Metallo-beta-lactamase producing isolates of *Escherichia coli* and *Klebsiella pneumoniae* and their Resistance Profiles in Enugu, Nigeria: A threat to public health

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ABSTRACT

Background: A potential threat to public health is the rapidly spreading enterobacteriaceae, especially *Escherichia coli* and *Klebsiella pneumoniae* which produce metallo-beta-lactamases (MBL). This study evaluated the prevalence of metallo-beta-lactamase (MBL) from clinical and non-clinical sources in Enugu Metropolis.**Methodology:** The study was conducted in the Microbiology Laboratory of the University of Nigeria Teaching Hospital, Ituku-Ozalla between October 2020 and July 2021. A total of 150 isolates including 85 and 65 isolates of *Escherichia coli* and *Klebsiella pneumoniae* respectively was recovered. Standard microbiology procedures were used to identify and characterize the isolates. Antimicrobial susceptibility was done using the Kirby-Bauer disc diffusion technique. Phenotypic detection of Metallo-beta-lactamase production was determined using Combined Disk Tests. **Results:** Imipenem resistance was detected in 22 (25.9%) isolates of *E. coli* and 18 (27.7%) isolates of *K. pneumoniae*. Of the 22 strains of *E. coli* that were imipenem resistant, 8 (9.4%) and 14 (16.5%) were found to be MBL producers and non-MBL respectively. Of the 18 strains of *Klebsiella pneumonia*e that were imipenem resistant, 10 (15.4%) were MBL producers and 8 (12.3%) were non-MBL producers. The highest prevalence of MBL was recovered from urine sources in both *E. coli* and *K. pneumonieae*. All MBL-producing isolates were multidrug resistant. **Conclusion:** The overall prevalence of MBL in this study was 12.0%. Public health is at risk due to the occurrence of metallo-beta-lactamase. Antimicrobial stewardship and the implementation of infection control strategies are required to halt the spread of these resistant bacteria in the environment. The use of antibiotics should be with utmost prudence.

Keywords: Antibiotic resistance, Carbapenemase, Metallo-beta-lactamase, phenotypic detection, *Escherichia coli, Klebsiella pneumoniae*

1. INTRODUCTION

Global health is challenged and threatened by the rise and spread of life-threatening infections caused by bacteria resistant to antibiotics [1]. Gram-negative bacteria such as *Acinetobacter baumannii* and Enterobacteriaceae, which is the most common in humans, are of special interest due to their increased resistance[2].The main challenge facing the healthcare sectors in developing countries like Nigeria is the rise and spread of multidrug-resistant (MDR) bacteria, especially *Escherichia coli* and *Klebsiella pneumoniae* harboring resistance genes. Accurate susceptibility testing, cautious use of currently available antibiotics and measurement of the prevalence of multidrug-resistant bacteria are necessary to reduce the potential risks associated with infections caused by multi-drug-resistant strains[3]. As a last resort, carbapenems like imipenem, meropenem, and ertapenem are used to treat infections caused by bacteria that are resistant to many antibiotics[4]. The primary reason for their exceptional antibacterial activity is their ability to swiftly penetrate gram-negative bacteria membrane and their resistance to most naturally occurring and acquired beta-lactamases, such as cephalosporinases and extended-spectrum beta-lactamases[5].However, bacteria have developed defense mechanisms against these carbapenems through adaptation.This resistance is associated either with the relationship of the over-expression of extended-spectrum *β*-lactamases with the efflux pump and impermeability or to the expression of *β*-lactamases hydrolyzing carbapenems, known as carbapenemases[6]. Selection pressure has, as predicted, led to the development of carbapenem resistance in bacterial pathogens[7, 8]. Though the situation in many countries is yet unknown, the prevalence of carbapenemases, which are typically located on mobile genetic elements, has the potential to be universal. Often, carbapenemase-producing bacterial isolates exhibit multiple antibiotic resistances[7].Carbapenemases fall under three of the four classes of β-lactamases in Ambler's classification (A, B, C, and D). Metallo-lactamases (MBLs) are a group of carbapenemases that are especially important to healthcare because of their ability to spread over the globe and the resulting limited number of therapeutic options that are accessible [9]. Zinc or another heavy metal is needed for the catalysis of these class B carbapenemases. MBLs have a wide range of substrates and are capable of catalyzing the hydrolysis of nearly all beta-lactam antibiotics, including carbapenems, except monobactams like aztreonam [7]. Furthermore, they are blocked by chelating agents such as ethylene-diamine tetra-acetic acid (EDTA)[3]. One further issue with strains that produce carbapenemase is that they are resistant to all of the strongest antibiotics, which means that colonization with them carries a significant risk of mortality[10]. The distribution of different carbapenemases produced by Gram-negative bacteria varies geographically[11].Standard antimicrobial susceptibility testing can be combined with early identification of particular carbapenem resistance mechanisms in resource-constrained settings by employing simple phenotypic methods. Test results can provide information that can help with the timely deployment of appropriate antibiotics, preventing and containing the spread of bacteria that are resistant to several drugs. Based on detection, suitable therapies may be selected [11].

Given the unmatched range of activity and resistance to therapeutic serine beta-lactamase inhibitors, MBLs are now considered a health risk and an important cause of the growing rates of morbidity and mortality. The gold standard for MBL detection and classification is still PCR-based genotyping; nevertheless, diagnostic laboratories still need to perform culture-based phenotypic testing to quickly identify MBL activity [12].The MBL enzyme can be identified phenotypically in pathogenic bacteria. The recognized techniques are the Modified Hodge Test (MHT), Double-Disc Synergy Test (DDST),

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Combined Disc Diffusion Test with Imipenem and EDTA (CDDT), and MBL E-Test [13, 14]. Since there isn't much information on MBL prevalence in Enugu, this study was carried out to determine how common MBLs are in isolates that are resistant to imipenem (carbapenem) from hospitals and other environmental sources in the city of Enugu. To prevent the spread of these infections, determine the best course of treatment, and put infection control measures in place, it is essential to identify MBL-producing organisms as early as possible.

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2. MATERIALS AND METHODS

2.1Study Design

The study was carried out from October 2020 to July 2021 at the University of Nigeria Teaching Hospital's Microbiology Laboratory in Ituku-Ozalla, Enugu.

2.2 Study Area

Enugu Metropolis is located in the Enugu State, South-East geopolitical Zone of Nigeria. There are 17 Local Government Areas in Enugu State. There are four Tertiary health care centers within the Metropolis. They include University of Nigeria Teaching Hospital (UNTH), Enugu, Federal Neuropychiatric Hospital, Enugu, Enugu (FNHE), and Enugu State University Teaching Hospital (ESUTH), National Orthopedic Hospital, Enugu (NOHE) that serve as referral and specialist centers for the southeast geopolitical zone.

2.3 Collection of Sample

Non-duplicate clinical bacterial isolates already processed in the microbiology laboratories of University of Nigeria Teaching Hospital (UNTH), Enugu, Enugu State University Teaching Hospital (ESUTH), National Orthopedic Hospital, Enugu (NOHE) and some private laboratories within Enugu Metropolis were collected aseptically and inoculated on nutrient agar slants. The isolates were from urine, wound, sputum, stool, and high vaginal swabs. Non-clinical isolates were from water, soya milk, and zobo drink and already processed in the Research Laboratories including Emmanuel Research Laboratory and University of Nigeria, Enugu Campus Microbiology Research laboratory. The isolates were randomly collected and taken to the Microbiology Laboratory of University of Nigeria Teaching Hospital Ituku-Ozalla.

2.4 Isolation of Bacteria

All the isolated organisms were brought to the laboratory and maintained in nutrient agar slants. A total of 600 isolates recovered from different sources were reactivated on MacConkey agar (Central Drug House (P) Ltd, and incubated for 24 hours at 37°C on various agar plates to obtain a pure culture.

2.5 Identification of Bacteria Isolates

Standard laboratory protocols for microbiology were employed to identify the isolates. Conventional microbiological techniques, such as colony morphology, Gram stain, biochemical testing, and the API 20E confirming procedure, were used to identify the isolates [15]. A total of 65 *Klebsiella pneumoniae* isolates and 85 *Escherichia coli* were identified.

2.6 Antimicrobial Susceptibility Test

In compliance with the Clinical Laboratory Standard Institute guidelines, the antibiotic susceptibility of each isolate was assessed using the Kirby Bauer disk diffusion method on Muller-Hinton agar (HiMedia Laboratories India) [16]. The antimicrobial used were Imipenem (10µg), cefoxitin (30µg), ofloxacin (5 µg),

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ciprofloxacin (5 µg), gentamicin (10 µg), amoxicillin/clavulanate (20/10 µg), nitrofurantoin (50 µg), ceftazidime (30 µg), cefixime (30 µg), cefotaxime (30 µg), and cefuroxime (30 µg). Mueller-Hinton agar plates were inoculated with a standardized suspension of each isolate comparable to 0.5 McFarland turbidity standards, and the antibiotics disc was aseptically placed on the agar plates. The plates were then incubated at 37°C for 18 to 24 hours. Following the 2015 CLSI recommendations, the Inhibition Zone Diameter (IZD) was measured and recorded after incubation[17].

2.7 Screening for carbapenemase production

MBL-producing *E. coli* and *K. pnuemoniae* resistant to imipenem were tested for MBL production. Isolates with reduced susceptibility to imipenem with an inhibition zone diameter of ≤ 21 mm were used as cut-off values according to CLSI guidelines [16].

2.8 Detection of metallo–beta-lactamase production

Combined Disc Diffusion Test (CDDT)

MBL production was phenotypically detected using the Combined Disc Diffusion Test as per Franklin *et al.* with slight modifications[18]. Two 10µg imipenem discs (one impregnated with 10µl of 750µg EDTA Sigma Chemicals, St. Louis, MO) were placed at a distance of 25mm apart on the Mueller Hinton (MH) agar medium inoculated with test organism standardized with 0.5 McFarland standards. After 24 hours of incubation at 37°C, the zones of inhibition around imipenem and imipenem + EDTA discs were compared. MBL production was deemed to have occurred when the zone diameter around the imipenem + EDTA discs increased by more than 4mm when compared to the imipenem alone. Before being added to the antibiotics disk(s), EDTA was tested on the isolate(s) alone to be sure it wouldn't suppress the test organism and result in a false positive [3].

2.9 Statistical Analysis

All statistical analyses were performed using SPSS for Windows version 22 (SPSS, Chicago, IL, USA). Descriptive statistics were used to describe categorical variables (frequencies and percentages). The Chi-square (X^2) test (at 95% confidence intervals) was used to compare the antimicrobial profile of bacterial isolates as well as the proportions of MBL producers between hospital and environmental isolates. P-value ≤ 0.05 is considered statistically significant.

3. RESULTS

The distribution of MBL and non-MBL-producing isolates based on the source of the isolate is shown in Table 1. Out of the 85 isolates of *E. coli* recovered, 22 (25.9%) had reduced susceptibility to imipenem. Of

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these 22 isolates 8 (9.4%) were MBL producers while 14 (16.5%) isolates were non-MBL producers. The highest prevalence of MBL producers was recovered from Urine and environmental samples 3(37.5%) followed by stool and HVS 1(12.5%) each. Statistically, there was no significant difference between MBL production and the source of the isolate. The overall prevalence of MBL was 9.4% (8/85).

Table1: Distribution of MBL and non-MBL producing *E. coli* Isolates based onsample source

Source of Isolate	Number of Isolate	No/ % Resistant to Imipenem	MBL Producers	Non-MBL Producers
Urine	34(40.0)	7 (31.8)	3(37.5)	4 (28.6)
HVS	20 (23.5)	4 (18.2)	1(12.5)	3 (21.4)
Stool	13 (15.3)	5 (22.7)	1(12.5)	4(28.6)
Non-clinical	18 (21.2)	6 (27.3)	3 (37.5)	3 (21.4)
Total	**85**	**22(25.9**	**8 (9.4)**	**14 (16.5)**
P= 0.920	**X^2= 3.86**			

The distribution of MBL and non-MBL-producing isolates of *K. pneumoniae* according to sources of isolates is shown in Table 2. Of the 18 (27.7%) isolates of *K. pneumoniae* that were resistant to imipenem, 10 (15.4%) were MBL producers while 8 (12.3%) were non-MBL producers. The highest prevalence of MBL was recovered from urine 4(40%), followed by sputum 3 (30%) and the least was from wound 1 (10%). There was no significant difference between MBL producers and the source of the isolates, P= 0.981.

Table 2: Distribution of MBL and non-MBL producing *K. pneumoniae* Isolates based on sample source

Source of Isolate	Number of Isolate	No/% Resistant to Imipenem	MBL Producers	Non-MBL Producers
Urine	35 (53.8)	7(38.9)	4 (40.0)	3 (37.5)
Sputum	12 (18.5)	5 (27.8)	3 (30.0)	2 (25.0)
HVS	6 (9.2)	1 (5.6)	0 (0.0)	1 (12.5
Wound	7 (10.8)	2 (11.1)	1(10.0)	1 (12.5)
Non-clinical	5 (7.7)	3 (16.7)	2(20.0)	1 (12.5)
Total	**65**	**18 (27.7)**	**10 (15.4)**	**8 (12.3)**
P = 0.981	**X^2 = 3.74**			

Table 3 is the in vitro antibacterial susceptibility and resistant profile of MBL and Non-MBL producing *E. coli*. MBL-producing isolates showed higher resistance in comparison to non-MBL producers with an average resistance prevalence of 76.3% as against 60.8%. MBL-producing isolates showed complete (100%) resistance to cefixime, ceftazidime, cefuroxime, cefotaxime, and augmentin. The most potent antibiotic was nitrofurantoin in both MBL and non-MBL producers 87.5% and 78.5% respectively.

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Table 3: In Vitro Antibacterial profile of MBL and Non-MBL isolates of *E. coli* (n=85)

	MBL Producers (n=8)		Non-MBL Producers (n= 14)	
Antibiotics	**No/% Susceptible**	**No/ % Resistant**	**No/% Susceptible**	**No/ % Resistant**
Cefixime	0 (0.0)	8 (100)	0 (0.0)	14 (100)
Ceftazidime	0 (0.0)	8 (100)	6 (42.9)	8 (57.1)
Cefuroxime	0 (0.0)	8 (100)	0 (0.0)	14 (100)
Cefotaxime	0 (0.0)	8 (100)	6 (42.9)	8 (57.1)
Cefoxitin	5 (62.5)	3 (37.7)	8 (57.1)	6 (42.9)
Ofloxacin	1 (12.5)	7 (85.5)	9 (64.3)	5 (35.7)
Augmentin	0 (0.0)	8 (100)	0 (0.0)	14 (100)
Ciprofloxacin	2 (25.0)	6 (75.0)	2 (14.3)	12 (85.7)
Gentamicin	2 (25.0)	6 (75.0)	6 (42.9)	8 (57.1)
Nitrofurantoin	7 (87.5)	1 (12.5)	11 (78.6)	3 (21.4)

Table 4 shows the in vitro antibacterial profile of MBL and non-MBL isolates of *K. pneumoniae.* MBL producers were 100% resistant to cefixime, ceftazidime, augmentin, and ofloxacin, 80% and 70% for cefotaxime and ciprofloxacin respectively. There was a high susceptibility to aminoglycoside gentamicin 80% and cefoxitin 70%. Of the 8 isolates that were non-MBL, there was high resistance of isolates to most of the antibiotics, 100% resistance to augmentin, 87.5 % resistance to cefixime, cefuroxime, cefotaxime, and ofloxacin. There was high susceptibility of non-MBL isolates to ciprofloxacin 87.5%, nitrofurantoin and gentamicin 75%, and cefoxitin 62.5%.

Table 4: In Vitro Antibacterial profile of MBL and Non-MBL isolates of *K. pneumoniae* (n=65)

	MBL Producers (n= 10)		Non-MBL Producers (n= 8)	
Antibiotics	**No/% Susceptible**	**No/ % Resistant**	**No/% Susceptible**	**No/ % Resistant**
Cefixime	0 (0.0)	10 (100)	1(12.5)	7 (87.5)
Ceftazidime	0 (0.0)	10 (100)	0 (0.0)	8 (100)
Cefuroxime	0 (0.0)	10 (100)	1(12.5)	7 (87.5)
Cefotaxime	2 (20.0)	8 (80.0)	1(12.5)	7 (87.5)
Cefoxitin	7 (70.0)	3 (30.0)	5 (62.5)	3 (37.5)
Ofloxacin	0 (0.0)	8 (80.0)	1(12.5)	7 (87.5)
Augmentin	0 (0.0)	10 (100)	0 (0.0)	8 (100)
Ciprofloxacin	3(30.0)	7 (70.0)	7 (87.5)	1 (12.5)
Gentamicin	8 (80.0)	2 (20.0)	6 (75.0)	2 (25.0)
Nitrofurantoin	4 (40.0)	6 (60.0)	6 (75.0)	2 (25.0)

DISCUSSION

Global public health is seriously threatened by the rapidly evolving and spreading enterobacteriaceae that produce MBL, especially *K. pneumoniae* and *E. coli*[19]. MBLs are beta-lactamase enzymes that hydrolyze carbapenems, such as imipenem, making them useless as therapeutics [7]. In our study, 26.7% (40/150)

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of the total isolates of *E. coli* and *K. pneumoniae* were found to be resistant to imipenem. Many researchers have reported the same. Kumarasamy *et al.*, 2010 in their research on the emergence of a new antibiotic resistance mechanism in India, Pakistan, and the United Kingdom reported a prevalence of 24% while Panchal *et al.*, 2017 reported 28% in India[20, 21]. However, Javed *et al., 2016* recorded lower prevalence of 11.81% in Pakistan [12]. Imipenem resistance was also found to be higher in *K. pneumoniae* than in *E. coli* 27.7% and 25.9% respectively, many researchers had reported the same[19, 22, 23]. The extensive use of carbapenems in the treatment of severe infections brought on by organisms that produce extended-spectrum beta-lactamases (ESBLs) has been a major factor in the rise of carbapenemases[24].The two main problems associated with carbapenemase production are their compromise to the effectiveness of antibiotics used as a last resort infection treatment and their ability to spread to different bacteria globally [7].

This study showed the prevalence of MBL in *K. pneumoniae* and *E. coli* to be 15.4%, and 9.4% respectively giving an overall prevalence of 12%. Mittal *et al.,* 2020 and Oberoi *et al.,* 2013 reported 23.37% and 22.72%respectively in India [25, 26] and Shanhandeh *et al,.* 2015 recorded 23.1% in Iran[27]. Bora *et al.*, 2014 reported a prevalence of 18.98% and 21.08% in *E coli* and *K. pneumoniae* respectively in Nepal [19]. However, research purportedly carried out in several nations revealed that the rates of MBL generation for *K. pneumoniae* and *E. coli* range from 33–36% and 13.4–61.5%, respectively [28]. This study demonstrates that there are wide regional variations. These differences might be due to variations in sample size, detection technique, and isolate sources [3].

In our study, MBL production was found to be higher in *K. pneumoniae* than in *E. coli.* Similar findings have been reported by many authors [19, 22, 29]. Higher resistance in *K. pneumoniae* may be due to alterations in cellular permeability caused by modifications in the AcrAB-TolC and KpnEF efflux pump systems, as well as the loss of the putative porin KpnO[30].

In this study, the highest rate of MBL-producing isolates was recovered from urine isolates 38.9% (7/18). This agrees with the 32.35% reported in India [25] but does not align with what was reported by Singh *et al.,* 2015 which recorded the highest prevalence of 62.5% in ET secretion [32] and Kamble *et al*., 2015 recorded the highest in pus at 42.55%[31]. Bora *et al., 2014* recorded the highest MBL-producing isolates from blood [19]. Our higher prevalence in urine could be due to the large number of urine isolates included in our study. In addition *E. coli* and *K. pneumoniae* are the common cause of urinary tract infectio n.

There is evidence linking carbapenemase producers to numerous additional non-beta-lactam resistance factors that contribute to MDR[6].The antibacterial profile showed that MBL producers displayed resistance to nearly all the commonly used antibiotics and this raises serious concerns. Many other investigations have found that MBL-positive isolates generally resist even carbapenems, employed as a last resort for treating MDR Gram-negative bacteria infections[33].There was complete resistance of MBL-producing isolates to cefixime, ceftazidime, cefuroxime, cefotaxime, and Augmentin. There was a high resistance of isolates to fluoroquinolones. According to our findings, all MBL-producing isolates are multidrug-resistant (MDR). This is consistent with the work of Bora *et al.*, 2014 [19].It has been reported that the prevalence of drug-resistant bacteria varies not only between nations but also between healthcare facilities therein. This variation may be partially attributed to variations in local prescribing practices of antibiotics and the efficacy of infection control programs among various healthcare facilities. Hospitalization and prior antibiotic use are also common risk factors linked to infection by multidrug-resistant bacteria [28]. These findings suggest that patients may be at risk because there are few available treatment options [19].

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5. Conclusion

Our research revealed that a sizable percentage of *K. pneumoniae* and *E. coli* produced MBL. It was found that every isolate that produced MBL was multidrug resistant. Consequently, it is increasingly necessary for diagnostic microbiology laboratories to routinely identify isolates of *K. pneumoniae* and *E. coli* that produce MBL. The combined disc test provides a reasonable alternative for phenotypic detection of MBL production in the absence of molecular detection and can be done in laboratories regularly. Routine surveillance is also necessary to develop appropriate empirical antibiotic therapy and control the spread of MBL-producing bacteria in our environment. This necessitates introduction of novel antibiotics with fresh mechanisms of action to stop the spread of MDR bacteria. Setting up an efficient infection control program is also crucial.

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