ORIGINAL ARTICLE

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

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Abstract

Emergence and spread of carbapenemase-producing *Enterobacteriaceae* (CPE) are two of the major problems currently threatening global public health. In Nigeria, interest in CPE is recent. In Sokoto, northwest Nigeria, there are 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 two hospitals between January and June 2019 were collected. Of these, 129 (44.2 %) and 19 (6.5%) were resistant to third-generation cephalosporin and carbapenems, respectively. RT-PCR revealed that 10 (7.8%), 19 (14.7%) and 46 (35.7%) of the third-generation cephalosporin-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 carbapenem-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 carbapenemase genes investigated, including bla_{VIM}, bla_{KPC} and bla_{IMP}, was detected in the remaining carbapenem-resistant isolates, suggesting a non-enzymatic mechanism. This study reports for the first time, the emergence of CPE in Sokoto state and the detection of NDM-producing *Citrobacter freundii* in Nigeria. The observed CPE in this study is a concern in a country where alternative antibiotics are rarely available.

Keywords: bla_{NDM-5}, $bla_{OXA-181}$, Carbapenem resistance, Carbapenemase genes, Nigeria
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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 Gram-negative bacteria [2]. Until the early 1990s, resistance to carbapenems has been mostly due to chromosomal β-lactamases [3]. A transferable plasmid-encoded carbapenemase (bla_{IMP-1}) emerged in Japan in 1990 [4]. Following this, there was rapid dissemination of carbapenemase-producing bacteria and this continues to be increasingly reported worldwide [5]. Other recognized carbapenem-resistance mechanisms include decreased outer membrane permeability and up-regulation of the efflux system with/without production of extended-spectrum β-lactamases (ESBLs) [6]. However, acquired carbapenemase production is the most clinically important carbapenem-resistance mechanism [5].

The emergence of carbapenemase-producing *Enterobacteriaceae* (CPE) is concerning, as it further limits options for

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treatment of infections due to multidrug-resistant 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 cause approximately 9300 infections in the USA, half of which usually result in death [11]. Moreover, longer duration of hospital stays and the consequently increased health-care costs are associated with carbapenem-resistant Gram-negative bacterial infections [12,13]. In the USA, bla_{KPC} is the most common carbapenemase gene, though recent emergence of bla_{NDM}-producing *Enterobacteriaceae* has been reported, particularly among individuals returning from regions where bla_{NDM} is endemic [14]. Moreover, despite the existence of wide geographical variation in the types of carbapenemase genes in Europe, bla_{KPC} and bla_{OXA-48} are generally the commonest carbapenemase enzymes [15,16]. In Nigeria, there is a dearth of data on the genetic diversity and spread of CPE. Interest in research on carbapenem resistance began recently. Most 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 were no data on the prevalence of CPE. In view of this, we aimed to investigate the prevalence of clinical *Enterobacteriaceae* isolates bearing carbapenemase genes in two-leading hospitals in Sokoto, northwest Nigeria and also to characterize 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 individuals attending the two main tertiary health-care 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 and Sokoto State Specialist Hospital are, respectively, 850-bed and 300-bed hospitals providing essential, specialized and referral medical and surgical services to residents of Sokoto state and patients from the 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 characterization. The identity of the isolates was confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics, Bremen, Germany) according to the previously described protocol [21].

Antibiotic susceptibility test

Antibiotic susceptibility test 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 Ltd, Basingstoke, 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 antibiotic susceptibility test results were interpreted according to EUCAST breakpoints. The imipenem and ertapenem MICs for isolates with reduced susceptibility to either imipenem or ertapenem by disc diffusion test were thereafter determined using the gradient diffusion tests (Etest®, bioMérieux, Marcy L'Étoile, 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 [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 enzyme production

The phenotypic detection of ESBL enzymes was performed using the double-disc synergy test by placing a β-lactamase inhibitor (amoxicillin–clavulanic and piperacillin-tazobactam) discs between two third-generation cephalosporins 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 third-generation cephalosporin- 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, USA) according to the previously described protocol [27]. The generated raw-read sequences were assembled using CODON CODE ALIGNER, v 9.0.1 (Codon Code Corp., Centerville, MA, 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

One of the leading objectives of this work was to investigate colistin resistance in these clinical isolates. Irrespective of the results of the phenotypic colistin-resistance test, qPCR was used to screen the whole collection of 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].

Results

The distribution of the clinical isolates recovered during clinical diagnostic testing in the two hospitals shows that most 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%).

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 and carbapenems, respectively. The distribution of the isolates is presented in Table 1. The third-generation cephalosporin-resistant isolates were *Citrobacter freundii* (*n* = 10), *Enterobacter cloacae* (*n* = 18), *Escherichia coli* (*n* = 51), *Klebsiella pneumoniae* (*n* = 28), *Morganella 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 third-generation cephalosporin-resistant bacteria were ESBL positive. The result of RT-PCR revealed that 10 (7.8%), 19 (14.7%) and 46 (35.7%) of the third-generation cephalosporin-resistant isolates harboured bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes, respectively (Table 1). Nine and eight of the isolates co-harboured bla_{TEM} and bla_{CTX-M} and bla_{SHV} and bla_{CTX-M}, respectively, and one of the isolates co-expressed all three ESBL genes.

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

The result of antibiotic susceptibility testing revealed that, with the exception of the naturally colistin-resistant strains of *Proteus*, *Morganella*, *Serratia* and *Providencia*, 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.

TABLE 1. Distribution of ESBL-producing and carbapenem-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

Abbreviations: 3GC, third-generation cephalosporins; CRE, carbapenem-resistant *Enterobacteriaceae*; ESBL, extended spectrum β-lactamase.

Discussion

Globally, carbapenem resistance is increasingly reported [30], but its prevalence varies from one geographical region to the other. In the present study, an overall prevalence 6.5% carbapenem resistance was reported. Previous reports across the country have established varying rates. For example, 28% carbapenem resistance was reported in the preceding year in one of the hospitals among carbapenem-naive patients [18]. Also, in a neighbouring West Africa country, Ghana, a rate of 66% carbapenem-resistant Gram-negative bacteria 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].

TABLE 2. Resistance phenotypes and carbapenemase genes identified in carbapenem-resistant isolates

Strain names	Hospital	Isolation sources	Bla_{GENES}	ERT MIC (mg/L)	IPM MIC (mg/L)	Antibiotic-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

Abbreviations: 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.

The observed resistance to the carbapenems in this study is troubling in a country where alternative antibiotics are rarely available [32]. Furthermore, the emergence and spread of carbapenem-resistant bacteria is more worrisome because of the 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]. β-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 individuals returning from international travel to regions like India and Europe where carbapenem resistance is endemic [35,36]. Importation of antibiotic-resistant bacteria across geographical borders has been documented [37].

In this study, the presence of bla_{NDM-5} and $bla_{OXA-181}$ 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_{NDM-5}, bla_{OXA-48} and $bla_{OXA-181}$ 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].

Although 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 findings, however, concur 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 the 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 the 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 findings of this study are of concern in a country where alternative antibiotics are rarely available.

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Conflicts of interest

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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