm	16mm	20mm	9mm	MβL detected
Klebsiella pneumoniae	15mm	16mm	22mm	26mm	9mm	MβL detected
Pseudomonas putida	15mm	17mm	24mm	25mm	9mm	MβL detected
Providencia stuartii	12mm	12mm	24mm	24mm	10mm	MβL detected
Escherichia coli	14mm	19mm	20mm	21mm	9mm	MβL detected
Enterobacter agglomerates	9mm	25mm	13mm	20mm	19mm	MβL detected
Escherichia coli	17mm	20mm	23mm	22mm	9mm	MβL detected
Pseudomonas aeruginosa	13mm	9mm	23mm	26mm	9mm	MβL detected
Pseudomonas aeruginosa	12mm	14mm	24mm	26mm	9m	MβL detected
Escherichia coli	9mm	9mm	16mm	21mm	10mm	MβL detected

Source: Research data (2019)

Table 2. **Molecular detection of MβL genes in samples**

Case Number	Sample	Organism	VIM	NDM	IMP
1	Wound	*Proteus mirabilis*	+	—	+
2	Wound	*Pseudomonas aeruginosa*	+	—	—
3	Urine	*Enterobacter agglomerans*	+	—	—
4	Wound	*Klebsiella pneumoniae*	+	—	—
5	Wound	*Pseudomonas aeruginosa*	+	—	—
6	Wound	*Pseudomonas aeruginosa*	+	+	—
7	Urine	*Providencia stuartii*	+	+	+
8	Urine	*Escherichia coli*	+	+	—
9	Urine	*Escherichia coli*	+	+	—
10	Wound	*Pseudomonas aeruginosa*	+	—	—
11	Wound	*Pseudomonas putida*	+	+	—
12	Urine	*Morganella morganii*	+	+	—

Source: Research data (2019)

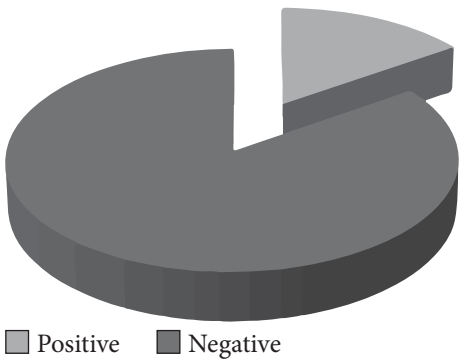

Fig. 3. Distribution of carbapenem-resistant organisms among Gram-negative isolates

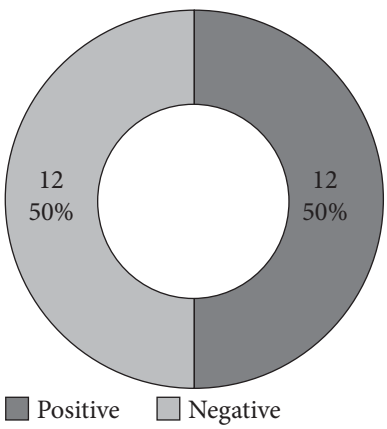

Fig. 4. Distribution of MβL among carbapenem-resistant organisms

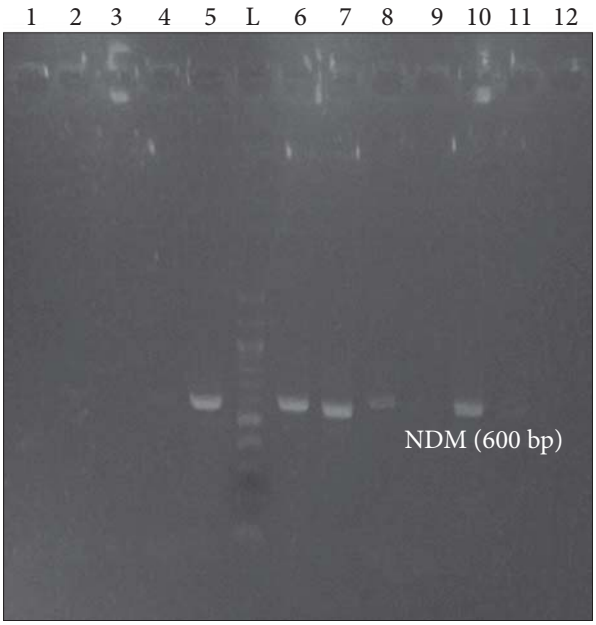

Fig. 5. Molecular detection of *NDM* genes from twelve MβL positive isolates

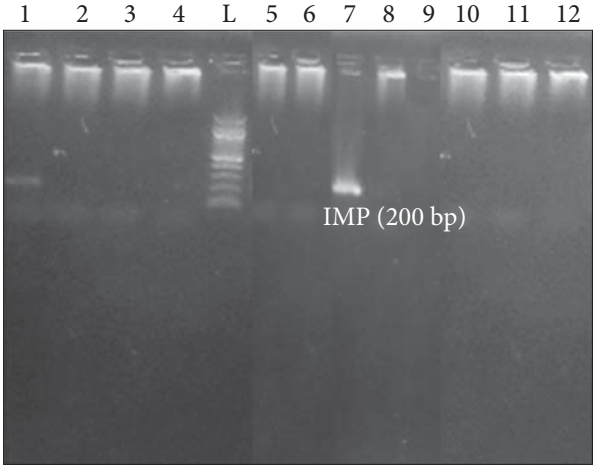

Fig. 6. Detection of *IMP* Genes from twelve MβL positive isolates

The distribution of MβL and its genes in samples is presented in Table 2. The MβL prevalence was higher in wound samples (7: 58.3%) than in urine samples (5: 41.7%) (Table 2). The *bla*VIM gene was detected in all 12 (100%) cases of MβL-producing isolates, while the *bla*NDM gene was present in 6 (50%) cases, though in

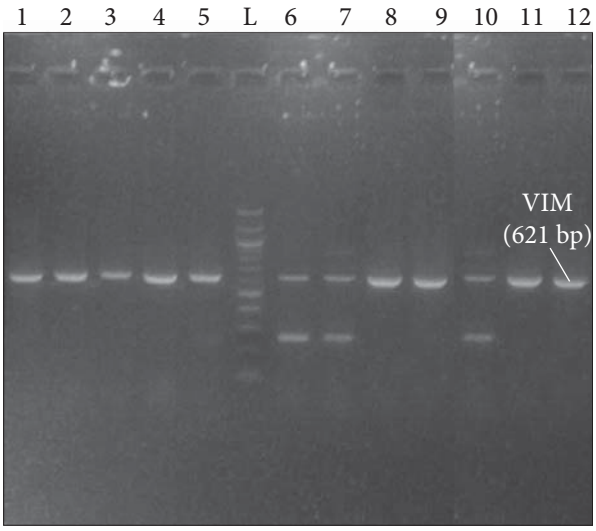

Fig. 7. Molecular detection of *VIM* gene from twelve MβL positive isolates

more urine samples. The *bla*IMP gene was found in 2 (16.7%) cases of the MβL-producing Gram-negative isolate which consisted of 1 urine and 1 wound samples each (Table 2).

The Agarose gel electrophoresis separation for detection of the genes among 12 Mβl positive isolates is shown in Figs. 5, 6 and 7. The amplified NDM gene in Lanes 5, 6, 7, 8, and 10 shows NDM gene bands at 600bp while lane L represents the 100bp molecular ladder (Fig. 5). The NDM gene was present in 6 cases. The IMP gene was detected in 2 cases, Lanes 1 and 7 show the IMP gene bands at 200bp while Lane L represents the 100bp molecular ladder (Fig. 6). The *VIM* gene was detected in all 12 (100%) cases, lanes 1—9 show *VIM* gene bands at 621 bp while lane L represents the 100 bp molecular ladder (Fig. 7).

Discussion. This study observed more isolates from females than from males within the age group of 35—44 years. Gram-negative isolates were the most common, accounting for 50.3% of samples. Similar findings have been reported by other researchers [18—20]. *E. coli* (33; 21%), *P. aeruginosa* (32; 20.4%), *K. pneumoniae* (27; 17.2%), *P. mirabilis* (19; 12.1%), *E. aerogenes* (12; 7.6%), *K. oxytoca* (12; 7.6%), and *M. mor-*

ganii (7; 4.5%) were the most frequently isolated Gram-negative bacteria, while less common isolates included *E. agglomerans*, *P. stuartii, P. putida, K. ornitholytica, S. maltophilia, A. baumanii,* and *K. aerogenes*. However, of particular interest in this study was the isolation of less frequently reported bacteria such as *S. maltophilia, P. stuartii,* and *K. ornitholytica*. *S. maltophilia* is a multidrug-resistant global opportunistic pathogen associated with a significant fatality/case ratio [21]. It is an emerging pathogen involved in the increasing incidence of both hospital and community-acquired infections, particularly among immunocompromised individuals [21]. It has been reported to be found in urine and wound cultures [21]. Physicians face a serious challenge in treating Stenotrophomonas infections due to its intrinsic resistance to many antibiotics and ability to acquire antibiotic resistance by multiple mechanisms [22]. *P. stuartii* is a Gram-negative opportunistic pathogen frequently isolated from the urinary tract of chronically catheterized patients [23]. There is limited information available regarding the pathogenicity of *K. ornitholytica*, a Gram-negative bacterium in the *Klebsiella* genus. Some studies suggest that *K. ornitholytica* may have a pathogenic potential as it is capable of producing virulence factors, including siderophores and exopolysaccharides [24]. Therefore, the use of a more sensitive biochemical identification system, Microbact 24E (Oxoid, UK), not routinely used in hospital laboratories, may explain the isolation of these rare organisms in this study, which has not been recorded in the service laboratories in the study area.

The study found high rates of resistance to commonly used antibiotics such as Gentamicin, Ciprofloxacin, Amoxicillin-Clavulanate, and Ceftriaxone among uropathogens and wound isolates. This resistance is likely due to the development of resistance genes by pathogens as a result of indiscriminate use or abuse of antibiotics. Gram-negative organisms showed high susceptibility to Imipenem, which may be due to limited use and abuse by patients. The result is consistent with other studies [25—27]. The moderate sensitivity of ertapenem is of concern, and the reason for this could be its acquisition of other resistance mechanisms. Also, *P. stuartii, P. aeruginosa*, *P. putida, E. coli, M. morganii, K. agglomerans* and *P. mirabilis* resisted all classes of antibiotics. This is indeed concerning as it suggests that there is a high prevalence of multi-drug resistant strains in the population and study area, which is likely due to the indiscriminate and/or non-prescription use of antibiotics. The implications of such high levels of antibiotic resistance are significant because treatment options for infections caused by these bacteria become limited, and patients may experience longer hospital stays, increased healthcare costs, and potentially worse outcomes. Furthermore, the development of new antibiotics is slow, and it is uncertain whether new antibiotics will be developed to address the increasing resistance problem [28].

This study found a 14.01% prevalence of carbapenem-resistant Gram-negative bacteria among isolates in the study area. This is consistent with previous studies conducted in Nigeria and other parts of Africa [20, 29, 30]. The emergence of carbapenem resistance in Gram-negative bacteria is a concerning global trend, and there are various factors contributing to its spread, including cross-resistance and patient travel from endemic areas. In addition, the localization of most carbapenemase genes on highly ambulatory genetic elements may contribute to the ease of acquisition and transmission of acquired carbapenemase resistance among bacterial isolates in a hospital [31].

The study found a 7.6% prevalence rate of metallo-beta-lactamase in the study area, which is comparable to similar studies in Ile-Ife [32, 33] but lower than in other studies in Sokoto, Nigeria [34]. The high prevalence of MβL in the study area may be due to the poor hygiene among medical personnel, and the ease of acquisition and transmission of carbapenem resis-

tance among bacterial isolates. The lower prevalence rates in other regions can be attributed to the low exposure of patients to carbapenems and improved hygiene measures. The predominant Gram-negative species found to produce MβL in this study was *P. aeruginosa*, which is similar to findings from other studies in Bayelsa [7]. However, other studies conducted in Ebonyi [35] and Sokoto [34] reported *E. coli* as the predominant MβL producer. The reasons for this difference can be due to changes in the pattern of bacterial infections or differences in the populations studied.

The phenotypic method used in this study had a high sensitivity and specificity in detecting MβL, which was confirmed by PCR. It is also interesting to note that this study found three different MβL genes (blaVIM, blaIMP, and blaNDM) in Uyo, which is the first report of all three genes in this area. The presence of all three genes in a single CR-GNB organism, *P. stuartii*, is particularly concerning as this could facilitate the rapid spread of carbapenem resistance. This finding is similar to a study done in the USA, where all three genes were also found [36].

The frequency of MβL genes in the study area Nigeria is similar to reports from other African countries such as Tanzania [37], Egypt [38], Uganda [39] and the Middle East [40], with *blaVIM* being the most common. The detection of *blaIMP* in a South Eastern Nigerian study indicates a regional variation. Pseudomonas species carrying *blaVIM* were isolated from wounds, highlighting the need for infection control practices. MβL production was detected in *P. stuartii, M. morganii*, and *P. mirabilis* not previously reported in Nigeria, likely due to the sensitivity and specificity of the detection kit used.

Conclusions. The study revealed that the prevalence of multidrug-resistant Gram-negative bacteria in the study area is high. The prevalence of carbapenem-resistant Gram-negative bacteria and metallo-beta-lactamase (MβL) in the study area is also concerning. Resistance to commonly used antibiotics like gentamicin, Ciprofloxacin, Amoxicillin-clavulanate, and Ceftriaxone is also high. Therefore, this study highlights the need for precautionary measures to prevent further increase. It is imperative to implement a comprehensive approach to address the threat of MDR pathogens in the study area. This should include reviewing infection control policies and antimicrobial prescription patterns to reduce selective pressure for the emergence and spread of MβL-producing organisms. Moreover, increased surveillance of MβL-producing organisms is necessary to prevent the possibility of an epidemic outbreak of MDR pathogens in the region. It requires a concerted effort to address this public health challenge, ensuring effective management of bacterial infections and reducing the burden of antimicrobial resistance.

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Author's Contribution

Unwana Ezekiel Akereuke: data gathering, interpretation of findings, final edit

Ifeanyi Abraham Onwuezobe, Ekom Ndifreke Edem: patient selection, manuscript preparation, study conception, overall supervision, final edit

Agantem Emmanuel Ekuma: patient selection, study conception, overall supervision, final edit

Ekom Ndifreke Edem: data gathering, patient selection, statistical analysis, final edit

Nsisong Sampson Uko, Rachel Sylvester Okon, Ene Bawonda, Edet Nsa Ekpenyong: data gathering, patient selection

Competing Interests. The authors do not have any conflict of interest to declare.

Ethical Approval. The protocol for the research project was approved by the Ethical Review Board at the University of Uyo Teaching Hospital, and all participants gave informed consent before inclusion in the study.

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МОЛЕКУЛЯРНИЙ ПРОФІЛЬ БАКТЕРІАЛЬНИХ ІЗОЛЯТІВ, ЩО ПРОДУКУЮТЬ МЕТАЛО-β-ЛАКТАМАЗУ З КЛІНІЧНИХ ЗРАЗКІВ; ПЕРСПЕКТИВИ ПІВДЕННОЇ НІГЕРІЇ

Однією з основних клінічних проблем резистентності до β-лактамних антибіотиків є метало-бета-лактамази (MβL) — група ферментів, що є підгрупою бета-лактамаз, які належать до групи B за класифікацією Амблера і спричиняють гідроліз карбапенемів. Дослідження було проведено з метою перевірки поширеності метало-β-лактамази (MβL) та її генів (*IMP, VIM* та *NDM*) серед грамнегативних ізолятів. **Методи.** 312 клінічних зразків (із сечі та ран) були культивовані; тестування чутливості до антибіотиків виконано з використанням звичайного методу дискової дифузії. Фенотипове виявлення MβL здійснювали за допомогою стандартних бактеріологічних методів, гени MβL ампліфікували з використанням попередньо визначених умов, встановлених на термоциклері AB19700 Applied Biosystem. **Результати.** Було виділено 157 (56.1 %) грамнегативних і 123 (43.9 %) грампозитивних мікроорганізмів. Найбільш поширеними були *Escherichia coli* 32 (11.4 %) та *Pseudomonas aeruginosa* 32 (11.4 %). *Providencia stuartii* 3 (1.1 %), *Klebsiella ornitholytica* 2 (0.7 %), *Stenotrophomonas maltophilia* 1 (0.4 %) були одними з менш поширених ізолятів. Штами були найбільш чутливими до іміпенему та ертапенему, а найбільш стійкими — до гентаміцину, амоксицилін-клавуланату та цефтріаксону. Дванадцять ізолятів (7.6 %) були ідентифіковані як продуценти MβL. Переважаючим геном був ген *VIM* (12: 100 %), за ним ішли ген *NDM* (6: 50%) та ген *IMP* (2: 16.7 %). **Висновки.** Виявлення генів *blaVIM*, *blaNDM* і *blaIMP* у місті Уйо викликає занепокоєння, і для запобігання спалахам інфекцій, викликаних MβL-продукуючими грамнегативними бактеріями, виділеними в Уйо (південна Нігерія), потрібно вжити належних заходів інфекційного контролю.

Ключові слова: *карбапенем, ефлюксна помпа, множинна лікарська стійкість (МЛС), метало-бета-лактамази (MβL), чутливість до антибіотиків.*