Original Article

Emergence of New-Delhi Metallo-Betalactamase-1 and Oxacillinase-48 Positive *Escherichia coli* in South-Eastern Nigeria

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Abstract

Objective: The spread of carbapenemase-producing *Enterobacteriaceae* is a global challenge that leads to an increase in health-care cost, treatment failures, high morbidity, and mortality. This study was aimed at determining the prevalence of New-Delhi Metallo Beta-Lactamase (NDM), and oxacillinase-48 (OXA-48) genes in clinical isolates of *Escherichia coli* obtained from a tertiary hospital in Nnewi, South-eastern, Nigeria. **Materials and Methods:** *E. coli* isolated from several clinical specimens including blood, urine, and wound swabs from patients receiving care at the hospital, were screened for resistance to meropenem and ertapenem antibiotics (Oxoid, UK) by the Kirby–Bauer disk-diffusion method. All isolates, which showed reduced sensitivity to the tested antibiotics, were then subjected to phenotypic confirmation of carbapenemase production using the Modified Hodge test. The NDM and OXA-48 genes were then detected using the polymerase chain reaction techniques. **Results:** Of the 187 *E. coli* isolates, 41 (21.9%) screened positive as suspected carbapenemase producers, while the prevalence of carbapenemase-producing *E. coli* in this study was 21/187 (11.23%). The prevalence of NDM and OXA-48 genes in the entire sample population was 3/187 (1.6%) and 12/187 (6.4%), respectively. **Conclusion:** The results obtained showed that NDM and OXA-48 carbapenemase-mediated resistance occurred in the study location. Hence, a reinforcement of infection prevention and control practices in the hospital will be required to curb the propagation of these resistant organisms.

Keywords: Beta-lactamase, carbapenemase, New-Delhi, Nigeria, oxacillinase

Introduction

Escherichia coli are one of the most frequently occurring members of the family of bacteria called the *Enterobacteriaceae* and are major multi-drug resistant pathogens of both community and hospital-acquired infections.[1] They have been renowned for the production of extended-spectrum β-lactamases (ESBL) and have in recent times also shown remarkable production of the carbapenemases leading to limited antibiotic treatment options.[1,2] This is particularly problematic in countries like Nigeria, where the carbapenems are considered agents of last resort in treating infections due to multidrug-resistant bacteria.[3]

Although ESBL production among *E. coli* isolates in Nigeria as well as globally are remarkably high, the burden of carbapenemase-producing *E. coli* in this environment is mostly unknown.[1-4] Moreso, only a few studies have reported the detection of these enzymes in the country.[1-4] This limitation increases when the focus is on the South-eastern parts of the country.

The objectives of this study were to determine the prevalence of New-Delhi Metallo Beta-Lactamase ((NDM) and oxacillinase-48 (OXA-48) genes in *E. coli* isolates from

Access this article online

Quick Response Code:

Website: www.njmonline.org

DOI: 10.4103/NJM.NJM_165_20

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How to cite this article: Ajuba IM, Akujobi CN, Aghanya IN, Ushie SN, Okoro AE, Ofiaeli RO, *et al*. Emergence of New-Delhi metallo-betalactamase-1 and oxacillinase-48 positive *Escherichia coli* in South-Eastern Nigeria. Niger J Med 2020;29:701-6.

Submitted: 31-Aug-2020 **Revised:** 12-Sep-2020
Accepted: 19-Nov-2020 **Published:** 24-Dec-2020

clinical specimens in a tertiary hospital in the South-eastern region of Nigeria and to determine the antibiotic susceptibility pattern of these isolates.

Materials and Methods

Study population

One hundred and eighty-seven nonduplicate *E. coli* isolates were collected consecutively from clinical specimens of patients submitted to the Medical Microbiology laboratory of Nnamdi Azikiwe University Teaching Hospital (NAUTH), a tertiary health institution and major referral centre serving individuals from most parts of South-East, Nigeria. The study was carried out between March and October 2018, and it included all Gram-negative bacilli isolated during the study duration.

Ethical consideration

This work received approval from the research and ethics committee of NAUTH, Nnewi, with reference number NAUTH/CS/66/VOL. 10/190/2017/100.

Specimen collection and transport

All the isolates used in this study were obtained from clinical specimens that had been collected and immediately transported in their appropriate specimen containers to the NAUTH medical microbiology laboratory for processing within 1 h of collection. Blood was collected aseptically in blood culture bottles containing brain heart infusion (Oxoid, UK). Urine was collected in a sterile wide bore and screw-capped containers, while wound swabs were collected using sterile single-use cotton swabs.

Bacteria isolation

Blood cultures were incubated aerobically at 35°C–37°C for 7days and sub-cultured onto chocolate agar and Mac Conkey agar (Oxoid, UK) on days 2, 5 and 7, whichever yielded growth first. Other specimens were cultured directly on Chocolate agar and Mac Conkey agar (Oxoid, UK) and incubated aerobically at 35°C–37°C for 18–24 h. Then growth was confirmed by visible colonies on the surface of the culture media.

Bacterial identification

The isolated organisms were Gram-stained, and all Gram-negative bacilli were then subjected to standard conventional biochemical tests for *E. coli* identification. These tests include lactose fermentation on Mac Conkey agar, indole production, citrate utilization, Voges Proskauer), and the hanging drop test for motility.[5] All motile, indole producing, lactose fermenting isolates which were negative for citrate utilization and Voges Proskauer tests were suspected to be *E. coli.* The suspected *E. coli* isolates were then confirmed as *E. coli by the* production of a greenish metallic sheen when sub-cultured on Eosin Methylene Blue Agar and incubated for 18–24 h at 37°C.[5]

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing (AST) was performed according to the techniques developed by Kirby–Bauer and described in the Clinical and Laboratory Standard Institute (CLSI) standards for AST.[6] The organisms were tested against the following antibiotics: ampicillin (10 µg), amoxicillin-clavulanate (20/10 µg), cefuroxime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), meropenem (10 µg), ertapenem (10 µg), doripenem (10 µg), gentamicin (30 µg), ciprofloxacin (5ug), and tetracycline (30 µg) (Oxoid Ltd UK). Incubation was performed at 37°C for 18–24 h. After incubation, the inhibition zone was measured for each antibiotic produced by the *E. coli* isolates was measured in millimetres, and this was interpreted as sensitive, intermediate, or resistant based on the CLSI standard interpretive criteria.[6] *E. coli* ATCC 25922 was used to control the quality of the test.[6]

Screening for suspected carbapenemase production

The isolates were screened for carbapenem resistance and hence possible carbapenemase producers according to the 2017 CLSI guidelines.[6] In this method, 10 µg carbapenem antibiotic (ertapenem and meropenem) discs (Oxoid, UK) were placed on the surface of Mueller Hinton Agar (MHA) (Oxoid, UK) plates inoculated with each isolate of *E. coli* and then incubated at 37°C for 24 h after which zones of inhibition were read off.[6] Isolates showing inhibition zones ≤22 mm or ≤21 mm in diameter for meropenem or ertapenem respectively were considered as suspected carbapenemase producers.[5,6] They were then subjected to phenotypic confirmation using the modified hodge test (MHT).[5,6] *E. coli* ATCC 25922 was used to control the quality of the screening test.

Phenotypic confirmation of carbapenemase production (modified hodge test)

In this method, a suspension of *E. coli* ATCC 25922 equivalent to 0.5 McFarland turbidity standard was prepared. The *E. coli* suspension was then diluted 1:10 by adding 0.5 ml of the *E. coli* suspension to 4.5 ml of saline. A lawn of the 1:10 dilution of *E. coli* ATCC 25922 was evenly streaked onto MHA plates using sterile cotton swabs and then allowed to dry for 3–5 min. Following this, one meropenem antibiotic disc (Oxoid, UK), was placed on the centre surface of the MHA plate. The test organisms were streaked in a straight line from one edge of each meropenem disc to the edge of the plate. Following this, the plates were incubated at 37°C for 24 h. After incubation, the plates were examined for a clover-leaf type indentation at the intersection of the test organism and *E. coli. ATCC 25922* within the zone of inhibition of the meropenem disc.[6] *Klebsiella pneumoniae* ATCC1705 and *K. pneumoniae* ATCC1706 were used as positive and negative controls.[6]

DNA extraction

Bacteria DNA from the *E. coli* isolates was extracted using a boiling method for DNA extraction.[7] Five hundred microlitre of an overnight broth culture of the bacterial isolate in Luria Bertani medium was added into an Eppendorf tube. To this, 1000 µl (i.e., 1 ml) of normal saline was added and correctly mixed using a vortex. Next was to spin the suspension at

14,000 rpm for 5 min. After spinning, the supernatant was discarded, while the sediments were resuspended in 1000 µl of normal saline and mix properly using a vortex mixer. This was repeated three times. After this, the supernatant was discarded again. The sediments were then resuspended in 500 µl of DNA elution buffer and vortexed. Then the mixture was heated on a heating block at 95°C for 20 min. The heated bacterial suspension was fast cooled on ice and spun for 3 min at 14,000 rpm. The supernatant containing the DNA was transferred to a 1.5 ml microcentrifuge tube and stored at-20°C for further downstream reactions.[7]

Molecular detection of New-Delhi metallo beta-lactamase and oxacillinase-48

Conventional polymerase chain reaction (PCR) was used to amplify genes encoding NDM and OXA-48 group carbapenemases employing the use of previously published protocols with slight modifications.[8,9] Specific Primer sequences for the detection of bla_{NDM} and bla_{OXA-48} (Inqaba, SA) were used [Table 1].

The PCR conditions were as follows: OXA-48; Initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 40 s, then final extension at 72°C for 5 min.[8] NDM; Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, extension, 72°C for 60 s, then final extension at 72°C for 5 min.[9] The PCR products were pipetted onto wells on a 1.5% agarose gel in an electrophoretic tank and resolved at 130V for 25 min and visualized on a blue light trans-illuminator.[8,9]

Results

Out of the 187 *E. coli* isolates, 41 (21.9%) were carbapenem-resistant, while 21 (11.3%) of the isolates were confirmed as carbapenemase producers. Urine was the most frequent specimen harbouring the *E. coli* isolates 115 (61.5%), followed by wounds 24 (12.8%). All phenotypically positive carbapenemase producers showed a clover-leaf type indentation on MHT [Figure 1]. NDM was detected in 3/187 (1.6%) of the *E. coli* isolates [Figure 2], while the OXA-48 gene was detected in 12/187 (6.4%) of the isolates [Figure 3].

The *E. coli* isolates were most susceptible to meropenem 170/187 (90.9%), and this was closely followed by ertapenem 163/187 (87.2%), and doripenem 152/187 (81.3%). The highest level of resistance was recorded against ampicillin 185/187 (99%), followed by tetracycline 180/187 (96.3%) [Table 2].

The prevalence of carbapenem-resistant *E. coli* in the total sample population was 41/187 (21.9%), while that of carbapenemase-producing *E. coli* was 21/187 (11.23%) [Table 3].

Discussion

The global emergence and dissemination of carbapenemases among Gram-negative bacteria are considered a significant public health challenge. Characterization of carbapenemase-producing *Enterobacteriaceae* has been done globally as well as in Nigeria. Still, information on the emergence and spread of these group of organisms in the South-eastern parts of Nigeria are limited.[1-4]

The antibiotic susceptibility patterns of the *E. coli* isolates revealed that the organisms had the highest resistance rates to Ampicillin 185/187 (99%), and Tetracycline 180/187 (96.3%), but reduced resistance to the carbapenem class of antibiotics (Meropenem, Doripenem, and Ertapenem), with meropenem being the least resistant of them 9/187 (4.8%). Similar findings were observed in Sudan and Mexico with the highest resistance rates observed in tetracycline (97.7%)[10] and ampicillin (70.8%)[11] respectively.

The carbapenem resistance prevalence ranging from 4.8% to 10.2% observed in this study was quite high for a drug

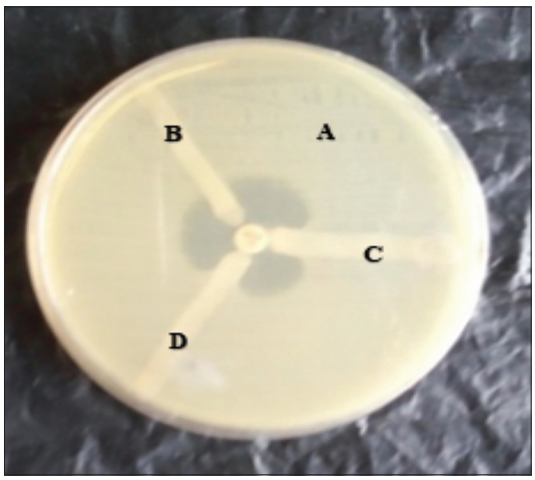

Figure 1: Modified hodges test. A is a lawn of *Escherichia coli* ATCC 25922; B is modified hodges test negative control (*Klebsiella pneumoniae* ATCC 1706); C is an *Escherichia* coli test isolate positive on modified hodges test, thus showing clover leaf appearance at its intersection with the inhibition zone of the lawn of *Escherichia coli* ATCC 25922; D is modified hodges test positive control (*Klebsiella pneumoniae* ATCC 1705)

Table 1: Primer sequences used in the study

Primers	Sequence (5′→3′)[a]	Location	Product size
NDM (F)	GCACACTTCCTATCTCGACATGC	bla_{NDM}	*209 bp*
NDM (R)	CCATACCGCCCATCTTGTCC	bla_{NDM}, reverse primer	
OXA-48 (F)	TTGGTGGCATCGATTATCGG	bla_{OXA-48}	*743 bp*
OXA-48 (R)	GAGCACTTCTTTTGTGATGGC	bla_{OXA-48}, reverse primer	

Table 2: Susceptibility patterns of the *Escherichia coli* isolates

Antibiotic	Number of isolates	Susceptible, *n* (%)	Intermediate, *n* (%)	Resistant, *n* (%)
Meropenem (MEM)	187	170 (90.9)	8 (4.3)	9 (4.8)
Doripenem (DOR)	187	152 (81.3)	16 (8.6)	19 (10.2)
Ertapenem (ETP)	187	163 (87.2)	11 (5.9)	13 (6.9)
Cefuroxime (CXM)	187	10 (5.4)	82 (43.9)	95 (51)
Cefotaxime (CTX)	187	81 (43.3)	20 (10.7)	86 (46)
Ceftazidime (CAZ)	187	93 (49.7)	15 (8.0)	79 (42.3)
Cefepime (FEP)	187	91 (48.7)	15 (8.0)	81 (43.3)
Ampicillin (AMP)	187	1 (0.5)	1 (0.5)	185 (99)
Amoxicillin-clavulanate (AMC)	187	75 (40.1)	52 (27.8)	60 (32.1)
Tetracycline (TE)	187	6 (3.2)	1 (0.5)	180 (96.3)
Ciprofloxacin (CIP)	187	24 (12.8)	43 (23)	120 (64.2)
Gentamycin (GM)	187	68 (36.4)	22 (11.76)	97 (51.9)

Table 3: Characteristics of the carbapenemase-producing *Escherichia coli* isolates

Isolate code	Source of isolation	Hospital unit	MHT	NDM detection	OXA-48 detection	Gel Electrophoresis lane
C30	Blood	FMW	Positive	Negative	Positive	1
C70	Urine	MMW	Positive	Negative	Positive	2
C158	Urine	GOPD	Positive	Negative	Positive	3
C64	Urine	MMW	Positive	Negative	Positive	4
C107	Blood	MMW	Positive	Negative	Negative	5
C80	Wound	A\E	Positive	Negative	Negative	6
C85	Urine	FMW	Positive	Negative	Positive	7
C105	Wound	SOP	Positive	Negative	Negative	8
C42	Wound	MMW	Positive	Positive	Positive	9
C69	Urine	GOPD	Positive	Negative	Negative	10
C29	Urine	GOPD	Positive	Negative	Positive	11
C67	Wound	MOP	Positive	Negative	Negative	12
C168	Wound	GOPD	Positive	Negative	Negative	13
C87	Urine	A\E	Positive	Negative	Negative	14
C63	Urine	FMW	Positive	Negative	Positive	15
C39	Urine	CHOP	Positive	Negative	Positive	16
C65	Urine	GOPD	Positive	Negative	Positive	17
C89	Wound	GOPD	Positive	Negative	Negative	18
C138	Urine	GOPD	Positive	Positive	Positive	19
C135	Urine	FMW	Positive	Positive	Negative	20
C91	Urine	FMW	Positive	Negative	Positive	21
Total			21	3 (1.6%)	12 (6.4%)	

GOPD: General out-patient department, MMW: Male medical ward, A\E: Accident and emergency, FMW: Female medical ward, SOP: Surgical out-patient, CHOP: Children out patient, MOP: Medical out patient

class which has been reserved for use only as a last resort. This was similar to other studies done in Kenya and Kuwait, wherein low resistance rates (5%–8%) to the carbapenems were observed.[12,13] A higher prevalence of 50/229 (21.8%) was observed in isolates from a surgical Intensive Care Unit of a hospital in Egypt.[14] This result, however, was in sharp contrast to an Egyptian study that revealed 100% resistance to meropenem.[15] The reduced rates of resistance to the carbapenems in this study were probably because of the high cost of purchase of carbapenems in Nigeria, which makes it less available for use, thus resulting in decreased selection pressure for development of resistance. Also, the varying antibiotic resistance rates observed may be attributed to varying levels of regulations regarding antibiotic usage in different countries.

The 11.23% prevalence of carbapenemase production in the *E. coli* isolates was slightly lower than the prevalence of carbapenemase-producing *E. coli* obtained in other Nigerian studies-Lagos (12.4%),[2] Kano (14%),[4] while also using various phenotypic detection techniques. A much lower prevalence of 3.5% was observed in Italy.[16] Contrasting rates were also observed in other studies with higher prevalences reported in Pakistan (69%)[17] and Tanzania (35%).[18] The varying results obtained from these studies may be attributed to differences in the phenotypic detection methods used, as well as different levels of

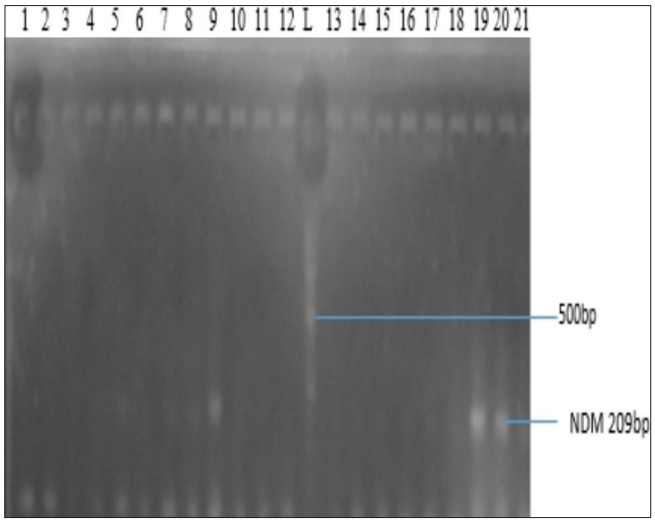

Figure 2: Agarose gel electrophoresis showing the amplified New-Delhi metallo beta-lactamase gene bands. Lanes 9, 19 and 20 represent the New-Delhi Metallo Beta-Lactamase bands at 209bp while lane L represents the 100bp molecular ladder

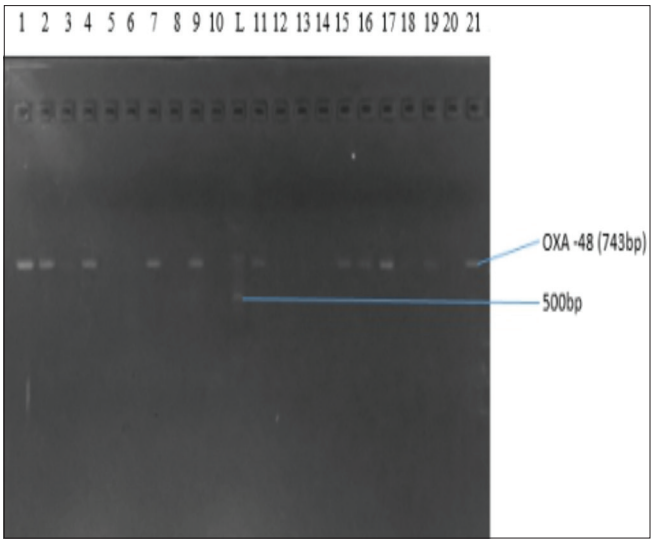

Figure 3: Agarose gel electrophoresis of the amplified oxacillinase-48 gene. Lanes 1–4, 7, 9, 11, 15–17, 19, and 21 represents the oxacillinase-48 gene bands at 743bp, while lane L represents the 100bp DNA ladder

application of antimicrobial stewardship programs in other settings.

The prevalence of NDM gene expression in *E. coli* clinical isolates in this study was similar to the prevalence of NDM observed in Kuwait (2.7%),[19] Uganda (2.6%)[20] and Pakistan (1.2%).[13] A higher prevalence of 12.2% was reported in Iran among clinical bacteria isolates,[21] but a slightly lower rate (0.8%) was noted among *E. coli* clinical isolates in Kano.[22] The observed prevalence of OXA-48 gene in *E. coli* clinical isolates was 6.4%. This prevalence was only slightly lower in a Lagos study (3.4%),[2] as well as in Tanzania (4.8%),[18] where the prevalence of OXA-48 gene was obtained from carbapenemase-producing *Enterobacteriaceae* isolates. A much higher rate was observed in Saudi Arabia.[23] Variations in the prevalence of these carbapenemase genes in the various regions may be due to varying sample sizes and specimens, cross border migrations and geographical distribution of NDM and OXA type genes, which contributes significantly to the types and percentage of carbapenemase enzymes being produced in each locality.

Conclusion

The findings from this study prove that NDM and OXA-48 carbapenemase mediated resistance occurred in the study location, and this may further limit treatment options in the region since these carbapenems are currently being used as agents of last resort in the south-eastern parts of Nigeria. Hence, a reinforcement of infection prevention and control practices in the hospital will be required to curb the propagation of these resistant organisms.

Acknowledgement

Special appreciations go to Prof. Tatfeng Mirabeau (Coordinator, Molecular Biology Laboratory, Niger Delta University, Bayelsa), for his assistance and directions in the molecular analysis aspects of the work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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