Phenotypic and genotypic characterization of clinical carbapenem-resistant Enterobacteriaceae isolates from Sokoto, northwest Nigeria

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Phenotypic and genotypic characterization of clinical carbapenems resistant *Enterobacteriaceae* isolates from Sokoto, northwest Nigeria

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Running title: Phenotypic and Genotypic characterization of carbapenemase-producing *Enterobacteriaceae*.

Abstract:

Emergence and spread of carbapenemase producing *Enterobacteriaceae* (CPE) is one of the major problems currently threatening the global public health. In Nigeria, interest on CPE begins recently. In Sokoto, northwest Nigeria, there is no data on the prevalence and mechanism underlying carbapenem resistance. In this study, we aimed to investigate the presence of clinical carbapenems resistant *Enterobacteriaceae* isolates in two leading hospitals in Sokoto, Northwest-Nigeria. A total of 292 non-duplicate *Enterobacteriaceae* isolated from clinical specimens processed in the diagnostic laboratories of the two hospitals between January and June 2019 were collected. Of these, 129 (44.2 %) and 19 (6.5%) were resistant to third generation cephalosporin (3CG) and carbapenems, respectively. The result of RT-PCR revealed that 10 (7.8 %), 19 (14.7 %) and 46 (35.7 %) of the 3GC resistant isolates harboured bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes, respectively. The modified Carba NP test result showed that only 7 (36.8 %) of the 19 carbapenems-resistant isolates were carbapenemase producing; among them bla_{NDM-5} and $bla_{OXA-181}$ genes were identified in five and two isolates, respectively. However, none of the investigated carbapenemase genes including bla_{VIM}, bla_{KPC}, and bla_{IMP} was detected in the remain carbapenems-resistant isolates suggesting therefore a non-enzymatic mechanism. This study reports for the first time, the emergence of CPE in Sokoto state and first detection of NDM-producing *Citrobacter freundii* in Nigeria. The observed CPE in this study is concerning in a country where alternative antibiotics is rarely available.

Keywords: Carbapenem resistance, Carbapenemase genes, bla_{NDM-5}, $bla_{OXA-181}$, Nigeria

Introduction

Carbapenems are highly effective β-lactam antibiotics introduced into clinical practices following the emergence of plasmids encoding for extended-spectrum β-lactamases [1]. The carbapenems are among the last resort armamentarium against infections due to multidrug resistant (MDR) Gram-negative bacteria [2]. Until the early 1990s, resistance to carbapenems has been mostly due to chromosomal β-lactamases [3]. The transferable plasmid encoded carbapenemase (*bla*$_{\text{IMP-1}}$) emerged in Japan in 1990 [4]. Following this, there was rapid dissemination of carbapenemase producing bacteria and it continues to be increasingly reported worldwide [5]. Other recognised carbapenem resistance mechanisms include decreased outer membrane permeability and up-regulation of efflux system with/without production of extended-spectrum β-lactamases (ESBLs) [6]. However, acquired carbapenemase production is the most clinically important carbapenem resistant mechanism [5].

The emergence of carbapenemases-producing *Enterobacteriaceae* (CPE) is concerning, as it further limits option for treatment of infections due to MDR Gram negative bacteria [7]. It has been described as an imminent threat to global public health with attendant morbidity and mortality [8–10]. Annually, carbapenem resistant Gram-negative bacteria (CR-GNB) cause approximately 9’300 infections in the United States, half of which usually result in death [11]. Moreover, longer duration of hospital stays and consequently increased healthcare cost is associated with CR-GNB infections [12,13]. In the United States, *bla*$_{\text{KPC}}$ is the most common carbapenemase genes, though recent emergence of *bla*$_{\text{NDM}}$ producing *Enterobacteriaceae* has been reported particularly among patients returning from *bla*$_{\text{NDM}}$ endemic region [14]. Moreover, despite that wide geographical variation exists in the types of carbapenemase genes in Europe, generally *bla*$_{\text{KPC}}$ and *bla*$_{\text{OXA-48}}$ are the commonest carbapenemase enzymes [15,16]. In Nigeria, there is dearth of data on the genetic diversity and spread of CPE. Interest in research on carbapenem resistance begins recently. Majority of studies on CPE have been limited to

phenotypic testing [17,18]. However, a few studies have used genotypic methods to establish the occurrence of carbapenemase genes among clinical and non-clinical bacterial isolates in Nigeria [19,20]. In Sokoto however, there was no data on the prevalence of CPE. In view of this, we therefore aimed to investigate the prevalence of clinical *Enterobacteriaceae* isolates bearing carbapenemase genes in two-leading hospitals in Sokoto, Northwest-Nigeria and also to characterise their molecular resistance mechanisms.

Materials and methods

Study area, design and period

This was a prospective, descriptive and epidemiological study conducted between January and July 2019 among patients attending the two main tertiary healthcare facilities in the capital of Sokoto state, Sokoto, Northwest Nigeria. The hospitals are the largest hospitals located within the state. The hospitals, Usman Danfodiyo University Teaching Hospital (UDUTH) and Sokoto State Specialist Hospital (SHS), are respectively 850 and 300-beds hospitals and rendering essential, specialized and referral medical and surgical services to residents of Sokoto state and patients from adjoining states of Zamfara, Kebbi and Niger within Nigeria and also to referral cases from the neighbouring Niger Republic.

Bacterial collection and identification

Non-duplicate clinical *Enterobacteriaceae* isolates were collected from the pool of biological specimens submitted and processed by the diagnostic microbiological laboratories of the two hospitals. The isolates were preliminarily identified by a combination of morphology and conventional biochemical tests for *Enterobacteriaceae* using the standard microbiological techniques. The isolates were then preserved on nutrient agar slants and subsequently shipped to Microbial Evolution and Phylogeny Infection, Institute Hospital University, Marseille in France for further characterisation. The identity of the isolates was confirmed using matrix-assisted laser

desorption ionisation time-of-flight mass spectrometry (Bruker Daltonics, Bremen, Germany) according to the protocol previously described [21].

Antibiotic Susceptibility Test

Antibiotic susceptibility test (AST) was carried out using the modified Kirby-Bauer disc diffusion method as outlined in the current European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, Version 9.0, 2019 [22]. The test was performed on Mueller–Hinton agar plates using the following commercially available discs (Oxoid limited, UK): carbapenems (imipenem and ertapenem); cephalosporins (ceftriaxone, cefalotine, and cefepime); fluoroquinolones (ciprofloxacin); aminoglycosides (amikacin and gentamicin); tetracycline (doxycycline). Others are trimethoprim-sulfamethoxazole, amoxicillin-clavulanate, fosfomycin and nitrofurantoin. The AST results were interpreted according to EUCAST breakpoints. The imipenem and ertapenem minimum inhibitory concentrations (MIC) for isolates with reduced susceptibility to either imipenem or/and ertapenem by disc diffusion test was thereafter determined using the gradient diffusion tests (Etest®, bioMérieux, Marcy L'Etoile, France). Any of the *Enterobacteriaceae* isolates that exhibits resistance to either of the carbapenem antibiotics (imipenem or ertapenem) would be regarded as carbapenem resistant as defined by the Centres for Disease Control and Prevention (CDC) [23]. The definition however requires reduced susceptibility to carbapenems other than imipenem for the trio of *Proteus* spp., *Morganella morganii* and *Providencia* spp. [23].

Phenotypic detection of ESBL and carbapenemase enzymes production

The phenotypic detection of ESBL enzymes was performed using the double-disk synergy test by placing a β-lactamase inhibitor (amoxicillin–clavulanic and piperacillin-tazobactam) discs between two third generation cephalosporins (3GCs) at a distance of 20 mm centre-to-centre.[24] Formation of a characteristic keyhole effect or champagne-cork shaped zone of inhibition between the discs was considered as a phenotypic indication of ESBL production. The carbapenem

resistant isolates were screened for phenotypic carbapenemase production using the modified Carba NP test as previously described [25].

Molecular characterization of ESBL and carbapenemase genes

The 3GC and carbapenem resistant isolates were screened for the presence of genes encoding ESBLs (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$) and carbapenemases (*bla*$_{OXA-48}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$ and *bla*$_{KPC}$), using the quantitative real-time PCR (qPCR) as previously described [26]. The qPCR positive isolates were confirmed by conventional PCR. The genetic variant of the carbapenemase genes was determined by sequencing of the positive PCR amplicons in both directions using the same set of standard PCR primers with BigDye Terminator on an automated ABI 3500XL genetic analyser (Applied Biosystems, Foster City, CA) according to the previously protocol described [27]. The generated raw read sequences were assembled using codon code aligner, v 9.0.1 (Codon Code Corp., Massachusetts, USA). The assembled sequences were identified by Blast analysis against the ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) database [28].

Genotypic investigation of colistin resistance mechanism

Moreover, one of the leading objectives of this work was to investigate colistin resistance in these clinical isolates. Irrespective of the results of phenotypic colistin resistance test, qPCR was used to screen the whole collection of the 292 *Enterobacteriaceae* isolates for mobilized colistin resistance (*mcr-1, mcr-2, mcr-3, mcr-4, mcr-5 and mcr-8*) genes, as previously described [29].

Result

The distribution of the clinical isolates recovered during clinical diagnostic testing in the two hospitals shows that majority of the isolates were recovered from urine 143 (49.0 %) and stool

76 (26.0 %) specimens. Others were obtained from sputum 21 (7.2 %), wound swab 29 (9.9 %) and ear swab 6 (2.1 %).

Out of the 292 *Enterobacteriaceae* isolated from the two hospitals during the study period, 129 (44.2 %) and 19 (6.5%) were resistant to third generation cephalosporin (3CG) and carbapenems, respectively. The distribution of the isolates is presented in Table 1. The third generation cephalosporins (3GC) resistant comprises *C. freundii* (*n*=10), *E. cloacae* (*n*=18), *E. coli* (*n*=51), *K. pneumoniae* (*n*=28), *M. morganii* (*n*=4), *Proteus mirabilis* (*n*=14), *Providencia rettgeri* (*n*=1) and *Providencia stuartii* (*n*=3). The phenotypic ESBL screening result showed that 36 of the 3GC resistant bacteria are ESBL positive. The result of RT-PCR revealed that 10 (7.8 %), 19 (14.7 %) and 46 (35.7 %) of the 3GC resistant isolates harboured bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$ genes, respectively (Table 1). While 9 and 8 of the isolates co-harboured bla_{TEM} and $bla_{CTX\text{-}M}$ and bla_{SHV} and $bla_{CTX\text{-}M}$, respectively, one of the isolates co-expressed the three ESBL genes.

The carbapenem resistant *Enterobacteriaceae* (CRE) on the other hand includes *Citrobacter freundii* (*n*=3), *Enterobacter cloacae* (*n*=6), *Escherichia coli* (*n*=8) and *Klebsiella pneumoniae* (*n*=2). The result of modified CarbaNP test however showed positive result for only 7 (36.8 %) of 19 CRE suggestive of carbapenemase expression in these isolates. As presented in Table 2, PCR and sequencing results revealed that the seven carbapenemase-producing strains harboured $bla_{NDM\text{-}5}$ (*n*=5) and $bla_{OXA\text{-}181}$ (*n*=2). However, 12 of the CRE isolates did not harbour any of the investigated carbapenemase genes suggestive of a non-enzymatic resistance mechanism in these isolates. Thus, we planned in perspective to investigate the carbapenems resistance mechanism in these isolates by whole genome sequencing (WGS) approach.

The result of antibiotic susceptibility testing revealed that, with the exception of the naturally colistin resistant strains of *Proteus*, *Morganella*, *Serratia* and *Providencia* isolates, none of the isolates was resistant to colistin. Molecular detection by PCR targeting six *mcr* gene variants was negative for all the 292 collected isolates in this study.

Discussion

Globally, carbapenem resistance is increasingly reported [30]. The prevalence of which varies from one geographical region to the other. In the present study, the overall prevalence 6.5 % carbapenem resistance was reported. Previous reports across the country have established varying rates. For example, 28 % carbapenem resistance rate was reported in the preceding year in one of the hospitals among carbapenem naïve patients [18]. Also, in a neighbouring west Africa country, Ghana, 66% CR-GNB rate has been reported [31]. Despite the poor drug regulatory system in Nigeria coupled with the lack of an established antibiotic stewardship, carbapenem use in both hospital and community is generally low, reserved as a last resort agent against life threatening infections by multidrug resistant bacteria [32]. The observed resistance to the carbapenems in this study is troubling in a country where alternative antibiotics is rarely available [32]. Furthermore, the emergence and spread of carbapenem resistance bacteria is more worrisome because of lack of laboratory capacity for its detection [32]. The emergence and dissemination of CRE in the present study may be attributed to a number of factors. The carbapenem resistance may have emerged independently as a result of selection pressure of overuse of β-lactam antibiotics [33]. Beta-lactam antibiotics are the most widely used, often inappropriately, antibiotics in both community and hospital settings in Nigeria [34]. In addition, the CRE could have been imported by patients returning from international travelling to regions like India and Europe where carbapenem resistance is endemic [35,36]. Reports of importation of antibiotic resistant bacteria across geographical border has been documented [37].

In this study, the presence of $bla_{\text{NDM-5}}$ and $bla_{\text{OXA-181}}$ accounted for carbapenem resistance in about a third of the CRE isolates. This corresponds to the findings of previous studies in different regions of Nigeria where $bla_{\text{NDM-5,}}$ $bla_{\text{OXA-48}}$ *and* $bla_{\text{OXA-181}}$ have been reported as the commonest carbapenemase genes [38,39]. Our findings however contrasted the report of a study in Maiduguri, northeast-Nigeria, where bla_{KPC} has been reported as the predominant

carbapenemase gene [40]. Other mechanisms such as ESBLs and/or plasmid AmpC enzyme production with reduced outer membrane permeability may be responsible for the carbapenem resistance in the remaining isolates [41].

While we did not find any isolates bearing *mcr* genes in this study, reports of clinical isolates from Nigeria harbouring plasmid encoding colistin resistance genes have begun to surface in the literatures.[42,43] Our finding however concurs with other studies where clinical bacterial isolates have been documented to be highly susceptible to colistin.[44,45]

This study is the first to comprehensively investigate molecular basis of resistance to carbapenems in northwest-Nigeria. The diversity of the strains investigated adds to the robustness of the study as previous studies concentrated on *K. pneumoniae* and *E. coli*. This permits the first detection of *Enterobacter cloacae* expressing NDM-carbapenemase in northwest-Nigeria and first description of carbapenemase producing *Citrobacter freundii* in Nigeria.

Conclusion:

Here, for the first time, we describe the emergence of CPE in Sokoto state and the first detection of NDM-producing *Citrobacter freundii* in Nigeria. The finding of this study is concerning in a country where alternative antibiotic is rarely available.

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Transparency declaration:

The authors declare that they have no competing interests.

Authors' contributions:

This study was designed by YKEI, BOO, JMR, and SMD. The experiment was conducted by AO and LZN. AO drafted the first manuscript which was revised by all authors. All authors have read and approved the final manuscript.

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Acinetobacter baumannii in Ladoke Akintola University Teaching Hospital, Osogbo, Nigeria . Eur J Microbiol Immunol 2016;6:238–43. https://doi.org/10.1556/1886.2015.00018.

322 Table 1: Distribution of ESBLs producing and carbapenems resistant *Enterobacteriaceae* isolates

	Number of 3GC resistant isolates	SHV	TEM	CTX-M	Number of CRE
Citrobacter freundii	10	0	0	2	3
Enterobacter cloacae	18	0	3	5	6
Escherichia coli	51	2	9	26	8
Klebsiella pneumoniae	28	5	4	10	2
Morganella morganii	4	0	0	0	
Proteus mirabilis	14	2	1	3	
Providencia rettgeri	1	0	1	0	
Providencia stuartii	3	1	1	0	
Total	129	10	19	46	19

323 3GC, Third generation cephalosporins; CRE, carbapenem resistant Enterobacteriaceae.

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Table 2: Resistance phenotypes and carbapenemase genes identified in Carbapenems resistant isolates

Strain names	Hospital	Isolation sources	*Bla*$_{GENES}$	ERT MIC (µg/ml)	IPM MIC (µg/ml)	Antibiotics resistance phenotype
Escherichia coli 13	SHS	Stool	*bla*$_{OXA-181}$	0.75 (R)	0.38 (S)	AMX AMC FEP TZP KF ERT SXT CIP DO
Escherichia coli 425	UDUTH	Urine	*bla*$_{OXA-181}$	0.75 (R)	1.5 (S)	AMX AMC FEP TZP KF ERT SXT CIP DO
Citrobacter freundii 448	UDUTH	Urine	*bla*$_{NDM-5}$	2 (R)	6 (R)	AMX AMC FEP TZP KF CRO ERT IMP SXT CIP DO CN
Citrobacter freundii 167	UDUTH	Urine	*bla*$_{NDM-5}$	4 (R)	6 (R)	AMX AMC FEP TZP KF CRO ERT IMP SXT CIP DO CN
Enterobacter cloacae 58	UDUTH	Urine	*bla*$_{NDM-5}$	8 (R)	16 (R)	AMX AMC FEP TZP KF CRO ERT IMP SXT CIP DO F CN
Enterobacter cloacae 116	UDUTH	Urine	*bla*$_{NDM-5}$	8 (R)	12 (R)	AMX AMC FEP TZP KF CRO ERT IMP SXT CIP DO AK
Enterobacter cloacae 138	SHS	Sputum	*bla*$_{NDM-5}$	4 (R)	8 (R)	AMX AMC FEP TZP KF CRO ERT IMP SXT CIP F CN

SHS, Specialist Hospital Sokoto; UDUTH, Usmanu Danfodiyo University Teaching Hospital Sokoto. AMX, Amoxicillin; AMC, Amoxicillin-clavulanic acid; FEP, Cefepime; CRO, Ceftriaxone; KF, Cefalotin; CN, Gentamicin; AK, Amikacin; DO, Doxycycline; CIP, Ciprofloxacin; ETP, Ertapenem; IPM, Imipenem; SXT, Trimethoprim-Sulfamethoxazole; FF, Fosfomycin; F, Nitrofurantoin. MIC, minimum inhibitory concentration; R, resistant; S, Susceptible