Whole genome-based characterization of extended-spectrum β-lactamase-producing *Enterobacter cloacae* from orthopedic patients and environment of a tertiary referral hospital in Tanzania

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ABSTRACT

Objectives: We investigated the genomic epidemiology of extended-spectrum β-lactamase-producing *Enterobacter cloacae* (ESBL-Ec) isolates from patients and hospital environment to better understand their distribution to help devising effective strategies for infection prevention and control.

Methods: We screened ESBL-Ec at Bugando Medical Center (BMC) in Mwanza, Tanzania. Rectal swabs from orthopedic patients on admission and swabs from the neighboring inanimate environment were collected. Following microbial culture, DNA was extracted from pure ESBL-Ec, and whole-genome sequencing was done. Sequence typing (ST), plasmid replicons, drug resistance, and virulence genes were deciphered using the Rapid Microbial Analysis Pipeline (rMAP).

Results: We obtained 209 ESBL isolates, of which 15 (7.2 %) were ESBL-Ec [8 (53.3 %) from patients and 7 (46.7 %) from the environment]. Seven isolates were novel and eight were diverse, each with a unique ST. All isolates harbored two to five β-lactamase genes, with the predominance of $bla_{CTX-M-15}$ (15/15), bla_{OXA-1} (14/15), bla_{TEM} (14/15) and bla_{ACT} (12/15). The most common non β-lactam drug resistance genes were *aac(3)-IIa* (14/15), *aac (6')-Ib-cr* (14/15), *fosA* (14/15), and *qnrB1* (12/15), *aph(3'')-Ib* (10/15) and *aph(6)-Id* (10/15). Eleven different types of plasmid replicons were identified in 14/15 of the isolates, harboring one to five plasmids, with the most common plasmids being *IncFII* (11/15) and *IncFIB* (10/15). All isolates harbored the outer membrane protein (*omp*A), and curli protein (*csg*) was in 14/15 isolates.

Conclusion: Admitted orthopedic patients and the hospital environment act as a reservoir of ESBL-Ec with diverse STs and endowed with drug resistance and arsenals of virulence genes, calling for their routine screening on admission for mitigation of potential subsequent infections.

1. Introduction

Enterobacter cloacae is a member of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*) group of pathogens which is part of the healthy gut microbiota of humans and animals and is widely distributed in the environment [1,2]. *E. cloacae is* considered to be an important opportunistic human pathogen that has

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emerged as a notorious cause of multidrug-resistant (MDR) nosocomial infections [3]. The emergence and spread of extended-spectrum β-lactamase-producing (ESBL-Ec) with extra non-β-lactam antimicrobial resistance exhausts the treatment options for patients infected with MDR *E. cloacae* [4]. More threatening, is the emergence of a super-resistance bacteria carbapenem-resistant *E. cloacae* that produces carbapenem-hydrolyzing β-lactamase rendering it ineffective to almost all β-lactams families [5,6]. MDR *E. cloacae* can colonize contact surfaces in hospitals and under suboptimal infection control measures in hospitals, the *E. cloacae* colonizing the hospital surfaces can cause subsequent nosocomial infections in vulnerable patient populations [5].

In communities, specifically in low- and middle-income countries, the rampant use of broad-spectrum antimicrobials, such as that observed during the COVID-19 pandemic era [7,8] may facilitate the selection and colonization of MDR *E. cloacae* in the human gut [4]. This results in forming a reservoir that can serve as an endogenous and exogenous source of invasive community-onset as well as nosocomial bloodstream infections, specifically in individuals with debilitating health conditions or hospitalized patients, such as reduced immunity, malignancy, diabetes, and severe illness requiring prolonged hospitalization and antimicrobial therapy like patients with open fractures [4,9]. The escalating burden of road traffic accidents in Sub-Saharan African countries (and Tanzania in particular), and its associated long-term disability and mortality further reiterates an urgent need to institute stringent infection prevention and control measures among orthopedic patients [10,11]. Compared to other ESKAPE members, *E. cloacae* is less studied, particularly on the virulence factors and plasmid replicon profiling.

Furthermore, the information on the distribution of β-lactamase genes and variants among *E. cloacae* is limited despite it being the common opportunistic pathogen and a frequent cause of MDR nosocomial infections. With the development of advanced genome sequencing and bioinformatics analysis thereof, it has become more practical to delineate the β-lactamase genes and their variants, identify the profiles of plasmid replicons, and the presence of drug resistance genes and virulence genes that play key roles in *E. cloacae* infectivity, adherence, invasion, toxin production, evasion of host immunity and persistence.

Understanding the distribution of sequence types (STs), plasmid replicons profiles, arsenals of drug resistance genes, and virulence genes among ESBL-Ec isolates from orthopedic patients and hospital environment is crucial for surveillance and informing strategies for control and prevention of this nosocomial MDR pathogen. Therefore, to better understand their distribution to help devising effective strategies for infection prevention and control, we used whole-genome sequencing (WGS) to unravel the β-lactamase genes and variants thereof, STs, plasmid replicon profiles, non-β-lactam drug resistance genes, and virulence genes of ESBL-Ec isolated from orthopedic patients and the hospital environment of Bugando Medical Center, a tertiary referral hospital in Mwanza, Tanzania.

2. Methods

2.1. Study design and settings

We conducted a cross-sectional study to undertake WGS to determine the ST, β-lactamase genes and variants thereof, non-β-lactam antimicrobial resistance genes, and virulence genes from the ESBL-Ec isolated from the orthopedic patients as well as from their inanimate hospital environment. This study was conducted at the Orthopedic wards of BMC, a tertiary referral hospital in Mwanza, Tanzania.

We screened a study population of 283 patients and 29 inanimate environments (walking clutches, wheelchairs, bathroom sinks, door knobs, floor below patients' beds, and bed rails) and obtained 88 environmental samples. We obtained 209 ESBL isolates, of which 15 (7.2 %) were *E. cloacae* [8 (53.3 %) from patients and 7 (46.7 %) were from hospital inanimate environment] as previously discussed in detail in our recent publication [12]. The selection of ESBL-Ec for WGS was based on

the fact that 3rd generation cephalosporin-resistant Enterobacterales are WHO priority 1 (Critical) pathogens requiring research and development for new antibiotics; and unlike other Gram-negative bacteria, limited studies exist for *E. cloacae* [13,14]. All 15 ESBL-Ec isolates were then taken for DNA extraction and WGS, and their sequences were analyzed in this study.

2.2. Data collection, DNA extraction, and whole-genome sequencing

We analyzed the whole-genome sequence data obtained from all ESBL-Ec isolates from the mother study titled "Understanding Transmission Dynamics and Acquisition of Antimicrobial Resistance at Referral Hospitals and Community Settings in East Africa using Conventional Microbiology and Whole-genome Sequencing" (Grant number GCA/AMR/rnd2/058). The confirmed 15 ESBL-Ec isolates were shipped to Earlham Institute, Norwich, located in the United Kingdom for WGS using the Low Input, Transposase Enabled (LITE) Illumina protocol on the Illumina NovaSeq 6000 platform with short paired-end 150 bp reads.

2.3. Bioinformatics analysis

The analysis of WGS data was done using our previously published Linux command line-based bioinformatics pipeline called "rMAP", the Rapid Microbial Analysis pipeline [15]. Briefly, the whole-genome raw sequences together with the *E. cloacae* reference sequence in fasta format (*E. cloacae* strain AF-401 chromosome, complete genome, Assembly: GCF_001896005., NZ_CP018254.1) were plugged into the rMAP pipeline. All raw sequences were checked for quality in the rMAP pipeline using the embedded FastQC version 0.11.9 [16] to generate individual sample reports using MultiQC version 1.9 [17] for aggregating all the multiple reports into one report. Trimmomatic version 0.39 [18] was used to trim off adapters and remove low-quality reads.

The trimmed reads were loaded into the Shovill version 1.0.9 [19], the Illumina short-read assembler for bacterial genomes for de-novo genome assembly. *K*-mer sizes 31, 55, 79, 103, and 127 were used to determine the optimum genome assembly. Pilon [20] was used for checking assembly errors, correcting ambiguous gaps, insertions, deletions and finally polishing the genomes. Then, the prodigal [21] was used to predict open reading frames from the assembled contigs, and for fast and efficient functional annotation of *E. cloacae* assembled genomes, we used Prokka version 1.14.6 [22].

Antimicrobial resistance profiling was done using AMRfinder version 3.8.4 [23] based on the ResFinder database [24] at default settings. Plasmid replicon profiles and virulence factors were determined from the assembled genomes using PlasmidFinder [25] and the Virulence Factor Database (VFDB) [26], respectively, using the ABRicate version 1.0.1 [27]. Multi-locus sequence typing (MLST) to determine the ST was performed using MLST version 2.19.0 [28] from the *E. cloacae* assembled contigs. Phylogenetic inference by maximum likelihood was performed using MAFFT version 7.471 [29], IQtree version 2.0.3 [30], vcf2phylip version 1.5 [31], and BMGE version 1.12 [32]. The rMAP pipeline collated all the individual variant call files (VCF) into a single VCF containing all the samples and their single nucleotide polymorphisms before being transposed by vcf2phylip into a multi-alignment fasta file. MAFFT software package was used to perform multiple sequence alignment; removal of ambiguously aligned reads as well as extraction of informative sites was performed to infer phylogeny using BMGE. IQtree was then used to test various substitution models and construct trees from the alignments using the maximum-likelihood method in 1000 bootstraps. The resulting trees were visualized in the form of rectangular phylograms and then annotated by iTOL [33] version 6.

3. Results

3.1. Sequence types (ST) and antimicrobial resistance determinants genes of ESBL-producing Enterobacter cloacae

Of the 15 ESBL-Ec isolates, seven (46.7 %) were novel isolates and the rest were so diverse that each isolate had its own ST. These STs were different between clinical and environmental samples. The seven ESBL-Ec isolates from environment were isolated from bathroom sinks (5), wheel chair (1) and ward floor (1). Of the 15 ESBL-Ec isolates from patients and hospital inanimate environment, there were six different β-lactamase genes observed with 14 genetic variants. All isolates harbored three to five β-lactamase gene variants, with bla_{CTX-M} (CTX-M-15 and 3) (15/15)[100 %], bla_{OXA-1} (14/15) [93.3 %] and bla_{ACT} (ACT-5, 6, 7, 9 and 16) (12/15) [80.0 %], bla_{TEM} (TEM-1A and 1B) (14/15)[93.3 %] being the most common β-lactamase genes. The most common non β-lactam drug resistance genes were $aac(3)$-IIa (14/15)[93.3 %], $aac(6')$-Ib-cr (14/15)[93.3 %], $fosA$ (14/15)[93.3 %], $qnrB1$ (12/15)[80.0 %], $aph(3'')$-Ib (10/15)[66.7 %], $aph(6)$-Id (10/15)[66.7 %], $tet(A)$ (8/15)[53.3 %], $sul1$ (8/15)[53.3 %], $sul2$ (8/15)[53.3 %] and $dfrA14$ (8/15)[53.3 %]. Tables 1 and 2.

3.2. Plasmid replicons profile of ESBL-producing Enterobacter cloacae

There were 11 different types of plasmid replicons identified in 93.3 % (14/15) of the genomes of the ESBL-Ec isolates and only one isolate did not harbor any plasmid replicon. The ESBL-Ec harbored one up to five plasmids with the most common plasmids being *IncFII* 11/15 (73.3 %) and *IncFIB* 10/15 (66.7 %). Table 1.

3.3. Virulence determinants genes of ESBL-producing Enterobacter cloacae

All ESBL-Ec harbored the outer membrane protein (*omp*A), and curli protein *(csg)* was observed in 14/15 (93.3 %) isolates. Other virulence genes observed encode for heat-stable enterotoxin-1 *(astA)* 6/15 (40.0 %), long polar fimbrial protein adhesin *(lpfABCE* operon*)* 5/15 (33.3 %), and at least one siderophore production systems (enterobactin [*entAB*] and/or salmochelin *iroBCDEN*]) 5/15(33.3 %). Tables 2 and 4.

Source of isolation, ST, β-lactamase genes, other drug resistance genes, plasmid replicon profiles, and virulence genes for each isolate and the overall distribution thereof are summarized in Tables 1–4.

Table 1
Distribution of 15 ESBL-producing *Enterobacter cloacae* isolates by Source, ST, β-lactamase genes, Other Drug resistance genes and Plasmid replicon profiles.

SN	ID	Source	ST	β-Lactamase genes	Other Drug resistance genes	Plasmid profile
1	ID001	Patient	125	bla_{ACT-9}; $bla_{CTX-M-15}$; bla_{OXA-1}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $dfrA14$; $fosA$; $qnrB1$; $tet(A)$	*IncFIB(pECLA)*; *IncFII (pECLA)*
2	ID002	Patient	826	$bla_{CTX-M-15}$; bla_{MIR-1}; bla_{OXA-1}; bla_{TEM-1B}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $aph(3'')$-Ib; $aph(6)$-Id; $fosA$; $sul2$; $tet(A)$	*Col440I*; *IncFIB*
3	ID013II	Patient	–	bla_{CMH-3}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1B}	$aac(3)$-IIa;$aac(6')$-Ib-cr; $ant(3'')$-Ia; $aph(3'')$-Ib; $aph(6)$-Id; $catA1$; $fosA$; $sul2$	*IncHI2*; *IncHI2A*
4	ID038	Patient	190	bla_{ACT-7}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1B}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $aph(3'')$-Ib;$aph(6)$-Id; $catA1$; $dfrA1$; $dfrA14$; $fosA$; $qnrB1$; $sul1$; $sul2$; $tet(A)$	*IncFII(pECLA)*; *IncHI2*; *IncHI2A*; *IncR*
5	ID086II	Patient	144	bla_{ACT-16}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1B}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $ant(3'')$-Ia; $aph(3'')$-Ib; $aph(6)$-Id; $catA1$; $dfrA14$; $fosA$; $qnrB1$; $sul2$; $tet(A)$	*IncFIB(pECLA)*;*IncFII (pECLA)*
6	ID149	Patient	–	bla_{ACT-6}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1A}	$aac(3)$-IIa;$aac(6')$-Ib-cr; $dfrA15$; $fosA$; $qnrB1$; $sul1$	*IncFIB(pB171)*; *IncFII(Yp)*
7	ID152	Patient	–	bla_{ACT-6}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1A}	$aac(3)$-IIa;$aac(6')$-Ib-cr; $dfrA15$; $fosA$; $qnrB1$; $sul1$	*IncFIB(pB171)*; *IncFII(Yp)*
8	ID259	Patient	414	bla_{ACT-6}; $bla_{CTX-M-3}$; bla_{TEM-1B}	$aadA2$;$aph(3'')$-Ib;$aph(6)$-Id; $dfrA12$; $fosA$; $qnrS1$; $sul1$	-
9	ORTHO013EI	Bathroom sink	–	bla_{ACT-6}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1A}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $dfrA15$; $fosA$; $qnrB1$; $sul1$	*IncFIB(pB171)*; *IncFII(Yp)*
10	ORTHO013EII	Bathroom sink	–	bla_{ACT-16}; bla_{ACT-6}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1A}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $aph(3'')$-Ib; $aph(6)$-Id; $catA2$; $dfrA14$; $dfrA15$; $qnrB1$; $sul1$	*IncFIB(pB171)*; *IncFIB(pECLA)*; *IncFII(pECLA)*; *IncFII(Yp)*
11	ORTHO014EII	Wheel-chair	102	bla_{ACT-5}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1B}	$aac(3)$-IIa;$aac(6')$-Ib-cr;$ant(3'')$-Ia; $aph(3'')$-Ib;$aph(6)$-Id;$catA1$; $dfrA14$; $fosA$; $qnrB1$; $sul2$; $tet(A)$	*Col440I*
12	ORTHO025E	Bathroom sink	–	$bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1B}	$aac(3)$-IIa; $aac(3)$-IId;$aac(6')$-Ib-cr; $ant(3'')$-Ia; $aph(3'')$-Ib;$aph(6)$-Id; $catA1$; $dfrA14$; $fosA$; $mph(A)$; $qnrB1$; $qnrE1$; $sul2$; $tet(A)$	*Col440I*; *IncFIB(K)*; *IncFII(Yp)*; *IncHI2*; *IncHI2A*
13	ORTHO039E	Ward Floor	1015	bla_{ACT-7}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1B}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $ant(3'')$-Ia; $aph(3'')$-Ib; $aph(6)$-Id; $catA1$; $dfrA14$; $fosA$; $qnrB1$; $sul2$; $tet(A)$	*IncFII(pECLA)*; *IncHI2*; *IncHI2A*
14	ORTHO042EI	Bathroom Sink	–	bla_{ACT-6}; $bla_{CTX-M-15}$; bla_{OXA-1}; bla_{TEM-1A}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $dfrA15$; $fosA$; $qnrB1$; $sul1$	*IncFIB(pB171)*; *IncFII (Yp)*
15	ORTHO047E	Bathroom Sink	515	$bla_{CTX-M-15}$; bla_{MIR-5}; bla_{OXA-1}; bla_{TEM-1B}	$aac(3)$-IIa; $aac(6')$-Ib-cr; $ant(3'')$-Ia; $aph(3'')$-Ib; $aph(6)$-Id; $catA1$; $dfrA1$; $dfrA14$; $fosA$; $mph(A)$; $qnrB1$; $sul1$; $sul2$; $tet(A)$	*IncHI2*; *IncHI2A*; *IncR*

Table 2

Distribution of 15 ESBL-producing *Enterobacter cloacae* isolates by Source, ST, and virulence genes.

SN	ID	Source	ST	Virulence genes
1	ID001	Patient	125	*csgG; ompA*
2	ID002	Patient	826	*csgG; ompA*
3	ID013II	Patient	–	*csgG; ompA*
4	ID038	Patient	190	*csgG; entB; iroB; iroC; iroD; iroE; iroN; ompA*
5	ID086II	Patient	144	*ompA*
6	ID149	Patient	–	*astA; csgG; lpfA; lpfB; lpfC; lpfE; ompA*
7	ID152	Patient	–	*astA; csgG; lpfA; lpfB; lpfC; lpfE; ompA*
8	ID259	Patient	414	*csgG; entB; ompA*
9	ORTHO013EI	Bathroom sink	–	*astA; csgG; lpfA; lpfB; lpfC; lpfE; ompA*
10	ORTHO013EII	Bathroom sink	–	*astA; csgG; lpfA; lpfB; lpfC; lpfE; ompA*
11	ORTHO014EII	Wheel-chair	102	*csgG; entA; ompA*
12	ORTHO025E	Bathroom sink	–	*csgG; entA; ompA*
13	ORTHO039E	Ward Floor	1015	*csgG; iroB; iroC; iroD; iroE; iroN; ompA*
14	ORTHO042EI	Bathroom Sink	–	*astA; csgG; lpfA; lpfB; lpfC; lpfE; ompA*
15	ORTHO047E	Bathroom Sink	515	*astA; csgG; ompA*

Table 3

Distribution of Drug resistance genes among 15 ESBL-producing *Enterobacter cloacae* isolates.

Gene	Antibiotic Resistance	Number (n)	Percent (%)
aac(3)-IIa	Gentamicin, tobramycin	14	93.3
aac(6′)-Ib-cr	Ciprofloxacin, gentamicin	14	93.3
fosA	Fosfomycin	14	93.3
qnrB1	Ciprofloxacin	12	80.0
aph(3″)-Ib	Streptomycin	10	66.7
aph(6)-Id	Streptomycin	10	66.7
tet(A)	Doxycycline, tetracycline	8	53.3
sul1	Sulfamethoxazole	8	53.3
sul2	Sulfamethoxazole	8	53.3
dfrA14	Trimethoprim	8	53.3
catA1	Chloramphenicol	7	46.7
ant(3″)-Ia	Streptomycin	6	40.0
dfrA15	Trimethoprim	5	33.3
mph(A)	Erythromycin, azithromycin, Spiramycin, telithromycin	2	13.3
dfrA1	Trimethoprim	2	13.3
catA2	Chloramphenicol	1	6.7
aac(3)-IId	Gentamicin, tobramycin	1	6.7
dfrA12	Trimethoprim	1	6.7
qnrE1	Ciprofloxacin	1	6.7
qnrS1	Ciprofloxacin	1	6.7
aadA2	Streptomycin	1	6.7

3.4. Phylogenetic relatedness of ESBL-producing Enterobacter cloacae

In phylogenic relatedness analysis, one isolate, ID086II with ST 144 from a patient accumulated more mutations than other ESBL-producing *Enterobacter cloacae* depicted in the phylogenetic tree. Furthermore, isolates from patients and those from the environment clustered in two different clusters (ID002 from the patient and ORTHO047E from the environment; ID0259 from the patient and ORTHO039E from the environment) (Fig. 1).

4. Discussion

Understanding the genetic diversity (ST), β-lactamase genes and

Table 4

Distribution of virulence genes among 15 ESBL producing *Enterobacter cloacae* isolates.

Virulence protein (Operon)	Virulence gene	Number (n)	Percent (%)
Outer membrane protein A	*ompA*	15	100.0
Curli protein	*csgG*	14	93.3
Heat-stable enterotoxin 1	*astA*	6	40.0
Long polar fimbrial protein (adhesin)	*lpaABCE operon*	5	33.3
Enterobactin biosynthetic system (*entA, entB* operon; *ironBCDEN* operon)	*entA*	2	13.3
	entB	2	13.3
	iroBCDEN	2	13.3

variants thereof, plasmid replicons profiles, drug resistance and arsenals of virulence genes of ESBL-Ec isolates from admitted patients and hospital environment is of great significance to prevention and control of this nosocomial MDR pathogens in a local setting. It also sheds light on the current global progress in the development of innovative strategies to combat this MDR and virulent nosocomial pathogen. In this study, we reveal ESBL-Ec with high frequency of several drug resistance genes beyond β-lactam drugs, virulence genes, and high content of plasmids, from patients as well as from the hospital environment, a situation that may predispose these patients with fractures to the acquisition of severe nosocomial infections and therefore sets an alarm for strengthening surveillance and nosocomial infection prevention measures on the need to expand the scope of priority pathogens by introducing a routine screening of *E. cloacae*.

We found a high diversity of genotypes as each isolate had its own ST indicating no evidence of clonal spread. However, 46.7 % of isolates were novel. Our finding that all isolates harbored three to five β-lactamase genes, with *blaCTX-M-15, blaOXA-1, blaTEM* and *blaACT* being the most common is similar to a recent study in Nepal [4]. The predominance of *blaCTX-M-15* gene variant is reiterated in the global review and meta-analysis [34]. The detection of multiple β-lactamase genes in extended-spectrum β-lactamase (ESBL)-producing *Enterobacter cloacae* from orthopedic patients and the environment of a tertiary referral hospital has significant clinical and public health implications. The presence of these multiple β-lactamase genes underscores the pathogen's capacity to degrade a broad range of β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems. This severely limits treatment options for infections, complicating patient management and leading to higher morbidity and mortality rates, particularly among vulnerable populations such as orthopedic patients undergoing invasive procedures.

Additionally, the findings suggest that the hospital environment, alongside the patients, can act as a reservoir for these resistant organisms. This raises the risk of nosocomial transmission of the bacteria, which could increase infection rates among hospitalized patients, especially those with compromised immune systems or undergoing surgery.

The presence of resistant ESBL-producing *Enterobacter cloacae* strains in the hospital environment further complicates infection prevention and control efforts. Addressing this challenge requires stricter hygiene protocols, regular surveillance, and robust antimicrobial stewardship to prevent outbreaks and control the spread of resistant bacteria. Moreover, the findings highlight the urgent need for hospitals to revise their antibiotic treatment guidelines, ensuring therapies remain effective against resistant strains. This may prompt a shift towards using alternative or combination therapies, which could be more costly and potentially more toxic.

On a larger scale, the identification of multiple β-lactamase genes points to the escalating challenge of antimicrobial resistance (AMR) in healthcare settings. It emphasizes the importance of continuous
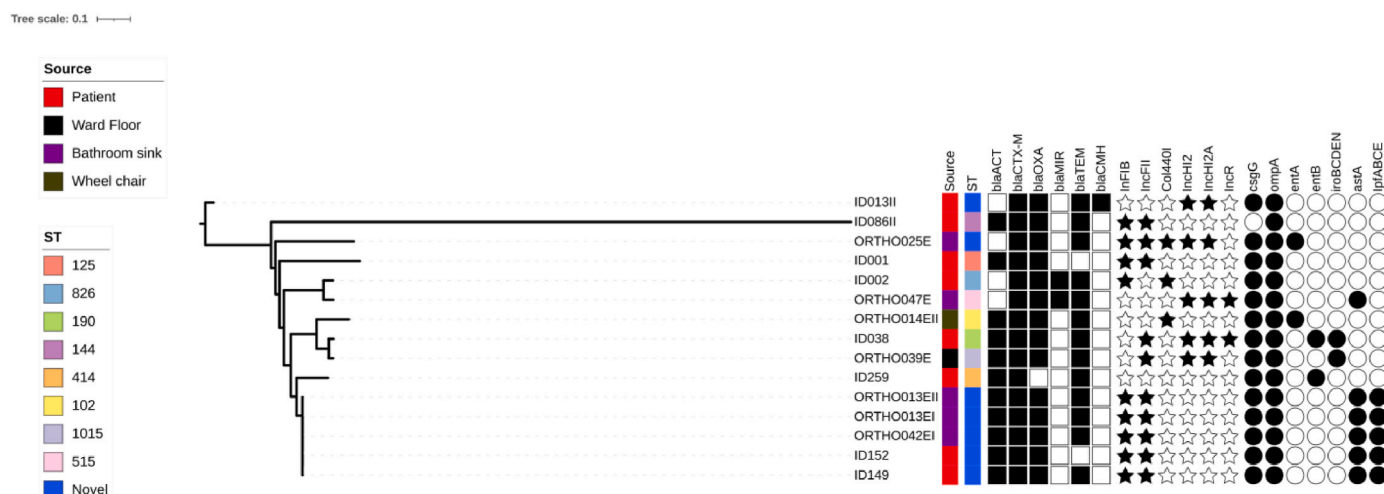
Fig. 1. Phylogenetic relatedness of ESBL-producing *Enterobacter cloacae* from patients and hospital environment of Bugando Medical Center, a tertiary hospital in Mwanza, Tanzania. ID086II with ST 144 from a patient accumulated more mutations than other ESBL-producing *Enterobacter cloacae*. Isolates from patients and those from the environment clustered in two different clusters (ID002 from the patient and ORTHO047E from the environment; ID0259 from the patient and ORTHO039E from the environment).

surveillance, rapid diagnostics, and the development of new antimicrobial therapies to combat these highly resistant organisms. There is a critical need for ongoing monitoring of drug resistance patterns in healthcare environments, along with the implementation of robust infection control measures, to protect patient health and prevent the spread of resistant pathogens.

Our study found that the most common non-β-lactam antimicrobial resistance genes were *aac(3)-IIa* (gentamicin, tobramycin), *aac(6')-Ib-cr* (aminoglycosides and fluoroquinolones) and *qnrB1* (ciprofloxacin), *fosA* (fosfomycin), *aph(3'')-Ib* (streptomycin), *aph(6)-Id* (streptomycin), *tet(A)* (doxycycline, tetracycline), *sul1* and *sul2* (sulfamethoxazole), and *dfrA14* and *dfrA15* (trimethoprim), *catA1* (chloramphenicol) and *ant (3'')-Ia* (streptomycin). This high frequency of resistance genes to aminoglycosides, quinolones, and sulfamethoxazole in ESBL-Ec isolated from orthopedic patients, and neighboring inanimate environment is alarming as this situation predisposes these patients with open fractures to the acquisition of severe nosocomial infections, a situation that sets an alarm for strengthening AMR surveillance and IPC.

Furthermore, the resistance of ESBL-Ec isolates to quinolones, aminoglycosides, and sulfamethoxazole, which are the front-line and second-line antibiotics currently used for treating common bacterial infections in Tanzania, emphasizes the need to continuously revise the local guidelines used for optimal empirical therapy for bacterial infections and devise proper control strategies and further research to combat antibiotic resistance. The presence of gene *aac (6')-Ib-cr* which encodes *aminoglycoside acetyltransferase* that increases minimum inhibitory concentration (MIC) against aminoglycosides and fluoroquinolones reveals a high prevalence of resistance to these two antimicrobials [35]. Furthermore, this high prevalence could be explained by the fact that these antibiotics are used as front-line drugs to treat infections such as UTI, bacteremia, and wound sepsis, which are the leading cause of hospital visits. This level of antimicrobial resistance of ESBL-Ec to these front-line antibiotics is of great concern as it limits the therapeutic options to treat these infections, and therefore highlights the growing threat of the emergence of pan-drug resistance in ESBL-Ec [5,6]. The finding of ESBL-Ec possessing several β-lactamase genes with multiple non β-lactam drug resistance could be due to the fact that *E. cloacae* reside in the gastrointestinal tract with other bacteria providing the suitable niche for horizontal gene transfer via conjugation.

In our study, only one ESBL-Ec did not harbor any plasmid, and the rest of the isolates 93.3 % harbored one up to five plasmids with the most common plasmids incompatibility groups being *IncFII* and *IncFIB* [4].

Our finding is similar to a previous study in Nepal and slightly different from the global distribution of plasmid replicons of ESBL-producing *E. cloacae,* in which the most common plasmid replicons were *IncHI2, IncHI2A, IncFII, IncCol, IncFIB,* and *IncR*. Furthermore, we observed the highest number of plasmids per isolate than that reported from a study done in Nigeria [36], which observed 3 plasmids per isolate compared to 5 plasmid replicons in our study. Of note, antibiotic-resistant genes encoding multiple resistance to antibiotic classes such as fluoroquinolones, aminoglycosides, and β-lactams borne on plasmids are capable of bacteria-to-bacteria transmission via horizontal gene transfer through conjugation among bacterial communities[37–39]. This poses a danger in the effort to control the spread of antibiotic resistance.

Our findings highlight that all ESBL-Ec isolates harbor the *ompA* gene encoding for outer membrane protein A. The other most common virulence genes observed encode for curli protein (*csg*), heat-stable enterotoxin 1 (*astA*), long fimbrial protein (*lpaABCDE* operon)*,* and siderophores (*entAB* encoding for enterobactin and for salmochelin) biosynthesis. The outer membrane protein (OmpA) is a major protein located on the outer surface of *E. cloacae* and the pathogen employs it to induce host damage. This study also shows that almost all isolates studied (93.3 %) have a gene encoding for curli protein (*csg*) and long polar fimbria (*lpfABCDE operon*) for adhesions. Adhesion allows *E. cloacae* to attach to the host surface, which is the first step in the colonization of their preferred host niche.

Iron is an essential trace element nutrient for humans and for nearly all pathogens to human [40], it is used for metabolic processes such as respiration, DNA synthesis and repair. Therefore, for a pathogen to acquire this essential iron nutrient, it must possess a mechanism to actively sequester iron from the host iron chelators protein in order to successfully colonize human. *E. cloacae* and other pathogens have evolved a mechanism to produce siderophores, which are small non-ribosomal peptide that sequester iron from the host chelators with incredible high iron binding affinity to outcompete human iron chelators [40]. The repertoire of siderophores expressed by a pathogen commensurate with the degree of virulence, pathogenicity and fitness for survival [41]. Furthermore, two out of five isolates harbored *astA* gene encoding for heat-stable enterotoxin 1, which is said to induce diarrhea in humans, especially children and young farm animals such as piglets and calves [42].

This study is limited by the low sample size of isolates investigated to limit the association between the β-lactamase genes and variants thereof with plasmid replicons. However, it sheds light on the content and

distribution of β-lactamase genes, STs, plasmid replicon profiles, drug resistance, and virulence genes from ESBL-Ec isolates from admitted patients and from hospital environment. Furthermore, this study provides critical information that is key to devising effective control and prevention strategies.

In conclusion, admitted orthopedic patients and the hospital environment harbor ESBL-producing *E. cloacae* with different STs and endowed with drug-resistance genes and arsenals of virulence genes, highlighting the necessity to routinely screen orthopedic patients.

5. Data availability statement

All source code for the rMAP pipeline, installation instructions, and implementation can be accessed via GitHub (https://github.com/GunzIvan28/rMAP). The source code is available on GitHub under the GPL3 license. Questions, bugs, or any other issues can be filed as GitHub issues. Although rMAP itself is published and distributed under a GPL3 license, some of its dependencies bundled within the rMAP volume are published under different license models.

The raw read files from this study are publicly available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the study BioProject ID: PRJNA971372 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA971372?reviewer=986g6bole2a90fj96k6a5uoet2).

Ethical approval

This study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Joint CUHAS/BMC Research and Ethics Committee (CREC/409/2019) and the National Health Research Ethics Review Committee of the National Institute for Medical Research (NIMR/HQ/R.8a/Vol.IX/3322) in Tanzania. Informed consent was obtained from all study participants. All information obtained was coded and kept confidential.

CRediT authorship contribution statement

Benson R. Kidenya: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing, Software, Supervision. **Gerald Mboowa:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing, Supervision. **Ivan Sserwadda:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Stephen Kanyerezi:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Esther Nakafu:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Inyasi Lawrence Akaro:** Data curation, Investigation, Methodology, Project administration, Writing – review & editing. **Baraka Mkinze:** Data curation, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. **Moses L. Joloba:** Investigation, Methodology, Project administration, Supervision, Writing – review & editing. **Jeremiah Seni:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

All Authors of this Manuscript declare that there is no any form of conflict of interest exists.

Abbreviations

AMR	antimicrobial resistance
CUHAS	Catholic University of Health and Allied Sciences
ESBL	extended-spectrum β-lactamase
ESBL-Ec	extended-spectrum β-lactamase producing *Enterobacter cloacae*
IPC	infection and prevention control
MDR	multi-drug resistance
DR	drug resistance
rMAP	Rapid Microbial Analysis Pipeline
STs	sequence types

References

[1] Keller R, Pedroso MZ, Ritchmann R, Silva RM. Occurrence of virulence-associated properties in Enterobacter cloacae. Infect Immun 1998;66:645–9.

[2] Dudley DJ, Guentzel MN, Ibarra MJ, Moore BE, Sagik BP. Enumeration of potentially pathogenic bacteria from sewage sludges. Appl Environ Microbiol 1980;39:118–26. https://doi.org/10.1128/aem.39.1.118-126.1980.

[3] Davin-Regli A, Pagès J-M. Enterobacter aerogenes and Enterobacter cloacae; versatile bacterial pathogens confronting antibiotic treatment. Front Microbiol 2015;6.

[4] Manandhar S, Nguyen Q, Nguyen Thi Nguyen T, Pham DT, Rabaa MA, Dongol S, et al. Genomic epidemiology, antimicrobial resistance and virulence factors of Enterobacter cloacae complex causing potential community-onset bloodstream infections in a tertiary care hospital of Nepal. JAC Antimicrob Resist 2022;4: dlac050. https://doi.org/10.1093/jacamr/dlac050.

[5] Davin-Regli A, Lavigne J-P, Pagès J-M. Enterobacter spp.: update on taxonomy, clinical aspects, and emerging antimicrobial resistance. Clin Microbiol Rev 2019; 32:e00002. https://doi.org/10.1128/CMR.00002-19.

[6] Annavajhala MK, Gomez-Simmonds A, Uhlemann A-C. Multidrug-resistant Enterobacter cloacae complex emerging as a global, diversifying threat. Front Microbiol 2019;10.

[7] Nandi A, Pecetta S, Bloom DE. Global antibiotic use during the COVID-19 pandemic: analysis of pharmaceutical sales data from 71 countries, 2020–2022. eClinicalMedicine 2023;57. https://doi.org/10.1016/j.eclinm.2023.101848.

[8] Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Aguilar GR, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet 2022;399:629–55. https://doi.org/10.1016/S0140-6736(21)02724-0.

[9] Sanders WE, Sanders CC. Circumventing antibiotic resistance in specialized hospital units. Clin Microbiol Infection 1997;3:272. https://doi.org/10.1111/j.1469-0691.1997.tb00613.x.

[10] Bonnet E, Lechat L, Ridde V. What interventions are required to reduce road traffic injuries in Africa? A scoping review of the literature. PLoS One 2018;13:e0208195. https://doi.org/10.1371/journal.pone.0208195.

[11] Sawe HR, Milusheva S, Croke K, Karpe S, Mohammed M, Mfinanga JA. Burden of road traffic injuries in Tanzania: one-year prospective study of consecutive patients in 13 multilevel health facilities. Emerg Med Int 2021;2021:4272781. https://doi.org/10.1155/2021/4272781.

[12] Seni J, Akaro IL, Mkinze B, Kashinje Z, Benard M, Mboowa G, et al. Gastrointestinal tract colonization rate of extended-spectrum beta-lactamase-producing gram-negative bacteria and associated factors among orthopaedic patients in a tertiary hospital in Tanzania: implications for infection prevention. Infect Drug Resist 2021;14:1733–45. https://doi.org/10.2147/IDR.S303860.

[13] Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis 2018;18:318–27. https://doi.org/10.1016/S1473-3099(17)30753-3.

[14] Seni J, Peirano G, Okon KO, Jibrin YB, Mohammed A, Mshana SE, et al. The population structure of clinical extra-intestinal Escherichia coli in a teaching hospital from Nigeria. Diagn Microbiol Infect Dis 2018;92:46–9. https://doi.org/10.1016/j.diagmicrobio.2018.04.001.

[15] Sserwadda I, Mboowa G. rMAP: the Rapid Microbial Analysis Pipeline for ESKAPE bacterial group whole-genome sequence data. Microb Genom 2021;7:000583. https://doi.org/10.1099/mgen.0.000583.

[16] Babraham bioinformatics - FastQC A quality control tool for high throughput sequence data n.d. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. [Accessed 13 June 2023].

[17] MultiQC n.d. https://multiqc.info/. [Accessed 13 June 2023].

[18] Bolger A.M., Lohse M., Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30(15):2114-2120. doi: 10.1093/bioinformatics/btu170. Epub 2014 Apr 1. PMID: 24695404; PMCID: PMC4103590.

[19] Seemann T. Shovill 2023. Published online June 12, 2023, https://github.com/tseemann/shovill. [Accessed 13 June 2023].

[20] Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One 2014;9:e112963. https://doi.org/10.1371/journal.pone.0112963.

[21] Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinf 2010;11:119. https://doi.org/10.1186/1471-2105-11-119.

[22] Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014; 30:2068–9. https://doi.org/10.1093/bioinformatics/btu153.

[23] AMRFinderPlus - pathogen Detection - NCBI n.d. https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/. [Accessed 13 June 2023].

[24] Bortolaia V, Kaas RS, Ruppe E, Roberts MC, Schwarz S, Cattoir V, et al. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother 2020; 75:3491–500. https://doi.org/10.1093/jac/dkaa345.

[25] Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 2014;58:3895–903. https://doi.org/10.1128/AAC.02412-14.

[26] Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, et al. VFDB: a reference database for bacterial virulence factors. Nucleic Acids Res 2005;33:D325–8. https://doi.org/10.1093/nar/gki008.

[27] Seemann T. ABRicate 2023. Published online June 12, 2023, https://github.com/tseemann/abricate. [Accessed 13 June 2023].

[28] Seemann T. mlst 2023. Published online June 6, 2023, https://github.com/tseemann/mlst. [Accessed 13 June 2023].

[29] Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 2002;30: 3059–66. https://doi.org/10.1093/nar/gkf436.

[30] Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, et al. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol 2020;37:1530–4. https://doi.org/10.1093/molbev/msaa015.

[31] Ortiz EM. vcf2phylip 2023. Published online June 12, 2023, https://github.com/edgardomortiz/vcf2phylip. [Accessed 13 June 2023].

[32] Criscuolo A, Gribaldo S. BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. BMC Evol Biol 2010;10:210. https://doi.org/10.1186/1471-2148-10-210.

[33] Letunic I, Bork P. Interactive Tree of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res 2021;49:W293–6. https://doi.org/10.1093/nar/gkab301.

[34] Bevan ER, Jones AM, Hawkey PM. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. J Antimicrob Chemother 2017;72: 2145–55. https://doi.org/10.1093/jac/dkx146.

[35] Dehbanipour R, Khanahmad H, Sedighi M, Bialvaei AZ, Faghri J. High prevalence of fluoroquinolone-resistant Escherichia coli strains isolated from urine clinical samples. J Prev Med Hyg 2019;60:E25–30. https://doi.org/10.15167/2421-4248/jpmh2019.60.1.884.

[36] Uzeh RE, Adewumi F, Odumosu BT. Antibiotic resistance and plasmid analysis of Enterobacteriaceae isolated from retail meat in Lagos Nigeria. One Health Outlook 2021;3:10. https://doi.org/10.1186/s42522-021-00042-x.

[37] Perez F, Bonomo RA. Can we really use ß-Lactam/ß-Lactam inhibitor combinations for the treatment of infections caused by extended-spectrum ß-lactamase–producing bacteria? Clin Infect Dis 2012;54:175–7. https://doi.org/10.1093/cid/cir793.

[38] Carattoli A. Plasmids and the spread of resistance. International Journal of Medical Microbiology 2013;303:298–304. https://doi.org/10.1016/j.ijmm.2013.02.001.

[39] Brolund A. Overview of ESBL-producing enterobacteriaceae from a nordic perspective. Infect Ecol Epidemiol 2014;4. https://doi.org/10.3402/iee.v4.24555.

[40] Cassat JE, Skaar EP. Iron in infection and immunity. Cell Host Microbe 2013;13: 509–19. https://doi.org/10.1016/j.chom.2013.04.010.

[41] Behnsen J, Raffatellu M. Siderophores: more than stealing iron. mBio 2016;7: e01906. https://doi.org/10.1128/mBio.01906-16.

[42] Pakbin B, Brück WM, Rossen JWA. Virulence factors of enteric pathogenic Escherichia coli: a review. Int J Mol Sci 2021;22:9922. https://doi.org/10.3390/ijms22189922.