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Polyclonal emergence of MDR *Enterobacter cloacae* complex isolates producing multiple extended spectrum beta-lactamases at Maputo Central Hospital, Mozambique

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Abstract

Enterobacter spp. are important nosocomial pathogens responsible of a wide variety of infections, mainly due to Extended Spectrum β-Lactamase (ESBL) producing isolates, constituting a global public health issue in terms of clinical treatment and infection control, especially in low-income countries, where last-line treatment is often unavailable and there is weak nosocomial surveillance. In this study, we conducted a phenotypic and molecular characterization of 8 clinical *Enterobacter* spp. strains, isolated from patient's blood in three hospitals in Mozambique. Isolates were identified by MALDI-TOF and antimicrobial Susceptibility Testing was performed by VITEK 2 system. Half of isolates were analyzed by PCR for β-lactamases genes, other isolates by Whole Genome Sequencing. We identified all isolates as *Enterobacter cloacae* complex (ECC), those from Maputo Central Hospital were polyclonal, multidrug resistant (5/8), and ESBL producers (50%), carrying $bla_{\text{CTX-M-15}}$ and different assortment of $bla_{\text{SHV-12}}$, $bla_{\text{TEM-1B}}$ and $bla_{\text{OXA-1}}$, and AmpCs $bla_{\text{CMH-3}}$, $bla_{\text{ACT-7}}$ and $bla_{\text{ACT-9}}$ genes. Resistance determinants linked to fluoroquinolone (*aac(6')Ib-cr* and *qnrB1*) and others antimicrobials were also found. Notably, one isolate showed phenotypically resistance to colistin, while another colistin susceptible isolate carried a silent *mcr-9* gene. ECC nosocomial surveillance is urgently needed to contain and prevent the dissemination of ESBLs producing clones, and *mcr-9* spread to other Enterobacteriaceae.

Keywords *E. cloacae* complex · $\text{ESBL}_{\text{CTX-M-15/SHV-12}}$ · *mcr-9* · Mozambique

1 Introduction

Enterobacter is a member of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), which includes the 6 most important nosocomial pathogens (Santajit and Indrawattana 2016; Davin-Regli et al. 2019).

Enterobacter spp., are mainly cause of nosocomial infections, including urinary tract infections (UTI), pneumonia, soft tissue infections, endocarditis and septicemia, while they are less commonly found in community-acquired infections (Ramirez and Giron 2020). *Enterobacter* spp. are associated to multidrug resistance (MDR) phenotypes thanks to their adaptation capability to the hospital environment and their ability to easily acquire resistance and virulence determinants through genetic mobile elements (Uhlemann 2019; Davin-Regli et al. 2019).

José Sumbana and Antonella Santona have contributed equally to the manuscript.

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Enterobacter spp. and in general all Enterobacteriaceae, are particularly resistant to beta-lactams, such as natural and synthetic penicillins and cephalosporins of 2nd and 3rd generation, due to the production of Expanded-Spectrum Beta-Lactamases (ESBLs) (Davin-Regli 2015).

The global emergence of ESBLs represents one of the greatest public health threats in hospitals, and $bla_{\text{CTX-M-15}}$ is the most common ESBL gene distributed globally in different clinical strains of Enterobacteriaceae (Rossolini et al. 2008; Sewunet et al. 2021; Awosile and Agbaje 2021) including *Enterobacter* spp. (Haenni et al. 2016).

Enterobacter spp. isolates harboring $bla_{\text{CTX-M-15}}$ and other antimicrobial resistance, including quinolone, aminoglycoside and more recently carbapenem and colistin determinants (Huang et al. 2012; Kananizadeh 2020), constitute a serious health problem due to the lack of treatments (Lim et al. 2010; Osei Sekyere 2016), and increased mortality worldwide (Fernández et al. 2015; Bonomo et al. 2018; Etemadi et al. 2020; Shawa et al. 2021).

In this study, we characterized 8 clinical isolates of *Enterobacter cloacae* complex strains isolated from bloodstream infections in 3 Mozambican hospitals, 4 of which were analyzed by PCR for β-lactamases genes and the other isolates by Whole Genome Sequencing (WGS).

To the best of our knowledge this is the first report describing clinical multidrug resistant ESBL-producing *Enterobacter cloacae* complex isolates in Mozambique.

2 Materials and methods

2.1 Bacterial isolates

Bacterial isolates were obtained from blood of individual patients at Maputo Central Hospital (MCH), Quelimane Provincial Hospital (QPH) and Quelimane Central Hospital (HCQ) mainly in 2018. Blood samples were collected by aseptically venipuncture in aerobic flasks (Becton–Dickinson, Franklin Lakes, NJ), and transported to the Microbiology Laboratory of Medicine Faculty of Eduardo Mondlane University (MLMF-UEM) and the hospital microbiology laboratories in Quelimane for 5 day culture in BACTEC 9050 instrument (Becton–Dickinson). Preliminary identification of isolates was done through Gram stain and subculture on MacConkey, Chocolate and Blood agar plates at 37 °C overnight, followed by conventional biochemical tests. Bacterial identifications were confirmed by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) (MALDI Biotyper, Bruker Daltonics Inc, USA) at the San Francesco hospital laboratory, Nuoro, Italy.

2.2 Antibiotic susceptibility testing

Vitek 2 compact system including specific card GN377 (bioMérieux, Marcy-l'Etoile, France) was used for Antibiotic Susceptibility Testing (AST) according to the guidelines of the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST_ 2018, http://www.eucast.org/clinical_breakpoints/).

2.3 Polymerase chain detection of β-lactamase genes

Isolates were tested by PCR for several resistance genes encoding for ESBLs (TEM, SHV, CTX-M, CTX-M-2, CTX-M-9, CTX-M-15, GES, VEB, and PER), AmpCs (MOXM, CITM, DHAM, ACC, EBCM, and FOXM) and Carbapenemases (KPC, OXA-48-like, IMP, VIM, and NDM) using specific primers and protocols (Perez-Perez and Hanson 2002; Dallenne et al. 2010; Hijazi et al. 2016) (Table S1a).

2.4 Whole genome sequencing

Bacterial DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), and then quantified for WGS using Nanodrop 1000 Spectrophotometer (ThermoFisher, USA). DNAs were sequenced on Illumina NextSeq platform, at a 30 × coverage (NGS Bio, San Francisco) to obtain short reads, which were assembled into contigs using de novo assembly, SPAdes 3.13.0. web-based tool.

Contigs were subjected to in silico analysis for searching antibiotic resistance determinants by ResFinder 3.2 and typing by MLST 2.0, PlasmidFinder 2.0 and pMLST 2.0, available at the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/).

3 Results

3.1 *E. cloacae* complex *isolates* identification and antimicrobial susceptibilities

Eight clinical *Enterobacter* spp. strains, isolated from blood in 2018 at the Maputo Central Hospital ($n = 5$), at Quelimane Provincial Hospital ($n = 2$) and at Quelimane Central Hospital ($n = 1$) were identified as *Enterobacter cloacae* complex (ECC) by MALDI-TOF.

The ECC isolates were resistant to trimethoprim–sulfamethoxazole (75%), gentamicin (50%), ciprofloxacin and fosfomycin (25%), colistin (13%), and all were susceptible to

Table 1 MIC values (mg/L) of antimicrobial agents for 8 *Enterobacter cloacae* complex isolates from Mozambican hospitals

Antimicrobial agents	Breakpoint for resistance (mg/L)	No. of resistant isolates (%)	MIC data (mg/L) range
Piperacillin–tazobactam	> 16	3 (38%)	≤ 4 to ≥ 128
Cefotaxime	> 2	4 (50%)	≤ 0.25 to ≥ 64
Ceftazidime	> 4	4 (50%)	≤ 0.12 to ≥ 64
Ertapenem	> 1	0	≤ 0.12
Meropenem	> 8	0	≤ 0.25
Amikacin	> 16	0	≤ 1 to 4
Gentamicin	> 4	4 (50%)	≤ 1 to ≥ 16
Ciprofloxacin	> 0.5	2 (25%)	≤ 0.06 to ≥ 4
Tigecycline	> 2	0	≤ 0.5 to 1
Fosfomycin	> 32	2 (25%)	≤ 0.16 to 128
Colistin	> 2	1 (13%)	≤ 0.5 to ≥ 16
Trimethoprim–sulfamethoxazole	> 4	6 (75%)	≤ 0.16 to ≥ 320

MIC Minimum inhibitor concentration

ertapenem, meropenem, amikacin and tigecycline (Table 1). Notably, 50% of isolates were MDR and 63% ESBL producers. The antimicrobial MICs are shown in Table 1.

3.2 Molecular typing and antimicrobial genetic determinants

Four Sequence Type (ST) were identified by in silico Multi Locus Sequence Type (MLST), including ST84, ST125 and two new ST, with new mutations in 3 (*dnaA*, *leuS* and *gyrB*) and 4 (*dnaA*, *leuS*, *pyrG* and *rplB*) alleles, respectively (under submission at the *Enterobacter cloacae* MLST database for STs assignation).

Fifty percent of ECC isolates, all from MCH, were ESBL producing strains (Table 1Sb). The ST84, ST125 clones and one untyped *E. cloacae* complex isolate (SSM111) carried $bla_{\text{CTX-M-15,}}$ and one also carried the bla_{SHV} gene. The bla_{SHV12} was also found in one of the new ST (SSM110). In addition, $bla_{\text{CMH-3}}$ ($n = 1$), $bla_{\text{ACT-7}}$ ($n = 1$), and $bla_{\text{ACT-9}}$ ($n = 1$) genes and other β-lactamase genes, $bla_{\text{TEM-1B}}$ ($n = 3$) and $bla_{\text{OXA-1}}$ ($n = 2$) were found (Table 1Sb).

Aminoglycoside and quinolone resistance were linked to aminoglycosides modifying enzymes [*aac(3)-IIa*, *aac(3)-IId*, *aac(6')-IIC*, *aph(3')-Ia*, *aph(3'')-Ib*, and *aph(6)-Id*] and plasmid mediated quinolone resistance genes [*aac(6')Ib-cr* and (*qnrB1*)], respectively, Moreover, we identified aminoglycoside adenyltransferases (*aadA1* and *aadA2*) encoding resistance to streptomycin/spectinomycin agents. Trimethoprim–sulfamethoxazole resistance determinants were *dfrA1* ($n = 1$), *dfrA14* ($n = 2$) and *dfrA19* ($n = 1$).

Furthermore, we also detected sulfonamides (*sul1* and *sul2*), tetracyclines [*tet(A)* and *tet(D)*], fosfomycin (*fosA*), chloramphenicols (*catA1*, *catA2* and *catB3*) and macrolides [*mph(A)* and *ere*(A)] resistance determinants (Table 1Sb).

The ST84 isolate showed colistin phenotypic resistance (MIC > = 16) without harboring plasmid acquired *mcr* colistin resistance genes, while one new ST isolate (SSM110) was susceptible to colistin even if harboring the *mcr-9* gene (Table S1b).

We also checked for *mgrB*, *pmrAB* and *phoQP* genes, described to be involved in colistin resistance in *Enterobacter* spp. The comparison analysis of each gene sequence of our isolates with the ATCC 13047 *E. cloacae* strain (NC_014121) showed several mutations with *pmrAB* having 81% of identity, but none sequences showed missense mutations in the corresponding translated proteins.

We also checked for the *qseCB* gene (linked with *mcr-9* functionality), in the *mcr-9* positive *E. cloacae* complex isolate (SSM110). We detected both *qseBC* genes (Node_2), showing 100% and 99% of nucleotide identity with *Enterobacter hormaechei subsp. hormaechei* strain 34,983, respectively. Of the 3 missense mutations (A353G, TQ376-77AR) on the QseC translated protein (AJB72359), only one (A267V) was also detected in the QseC protein of *Enterobacter hormaechei* CFSAN080736 strain (HAZ0554290).

The CTX-M-15 and SHV-12 ESBLs were likely associated with *Inc*HI2 or *Inc*HI2A (Type 1) plasmids as well as the *mcr-9* gene. *Inc*FIB and *Inc*FII plasmid replicons were also found (Table S1b).

4 Discussion

The global emergence and spread of multidrug resistant Gram-negative pathogens producing β-lactamases, including ESBL, AmpC and carbapenemase, has become a serious public health problem because of their association with nosocomial and community-acquired infections worldwide

(Jacoby and Munoz-Price 2005; Nordmann et al. 2011; Beyene et al. 2019).

ESBLs are often associated with other resistance genes (e.g., quinolone PMQR genes, aminoglycoside genes), mainly found within conjugative plasmids or other mobile genetic elements, which can be transmitted intra and interspecies, conferring resistance to antimicrobials extensively used in human and animals (Jiang et al. 2008; Rozwandowicz et al. 2018). This constitutes a worrisome situation especially in low-income countries, where last-line treatments are often unavailable (Meunier et al. 2017; Frost et al. 2019; Annavajhala et al. 2019; Nishida et al. 2020). Moreover, the plasmid-mediated polymyxin resistance (*mcr* genes), are also increasing worldwide (Nang et al. 2019), further decreasing available therapeutic choices.

The emergence of ESBLs among *Enterobacteriaceae*, have been observed mainly in *Klebsiella* spp., *E. coli* but also in *Enterobacter* spp., *Proteus* spp., *Morganella* spp., *Citrobacter* spp., *Providencia* spp., and *Salmonella* spp (Pitout et al. 2005; Haenni et al. 2016).

CTX-M-15 is the most widely distributed $bla_{\text{CTX-M}}$ gene on a global scale among *Enterobacteriaceae,* it has been associated with other ESBLs including carbapenemase and also with colistin resistance determinants (Ribeiro et al. 2016; Zeynudin et al. 2018; Soliman et al. 2020; Awosile and Agbaje 2021).

In this study, we reported the presence of a polyclonal MDR *Enterobacter cloacae* complex circulating in the Maputo Central Hospital, Mozambique. Four out of 5 isolates were ESBL producing strains carrying $bla_{\text{CTX-M-15,}}$ bla_{SHV} genes and additional determinants involved in β-lactam resistance, including $bla_{\text{CMH-3,}}$ $bla_{\text{ACT-7}}$, $bla_{\text{ACT-9,}}$ $bla_{\text{TEM-1B}}$, and $bla_{\text{OXA-1}}$ genes.

CTX-M-15 has been reported in Mozambique, particularly in *Klebsiella pneumoniae* and *E. coli* isolates, associated with invasive and non-invasive infections (Pons et al. 2015; Guiral et al. 2018). In addition, recent studies carried out at MCH reported the dominance of CTX-M-15 and AmpC-genes in *E. coli* isolated from urine and blood cultures (Estaleva et al. 2021) and the occurrence of a pandemic *E. coli* ST405 clone coharboring $bla_{\text{NDM-5}}$ and $bla_{\text{CTX-M-15}}$ (Sumbana et al. 2021).

Always in Maputo, $bla_{\text{CTX-M-15}}$ producing *E. coli* and *Klebsiella* spp., also harboring AmpC genes, were detected from colonized university students (Chirindze et al. 2018).

To date, no ESBL genes were reported in *Enterobacter* spp. in Mozambique, even if third and fourth generation cephalosporin resistant and MDR *Enterobacter* spp. isolates were phenotypically detected, in both pediatric and adult wards at MCH, resulting the most resistance species among enterobacteria at MCH (Mahaluca et al. 2019).

Our study showed *E. cloacae* complex isolates harboring $bla_{\text{CTX-M-15}}$, circulating at the MCH. In the same hospital, *Klebsiella* spp. and *E. coli* isolates, carrying the $bla_{\text{CTX-M-15}}$ were isolated since 2015 (Estaleva et al. 2021; Sumbana et al. 2021), which may have contributed to the spread of the resistance. These finding reinforce the idea that at MCH, penicillins and beta-lactam antibiotics are not suitable for the treatment of infections caused by *Enterobacteriaceae*.

ESBL producing *E. cloacae* complex isolates from this study were MDR, carrying multiple aminoglycoside modifying enzymes and quinolone determinants, further narrowing the therapeutic choices.

Moreover, one isolate (ST84) showed phenotypically resistance to colistin, while a new ST colistin susceptible isolate, carried a silent *mcr-9* gene.

Colistin resistance can be mediated by chromosomal genes (*phoPQ*, *pmrAB*, and *mgrB*), altering the structure of lipopolysaccharides (Esposito et al. 2017; Osei Sekyere 2019), and by plasmid-mediated mobilized colistin-resistance *mcr*-like genes (namely, from *mcr-1* to *mcr*-10) (Osei Sekyere 2019; Wang et al. 2020), where *mcr-1* is the predominant genetic variant in human and other sources in Africa (Olowo-okere and Yacouba, 2020).

Here, we did not find any missense mutation in the PhoPQ, PmrAB two-Component regulatory systems nor in MgrB, the negative feedback regulator of the PhoQ-PhoP signaling system. This validated a recent study, which showed that, unlike in *Klebsiella* spp. and *E. coli,* the PhoQ and PmrB proteins were not overexpressed in *E. cloacae* colistin-resistant isolates, suggesting that the colistin resistance mechanisms might be different in *E. cloacae* when compared to other gram-negative bacteria (Hong and Ko 2019).

Therefore, other mechanisms in *Enterobacter* spp., including overexpression of efflux-pump or overproduction of capsule (Olaitan et al. 2014; Aghapour et al. 2019) may be involved in colistin chromosomal resistance in our isolates and further studies are necessary to clarify the adaptation mechanisms involved in colistin resistance.

We also reported the occurrence of the *mcr-9* gene from a colistin-susceptible *E. cloacae* complex isolate, carrying other antimicrobial resistance genes.

The gene *mcr-9,* mainly carried in *Inc*HI2 plasmids (Li et al. 2020), has been reported in several countries including USA, China, Sweden, and France with human and animal origins (Li et al. 2020; Börjesson et al. 2020). In accordance with our findings, *Enterobacter* spp. harboring *mcr-9,* without expressing the gene product, was previously reported from a pediatric patient in United States hospitals (Kananizadeh 2020) and from Japan (Chavda et al. 2019). The lack of the two potential regulatory genes system (*qseCB*), was shown to play a role in the inducibility of *mcr-9* (Kieffer et al. 2019; Kananizadeh 2020). However, other components as yet undetermined including genes or molecules might

regulate *mcr-9* expression (Clarke and Sperandio 2005; Kananizadeh 2020).

In this study, we detected both *qseCB* genes in the *mcr-9* positive *E. cloacae* complex isolate, showing missense mutations on the QseC protein. However, additional studies are necessary to clarify if these mutations or other mechanisms are implicated in the lack of functionality of the *mcr-9* gene.

The silence of *mcr-9* gene constitutes a concern, since increases MIC have been noted following colistin exposure (Kieffer et al. 2019), and these bacteria may serve as reservoirs of colistin antibiotic resistance without being detected phenotypically. In these isolates, also the use of polymyxins is strongly discouraged.

Bacterial strategies of resistances to polymyxins, including alterations of lipopolysaccharides, utilization of efflux pumps and capsule formation (Olaitan et al. 2014) could also play an important role in *Enterobacteriaceae* isolates at MCH suggesting antimicrobial surveillance reinforcement.

5 Conclusion

There is the need of a rational use of cephalosporins at MCH, due to the high β-lactamases (ESBLs and AmpCs) presence in Enterobacteria and a rational use of colistin since it could activate the silence *mcr-9* gene found in a ECC isolate.

An improvement of the hygiene rules and a large-scale epidemiological surveillance are strongly recommended at MCH to avoid the dissemination of ESBLs and *mcr-9* plasmid among Enterobacteria.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12210-021-01039-4.

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Declarations

Conflict of interest There is no conflict of interest.

Ethics approval and bacterial identification The study was approved by National Health Bioethics Committee (CNBS) of Mozambique with (Ref 78/CNBS/2017) reference.

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