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Screening and Characterization of Multidrug-Resistant Gram-Negative Bacteria from a Remote African Area, São Tomé and Príncipe

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ABSTRACT The occurrence of resistance to last-resort antibiotics was evaluated among *Enterobacteriaceae* isolates recovered from hospitalized children in a remote African archipelago, São Tomé and Príncipe, where there is limited access to those antibiotics. Fifty patients were screened for colonization by carbapenem-, pan-aminoglycoside-, or polymyxin-resistant *Enterobacteriaceae*. A total of 36 isolates (including 30 *Escherichia coli* and 4 *Klebsiella pneumoniae*) were recovered from 23 patients, including 26 isolates harboring the $bla_{OXA-181}$ carbapenemase gene, a single isolate harboring the 16S rRNA methylase gene *rmtB* encoding pan-resistance to aminoglycosides, and 8 isolates coharboring both genes. A single isolate possessed the plasmid-borne colistin resistance gene *mcr-1*. A high clonal relationship was found for OXA-181-producing *E. coli* (4 clones), and conversely, three of the four OXA-181-producing *K. pneumoniae* isolates were clonally unrelated. This study overall showed a high prevalence of resistance to last-resort antibiotics in this country, where no epidemiological data were previously available.

KEYWORDS aminoglycosides, carbapenemase, colistin, MCR, São Tomé and Príncipe, carbapenem

The increasing occurrence of resistance to last-resort drugs in Gram-negative bacteria is one of the major public health concerns, particularly in regard to *Enterobacteriaceae*. Carbapenems are among those last-resort antibiotics, because of their wide range of activity (1), but their effectiveness may be compromised by the production of carbapenemases (2). Acquired carbapenemases in *Enterobacteriaceae* belong to three different Ambler classes (3): 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 (with OXA-48 and OXA-181 variants being predominant) (4).

OXA-48, originally identified in a *Klebsiella pneumoniae* clinical isolate in Turkey, is now known to be widespread among enterobacterial species in Europe and in several African countries (5, 6). Many nosocomial outbreaks due to OXA-48-producing *K. pneumoniae* have been reported, but OXA-48 producers may also be detected in the community, especially in *Escherichia coli* (4). OXA-181 is a close derivative of OXA-48 (7) that has been identified in the Indian subcontinent, in China, and in some African

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countries. It differs from OXA-48 by four amino acids while sharing a very similar hydrolytic profile (8).

The epidemiology of carbapenemases in Africa remains quite unknown, since some of the local reports correspond to scattered cases only, and some other external reports are based on observations made in other continents after patient transfers. Nonetheless, OXA-48-like and NDM-like enzymes seem to be the main carbapenemases identified in this continent (4), with the exception of South Africa, where NDM and KPC producers have additionally been found (9, 10). Isolates producing OXA-48-like enzymes seem to be predominant in the Northern part of the continent (4, 10), and strains producing OXA-181 are widespread in South Africa, Nigeria, and Angola (11–13). All of those areas are in mainland Africa and have connections to many other countries, which may explain the recurrent importations and spread of those resistance determinants.

Our aim was to investigate a remote African area, with limited population exchanges and limited access to antibiotic usage. Hence, we investigated the occurrence and genetic characteristics of either carbapenem-, pan-aminoglycoside-, or polymyxin-resistant *Enterobacteriaceae* recovered from rectal samples of pediatric patients in a hospital of São Tomé and Príncipe.

RESULTS

From the 50 patients, a total of 36 isolates were recovered on the different selective media used. Those 36 isolates were recovered from 23 different patients, who were all hospitalized for more than 48 h. When several isolates presented the exact same phenotype, only one was retained for further analysis. A total of 34 carbapenem-resistant enterobacterial isolates were recovered, of which 30 were *Escherichia coli* and 4 were *K. pneumoniae*. The rate of colonization by carbapenem-resistant *Enterobacteriaceae* among patients was therefore 44% (22/50). They were all found to produce the same carbapenemase, OXA-181. Nine isolates (recovered from eight patients; colonization rate, 16%) were found to be pan-resistant to aminoglycosides, and all possessed the *rmtB* 16S rRNA methylase gene. Noticeably, 8 of the 9 *rmtB*-positive strains coharbored the $bla_{\text{OXA-181}}$ gene.

A single colistin-resistant *E. coli* isolate was also recovered, being positive for the *mcr-1* gene. That isolate did not exhibit any other resistance, being fully susceptible to β-lactams.

A majority of the $bla_{\text{OXA-181}}$-positive isolates copossessed the extended-spectrum β-lactamase (ESBL)-encoding gene $bla_{\text{CTX-M-15}}$. All the 30 $bla_{\text{OXA-181}}$-positive *E. coli* isolates coharbored $bla_{\text{TEM-1}}$, and 60% (18/30) coharbored $bla_{\text{CTX-M-15}}$. Moreover, 8 of those 18 $bla_{\text{CTX-M-15}}$- and $bla_{\text{OXA-181}}$-positive isolates also harbored the RMTase gene *rmtB*. Four of the five *K. pneumoniae* isolates harbored the $bla_{\text{OXA-181}}$ gene, with three of them copossessing $bla_{\text{TEM-1}}$, and a single isolate coharboring $bla_{\text{CTX-M-15}}$. The only *K. pneumoniae* strain that did not possess $bla_{\text{OXA-181}}$ coharbored the $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, and *rmtB* genes (Table 1).

Pulsed-field gel electrophoresis (PFGE) analysis showed a quite high clonality rate among the *E. coli* isolates (data not shown), with four clones (EC-1 to EC-4) identified from the 31 strains. *E. coli* clones EC-1 ($n = 12$), EC-2 ($n = 10$), and EC-3 ($n = 8$) all produced the carbapenemase OXA-181. *E. coli* EC-3 additionally possessed the 16S rRNA methylase gene *rmtB*. Only a single strain was associated with EC-4, corresponding to the *mcr-1*-positive *E. coli* isolate. By using multilocus sequence typing ([MLST] considering alleles *adk-fumC-gyrB-icd-mdh-purA-recA*), we found that those four clones actually corresponded to four novel sequent types (STs), being, however, quite closely related (single locus variants [SLV]) to some previously reported. Indeed, EC-1 (MLST: 550/11/4/8/8/13/2) is an SLV of ST1163, EC-2 (8/977/5/220/8/8/2) an SLV of ST410, EC-3 (20/45/56/106/7/534/46) an SLV of ST167, and EC-4 (635/4/12/1/20/18/7) an SLV of ST1408.

In contrast to that for *E. coli*, a high clonal diversity was identified among the *K. pneumoniae* isolates, with 4 different PFGE profiles identified among the five isolates

TABLE 1 Genetic features associated with the isolates in this collection

Strains[a]	Species	Resistance determinants	Plasmid size (kb)	Incompatibility group[b]	PFGE profile	MIC (μg/ml)[c]					
						IPM	MEM	CAZ	AMK	GEN	CST
Carbapenem resistant											
STP1	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP2	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP3	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP4A	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP5	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP6	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP7	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP8	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP9A	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP10A	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP11A	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP12A	*E. coli*	OXA-181, TEM-1	66	IncX3	EC-1	16	4	32			
STP13A	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP14A	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP15A	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP4B	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP16A	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP9B	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP17	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP18	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP19	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP20	*E. coli*	OXA-181, CTX-M-15, TEM-1	60	IncX3	EC-2	32	8	256			
STP11B	*E. coli*	OXA-181, CTX-M-15, TEM-1, RmtB	ND[d]	IncX3	EC-3	16	2	256			
STP14B	*E. coli*	OXA-181, CTX-M-15, TEM-1, RmtB	ND	IncX3	EC-3	16	2	256			
STP21	*E. coli*	OXA-181, CTX-M-15, TEM-1, RmtB	ND	IncX3	EC-3	16	2	256			
STP22A	*E. coli*	OXA-181, CTX-M-15, TEM-1, RmtB	ND	IncX3	EC-3	16	2	256			
STP15B	*E. coli*	OXA-181, CTX-M-15, TEM-1, RmtB	ND	IncX3	EC-3	16	2	256			
STP16B	*E. coli*	OXA-181, CTX-M-15, TEM-1, RmtB	ND	IncX3	EC-3	16	2	256			
STP10B	*E. coli*	OXA-181, CTX-M-15, TEM-1, RmtB	ND	IncX3	EC-3	16	2	256			
STP11C	*E. coli*	OXA-181, CTX-M-15, TEM-1, RmtB	ND	IncX3	EC-3	16	2	256			
STP11D	*K. pneumoniae*	OXA-181, CTX-M-15	66	IncX3	KP-1	32	8	256			
STP12B	*K. pneumoniae*	OXA-181, TEM-1	66	IncX3	KP-2	64	8	0.25			
STP13B	*K. pneumoniae*	OXA-181, TEM-1	66	IncX3	KP-3	16	4	256			
STP9C	*K. pneumoniae*	OXA-181, TEM-1	66	IncX3	KP-3	16	4	256			
Pan-aminoglycoside resistant											
STP23	*K. pneumoniae*	TEM-1, CTX-M-15, RmtB	ND	ND	KP-4			256	256	256	
Colistin resistant											
STP22B	*E. coli*	MCR-1	54	IncX4	EC-4						32

[a]Different numbers indicate different patients and associated letters, if any, indicate different isolates recovered from the same patient.
[b]Incompatibility group of the plasmid harboring the carbapenemase gene.
[c]IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; AMK, amikacin; GEN, gentamicin; CST, colistin.
[d]ND, not determined.

(KP-1 to KP-4). MLST identified those four clones corresponding to four distinct STs, namely, ST18, ST3346, ST1035, and ST3347.

Mating-out assays followed by plasmid analysis revealed a single ca. 64-kb $bla_{OXA-181}$-bearing plasmid possessing an IncX3 backbone in all the $bla_{OXA-181}$-positive isolates (data not shown). The *mcr-1* gene was identified on a 54-kb plasmid, possessing an IncX4 backbone (data not shown). Mating-out assays and electro-transformation assays performed with *E. coli* isolates coproducing OXA-181 and RmtB remained unsuccessful.

PCR mapping of the $bla_{OXA-181}$-positive isolates was performed to verify whether the close vicinity of the carbapenemase gene was actually similar to that previously identified on plasmid pOXA-181 recovered in Angola (13). As observed with pOXA-181, the insertion sequence IS*Kpn19* was identified upstream of the $bla_{OXA-181}$ gene in all strains. Conversely, the IS*3000* element identified downstream of the $bla_{OXA-181}$ gene in pOXA-181 was not found in our collection.

DISCUSSION

The dissemination of last-resort-antibiotic-resistant bacteria is becoming one of the most important health issues worldwide. In this study, we evaluated the prevalence of clinically relevant antibiotic resistance traits among *Enterobacteriaceae* recovered from patients in the main hospital of São Tomé and Príncipe. We identified a high rate of colonization by carbapenemase-producing enterobacterial isolates (44% of the patients), with OXA-181 being the only carbapenemase identified. This observation is of note, considering that no patient received carbapenem-based antibiotherapy in that hospital, where such antibiotics are not available.

The presence of the $bla_{OXA-181}$ gene is remarkable, since it has been previously identified in other African countries, including Angola, Nigeria, South Africa, and across several Northern African countries. We recently conducted similar studies in one pediatric hospital in Angola, another Portuguese-speaking African country (13, 14). In those studies, a large number of carbapenemase-producing isolates also possessed the $bla_{OXA-181}$ gene, although bla_{NDM}-like genes were also commonly identified. Of note, the four $bla_{OXA-181}$-positive *K. pneumoniae* isolates recovered corresponded to three distinct clones, therefore ruling out a nosocomial transmission in most cases. Those strains did not correspond to known STs identified elsewhere.

In contrast, a clonal relationship was found among the numerous $bla_{OXA-181}$-positive-producing *E. coli* recovered. Considering that most patients had been hospitalized for quite a long period of time, and owing to the very poor conditions of hospitalization of those patients (very limited nursing staff, poor hygiene within the wards, etc.), it is likely that nosocomial transmission was quite frequent in that hospital, explaining such clonal dissemination and such a high rate of multidrug-resistant isolates.

One of the *E. coli* clones (EC-2) possessing $bla_{CTX-M-15}$ was found genetically close to an ST410 background. This is noteworthy, since a series of $bla_{CTX-M-15}$- and $bla_{OXA-181}$-positive ST410 *E. coli* isolates have been identified in Italy (15) and Denmark (16). This clone is also predominant among OXA-181 producers in Canada (17). Furthermore, by investigating ESBL-producing *E. coli* isolates of diverse sources in Germany (wildlife, environment, animal, and humans), Schaufler et al. (18) identified this same ST410 clone in all those sources, highlighting its very wide dissemination.

Of note, a similar self-conjugative $bla_{OXA-181}$-bearing IncX3-type plasmid was identified in São Tomé and Príncipe and in Angola. This plasmid was identified in variable species and strain backgrounds, highlighting its wide spread. Even though this plasmid did not carry any other resistance determinant, and even though OXA-181 does not confer resistance to those molecules, most of the isolates were resistant to broad-spectrum cephalosporins. The reason was that those isolates coharbored an ESBL-encoding gene, namely, the worldwide spread $bla_{CTX-M-15}$ gene, which is also commonly identified coassociated with bla_{OXA-48}-bearing plasmids (4).

Interestingly, the genetic environment of the $bla_{OXA-181}$ gene shared some elements

with those identified in China (19) and in Angola (13, 14). That gene was identified on an IncX3-type plasmid, and upstream it was flanked by IS*Ecp1* truncated by IS*3000*. Remarkably, IncX3-type plasmids harboring the $bla_{OXA-181}$ gene seem to be widespread in Asia. A recent study showed that $bla_{OXA-181}$-positive isolates in Switzerland from fresh vegetables originating from Asia indeed harbored similar genetic features (20). Furthermore, eight isolates in our collection were positive for the 16S rRNA methylase gene *rmtB*, which is also widespread in China, and particularly, among animal isolates (21, 22).

Our data support the importance of surveillance of multidrug-resistant Gram-negative bacteria. This study was done in a remote environment, in a hospital where broad-spectrum and last-resort antibiotics are not available (particularly carbapenems) but where carbapenemase genes are widespread. It further highlights that Africa may act as an important reservoir for OXA-181 producers, as likewise, Asia. Owing to the fact that OXA-181 (like OXA-48) has a peculiar hydrolytic spectrum, including penicillins and carbapenems but sparing broad-spectrum cephalosporins, and is resistant to usual β-lactamase inhibitors (clavulanic acid and tazobactam), we might speculate that the driving factors of selection of this carbapenemase could simply be corresponding to penicillins or penicillin-β-lactamase inhibitor combinations in that geographical area.

MATERIALS AND METHODS

Bacterial isolates and susceptibility testing. A total of 50 rectal swabs were collected from hospitalized children ($n = 50$) during 3 days in March 2018 at the Ayres de Menezes Hospital of Sao Tome, Sao Tome and Principe. Samples were collected from different wards, from children suffering from diverse diseases, including respiratory failures, burns, HIV-related immunodeficiency, malaria, and trauma. Whether patients previously received an antibiotic regimen was quite unclear; nonetheless, it is worth highlighting that among the few antibiotics available, there were amoxicillin, cefuroxime, ciprofloxacin, erythromycin, trimethoprim-sulfamethoxazole, and gentamicin. The samples were incubated in Luria-Bertani (LB) broth (5 ml) overnight. From each sample, one calibrated loop (10 μl) was plated on each of the three selective media, i.e., (i) CHROMagar mSuperCARBA (CHROMagar, Paris, France) (23), (ii) SuperAminoglycoside (Drigalski agar supplemented with 30 μg/ml gentamicin, 30 μg/ml amikacin, 10 μg/ml vancomycin, and 5 μg/ml amphotericin B) (24), and (iii) SuperPolymyxin (eosin methylene blue agar supplemented with 1 μg/ml colistin, 10 μg/ml daptomycin, and 5 μg/ml amphotericin B) (25). The isolates that were selected were identified at the species level using the API20E system (bioMérieux, La Balme-les-Grottes, France). Antimicrobial susceptibility testing was performed using the disc diffusion method, and interpreted according to the CLSI recommendations (26), except for colistin, for which susceptibility was only evaluated by broth microdilution (BMD). Carbapenemase activity was assessed by using the Rapidec Carba NP test (27) for each of the isolates growing on the mSuperCARBA plates. MICs were determined by BMD as recommended by EUCAST (www.eucast.org).

Molecular analysis. The identification of carbapenemase and extended-spectrum β-lactamase (ESBL) genes was performed by PCR using previously reported primers (28–31). Additionally, the aminoglycoside resistance 16S rRNA methylase genes (*armA*, *rmtA* to *rmtG*, and *npmA*) and the colistin resistance *mcr*-like genes (*mcr-1* to *mcr-5*) were searched by PCR (32–35). All positive PCR amplicons were sent for sequencing (Microsynth, Balgach, Switzerland).

Clonal relationship of the isolates was evaluated by pulsed-field gel electrophoresis (PFGE). Total DNA from *K. pneumoniae* isolates and *E. coli* isolates was digested using the XbaI enzyme (New England BioLabs, Ipswich, USA). The generated fragments were separated by PFGE using a CHEF-DR III System (Bio-Rad, Cressier, Switzerland), creating a unique PFGE profile for each clonal strain. Multilocus sequence typing (MLST) (36) was performed for one strain from each of the unique PFGE profiles. Sequence types (STs) were investigated using the online databases (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html and https://enterobase.warwick.ac.uk/) for *K. pneumoniae* and *E. coli* isolates, respectively.

DNA of plasmids harboring the $bla_{OXA-181}$, *mcr-1*, or *rmtB* genes was extracted using the Kieser extraction method (37). Extracted plasmid DNA was then electroporated into *E. coli* TOP10 and selected on LB agar plates supplemented either with temocillin (50 μg/ml) for OXA-181 producers, colistin (1 μg/ml) for MCR-1 producers, and amikacin (50 μg/ml) plus gentamicin (50 μg/ml) for RmtB producers. Plasmid size was evaluated by agarose gel electrophoresis using *E. coli* NCTC50192 as a size control. It harbors four plasmids with sizes of 154, 66, 48, and 7 kb. Plasmids were characterized by PCR-based replicon typing (PBRT) (38). The PCR scheme was complemented with primers specific for the 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′) (13), as well as primers specific for IncX4 plasmids (39). The genetic environment of $bla_{OXA-181}$ was investigated by PCR mapping with primers for insertion sequences IS*Kpn19* and IS*3000*, taking in account that those mobile elements have been previously identified in the close vicinity of the $bla_{OXA-181}$ gene (7, 40).

Mating-out experiments. The azide-resistant *E. coli* strain J53 was used as the recipient in conjugation assays. Donors and recipient strains were incubated separately overnight in LB broth (5 ml). After

incubation, the donor strain and recipient were combined at a ratio of 9:1 (donor/recipient) and centrifuged. The supernatant was removed, and the pellet was resuspended in 1 ml of LB broth, which was plated on a 0.45-μm conjugation filter on an LB agar plate. The plates were incubated for 3 h and then plated on LB agar supplemented with sodium azide (100 μg/ml) and temocillin (50 μg/ml) for $bla_{OXA-181}$-positive plasmids, colistin (1 μg/ml) for *mcr-1*-positive plasmids, and gentamicin (50 μg/ml) plus amikacin (50 μg/ml) for *rmtB*-positive plasmids. Susceptibility testing was performed for *E. coli* transconjugants for which the positivity for the respective resistance genes was checked by PCR.

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An international patent form has been filed on behalf of the University of Fribourg (Switzerland) corresponding to the SuperPolymyxin selective medium.

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