DETECTION OF PLASMID-BORNE NDM-1 GENE IN CLINICAL ISOLATES OF ENTEROBACTERIACEAE AND THEIR CARBAPENEM ANTIBIOGRAM IN CROSS RIVER STATE, NIGERIA

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ABSTRACT
Background: Carbapenemase-producing Enterobacteriaceae (CPE) are considered by the World Health Organization to be a critical global health concern. New Delhi Metallo-beta-lactamase (NDM) enzymes are capable of conferring resistance to almost all β-lactam antimicrobial drugs which are often considered drugs of last resort for the treatment of serious infections.
Aim: This study investigated the presence of *bla*$_{\text{NDM-1}}$ gene on plasmids of multiple antibiotic resistant clinical isolates of Enterobacteriaceae in Cross River State, Nigeria.
Methodology: Seventy-nine Enterobacteriaceae which were obtained from urine and stool samples of patients in secondary and tertiary hospitals in Cross River State, Nigeria, were identified and tested for their susceptibility to three carbapenem antibiotics. Their ability to produce carbapenemase was determined by the Modified Hodges Test (MHT), re-modified Hodges Test (rMHT) and PCR.
Results: Two *Klebsiella pneumoniae* isolates from two separate urine samples obtained from two patients who had previously visited India, harboured the *bla*$_{\text{NDM-1}}$ gene; both were resistant to the three carbapenems tested.
Conclusion: The detection of *bla*$_{\text{NDM-1}}$ gene in Enterobacteriaceae confirms the circulation of the gene in Calabar. It further underscores the origin of the gene and its rapid spread. This has grave public health implications for Nigeria as India remains a major medical tourism destination for Nigerians.
Keywords: plasmid, NDM-1 gene, Enterobacteriaceae, carbapenem, Cross River State, carbapenem

INTRODUCTION
Enterobacteriaceae that produce carbapenemases which hydrolyze carbapenem β-lactam antibiotics are increasingly being reported (Queenan and Bush, 2007). Carbapenemases produced by a wide range of bacteria can hydrolize antibiotics containing beta-lactam rings, including carbapenems, even more than extended-spectrum beta-lactamases (ESBLs) (Kyung *et al.*, 2022). Of all beta-lactamases which are categorized into Ambler classes A through D, three classes, namely, A, B and D, contain carbapenemases (Kopotsa *et al.*, 2019; Kyung *et al*, 2022). Among class B carbapenemases, New Delhi metallo-beta-lactamase (NDM) is known to be more effective than other groups, but can be inhibited by metal chelators like EDTA and mercaptopropionic acid (Somboro *et al.*, 2018; Kyung *et al*., 2022).
Acquisition of NDM-1 has been associated with medical tourism in India and it has been reported in several countries of the world (Walsh and Toleman, 2011).

The origin of 'New Delhi Metallo-β-lactamase-1' (NDM-1) enzyme was traced to the Indian subcontinent in 2009 (Yong *et al.*, 2009), borne by a Swedish patient who was hospitalized in India; the aetiology of his urinary tract infection was *Klebsiella pneumoniae* (Somboro *et al.*, 2018; Kyung *et al*., 2022). Bacteria producing this enzyme have the ability to resist antibiotic treatment, including those used as last line drugs (carbapenems) for the treatment of bacterial infections (Toleman *et al.*, 2012). NDM-1-carrying bacteria are also referred to as 'the new superbug' [Centers for Disease Control and Prevention (CDC), 2010] by many researchers because the enzyme confers high resistance determinants on bacteria. Several members of the family Enterobacteriaceae such as *Escherichia coli, Klebsiella pneumoniae*, *Enterobacter aerogenes* and *Proteus* species have been associated with the production of NDM-1 (CDC, 2010), although, the enzyme is produced by a wide range of bacteria, including *Pseudomonas* species and *Acinetobacter baumannii* (Kumarasamy *et al.*, 2010; Nordmann *et al.*, 2011). The $bla_{\text{NDM-1}}$ is plasmid mediated and can disseminate at a fast rate between bacterial species (Carattoli *et al.*, 2012; Ho *et al.*, 2011; Sekizuka *et al.*, 2011).

NDM has spread globally as a result of its location on mobile genetic elements such as plasmids, transposons and integrons (Kyung *et al.*, 2022). The $bla_{\text{NDM-1}}$ gene was first detected in a 180kb plasmid (Yong *et al.*, 2009; Zenati *et al.*, 2019), but later on discovered on other plasmids which range from 50-500 kb of a wide range of Gram-negative species (Kumarasamy *et al.*, 2010; Naeem *et al.*, 2021). NDM-encoding genes are highly transmissible, often located on plasmids harbouring several antibiotic resistance genes. Outbreaks of NDM-producing bacteria, either clonal or through the dissemination of successful plasmids, are increasingly been reported (Wailan *et al.*, 2016; Otter *et al.*, 2017; Politi *et al.*, 2019). Treatment options for infections caused by NDM-producing bacteria are very limited, particularly because they often harbor other resistance genes. For example, there are notable associations between bla_{NDM} genes and plasmid-borne ESBLs and pAmpC encoding genes (especially $bla_{\text{CTX-M}}$ and bla_{CMY}) that result in resistance to aztreonam (Wu *et al.*, 2019; Findlay *et al.*, 2021)

Production of carbapenemases by bacteria can be tested using several phenotypic methods including Modified Hodges Test (MHT), re-Modified Hodges Test (rMHT), Combined Disk Test (CDT) and Double Disk Synergy Test (DDST), as recommended by Clinical Laboratory Standards Institute (CLSI) (CLSI, 2010). However, all these methods only screen for a phenotype and will not distinguish between the various types of carbapenemases. Molecular testing is recommended to characterize metallo-β-lactamases and to detect NDM-1 gene (Solanki *et al.*, 2014). Till date, very few studies have molecularly characterized carbapenemase enzymes to detect the NDM-1 gene. The present study investigated the presence of $bla_{\text{NDM-1}}$ on plasmids of multiple antibiotic resistant clinical isolates of Enterobacteriaceae bacilli in Cross River State, Nigeria.

MATERIALS AND METHODS

Study Area

This study was carried out in the three Senatorial Districts of Cross River State, Nigeria.

Ethical considerations

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Medical Research Ethics Committee of the Cross River State Government of Nigeria (Reference Number: CRS/MH/CGS/E-H/018/Vol. II/116)and the Ethics Research Committee of the University of Calabar Teaching Hospital, Calabar, Cross River State (Reference Number: UCTH/HREC/33/326). Informed consent from study participants was obtained before any participant was recruited to the study; original copies of informed consent forms are securely stored in the lead author's office.

Isolation of Enterobacteriaceae

A total of 79 non-duplicate Enterobacteriaceae were isolated from 405 urine and 195 stool samples of in-patients and out-patients attending selected health facilities in Cross River State. All urine isolates (85/405) were obtained by inoculating urine samples onto Meropenem-Supplemented Cysteine-Lactose Electrolyte-Deficient (MS-CLED) agar, while those from stool (44/129) were obtained by initially inoculating stool samples into selenite-F broth and the growth sub-cultured onto meropenem - supplemented deoxycholate citrate agar (MS-DCA). Pure isolates were maintained on nutrient agar slants overlaid with liquid paraffin. All clinical isolates were identified using the GNB Microbact 24E (Oxoid, Basingstoke, UK).

Carbapenem antibiotic susceptibility testing

Three carbapenems (ertapenem, imipenem and doripenem) were used to carry out antibiotic susceptibility testing on the isolates using the Kirby-Bauer disc diffusion technique. Sterile swabs were used to inoculate isolates grown in tryptone soy broth which showed comparable turbidity with 0.5 McFarland Standard on Mueller Hinton agar plate. Antibiotic discs were applied and the plates incubated at 37^{O}C for 24 hours. All zone diameters were measured with a ruler and results were recorded as sensitive or resistant using interpretative criteria according to Clinical Laboratory Standard Institute (CLSI, 2010).

Phenotypic testing for carbapenemase production

All isolates were phenotypically tested for carbapenemase production using modified Hodges Test (MHT) (CLSI, 2010) and re-Modified Hodges Test (rMHT). A suspension of 0.5 McFarland standard dilution of *Escherichia coli* 25922 (indicator strain) was used to inoculate Mueller Hinton agar plates by spread plate method and a 10µg ertapenem disc placed at the center of the inoculated plate. The test isolates were heavily inoculated in a straight line from the edge of the ertapenem disc to the edge of the plate as described by CLSI (CLSI, 2010). For the rMHT, zinc solution was added to the ertapenem disc as described (Rai *et al.*, 2011). *Klebsiella pneumoniae* ATCC BAA-1705 and 1706 served as positive and negative controls respectively. A cloverleaf-type indentation at the intersection of the test isolates and the indicator organism within the zone of inhibition of the ertapenem antibiotic disc indicated a positive MHT and rMHT.

Extraction of plasmids and PCR detection of the *bla*$_{\text{NDM-1}}$ gene

Plasmid DNA of test isolates was extracted using the Plasmid Miniprep Kit (Aidlab, China) according to manufacturer's instructions. Target genes were amplified using NDM-1-F primers: GCATAAGTCGCAATCCCCG and NDM-1-R primers: CTTCCTATCTCGACATGCCG (Biolabs, New England, USA) designed to amplify a 237bp region of the plasmid. PCR was carried out on plasmid DNA using a thermal cycler (Bio Rad, Hercules, USA) after which electrophoresis of amplified DNA products was carried out in 2% agarose gel, with a 1kbp DNA ladder (Aidlab, China) and visualized using a transilluminator (UVP, Porklington, UK).

Statistical analysis

Results obtained from this study were expressed in percentages, charts and arithmetic mean.

RESULTS

The predominant isolates, in this study, were *K. pneumonia* (40%) and *E. coli* (31.6%) (Table 1) In this study, the least-susceptible isolates to all three carbapenems were *Providencia stuartii* with susceptibility ranging from 25-50%. *K. pneumonia* and *E. coli* were at least 70% susceptible to all three carbapenems. The only *Proteus mirabilis* isolate was not susceptible to Ertapenem, while all 4 *Serratia rubidaea* were susceptible to the three carbapenems (Figure 1).

Carbapenem Resistance Index (CRI) of 0.7 and above was seen in 16% of *K. pneumoniae*, 28% of *E. coli*, 50% of *Providencia stuartii* and 23% of *Proteus vulgaris* (Figure 2).

The carbapenemase enzyme was detected in 41 out of 79 isolates; 26 (32.9%) were positive by both methods, while 15 (19%) were positive by either MHT or rMHT. Thirty eight (48.1%) of the 79 isolates were negative by both methods (Table 2).

The 237bp NDM-1 gene was detected in an electrophoretic gel at position 237 of the DNA Ladder used (Plate 2). The two NDM-1 gene positive *K. pneumoniae* isolates were obtained from urine samples of two male participants.

Table 1: Distribution of carbapenemase-producing Enterobacteriaceae by sample type

Clinical Isolates	**Prevalence No. (%)**	**Urine No. (%)**	**Stool No. (%)**
Klebsiella pneumoniae	32 (40.5)	14 (34.1)	18 (47.4)
Escherichia coli	25 (31.6)	12 (29.3)	13 (34.2)
Proteus vulgaris	13 (16.5)	10 (24.4)	3 (7.9)
Proteus mirabilis	1 (1.3)	1 (2.4)	0 (0)
Providencia stuartii	4 (5.1)	3 (7.3)	1 (2.6)
Serratia rubidaea	4 (5.1)	1 (2.4)	3 (7.9)
Total	**79**	**41 (52)**	**38 (48)**

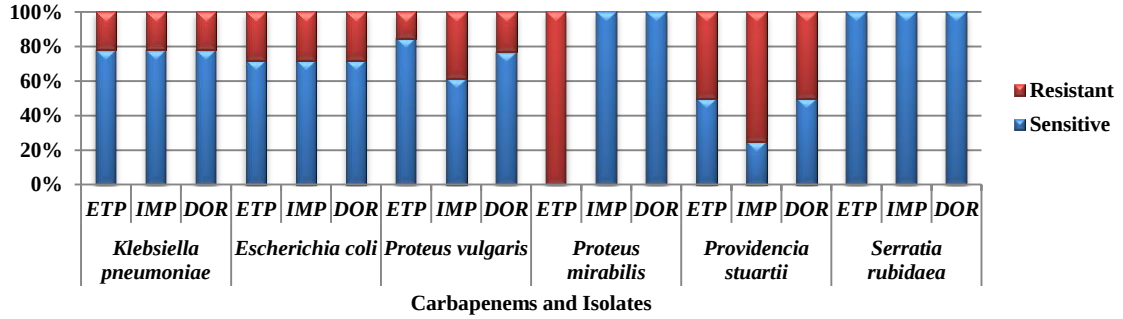

FIGURE 1: Carbapenem antibiotic resistance patterns of clinical isolates

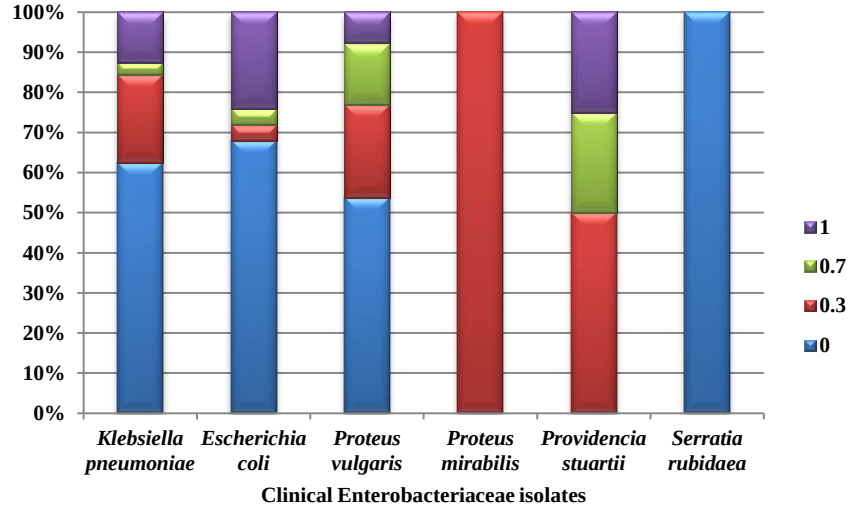

FIGURE 2: Carbapenem Resistance Index (CRI) of clinical isolates

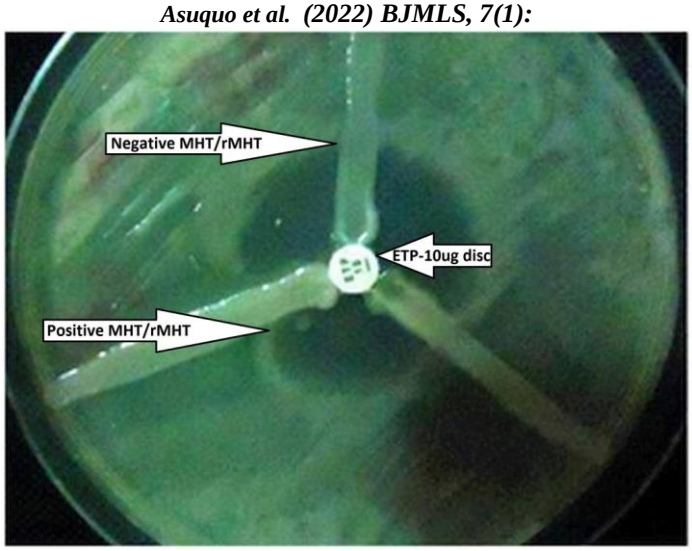

Plate 1: Positive Modified/re-Modified Hodges test result

Table 2: Carbapenemase production by clinical isolates using Modified and Re-Modified Hodge Tests

Clinical isolates	No (%) of isolates	MHT (+)/RMHT (+) No (%)	MHT (-)/RMHT (-) No (%)	MHT (+)/RMHT (-) No (%)	MHT (-)/RMHT (+) No (%)
Klebsiella pneumoniae	32 (40.5)	11 (42.3)	16 (42.1)	4 (44.4)	1 (16.7)
Escherichia coli	25 (31.6)	5 (19.2)	16 (42.1)	1 (11.1)	3 (50.0)
Proteus vulgaris	13 (16.5)	6 (23.1)	4 (10.5)	3 (33.3)	0 (0)
Proteus mirabilis	1 (1.3)	0 (0)	1 (2.6)	0 (0)	0 (0)
Providencia stuartii	4 (5.1)	3 (11.5)	1 (2.6)	0 (0)	0 (0)
Serratia rubidaea	4 (5.1)	1 (3.8)	0 (0)	1 (11.1)	2 (33.3)
Total	**79**	**26 (32.9)**	**38 (48.1)**	**9 (11.4)**	**6 (7.6)**

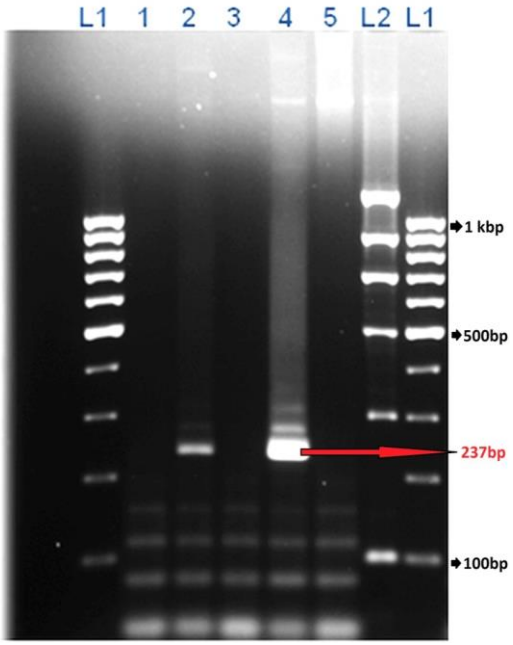

Plate 2: Gel electrophoregram of DNA amplicons (Wells 2 and 4 show NDM-1 positive samples; wells 1, 3 and 5 are NDM-1 negative samples; L1 and L2 are different sizes of DNA ladder).

DISCUSSION

Bacteria producing the NDM-1 enzyme have the ability to resist antibiotic treatment, including those drugs used as last line drugs (carbapenems) in the treatment of bacterial infections (Walsh *et al.*, 2005). The epidemiological link of NDM-1 with the Indian subcontinent is based on the fact that bacteria producing NDM-1 are often isolated from patients with a history of travel to India especially for medical tourism (Walsh and Toleman, 2011; Walsh and Toleman, 2012).

This study detected this highly resistant gene among clinical isolates in Calabar, Nigeria. Enterobacteriaceae isolated from subjects had variable susceptibilities to carbapenems and other antibiotics. Some of the isolates (12; 15.2%) [Figure 2], were completely resistant to carbapenems (with CRI of 1.0 each), which are considered last line drugs in the treatment of multidrug-resistant bacterial infections. This indicates high level of carbapenemase production, especially by *K. pneumoniae* (16/32), *Proteus vulgaris* (9/13), *Providencia stuartii* (3/4) and *Serratia rubidaea* (4/4) [Table 2]. The *K. pneumoniae* and *E. coli* isolates in this study exhibited high level resistance to carbapenems as previously reported (Kumarasamy *et al.*, 2010; Manchanda *et al.*, 2010).The low susceptibility of isolates to carbapenems, cephalosporins and quinolones by these isolates (data not shown) underscores the current antibiotic resistance pandemic, The two isolates were however sensitive to tigecyline (data not shown). Nordmann *et al.* (2009) have previously reported the presence of *K. pneumonia* carbapenemase (KPC) in *K. pneumoniae* isolates, while Cornaglia *et al.* (2011) and Miriagou *et al.* (2010) also demonstrated the presence of three metallo-β-lactamase genes (VIM, IMP and NDM) in *K. pneumoniae* and *E. coli*, indicating high level of resistance to last resort antibiotics in Europe. Carbapenems are considered the last-resort antibiotics for multidrug-resistant (MDR) Gram negative bacteria (Kyung *et al.*, 2022).

The performance of rMHT in this study was slightly higher than MHT corroborating the work of Rai *et al.* (2011) in which addition of zinc enhanced the activity of metallo-β-lactamase *in vitro*, thereby increasing the sensitivity of the detection method.

Of the 79 Enterobacteriaceae tested, the $bla_{\text{NDM-1}}$ gene was detected in two *K. pneumoniae* isolates (2.5%) by PCR only [Figure 2] as asserted by Solanki *et al.* (2014) that only PCR can detect specific enzymes; Ogbolu and Webber (2014) recorded a prevalence rate of 5.5% in some Nigerian tertiary hospitals. *Klebsiella* species were named only second to *Pseudomonas aeruginosa* as the highest carrier of the NDM-1 gene in Tamil Nadu, India, by Manohar *et al.* (2020). Isolation of NDM-1 positive *K. pneumoniae* from patients with travel history to India in this study agrees with previous studies (Walsh and Toleman, 2012; Johnson and Woodford, 2013) that linked NDM-1 to medical tourism in India. One of the individuals harbouring the gene in the study was never hospitalized in India, suggesting that NDM-1 is not only hospital-acquired, but also community-acquired (Walsh and Toleman, 2011). Another study in Nigeria detected the gene in patients with no history of travel to India or outside Nigeria (Uwaezuoke *et al.*, 2017). The ever-increasing global spread of carbapenem-producing Enterobacteriaceae (CPE), more especially *Escherichia coli* and *Klebsiella pneumoniae*, is currently considered a public health threat to humans and animals; these bacteria are listed among the priority one critical pathogens by the World Health Organization (WHO, 2017).

Other reports that detected the NDM-1 gene from Nigeria include those from the North-East region (Mohammed *et al.*, 2015) and from some tertiary hospitals in Nigeria (Ogbolu and Webber, 2014). More studies are emerging that report the presence of the gene in humans in the North East and

Central regions of Nigeria (Abdullahi *et al.*, 2017; Uwaezuoke *et al.*, 2017) and from poultry and rats living in poultry houses in the South-East and South-Western regions (Ogunleye *et al.*, 2016; Ogunleye and Jemilehin, 2016). To the best of our knowledge, this is the first report of NDM-1-producing Enterobacteriaceae in Cross River State, Nigeria.

CONCLUSION

The study revealed the presence of NDM-1 in Calabar, Nigeria, and further gives credence to its origin as the gene was detected in patients with travel history to India. Its presence in Nigeria is a serious public health problem since bacteria producing the enzyme are resistant to all antibiotics in common use.

RECOMMENDATIONS

Treatment failure in patients especially after the use of last line treatment drugs should trigger investigations into carriage of the NDM-1 genes by such patients. Further infection control measures including management under isolation may prevent further dissemination. Already, the lack of legislation for use of antibiotics has rendered useless most first line drugs for treatment of infectious diseases. Multiple antibiotic resistant isolates from patients should be investigated for NDM-1. Patients with NDM-1-positive strains should be managed under isolation.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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