DETECTION OF CARBAPENEM-RESISTANT GENES ASSOCIATED TO HOSPITAL-ACQUIRED GRAM-NEGATIVE BACTERIAL PATHOGENS FROM A TERTIARY HOSPITAL IN LAGOS, NIGERIA

ABSTRACT

Aims: To determine carbapenem-resistant genes associated to hospital-acquired Gram-negative bacteria pathogens from a tertiary hospital in Lagos, Nigeria.

Study Design: Prospective cross-sectional experimental study

Place and Duration of study: Lagos State University Teaching Hospital Ikeja, Lagos and Nigerian Institute of Medical Research (NIMR), Lagos, between February 2023 and April 2024.

Methodology: We collected 162 Gram-negative bacteria isolates from urine and wound cultures of patients suspected of having hospital-acquired infections. Phenotypic identification of the isolates was by standard microbiological procedures. Carbapenem resistance was determined by Kirby-Baurer disc diffusion method using Meropenem and Imipenem. Confirmation of the strains of carbapenem-resistant bacteria isolates was by gene sequencing. The carbapenem-resistant genes of the bacterial strains were detected by real-time Polymerase Chain Reaction (PCR) using carbapenemase primers.

Results: Out of 162 bacterial isolates, 13(8%) bacterial isolates were resistant to Meropenem and Imipenem. New Dehli Metallo-β-lactamase (*bla*$_{NDM}$) was detected in 6(46%) of the carbapenem-resistant isolates while Verona integron-encoded Metallo-β-lactamase (*bla*$_{VIM}$) was detected in 2(15%). Carbapenemase genes were detected in 54% (7/13) of the carbapenem-resistant isolates. A strain of *Enterobacter hormaechei* harboured *bla*VIM and *bla*NDM genes.

Conclusion: The presence of these carbapenem-resistant genes in these pathogens is a public health threat. This study has provided knowledge of carbapenem-resistant genotypes of hospital-acquired bacterial pathogens in the study population. There should be renewed clinical monitoring of these pathogens and further studies on other mechanisms of carbapenem resistance.

Keywords: Hospital-acquired, Carbapenem-resistant, Isolates, Metallo-β-lactmases, Pathogens

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1. INTRODUCTION

Hospital-acquired or healthcare-associated infections (HAI) are nosocomial infections and also include workplace infections that affect hospital workers. They are one of the commonest harmful events that pose a risk to the wellbeing of patients and have huge socioeconomic implications [1].These infections have a prevalent rate of 40% in developing nations and 5% in developed nations [2]. Antibiotics resistance is a worldwide problem in the public health sector which has increased due to overuse of antibiotics. In the U.S, the Centre for Disease Control (CDC) 2019 Antibiotic Resistance (AR) threats report, stated that at least 2.8 million people are infected with antibiotic-resistant infections every year and more than 35,000 people die as a result [3]. Beta-lactam antibiotics are one of the major pillars of antimicrobial therapy due to their high potency and tolerability [4].

Carbapenems are reserve drugs for treatment of multidrug-resistant infections and carbapenem-resistant Gram-negative bacterial infections are rapidly increasing resulting to major public health concern globally. The presence of Extended Spectrum Beta Lactamases (ESBL) and carbapenemases has led to

widespread of antibiotics resistance among Enterobacterales [5]. Carbapenemase-producing Enterobacterales is globally spread and among the top three drug-resistant bacteria in dire need of new antibiotics [6]. Carbapenem-resistant Enterobacterales (CRE) are highly active against most antibiotics and they cause infections that have higher mortality than the carbapenem-susceptible Enterobacterales [7]. Gram negative bacterial isolates obtained from urine and wound cultures of patients admitted in the hospital for 48 or more hours that are showing clinical symptoms for nosocomial infections and had no evidence of this infection on admission were used in this study.

Carbapenem resistance in Gram negative bacteria remains an emerging and significant public health threat especially in low- and medium-income countries like Nigeria [8]. In Nigeria several studies have reported clear evidence of carbapenem resistance, however there is paucity of information on molecular characterization and profiling of carbapenem-resistant genes and their adverse effects in hospital environments [10] [9]. The detection of these genes will aid in understanding their epidemiology which is essential in controlling the spread of carbapenem resistance. This study aims to detect carbapenem-resistant genes associated to hospital-acquired carbapenem-resistant Gram-negative bacteria pathogens in the study population.

2. MATERIALS AND METHODS

2.1 Study design and setting

This study was a prospective cross-sectional experimental study involving bacteriological analysis. It was carried out at Lagos State University Teaching Hospital Ikeja, Lagos from February 2023 to April 2024. Lagos State University Teaching Hospital (LASUTH) is a state owned tertiary Hospital located in south-western Nigeria and serves as a referral centre for patients in Lagos and its environs. The detection of carbapenem-resistant genes was by PCR at Nigeria Institute of Medical Research (NIMR), Lagos.

2.2 Ethical Approval

This study was approved by Lagos State University Teaching Hospital Ethical Committee Reference number: LREC/06/10/2012. Informed consent was obtained from participants.

2.3 Study Population

Gram-negative bacteria isolates from wound and urine cultures of patients suspected of having hospital-acquired infections was used for the study. A total of 162 bacteria isolates were obtained

2.4 Study Criteria

2.4.1 Inclusion criteria: Gram negative bacterial isolates obtained from urine and wound cultures of patients admitted in the hospital for 48 or more hours that are showing clinical symptoms for nosocomial infections and had no evidence of this infection on admission were used in this study.

2.4.2 Exclusion criteria: Bacterial isolates from cultures of patients admitted in less than 48 h or patients not showing clinical symptoms for nosocomial infections were not used in this study.

2.5 Bacterial Isolates Collection and Identification

Different clinically significant isolates of Gram-negative bacteria from cultures of urine and wound samples of in-patients submitted to the Medical Microbiology and Parasitology Unit Laboratory of Lagos State University Teaching Hospital, (LASUTH), Lagos, were collected in Nutrient agar slants. Bacterial isolates collected in Nutrient agar slants were further characterized by streaking them on Nutrient agar plates and incubated for 24 h at 37^{o}C. This was done to get pure colonies of the isolates prior to

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confirmation of their identities using selective/differential diagnostic media and biochemical tests according to standard microbiological protocols [11].

2.6 Carbapenem susceptibilty screening tests

Phenotypic screening for carbapenem resistance was by Kirby-Bauer disc diffusion method using Meropenem (10µg) and Imipenem (10µg) discs. Distinct colonies of bacteria isolates on 18-24 h culture plate were suspended in 4 ml of sterile normal saline using a sterile inoculation loop. The bacteria suspension was standardized by comparing it with 0.5 McFarland standards. Sterile cotton swab stick was dipped into the standardized inoculums suspension and used to streak the entire surface area of sterilized Mueller-Hinton agar plates. The antibiotic discs were placed on the surface of the inoculated agar plates using sterile forceps and the plates were incubated at 37^{o}C for 24 h.

The zones of inhibition were measured and results were interpreted based on standard reference values of the Clinical and Laboratory Standards Institute (CLSI) guidelines. Bacteria Isolates were classified as resistant, intermediate or susceptible based on CLSI guidelines [12]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains for the Enterobacterales and non-Enterobacterales respectively. Bacterial Isolates were recorded as carbapenem-resistant if they showed simultaneous resistance to meropenem and imipenem [10]. The carbapenem-resistant bacteria isolates strains were identified by gene sequencing using ABI 3500XL Genetic analyzer.

2.7 Detection of Carbapenem-resistant genes

For bacterial DNA extraction, the isolates were cultured on nutrient agar plates and incubated at 37^{o}C for 20-24 h. A colony from the nutrient agar plate was inoculated into nutrient broth for DNA isolation, the cultures were grown overnight. The NIMR genomic DNA extraction kit was used for the extraction of genomic DNA. It is a spin column based DNA purification kit for isolation of genomic DNA from bacteria cells and the extracted DNA is suitable for all downstream applications. The carbapenem-resistant gene in the carbapenem-resistant bacteria isolates were detected using PCR and carbapenem-resistant primers *bla*NDM (603bp), *bla*VIM(437bp), *bla*IMP(387bp) and *bla*KPC (353bp) (listed in Table 1). The FIREPol Master Mix **(**Manufactured by Solis BioDyne) ready-to-load is a ready-to-use concentrated solution containing all reagents required for PCR (except template, primers and water), with compound for direct loading onto agarose gel and two dyes (blue and yellow) to monitor progress during electrophoresis. A 20 µl reaction mix was prepared based on the manufacturer's instructions. A negative control made up of the reaction mixture with water instead of DNA was added in each run. The *bla*KPC and *bla*IMP PCR program consists of initial denaturation at 94^{o}C /3min, followed by 30 cycles of denaturation (94^{o}C /1min), annealing (*bla*KPC 62^{o}C, *bla*IMP54^{o}C at 1min), elongation (72^{o}C/1min), final elongation (72^{o}C/10min) and hold at 4^{o}C/4min. The *bla*NDM and *bla*VIM duplex PCR program consists of an initial denaturation process at 95^{o}C/5min, followed by 30 cycles of denaturation (95^{o}C/30s), annealing (54^{oC}/40s) and elongation process (72^{o}C/1min 40s). Then, a final elongation at 72^{o}C/10min and holding time at 4^{o}C / >10min [13]. The amplicons were analyzed and visualized using a DNA ladder via gel electrophoresis on 1.5% (w/v) agarose gel in 0.5 x TAE buffer at a constant voltage of 100 V for 30 min.

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TABLE1. Oligonucleotides used in this study

S/N	SEQUENCE ID	SEQUENCE	LENGTH	Tm°C	SIZE (bp)	REFERENCE
1	*bla*NDM F	ACTTGGCCTTGC TGTCCTT	19	57	603	Bogaerts et al., (2013)
2	*bla*NDM R	CATTAGCCGCTG CATTGAT	19	55	,,	Bogaerts et al., (2013)
3	*bla*VIM F	TGTCCGTGATG10 GTGATGAT	20	57	437	Bogaerts et al., (2013)
4	*bla*VIM R	ATTCAGCCAGAT CGGCATC	19	57	,,	Bogaerts et al., (2013)
5	*bla*KPC F	TCGCCGTCTAGT TCTGCTGTCTTG	24	74	353	Bogaerts et al., (2013)
6	*bla*KPC R	ACAGCTCCGCCA CCGTCAT	19	62	,,	Bogaerts et al., (2013)
7	*bla*IMP F	ACATGGCTTGATT GTGCTTG	20	55	387	Bogaerts et al., (2013)
8	*bla*IMP R	GGTTTAACAAAG CAACCACC	20	55	,,	Bogaerts et al., (2013)

Note: 1-8, Adapted from "Validation of carbapenemase and extended-spectrum -lactamase multiplex endpoint PCR assays according to ISO 15189." [14]

2.9 Statistical Analysis

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Results were summarized using descriptive statistics (percentages and frequencies). Tables were used to display distribution of the carbapenemase encoding genes found in the selected Gram-negative bacteria pathogens in this study.

3. RESULTS

A total of 162 hospital-acquired Gram-negative bacteria isolates were collected for this study. Among the 162 bacteria isolates, I3 (8%) of the isolates were simultaneously resistant to Meropenem and Imipenem. The gel electrophoresis of the carbapenem-resistant bacteria PCR amplicons using *bla*KPC (353bp) and *bla*IMP (387bp) primers showed no significant amplification. While the carbapenem-resistant bacteria PCR amplicons using *bla*NDM (603bp) and *bla*VIM (437bp) primers showed significant amplification in many of the PCR amplicons. The bacteria PCR amplicons that showed presence of carbapenem-resistant gene are Z_2-NDM, C_2-VIM, J_3-VIM and NDM, T_2-NDM, C- NDM, B-NDM, X_2-NDM while I_3, N_6, NAF, N_9 and M_3 showed no significant amplification. Gel electrophoresis of the carbapenem-resistant bacteria PCR amplicons using *bla*NDM and *bla*VIM primers is as presented in (figure 1). New Dehli Metallo beta-lactamase gene (NDM) was identified in 6/13(46%) carbapenem-resistant bacteria isolates while Verona Integron-encoded Metallo beta-lactamase (VIM) gene was identified in 2/13(15%) as shown in (Table 2). The *bla*NDM and *bla*VIM are the carbapenem-resistant genes identified in this study and they have a frequency of 6/8(75%) and 2/8(25%) respectively. Among the carbapenem-resistant nosocomial Enterobacterales isolates, (7/13, 54%) were carrying carbapenem-resistant genes. Two carbapenem-resistant genes were harboured by a strain of *Enterobacter hormaechei*

TABLE 2 .Types/Sources of carbapenem-resistant genes in Gram-negative hospital-acquired bacterial pathogens in this study

Bacterial Isolate	Sources	Type of carbapenem-resistant gene
Z_2 *Escherichia coli*	Urine	*bla*NDM
C_2 *Alcaligenes faecalis*	Urine	*bla*VIM
J_3 *Enterobacter hormaechei*	Urine	*bla*VIM, *bla*NDM
T_2 *Pseudomonas aeruginosa*	Urine	*bla*NDM
C *Escherichia coli*	Urine	*bla*NDM
B *Providencia stuartii*	Urine	*bla*NDM
X_2 *Pseudomonas aeruginosa*	Urine	*bla*NDM

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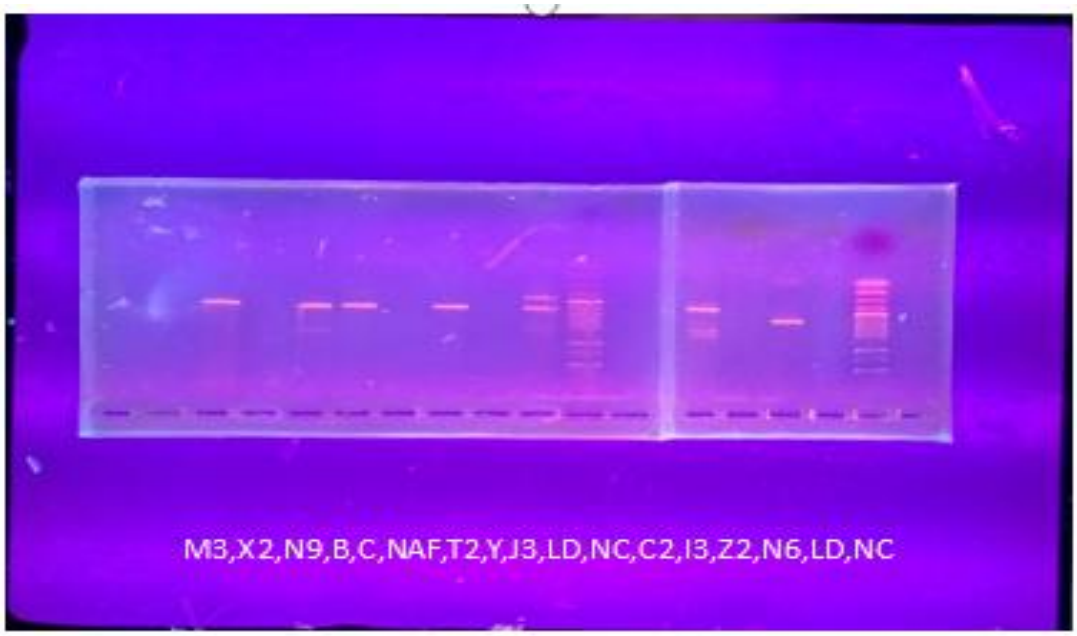

Figure 1: Gel electrophoresis of carbapenem-resistant gene duplex PCR amplicons using *bla*NDM (603bp) and *bla*VIM (437bp) primers. Note: LD-Ladder, NC-negative control, carbapenem resistant genes were present in Z_2, C_2, J_3, T_2, C, B, X_2 respectively, while carbapenem-resistant genes were not identified in N_6, I3, Y, NAF, N9 and M_3 respectively.

4. DISCUSSION

This study reported the occurrence and types of carbapenem-resistant genes present in hospital-acquired carbapenem-resistant Gram-negative bacteria pathogens using real time PCR. For the carbapenem susceptibility tests performed, the criteria used for carbapenem resistance was strictly based on simultaneous resistance of bacteria isolate to Imipenem and Meropenem [10] [15]. There has been an increase in the prevalence of acquired metallo-beta-lactamases globally though there is paucity of data from Africa, particularly in Nigeria [15]. In Nigeria, there have been various reports on prevalence of carbapenem-resistant Enterobacterales from many parts of the country [9]; this necessitated the need for this study on the profile of genes responsible for carbapenem resistance. The absence of carbapenem-resistant genes in the bacterial isolates that were phenotypically resistant to carbapenems suggests that other antibiotic resistant mechanism might be at play [16]. These resistant mechanisms include efflux pumps activities, impermeability of the outer membranes, target site modification and other carbapenemases.

New Dehli metallo-beta-lactamase gene (*bla*NDM) was the most prevalent carbapenem-resistant gene in this study. The *bla*NDM is globally distributed and has been reported in US, European and African nations [17]. Metallo-beta-lactamases have the ability to hydrolyze most beta-lactam antibiotics, ubiquitous in hospital setting and their encoding genes are easily transferred [4]. NDM gene is transmitted within bacteria species by plasmid through horizontal transfers and studies have shown that *E.coli* spp are among the highest carriers [18][19] This is comparable with the findings in this work and is suggestive of hospital transmission of a common multidrug resistant strain. *Enterobacter hormaechei*, a clinically important nosocomial pathogen, have both Verona integron-encoded metallo-beta-lactamase(VIM) and

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NDM genes, this is comparable to other research findings mainly in Asia where carbapenem-resistant *E.hormaechei* carrying multiple carbapenamases have been reported [21],[20],[22],[23]. Although in Nigeria, there is paucity of information on *bla*VIM in hospital-acquired *E.hormaechei* infections.

The *bla*VIM gene was first detected in Italy in 1999 from a clinical sample and has subsequently been reported around the Mediterranean region but its detection in Lagos might be a case of imported antibiotic resistance strains due to international travels by Nigerians to such regions (Isreal, Rome and Saudi Arabia) for pilgrimage.

The *bla*VIM is also present in *Alcaligenes faecalis,* this gene is responsible for the carbapenem resistance by this pathogen and this is similar to published study results on drug resistant *Alcaligenes faecalis* [24]. There has been paucity of reports on *bla*VIM in Nigeria, though the north has few reports [18];[25], the South has very poor known blaVIM report from clinical sources. This report on *bla*VIM from this study exposes the threat of this gene to patients and hospital environment in the study population. The prevalence of some carbapenem-resistant genes may differ within hospitals and communities due to the unique nature of antimicrobial resistant genes which can easily disseminate among different bacterial species through horizontal transfers.

5. CONCLUSION

This study has provided knowledge of phenotypes and genotypes of hospital-acquired carbapenem-resistant Enterobacterales in the study population which will further aid in understanding emerging resistance and new resistance determinants. The carbapenem-resistant genes identified in this study were *bla*VIM and *bla*NDM. There should be renewed clinical monitoring of these genes since they can survive in healthcare environments. There should be more studies on the new variants of metallo-beta-lactamases so as to aid in the development of inhibitors against them. The reports from this study will aid the relevant government agencies in planning, advocacy and developing a robust antibiotic stewardship intervention and infection control programs. Furthermore, there should be more research on the precise mechanism for antibiotic resistance in carbapenem-resistant nosocomial pathogens in other to achieve narrow-spectrum targeted-treatment approaches.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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