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Short Communication

High proportion of carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* among extended-spectrum β-lactamase-producers in Nigerian hospitals

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ABSTRACT

Objectives: Carbapenem-resistant Enterobacterales are a global problem, however little is known about the burden and origin of carbapenem resistance in Africa. The objectives of this study were to determine the proportion of carbapenem-resistant isolates among extended-spectrum β-lactamase-producing Enterobacterales (ESBL-E), to identify the underlying mechanisms of resistance and to assess the population structure of carbapenem-resistant isolates from Nigeria.

Methods: ESBL-E isolates (n = 175) from infections were collected at four hospitals in Lagos, Nigeria, from July 2016 to January 2018 and were screened for carbapenem resistance using a VITEK® 2 automated system. All carbapenem-resistant ESBL-E (CRE) were screened for *bla*$_{KPC}$, *bla*$_{CTX-M}$, *bla*$_{CMY-2}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{OXA-181}$ and *bla*$_{OXA-48}$ genes. Genotyping of randomly selected isolates was performed by whole-genome sequencing.

Results: The isolates included *Escherichia coli* (n = 113; 64.6%) and *Klebsiella pneumoniae* (n = 62; 35.4%). Of the 175 ESBL-E isolates, 48 (27.4%) were resistant to carbapenems (15 *E. coli* and 33 *K. pneumoniae*). CRE isolates carried *bla*$_{NDM}$ (n = 30; 62.5%), *bla*$_{NDM}$ + *bla*$_{OXA-181}$ (n = 10; 20.8%), *bla*$_{OXA-181}$ (n = 2; 4.2%) and *bla*$_{NDM}$ + *bla*$_{OXA-48}$ (n = 1; 2.1%); no carbapenemase gene was detected in 5 isolates (10.4%). The isolates showed low diversity and were mainly associated with multilocus sequence typing (MLST) sequence types ST410 for *E. coli* and ST395 and ST147 for *K. pneumoniae*.

Conclusion: Carbapenem resistance is frequent among ESBL-E in Nigeria and is mainly associated with *bla*$_{NDM}$. Genotyping suggested that the observed clones possibly originated from Southeast Asia.

1. Introduction

Escherichia coli and *Klebsiella pneumoniae* are major multidrug-resistant pathogens of community- and hospital-acquired infections. They can produce extended-spectrum β-lactamases (ESBLs) and carbapenemases, leading to limited antimicrobial treatment options [1]. This is particularly worrisome in resource-limited settings where antimicrobial agents of last resort (e.g. colistin, tigecycline and ceftazidime/avibactam) are generally not affordable.

ESBL-producing isolates are frequently found among clinical isolates of *E. coli* (5–25%) and *K. pneumoniae* (12.6–29.8%) from sub-Saharan Africa [2]. Some studies have reported the presence of carbapenemases in Africa (e.g. *bla*$_{OXA-181}$, *bla*$_{OXA-48}$, *bla*$_{NDM-1}$, *bla*$_{VIM}$, *bla*$_{GES}$ and *bla*$_{KPC}$), but the burden and molecular epidemiology of carbapenem resistance among clinical ESBL-producing Enterobacterales (ESBL-E) is mostly unknown [3,4].

The objectives of the study were to determine the proportion of carbapenem-resistant isolates among clinical ESBL-E in Nigeria, to identify the underlying mechanisms of carbapenem resistance and to assess the population structure of the detected carbapenem-resistant isolates.

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2. Materials and methods

2.1. Bacterial isolates

A total of 387 isolates of *K. pneumoniae* (n = 193) and *E. coli* (n = 194) from infections that were resistant to any cephalosporin were screened for ESBL production. The isolates had been prospectively collected at the National Orthopaedic Hospital (n = 118), Lagos State University Teaching Hospital (n = 33), Lagos University Teaching Hospital (n = 221) and Federal Neuro-Psychiatric Hospital (n = 15) in Lagos, Nigeria, from July 2016 to January 2018. The National Orthopaedic Hospital (450 beds) is a referral hospital for orthopaedic surgery. Lagos State University Teaching Hospital (774 beds) is a state-owned tertiary institution attached to Lagos State University. Lagos University Teaching Hospital is a tertiary hospital (761 beds) affiliated to the University of Lagos College of Medicine. The Federal Neuro-Psychiatric Hospital is a specialist hospital for psychiatric treatment with 15 wards and 357 beds.

Only isolates resistant to third-generation cephalosporins were included in the final analysis. Duplicate isolates and isolates from colonisation were excluded. Species identification was done by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry mass spectrometry (MALDI-TOF/MS) (Microflex LT; Bruker Daltonik GmbH, Bremen, Germany).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed for all isolates using a VITEK®2 automated system with VITEK®2 AST N214 card (bioMérieux, Marcy-l'Étoile, France). Susceptibility to ceftolozane/tazobactam and ceftazidime/avibactam was tested by gradient diffusion tests (Etest®; bioMérieux). Since aztreonam can be active against metallo-β-lactamase (MBL)-producing isolates but is inactivated by β-lactamases other than MBLs (which in turn can be inactivated by avibactam or tazobactam), the activity of ceftazidime/avibactam/aztreonam and piperacillin/tazobactam/aztreonam was tested in carbapenem-resistant ESBL-E (CRE). For that purpose, aztreonam (3 g/L) was dissolved in water and 10 μL of the solution containing 30 μg of aztreonam was placed on ceftazidime/avibactam disks (10/4 μg) or piperacillin/tazobactam disks (30/6 μg) [5]. The diameter of the zone of inhibition was interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST v.9.0, January 2019) clinical breakpoints for aztreonam (susceptible, ≥26 mm; resistant <21 mm). Isolates categorised as 'susceptible, increased exposure' were considered susceptible when reporting resistance rates.

The ESBL phenotype was confirmed by double-disk diffusion test (MASTDISCS® D67C; Mast Group Ltd., Bootle, UK). Carbapenem resistance was further analysed using a carbapenemase detection kit (MASTDISCS® D70C; Mast Group Ltd.).

2.3. Detection of antimicrobial resistance genes

All CRE were screened for $bla_{\text{KPC-2–15}}$, $bla_{\text{VIM-1–37}}$, $bla_{\text{NDM-1–7}}$, $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-181}}$ by isothermal amplification (eazyplex® SuperBug CRE Kit; AmplexDiagnostics GmbH, Gars-Bahnhof, Germany) and for $bla_{\text{CTX-M}}$, bla_{IMP} and $bla_{\text{CMY-2}}$ by conventional PCR [6–8]. In addition, 24 randomly selected CRE were sequenced and their genomes were screened for antimicrobial resistance genes using ResFinder v.2.1 with a 98% ID threshold and 60% minimum length (Supplementary Table S1) [9].

2.4. Genotyping

The revised Clermont typing scheme was used for *E. coli* phylogrouping [10]. A randomly selected subset of carbapenem-resistant *E. coli* and *K. pneumoniae* (50% of isolates from each species and 50% of isolates from each hospital) were subjected to whole-genome sequencing (WGS) on an Illumina MiSeq or NextSeq sequencing platform (Illumina Inc., San Diego, CA, USA) [11]. A minimum spanning tree was constructed using SeqSphere+ with the 'pairwise ignoring missing values' option using allelic profiles of all genes detected in the WGS data [core genome multilocus sequence typing (cgMLST)]. For *E. coli* up to 4671 genes present in the genome of reference strain Sakai (GenBank accession no. **NC_002695**), and for *K. pneumoniae* up to 4728 genes present in the genome of reference strain MGH 78578 (GenBank accession no. **NC_009648**) were extracted from WGS data. Classical MLST data were extracted in silico from WGS data. Raw reads were deposited in the European Nucleotide Archive under study accession no. **PRJEB27707**.

2.5. Plasmid stability test

Plasmid stability of all CRE was tested in duplicate using the modified 'naïve test' [12]. Briefly, isolates were cultured in brain–heart infusion broth with meropenem (8 mg/L) followed by incubation without meropenem. The culture was titrated on permissive agar (Mueller–Hinton agar II; BD, Heidelberg, Germany) and restrictive agar (CARB ID; bioMérieux). The CARB ID plate has good sensitivity and specificity to detect major carbapenemases (e.g. bla_{NDM} and bla_{OXA}) [13].

2.6. Statistical analysis

Antimicrobial resistance rates between carbapenem-susceptible and -resistant ESBL-E were compared using χ^2 test or Fisher's exact test, where appropriate, as implemented in 'R' (package epiDisplay; significance level = 0.05).

3. Results

Of the 387 isolates with resistance to any cephalosporin, 175 were confirmed to be ESBL-producers and were included in this study, including 113 ESBL-producing *E. coli* and 62 ESBL-producing *K. pneumoniae*. The median (range) age of patients was 43 years (0.01–79 years) and 89 (50.9%) were female. The majority of isolates were derived from urine (n = 85; 48.6%), followed by wound (n = 69; 39.4%), blood culture (n = 8; 4.6%), lower respiratory tract (n = 4; 2.3%) and others (n = 9; 5.1%).

Of the 175 ESBL-E isolates, 48 (27.4%) were resistant to carbapenems (15 *E. coli* and 33 *K. pneumoniae*). Whilst the susceptibility rate of piperacillin/tazobactam/aztreonam in CRE was low (4.2%), a high rate was found for ceftazidime/avibactam/aztreonam (75 %, Table 1). The phenotypic carbapenemase detection kit identified MBLs in the majority of CRE (44/48; 91.7%), followed by AmpC/porin loss (3/48; 6.3%) or ambiguous (1/48; 2.1%). Carbapenem-resistant *E. coli* were positive for bla_{NDM} + $bla_{\text{OXA-181}}$ (n = 10), bla_{NDM} (n = 3) and $bla_{\text{OXA-181}}$ (n = 2). Carbapenem-resistant *K. pneumoniae* carried bla_{NDM} (n = 27) or bla_{NDM} + $bla_{\text{OXA-48}}$ (n = 1); no carbapenemases were detected in 5 isolates.

CMY-2 was detected in 26 isolates including 11 carbapenem-resistant isolates (Table 1).

A plasmid stability test was performed on all carbapenem-resistant isolates to assess the stability of carbapenem resistance gene expression after passaging, as high plasmid instability would restore carbapenems as a treatment option. A large proportion of carbapenem-resistant isolates (n = 48) grew on permissive agar (median 377 CFU, range 150–733 CFU) whereas a smaller number grew on restrictive agar (median 349 CFU, range 0–618 CFU).

Table 1
Antimicrobial resistance rates and antimicrobial resistance genes of extended-spectrum β-lactamase (ESBL)-producing and carbapenem-resistant Enterobacterales.

	Total (n = 175) [n (%)]	Carbapenem-susceptible ESBL-E (n = 127) [n (%)]	Carbapenem-resistant ESBL-E (n = 48) [n (%)]	Odds ratio (95% CI)	P-value
Antimicrobial resistance rate					
Piperacillin	175 (100)	127 (100)	48 (100)	NA	NA
Piperacillin/tazobactam	104 (59.4)	56 (44.1)	48 (100)	NA	<0.0001
Piperacillin/tazobactam/aztreonam	–	–	46 (95.8)[a]	–	–
Aztreonam	172 (98.3)	126 (99.2)	46 (95.8)	5.5 (0.5– 61.9)	0.2
Cefotaxime	175 (100)	127 (100)	48 (100)	NA	NA
Ceftazidime	159 (90.9)	111 (87.4)	48 (100)	NA	0.007
Ceftazidime/avibactam	47 (26.9)	1 (0.8)	46 (95.8)	0 (0–0)	<0.0001
Ceftazidime/avibactam/aztreonam	–	–	12 (25.0)[b]	–	–
Ceftolozane/tazobactam	89 (50.9)	41 (32.3)	48 (100)	NA	<0.0001
Cefepime	136 (77.7)	88 (69.3)	48 (100)	NA	<0.0001
Imipenem	36 (20.6)	0 (0)	36 (75.0)	NA	<0.0001
Meropenem	39 (22.3)	0 (0)	39 (81.3)	NA	<0.0001
Amikacin	28 (16.0)	4 (3.1)	24 (50.0)	0.03 (0.01– 0.1)	<0.0001
Gentamicin	130 (74.3)	101 (79.5)	29 (60.4)	2.7 (1.3–5.5)	0.007
Tobramycin	151 (86.3)	105 (82.7)	46 (95.8)	0.2 (0.05– 0.9)	0.03
Ciprofloxacin	167 (95.4)	119 (93.7)	48 (100)	NA	0.1
Moxifloxacin	174 (99.4)	126 (99.2)	48 (100)	NA	1
Tigecycline	44 (25.1)	15 (11.8)	29 (60.4)	0.1 (0.04–0.3)	<0.0001
Fosfomycin	19 (10.9)	4 (3.1)	15 (31.3)	0.1 (0.02–0.2)	<0.0001
Trimethoprim/sulfamethoxazole	167 (95.4)	119 (93.7)	48 (100)	NA	0.08
Antimicrobial resistance genes					
$bla_{\text{CTX-M}}$	33 (18.9)	28 (22.0)	5 (10.4)	0.4 (0.2–1.1)	0.08
$bla_{\text{CMY-2}}$	26 (14.9)	15 (11.8)	11 (22.9)	2.2 (0.9–5.3)	0.07
bla_{NDM}	–	–	41 (85.4)	–	–
$bla_{\text{OXA-48}}$	–	–	1 (2.1)	–	–
$bla_{\text{OXA-181}}$	–	–	12 (25.0)	–	–
bla_{IMP}	–	–	0 (0)	–	–

OR, odds ratio; CI, confidence interval; NA, not applicable.
[a] Eleven isolates were positive for $bla_{\text{CMY-2}}$.
[b] Nine isolates were positive for $bla_{\text{CMY-2}}$.

Apparently, a median of 13.7% of CFU (range 0–100%) did not express the carbapenem resistance gene or lost the plasmid after one passage. The only isolate that did not grow on the restrictive medium was a $bla_{\text{OXA-181}}$-positive isolate.

The predominant phylogroups of ESBL-producing *E. coli* were B2 (n = 65; 57.5%), followed by clade I or II (n = 16; 14.2%), E (n = 12; 10.6%), F (n = 6; 5.3%), A (n = 3; 2.7%), C (n = 2; 1.8%) and B1 (n = 1; 0.9%). Eight isolates (7.1%) belonged to an unknown phylogroup.

WGS revealed low diversity among 24 randomly selected carbapenem-resistant isolates, with a predominance of MLST sequence type ST410 in *E. coli* (7/8). The predominant sequence types in *K. pneumoniae* were ST395 (7/16) and ST147 (5/16) (Fig. 1). These 24 carbapenem-resistant isolates were additionally screened for antimicrobial resistance genes using ResFinder and a high diversity (n = 16) of aminoglycoside resistance genes was found [*aadA1*, *aadA2*, *aadA5*, *aadA13*, *aadA16*, *aac(3)-IIa*, *aac(3)-IId*, *aac(6')-Ib*-suzhou, *aac(6')-Ib*-cr, *aac(6')-Ib3*, *armA*, *aph(3")-Ia*, *aph (3")-Ib*, *aph(3')-VI*, *aph(6)-Id* and *rmtC*] (Supplementary Table S1). The most common ESBL gene in this random sample was $bla_{\text{CTX-M-15}}$ (20/24). The underlying mechanisms of quinolone resistance were *oqxA*, *oqxB* and *qnrS1*. All bla_{NDM}-positive isolates of this random subset carried $bla_{\text{NDM-1}}$ (20/24). See Supplementary Table S1 for detailed antimicrobial resistance genes of each isolate.

The indistinguishable *E. coli* ST410 and *K. pneumoniae* ST395 from three hospitals suggest either interhospital spread of these isolates or a common source. Among the bacteria that underwent WGS, the *E. coli* isolates were collected between July 2016 and January 2018 whilst the *K. pneumoniae* isolates were detected between April 2016 and December 2017.

4. Discussion

In this study, 27.4% (48/175) of ESBL-producing isolates were carbapenem-resistant, of which 85.4% (41/48) were positive for bla_{NDM}. The extent of carbapenem resistance becomes even more evident considering the overall high proportion of ESBL-producers among *E. coli* (48.8%) and *K. pneumoniae* (37.5%) in the study region [14].

The high resistance rates to ceftazidime/avibactam and ceftolozane/tazobactam in carbapenem-resistant isolates are in line with a high proportion of bla_{NDM}. This is consistent with an analysis of 38 266 Enterobacteriaceae isolates that revealed a predominance of bla_{NDM} in MBL-producing isolates in the Middle East/Africa (85.2%) [15].

However, the combination of ceftazidime/avibactam with aztreonam could be a promising approach in future as it showed good activity in 75.0% of carbapenem-resistant isolates (Table 1).

The modified plasmid stability test suggested a reduction in carbapenem resistance in CRE by 13.7% after one passage. The plasmid stability test was originally developed for a negative selection system (selection of isolates without the resistance gene) [12]. Since a positive selection approach (i.e. selection of isolates with resistance genes) was used in the current study, the plasmid loss frequency may have been overestimated due to low or non-expression of antimicrobial resistance genes.

All bla_{NDM}-positive isolates that were randomly selected for WGS carried $bla_{\text{NDM-1}}$. The majority of bla_{NDM}-positive *E. coli* belonged to ST410 (Fig. 1). To date, there are only sporadic reports of $bla_{\text{NDM-1}}$-positive *E. coli* ST410 from Egypt, England, Norway and Southeast Asia (e.g. India, Myanmar, China) [16,17]. Similarly, $bla_{\text{NDM-1}}$-positive *K. pneumoniae* ST395 is also mainly found in China [18].

Some limitations of this study need to be addressed. First, since the only inclusion criterion was resistance to third-generation cephalosporins, ESBL-negative isolates producing OXA-48-like carbapenemases that do not hydrolyse broad-spectrum cephalosporins might have been missed. Second, owing to limited funding, we were unable to perform WGS on all isolates. Third, the number of isolates from each hospital was unequal as two of four hospitals were overrepresented. This bias might point towards an

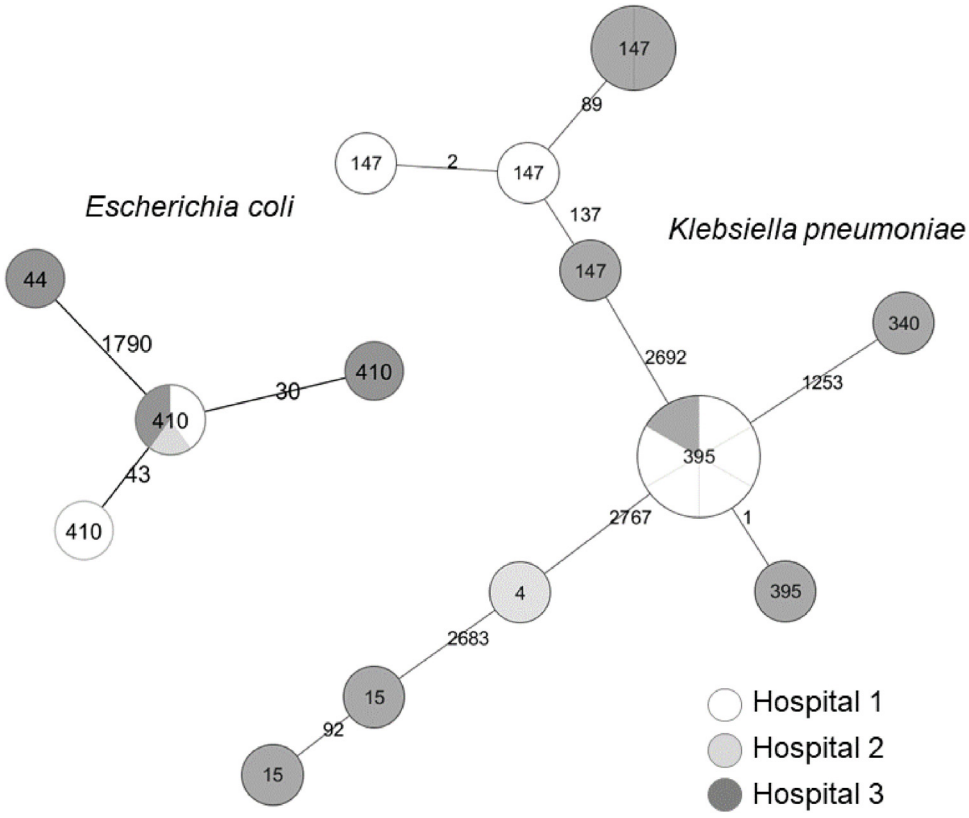

Fig. 1. Phylogenetic relationship of carbapenem-resistant extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* from three hospitals in Lagos, Nigeria. The minimum spanning tree was constructed based on the allelic profile of the respective core genome multilocus sequence typing (cgMLST) scheme. Each node is labelled with the MLST sequence type. The hospitals are coded with grey shading. The size of the node corresponds to the number of isolates. The number between nodes indicates the number of differing alleles. For *E. coli* up to 4671 genes present in the genome of reference strain Sakai (GenBank accession no. **NC_002695**), and for *K. pneumoniae* up to 4728 genes present in the genome of reference strain MGH 78578 (GenBank accession no. **NC_009648**) were extracted from whole-genome sequencing data.

ongoing (not yet detected) outbreak. Fourth, instead of EUCAST screening breakpoints, EUCAST clinical breakpoints were applied to identify carbapenem-resistant isolates. Therefore, additional carbapenem-resistant isolates might have been missed. However, since the lowest meropenem minimum inhibitory concentration (MIC) that can be detected by VITEK®2 is 0.25 mg/L, we were unable to apply the screening cut-off value of 0.125 mg/L.

In conclusion, the majority of carbapenem-resistant *E. coli* and *K. pneumoniae* from Nigeria carrying bla_{NDM} may have come from Southeast Asia.

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Competing interests

FS has received grants from MSD Sharp & Dohme GmbH and Pfizer Inc. All other authors declare no competing interests.

Ethical approval

Ethical approval was provided by the Health Research Ethics Committee of the College of Medicine, University of Lagos (Lagos, Nigeria) [CMUL/HREC/05/17/136].

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.09.007.

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