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High Prevalence of Carbapenemase-Producing *Enterobacteriaceae* among Hospitalized Children in Luanda, Angola

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This study aimed to evaluate the prevalence of carbapenemase-producing *Enterobacteriaceae* in Luanda, Angola. A total of 157 rectal samples were collected from children visiting a pediatric hospital in Luanda in March 2015. Fifty-seven imipenem-nonsusceptible enterobacterial isolates were recovered, most of which were non-clonally related. The $bla_{OXA-181}$ (50/57) and bla_{NDM-1} (7/57) carbapenemase genes were identified. Notably, OXA-181-producing *Escherichia coli* isolates rarely coproduced extended-spectrum β-lactamases and consequently remained susceptible to broad-spectrum cephalosporins. The $bla_{OXA-181}$ gene was always located on an IncX3 plasmid, while the bla_{NDM-1} gene was located on either IncFIA or IncA/C plasmids. The study identified a high prevalence of OXA-181 among hospitalized children in Angola.

The increasing occurrence of carbapenem resistance in Gram-negative bacteria is one of the major public health problems involving *Enterobacteriaceae*. Carbapenem resistance arises from two main mechanisms: decreased uptake of carbapenems by porins associated with overexpression of a β-lactamase with weak carbapenemase activity, and the acquisition of carbapenemases (1). Carbapenemases belong to three different Ambler classes (2), with class A including the serine carbapenemases KPC, NMC/IMI, and SME; class B including the metallo-β-lactamases VIM, IMP, and NDM; and class D including OXA-48-like β-lactamases (OXA-48, OXA-181, etc.). Since the initial report of OXA-48 from a *Klebsiella pneumoniae* isolate in 2003 (3), OXA-48-like enzymes have been recovered in a variety of *Enterobacteriaceae* worldwide, particularly within the Mediterranean area, including southern Europe and northern Africa, but also in the Middle East (4). Variants of OXA-48 have been described (5), including OXA-181, which differs from OXA-48 by 4 amino acids while sharing a very similar hydrolytic profile (6). Bacteria producing OXA-48-like β-lactamases do not always exhibit high levels of resistance to carbapenems, making their detection challenging (4). The epidemiology of carbapenemases in Africa remains mostly unknown, with the exception of OXA-48, which was identified in the northern part of the continent (Algeria, Egypt, Morocco, and Tunisia) (4, 7), and some reports of KPC- and NDM-like enzymes in South Africa (7). However, there are limited data about the occurrence of carbapenemase producers in most of the other African countries. Therefore, we investigated the occurrence and molecular characteristics of carbapenemase-producing enterobacterial isolates recovered from rectal samples from children visiting a pediatric hospital in Luanda, Angola.

MATERIALS AND METHODS

Bacterial isolates and susceptibility testing. A total of 157 rectal swabs were collected on 30 and 31 March 2015 from hospitalized patients ($n = 100$) and from ambulatory patients ($n = 57$). The samples were incubated in Luria-Bertani (LB) broth supplemented with ertapenem (0.25 μg/ml) for 10 h. From each broth, a calibrated inoculated loop (10 μl) was plated onto ChromID CarbaSmart selective medium (bioMérieux, La Balme-les-Grottes, France) to select for carbapenem-resistant isolates. The isolates were identified at the species level using the API20E system (bioMérieux). Antimicrobial susceptibility testing was performed according to the disc diffusion method following the CLSI recommendations (8) and using cation-adjusted Mueller-Hinton (MH) plates (Bio-Rad, Cressier, Switzerland).

MICs were determined by Etest (bioMérieux, La Balme-les-Grottes, France) for imipenem, meropenem, and ceftazidime. Carbapenemase activity was assessed using the Rapidec Carba NP test (9) (bioMérieux) for each isolate growing on the ChromID CarbaSmart plate.

Molecular analysis. Carbapenemase- and extended-spectrum β-lactamase (ESBL)-encoding genes were identified by PCR amplification using specific primers as described previously (10–12), followed by sequencing (Microsynth, Balgach, Switzerland).

Considering the high-level resistance to all aminoglycosides observed for some isolates, a search of 16S rRNA methylase-encoding genes was performed by multiplex PCR as described previously (13). Similarly, a search of plasmid-mediated Qnr-like encoding genes involved in reduced susceptibility to quinolones was performed by multiplex PCR, as described previously (14). Finally, a search of the plasmid-mediated colistin resistance *mcr-1* gene was performed by real-time PCR (15).

The clonal relationship of the isolates was evaluated by pulsed-field gel electrophoresis (PFGE). Total DNA from *K. pneumoniae* isolates and *Escherichia coli* isolates was digested by using the XbaI enzyme (New England BioLabs, Ipswich, MA, USA). The generated fragments were separated by PFGE using a CHEF-DR III System (Bio-Rad), followed by multilocus sequence typing (MLST) (16) for one strain of each different PFGE profile. Sequence types (STs) were assigned using the databases (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/ and http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html) for *E. coli* isolates and for *K. pneumoniae* isolates, respectively. DNAs of plasmids harboring the $bla_{OXA-181}$ gene were extracted using the Kieser extraction method (17), electroporated into *E.*

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coli TOP10, and selected on LB agar plates supplemented with temocillin (50 μg/ml), which is selective for OXA-48-like β-lactamase producers. Plasmid sizes were evaluated by agarose gel electrophoresis using *E. coli* NCTC50192 harboring four plasmids of 154, 66, 48, and 7 kb as plasmid size markers. Plasmids carrying $bla_{OXA-181}$ were characterized by PCR-based replicon typing (PBRT) as described previously (18) by including primers specific for IncX3-type plasmid backbones (IncX3 Fw, 5′-GAG GCT TAT CGT GAA GAC AG-3′; IncX3 Rv, 5′-GAA CGA CTT TGT CAA ACT CC-3′) and by restriction fragment length polymorphism (RFLP) using restriction enzymes PvuII and HindIII, respectively. The genetic environment of the $bla_{OXA-181}$ gene was investigated by PCR mapping with primers specific for insertion sequence IS*Ecp1*, IS*Kpn19*, or IS*3000*, as previously described (6, 19), since previous studies showed they might be located upstream of $bla_{OXA-181}$.

Conjugation experiments. Mating-out assays were performed using the azide-resistant *E. coli* J53 as the recipient. *E. coli* J53 and $bla_{OXA-181}$-carrying donors were separately inoculated overnight into LB broth and incubated. The samples were then mixed at a ratio of 10:1 (donor/recipient) for 5 h and plated onto LB agar plates supplemented with temocillin (50 μg/ml) and sodium azide (100 μg/ml). Susceptibility testing was performed for all *E. coli* transconjugants, and positivity for $bla_{OXA-181}$ was checked by PCR.

Ethical approval. The protocol was approved by the institutional review board of the hospital. Informed consent was obtained from the guardians of the children after a verbal presentation of the purpose, method, and design of the study.

RESULTS

All the patients were children from 3 months to 13 years old. They were visiting the hospital for several reasons, either for treating infections (pneumonia, peritonitis, malaria, and hepatitis) or for surgery, tumors, leukemia, malnutrition, or renal failure. Limited data in relation to antimicrobial therapy were available; the only important issue was that penicillins and broad-spectrum cephalosporins are quite often used in the hospital, but none of the children had previously received carbapenems.

From the 157 patients, a total of 57 imipenem-nonsusceptible and carbapenemase-producing enterobacterial isolates were isolated (Table 1). They were recovered from a total of 48 patients; 9 patients were colonized with two different carbapenemase producers. Out of the 57 community patients screened, only a single carbapenemase producer was recovered, while 42 out of the 100 hospitalized patients were colonized by at least one carbapenemase-producing isolate.

Among the 57 carbapenemase-producing isolates, 50 were found positive for the $bla_{OXA-181}$ gene and 7 were positive for the bla_{NDM-1} gene. None of the isolates coproduced two carbapenemases. Among the $bla_{OXA-181}$-positive enterobacterial isolates, 25 *E. coli* isolates, 24 *K. pneumoniae* isolates, and a single *Enterobacter cloacae* isolate were identified. The seven bla_{NDM-1} producers were *E. coli* ($n = 4$), *K. pneumoniae* ($n = 1$), *Providencia stuartii* ($n = 1$), and *Providencia rettgeri* ($n = 1$). Moreover, six out of the seven bla_{NDM-1}-positive isolates coharbored the *rmtB* or *rmtH* 16S rRNA methyltransferase gene (Table 1). No isolate was positive for the *mcr-1* gene.

Notably, the majority of the isolates belonging to either the *E. coli* or *K. pneumoniae* species exhibited low carbapenem MIC values, in particular for imipenem, with most isolates showing MIC values of 0.5 or 1 μg/ml.

The majority of the OXA-181-producing *E. coli* isolates (64%) did not coproduce any ESBL and remained susceptible to broad-spectrum cephalosporins, whereas almost all the *K. pneumoniae* isolates (96%) were ESBL producers (Table 1).

Mating-out assays followed by plasmid analysis revealed four different-size plasmids (ca. 30 kb, 64 kb, 70 kb, and >150 kb) carrying the $bla_{OXA-181}$ gene (Table 1). *E. coli* transconjugants harboring the 30-kb plasmid showed slightly higher MICs of carbapenems than the other transconjugants (4- and 2-fold-increased MICs for imipenem and meropenem, respectively), suggesting that the corresponding plasmid type could likely be present at higher copy numbers and therefore could be enhancing the expression of the carbapenemase gene. Notably, although no additional β-lactamase gene was identified on the other two plasmid scaffolds, PCR amplification showed that the 150-kb plasmid bearing the $bla_{OXA-181}$ gene coharbored the bla_{TEM-1} and $bla_{CTX-M-15}$ β-lactamase genes.

PCR mapping performed on all the positive isolates revealed that a remnant of the IS*Ecp1* element was located upstream of the $bla_{OXA-181}$ gene, as previously reported on the IncX3-type plasmid pOXA-181 from a Chinese *E. coli* isolate (20), with IS*Ecp1* being truncated by the insertion of IS*3000*. Downstream of $bla_{OXA-181}$, and similar to what was observed on pOXA-181, IS*Kpn19* was identified. Further downstream, the *qnrS1* gene, encoding resistance to quinolones and found on pOXA-181, was identified. Overall, the same structure as that identified on pOXA-181 was detected.

The mating-out assays were successful for all the carbapenemase-producing isolates as donors, except for a single $bla_{OXA-181}$-positive *K. pneumoniae* isolate (Table 1). PBRT analysis showed that all the different-size plasmids carrying the $bla_{OXA-181}$ gene belonged to the same IncX3 group, and RFLP confirmed that they shared a common scaffold structure, with similar-size bands (data not shown). The bla_{NDM-1} gene was identified on an IncFIA-type plasmid for three *E. coli* isolates and on an IncA/C-type plasmid for a single *P. rettgeri* isolate. PFGE and MLST analyses identified nine different *E. coli* clones and 10 different *K. pneumoniae* clones (Table 1).

DISCUSSION

Here, we report a high rate of recovery of carbapenemase-producing enterobacterial isolates from children in Angola and an important dissemination of the $bla_{OXA-181}$ carbapenemase gene, which might be considered endemic in that geographical area, through the diffusion of conjugative plasmids. Indeed, 27.4% of the screened individuals were found positive for carbapenemases, and 88% of those carbapenemase-producing isolates harbored the $bla_{OXA-181}$ gene. This is particularly noteworthy considering that none of the patients had received any current or past carbapenem-based antimicrobial therapy.

The occurrence of NDM-1-producing isolates is also noteworthy, since the identification of NDM-1 producers in Tunisia, Morocco, Algeria, and South Africa shows that this carbapenemase, known to be widespread in the Indian subcontinent, may also be widespread in Africa.

The rare association of the $bla_{OXA-181}$ gene with an ESBL-encoding gene in the *E. coli* isolates contrasts with the frequent associations observed among bla_{OXA-48}-positive *K. pneumoniae* isolates, in particular with the $bla_{CTX-M-15}$ ESBL gene. This further underlines the fact that the bla_{OXA-48} and $bla_{OXA-181}$ genes, although structurally related, have distinct origins and epidemiologies.

TABLE 1 Genetic features associated with the carbapenemase-producing isolates

Strain	Species	Resistance determinants	Plasmid size (kb)	Incompatibility group[a]	PFGE profile	ST	Hospitalization unit	Resistance[b] phenotype	MIC (μg/ml)[c]		
									IPM	MEM	CAZ
E1	*E. coli*	OXA-181, QnrS	64	IncX3	A1	ST5692	Acute care	CIP	1	0.5	0.19
E2	*E. coli*	OXA-181, CTX-M-15, QnrS	64	IncX3	A2	ST5692	Acute care	CAZ-CIP	0.5	0.25	64
E3	*E. coli*	OXA-181, QnrS	64	IncX3	A2	ST5692	Special care	CIP	1	0.5	0.19
E4	*E. coli*	OXA-181, QnrS	64	IncX3	A2	ST5692	Special care	CIP	0.5	0.25	0.19
E5	*E. coli*	OXA-181, QnrS	64	IncX3	A2	ST5692	Pediatrics	CIP	0.5	0.25	0.19
E6	*E. coli*	OXA-181, QnrS	64	IncX3	B3	ST38	Pediatrics	CIP	1	0.5	0.19
E7	*E. coli*	OXA-181, QnrS	64	IncX3	C	ST3258	Surgery	CIP	1	0.25	0.19
E8	*E. coli*	OXA-181, CTX-M-15, QnrS	64	IncX3	D	ST361	Pneumology	CAZ-CIP-GEN-KAN-TOB	1	0.5	4
E9	*E. coli*	OXA-181, CTX-M-15, QnrS	64	IncX3	F	ST361	Special care	CAZ-CIP	0.5	0.25	2
E10	*E. coli*	OXA-181, QnrS	64	IncX3	G	ST5692	Special care	CIP	1	0.25	0.19
E11	*E. coli*	OXA-181, CTX-M-15, QnrS	64	IncX3	H	ST3268	Ambulatory	CAZ-CIP	1	1	16
E12	*E. coli*	OXA-181, CTX-M-15, QnrS	64	IncX3	I	ST167	Pediatrics	CAZ-CIP-KAN-TOB	0.5	0.25	128
E13	*E. coli*	OXA-181, CTX-M-15, QnrS	64	IncX3	J	ST5703	Pediatrics	CAZ-CIP-KAN-TOB-GEN	1	0.5	32
E14	*E. coli*	OXA-181, CTX-M-15, QnrS	30	IncX3	A1	ST5692	Acute care	CAZ-CIP-TOB	0.5	0.5	256
E15	*E. coli*	OXA-181, QnrS	30	IncX3	A1	ST5692	Surgery	CIP	0.5	0.25	0.19
E16	*E. coli*	OXA-181, QnrS	30	IncX3	A1	ST5692	Pneumology	CIP	0.5	0.25	0.19
E17	*E. coli*	OXA-181, QnrS	30	IncX3	A1	ST5692	Special care	CIP	0.5	0.25	0.19
E18	*E. coli*	OXA-181, QnrS	30	IncX3	A1	ST5692	Special care	CIP	1	0.25	0.19
E19	*E. coli*	OXA-181, QnrS	30	IncX3	A1	ST5692	Special care	CIP	1	0.25	0.19
E20	*E. coli*	OXA-181, QnrS	30	IncX3	A1	ST5692	Special care	CIP	1	0.25	0.19
E21	*E. coli*	OXA-181, QnrS	30	IncX3	B1	ST38	Pediatrics	CIP	1	0.5	0.25
E22	*E. coli*	OXA-181, QnrS	30	IncX3	B1	ST38	Pediatrics	CIP	2	1	0.25
E23	*E. coli*	OXA-181, CTX-M-15, QnrS	30	IncX3	B1	ST38	Pediatrics	CAZ-CIP	2	1	128
E24	*E. coli*	OXA-181, CTX-M-15, QnrS	30	IncX3	B2	ST38	Pediatrics	CAZ-CIP	1	0.5	128
E25	*E. coli*	OXA-181, QnrS	30	IncX3	E	ST5692	Special care	CIP	1	0.5	0.19
E26	*E. coli*	NDM-1, CTX-M-15, RmtB	100	IncFIA	ND	ST5079	Pneumology	CAZ-CIP-GEN-TOB-AMK	8	32	256
E27	*E. coli*	NDM-1, CTX-M-15, RmtB	100	IncFIA	ND	ST5079	Pneumology	CAZ-CIP-GEN-TOB-AMK	8	32	256
E28	*E. coli*	NDM-1, CTX-M-15, RmtB	100	IncFIA	ND	ST5079	Pneumology	CAZ-CIP-GEN-TOB-AMK	16	32	256
E29	*E. coli*	NDM-1, CTX-M-15, RmtB	100	IncFIA	ND	ST5693	Pediatrics	CAZ-CIP-GEN-TOB-AMK	8	8	256
K1	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	>150	IncX3	D	ST1301	Pneumology	CAZ-CIP-GEN-TOB	1	0.5	32
K2	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Acute care	CAZ-CIP-GEN-TOB	1	1	128
K3	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Acute care	CAZ-CIP-GEN-TOB	1	1	128
K4	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Acute care	CAZ-CIP-GEN-TOB	0.5	0.5	128
K5	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Acute care	CAZ-CIP-GEN-TOB	128	128	128
K6	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Acute care	CAZ-CIP-GEN-TOB	128	128	128
K7	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Surgery	CAZ-CIP-GEN-TOB	1	1	128
K8	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Surgery	CAZ-CIP-GEN-TOB	0.5	0.5	128
K9	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Pediatrics	CAZ-CIP-GEN-TOB	64	64	128
K10	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Pediatrics	CAZ-CIP-GEN-TOB-AMK	64	64	128
K11	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Pediatrics	CAZ-CIP-GEN-TOB	1	0.5	64
K12	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Pediatrics	CAZ-CIP-GEN-TOB	4	8	128
K13	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	A	ST2094	Pediatrics	CAZ-CIP-GEN-TOB	16	8	128
K14	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	B	ST2074	Surgery	CAZ-CIP-GEN-TOB	0.5	0.5	2
K15	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	B	ST2074	Surgery	CAZ-CIP-GEN-TOB	0.5	0.5	128
K16	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	C	ST2092	Surgery	CAZ-CIP-GEN-TOB	1	1	128
K17	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	C	ST2092	Surgery	CAZ-CIP-GEN-TOB	0.5	0.5	128
K18	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	30	IncX3	C	ST34	Special care	CAZ-CIP-GEN-TOB	1	1	16
K19	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	C	ST231	Pediatrics	CAZ-CIP-GEN-TOB	1	1	64
K20	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	E	ST2093	Pneumology	CAZ-CIP-TOB	1	1	128
K21	*K. pneumoniae*	OXA-181, QnrS	64	IncX3	F	ST2093	Pneumology	CIP	0.5	0.5	0.19
K22	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	G	ST2074	Pneumology	CAZ-CIP-GEN-TOB	0.5	0.5	2
K23	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	H	ST45	Pneumology	CAZ-CIP-GEN-TOB	0.5	0.5	128
K24	*K. pneumoniae*	OXA-181, CTX-M-15, QnrS	64	IncX3	I	ST2094	Special care	CAZ-CIP-GEN-TOB	128	128	128
K25	*K. pneumoniae*	NDM-1, CTX-M-15	120			ST15	Treatment 1 room A	CAZ-CIP-GMI-TMN	8	4	>256
Cl1	*E. cloacae*	OXA-181, QnrS	64	IncX3	ND		Pediatrics	CIP	1	0.25	1
PR1	*P. rettgeri*	NDM-1, CTX-M-15	100	ND	ND		Pediatrics	CAZ-CIP-GEN-TOB	8	8	256
PS1	*P. stuartii*	NDM-1, CTX-M-15	100	IncA/C	ND		Acute care	CAZ-CIP-GEN-TOB	32	16	256

[a] Incompatibility group of the plasmid harboring the carbapenemase gene.
[b] Coresistances provided by the carbapenemase-encoding plasmids. AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin.
[c] IPM, imipenem; MEM, meropenem.

According to our data, the high rate of OXA-181 producers in Luanda results from the spread of some predominant *E. coli* and *K. pneumoniae* clones, but also from the dissemination of a self-conjugative IncX3-type plasmid among different enterobacterial isolates.

The plasmids harboring the $bla_{\text{OXA-181}}$ gene were different in size; nevertheless, they all shared the same backbone. It might be hypothesized that they derive from a common IncX3 conjugative plasmid. Notably, although all $bla_{\text{OXA-181}}$-positive plasmids co-harbored the *qnrS1* gene conferring reduced susceptibility to

quinolones, no additional resistance markers were detected, with the exception of a single 150-kb plasmid identified in only one isolate that coharbored the bla_{TEM-1} and $bla_{CTX-M-15}$ β-lactamase genes. In addition, this 150-kb plasmid was not self-conjugative, in contrast to the other $bla_{OXA-181}$-bearing plasmids identified. This might be the consequence of the insertion of the two additional β-lactamase genes in a region of the plasmid that is crucial for its conjugative property.

Interestingly, the genetic environment of the $bla_{OXA-181}$ gene was identical to that identified recently by Liu et al. (20) in China, also on an IncX3-type plasmid, with the $bla_{OXA-181}$ gene flanked upstream by IS*Ecp1* truncated by IS*3000* and downstream by IS*Kpn19* followed by the *qnrS1* gene. In other reports from different parts of the world, the $bla_{OXA-181}$ gene was identified on different plasmid backbones, including IncN and IncT plasmids (19, 21). IncX3-type plasmids harboring the $bla_{OXA-181}$ gene seem to be predominant in Asia. Indeed, a recent study showed that OXA-181-producing isolates had been imported into Switzerland on fresh vegetables originating from Asia (22).

Our data support the importance of active and continuous surveillance of carbapenemase-producing Gram-negative bacteria in health care facilities. They also show that not only Asia, but also Africa, may act as an important reservoir of OXA-181.

Altogether, most isolates coharbored a high number of resistance determinants that represent a major source of concern in terms of public health.

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