Genetic analysis of extended-spectrum β-lactamase-producing *Enterobacteriaceae* from humans and poultry in Zambia.

(ザンビアのヒトおよび家禽から分離された基質特異性拡張型βラクタマーゼ産生腸内細菌の遺伝学的解析)

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Contents

Abbreviations

AGP	Antimicrobial growth promoter
AMR	Antimicrobial resistance
BES	Brazilian extended-spectrum β-lactamase
CLSI	Clinical and laboratory standards institute
CSF	Cerebrospinal fluid
CTX-M	Cefotaximase-Munich
ESBL	Extended-spectrum β-lactamase
GDP	Gross domestic product
HGT	Horizontal gene transfer
IS	Insertion sequence
LB	Luria broth
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MLF	Ministry of livestock and fisheries
MLST	Multilocus sequence typing
OD	Optical density
OXA	Oxacillinase
PMQR	Plasmid-mediated quinolone resistance
PCR	Polymerase chain reaction
PER	Pseudomonas extended resistance
PPV	Positive predictive value
QRDR	Quinolone resistance determining region
SHV	Sulphydryl variable
TEM	Temoneira
TU	Translocatable unit
UTH	The university teaching hospital
VEB	Vietnam extended-spectrum β-lactamase
WGS	Whole-genome sequencing
ZNPHI	The Zambia national public health institute

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Unit measure abbreviations

%	percent
g	gram
kb	kilobase
ml	milliliter
ng	nanogram
µg	microgram
µl	microliter

Notes

The contents of Chapter I have been published in *BMC Antimicrobial Resistance and Infection Control*.

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Preface

Antimicrobials have significantly improved the treatment of many life-threatening bacterial diseases, leading to increased life expectancy [1]. However, as Fleming warned in 1945 [2], misuse and overuse of antimicrobials have accelerated the emergence and evolution of antimicrobial resistance (AMR). The clinical, social, and economic impacts of AMR are profound and enormously worrisome. Over 2.8 million AMR-related cases are reported every year in the United States alone, with over 35,000 resulting in mortality [3]. The year-on-year rise in AMR incidence is attributable to modern anthropogenic elements linked to social, cultural, economic, and political factors. For instance, inaccurate or delayed diagnosis resulting from limited laboratory capacity may compel clinicians to make treatment decisions on a presumptive basis [4], leading to antibiotic overuse and AMR surge. Also, behavioral factors such as self-medication, entwined with a lack of political will to regulate access to antibiotics, have worsened the AMR burden [5]. Additionally, the growing demand for animal-source food due to the steady expansion of the world population promotes antimicrobial usage in livestock feed [6], further exacerbating the problem.

While AMR has a broad scope encompassing several pathogens, multidrug-resistant (MDR) Gram-negative bacteria are most dreaded because of their health impacts in developed and developing countries. Most MDR *Enterobacteriaceae* produce hydrolytic enzymes called extended-spectrum β-lactamases (ESBLs) that degrade β-lactam antibiotics to render them ineffective. Worldwide, over 1.5 billion people are colonized with ESBL-producing strains [7], making ESBL-mediated AMR a critical threat to human health. Despite the many ESBL classes described, the CTX-M-type ESBLs dominate, with reports documented on every populated continent [8, 9]. The CTX-M-type enzymes are encoded by *bla*$_{\text{CTX-M}}$ genes, which usually exist on plasmids with other AMR genes, thus explaining the associated MDR. While controversial, it is believed that most MDR plasmids are costly for the bacterial hosts and tend to be lost in the absence of antibiotic selection [10], making the basis for advocating for antimicrobial stewardship. However, chromosomal *bla*$_{\text{CTX-M}}$ genes have also been reported [11, 12], though their association with MDR is still poorly studied.

Strains carrying *bla*$_{\text{CTX-M}}$ genes are usually found in clinical samples, but non-human sources like food animals have also been identified as reservoirs [13]. Generally, poultry is considered an important hazard for *bla*$_{\text{CTX-M}}$ genes [14], causing zoonotic transmission via the food chain or direct contact. Although research on CTX-M-type ESBLs from the human-animal interface has advanced in Western countries, such studies are relatively rare in sub-

Saharan Africa. Nevertheless, a few integrated studies in Africa have compared human strains to those obtained from poultry [4], despite the One Health concept being relatively new on the continent. In Zambia, a few PCR-based studies have independently characterized CTX-M-producing strains from humans [15, 16] and poultry [17]. However, there is no information linking the transmission of *bla*$_{\text{CTX-M}}$-carrying strains across these ecological niches in Zambia. Given the potential of chickens to transmit *bla*$_{\text{CTX-M}}$-harboring MDR isolates, it is crucial to evaluate the molecular relatedness of *bla*$_{\text{CTX-M}}$-carrying strains from humans and poultry in Zambia.

Chapter I describes the prevalence and diversity of *bla*$_{\text{CTX-M}}$ genes and *bla*$_{\text{CTX-M}}$-carrying chromosomal insertions among clinical strains in three *Enterobacteriaceae* species. The strains were resistant to multiple antibiotics of clinical importance, and the MDR insertions seemed to have been mobilized by the insertion sequence IS*Ecp1*. To verify the epidemiological linkage between poultry and humans in Lusaka, Zambia, Chapter II provides evidence suggesting the clonal transmission of MDR *Escherichia coli* between the two niches.

CHAPTER I:

Novel chromosomal insertions of IS*Ecp1-bla*$_{\text{CTX-M-15}}$ and diverse antimicrobial resistance genes in Zambian clinical isolates of *Enterobacter cloacae* and *Escherichia coli*

Summary

Forty-six cefotaxime-resistant *Enterobacteriaceae* isolates from Zambian hospital patients were sequenced on MiSeq and MinION platforms and reconstructed to nearly complete genomes. Phylogenetic analysis and hierarchical clustering suggested the clonal spread of the strains among patients. *In silico* genotyping detected four alleles of the *bla*$_{\text{CTX-M}}$ gene and 54 other antimicrobial resistance (AMR) genes across 45/46 (97.8%) isolates. The *bla*$_{\text{CTX-M}}$ gene existed on plasmids in 38/45 (84.4%) strains and on chromosomes in the remaining 7/45 (15.6%). In one *Enterobacter cloacae* and three *Escherichia coli* strains, the *bla*$_{\text{CTX-M-15}}$ gene was found on large (> 10 kb) chromosomal insertions bordered by the IS*Ecp1* insertion sequence at one end. The nucleotide sequences of these insertions resembled previously reported plasmids and harbored multiple AMR genes that correlated with the observed phenotypic AMR profiles. These results revealed the coexistence of IS*Ecp1-bla*$_{\text{CTX-M-15}}$ with multidrug resistance (MDR) determinants on the chromosomes of *E. cloacae* and *E. coli*, signifying that IS*Ecp1*-mediated transposition may be essential for the mobilization of various AMR genes from plasmids to chromosomes. Retaining such insertions in the chromosome may enhance the persistence and dissemination of MDR clones, regardless of selection pressure.

Introduction

The growing use of antimicrobials in humans, animals, and agriculture has culminated in selecting drug-resistant microorganisms. Despite significant strides in developing novel antimicrobials, many human infectious diseases are increasingly difficult to treat, making antimicrobial resistance (AMR) a central issue in public health. Some experts regard AMR as the next great catastrophe for humanity as they project it to kill 10 million people every year by 2050 [18]. In Gram-negative bacteria, most AMR cases are related to the production of extended-spectrum β-lactamases (ESBLs). The emergence of ESBL-mediated resistance globally affects every populated continent and represents a significant form of resistance in communities [19, 20] and hospital settings [21].

ESBLs are bacterial enzymes capable of hydrolyzing penicillins, first, second, and third-generation cephalosporins, and aztreonam (but not cephamycins or carbapenems) and therefore conferring resistance to these antibiotics [22]. Thus, ESBL-producers pause serious therapeutic challenges to clinicians during patient management. The ESBL burden is significantly higher among inpatients than those not hospitalized [23] due to the heavy use of antibiotics in hospitals [24]. Escalated hospital ESBL levels have been reported in industrialized countries such as France and China, at 17.7% [25] and 68.2% [26], respectively. Similarly, ESBL is a significant problem among hospitals in developing countries, as evidenced by studies in Mali [27], Burkina Faso [28], and Cote d'Ivoire [29], showing prevalence figures of 62.3%, 70 %, and 86%, respectively. In Zambia, a few PCR-based studies have attributed the observed AMR to ESBLs [15, 16]. However, detailed molecular information characterizing ESBL-producing strains is still lacking.

Although ESBLs only emerged in 1985 [30], there were over 150 documented ESBL variants by the beginning of the 21st century [31], most of which were derivatives of Temoneira types 1 and 2 (TEM-1 and TEM-2), and Sulphydryl variable type 1 (SHV-1) [32, 33]. In addition, non-TEM and non-SHV ESBLs have also emerged, including Cefotaximase-Munich (CTX-M-type), Oxacillinases (OXA-type), Vietnam ESBLs (VEB-type), Brazilian ESBLs (BES-type), and Pseudomonas Extended Resistance (PER-type) β-lactamases. Notably, the frequency of CTX-M-type ESBLs has increased in contemporary times, with over 170 variants [34], making them the most abundant type worldwide [7]. The observed predominance of CTX-M-type ESBLs has been attributed to the success of the *Escherichia coli* O25b:H4-ST131 pandemic clone, which usually carries the plasmid-borne *bla*$_{\text{CTX-M-15}}$ gene [35]. Furthermore, the dissemination of *bla*$_{\text{CTX-M}}$ genes is also propelled by horizontal

gene transfer (HGT) via mobile genetic elements such as insertion sequences (IS) (e.g., IS*Ecp1*, IS*1*, IS*5*, and IS*26*) [36]. In most studies, the IS*Ecp1* insertion sequence has been detected upstream of *bla*$_{\text{CTX-M}}$ genes [37-39], highlighting its role in mobilizing the *bla*$_{\text{CTX-M}}$ genes. As transposons require two inverted repeats (IR) for recognition by the transposase, it is thought that the IS*Ecp1* transposase can use one IR and another sequence related to the other IR to effect the transposition of large genetic elements [40]. Moreover, the IS*Ecp1* has also been shown to be a strong promoter for expressing the *bla*$_{\text{CTX-M}}$ genes [41].

bla$_{\text{CTX-M}}$ genes are generally encoded on large plasmids, which usually carry other AMR genes, leading to multidrug resistance (MDR). While plasmids may confer an ability to withstand antimicrobials, they are perceived to be costly in antibiotic-free environments [42], making antimicrobial stewardship a viable approach to combatting *bla*$_{\text{CTX-M}}$-associated MDR. However, chromosomal *bla*$_{\text{CTX-M}}$ genes are increasingly reported among various *Enterobacteriaceae* [43-48], though their coexistence with other AMR genes is poorly studied. With more reports of the chromosomally located *bla*$_{\text{CTX-M}}$, exploring possible links between chromosomal *bla*$_{\text{CTX-M}}$ and MDR is paramount. This study intended to provide a benchmark for the comparative molecular epidemiology of chromosomal *bla*$_{\text{CTX-M}}$-associated MDR in Zambia and beyond.

By analyzing the genomes and AMR profiles of *Enterobacteriaceae* isolates from Zambian hospital patients, clonal expansion was identified as the primary mode of *bla*$_{\text{CTX-M}}$ spread. Additionally, the nearly complete genome sequences allowed identifying chromosomal insertions of plasmid origin harboring IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ and various AMR genes among *Enterobacter cloacae* and *E. coli*. Furthermore, the strains carrying these insertions displayed MDR phenotypes consistent with the AMR genes in the insertions. These findings suggest that IS*Ecp1*-mediated transposition facilitates the spread of MDR determinants among *Enterobacteriaceae*. Furthermore, the stable maintenance of these chromosomal insertions may enhance the persistence of MDR *Enterobacteriaceae* species.

Materials and methods

Strain selection

From June to October 2018, 46 non-repeated cefotaxime resistant *Enterobacteriaceae* strains were isolated from various clinical sources among patients admitted to the University Teaching Hospital (UTH), Zambia. The strains were isolated from samples collected by hospital clinicians during routine patient care investigations as follows: blood (1), cerebrospinal fluid (CSF) (2), high vaginal swab (1), pus (4), sputum (4), stool (30), and urine (5). To confirm cefotaxime resistance, each strain was plated on LB agar containing 1 μg/ml cefotaxime (Sigma-Aldrich, USA).

Determination of minimum inhibitory concentration (MIC)

MICs of nine antimicrobials were determined by subjecting the strains to broth microdilution using breakpoints specified in Table 1. To this end, the strains were cultured overnight in cefotaxime (1 μg/ml) supplemented LB. The cultures were diluted 10^4-fold, added in triplicate to different antibiotic concentrations in a 96-well plate, and incubated aerobically at 37°C for 18 hours while shaking at 1,600 rpm. Bacterial growth was determined by measuring optical densities at 595 nm (OD_{595}) using the Multiskan FC Microplate Photometer (Thermo Scientific, USA). Positive bacterial growth was considered when the OD_{595} value was at least 0.1, while MIC was defined as the least antibiotic concentration for which the OD_{595} value was less than 0.1. Quality control was performed using the two reference strains, *E. coli* MG1655 and *E. coli* 10-β (NEB, USA).

Table 1. Antimicrobials used in this study

Antimicrobial	Class	Solvent	S	I	R
Ampicillin sodium	β-lactam	DW	≤ 8	16	≥ 32
Cefotaxime sodium	β-lactam	DW	≤ 1	2	≥ 4
Chloramphenicol	Amphenicol	100% ethanol	≤ 8	16	≥ 32
Ciprofloxacin	Quinolone	0.1 M NaOH	≤ 0.25	0.5	≥ 1
Colistin sulfate	Polymyxin	DW	≤ 2	-	≥ 4
Doxycycline hyclate	Tetracycline	DW	≤ 4	8	≥ 16
Gentamicin sulfate salt	Aminoglycoside	DW	≤ 4	8	≥ 16
Imipenem monohydrate	Carbapenem	1 M MOPS	≤ 1	2	≥ 4
Nalidixic acid free acid	Quinolone	0.1 M NaOH	≤ 16	-	≥ 32
Nitrofurantoin	Nitrofuran	DMF	≤ 32	64	≥ 128

NB; All antimicrobials were purchased from Sigma-Aldrich®. LB broth (Difco™) was used.
S; susceptible. I; intermediate. R; resistant.
DW; distilled water. DMF; dimethyl formamide. MOPS; 3-(N-morpholino)propanesulfonic acid, pH 6.8.
Breakpoints are expressed in μg/ml.

Growth rate determination

Growth monitoring was performed by preparing bacterial cultures in antibiotic-free LB and measuring the OD_{600} continuously for 16 hours. Briefly, OD monitoring was performed on duplicates of 10^3-fold-diluted overnight cultures in a 96-well plate at 37°C using the Varioskan LUX Multimode Microplate Reader (Thermo Scientific, USA) while shaking at 600 rpm. The obtained data were used to fit parametric models using the R package grofit version 1.1.1 [49], and the gradients of the fitted lines represented growth rates.

Whole-genome sequencing

The strains were cultured overnight in LB containing 1 μg/ml cefotaxime, and genomic DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). Libraries were prepared using NexteraXT (Illumina, USA) and subjected to sequencing using MiSeq (Illumina), resulting in paired-end reads (2 x 300 bp). Poor-quality reads and adapter sequences were trimmed using Trim Galore version 0.4.2 with options of "--paired --nextera" (https://github.com/FelixKrueger/TrimGalore). The aforesaid genomic DNA was also sequenced with MinION (Oxford Nanopore Technologies, United Kingdom) using Rapid Barcoding Kit (SQK-RBK004) and flowcell R9.5 (FLO-MIN107) to obtain long reads. The long reads were basecalled using Guppy Basecalling Software version 3.4.5 and then assembled using Canu version 1.8 [50], specifying "corOutCoverage = 1000 genomeSize = 6m". Gepard version 1.40 [51] was then used to identify and trim redundant repeats at terminal ends of contigs, followed by base-error correction with trimmed Illumina reads using Pilon version 1.23 [52]. Chromosomal sequences were defined as contigs longer than 2 Mb, while plasmids were identified by the presence of plasmid replicons on contigs smaller than 500 kb. The rest of the sequences were screened for redundancy through BLASTn searches against a local database created for each strain using known chromosomal and plasmid contigs. Query sequences matching the database with ≥ 70% identity were defined as redundant and eliminated from the data pool. Nonredundant query sequences were screened further using NCBI BLASTn searches against the nt database. A contig was considered chromosomal if ≥ 7 of the top 10 hits were chromosomes and plasmid-based if ≥ 7 of the top 10 were plasmids.

Phylogenetic analysis

To elucidate the genetic similarity among strains, whole genome-based phylogenetic analysis was conducted using Parsnp version 1.2 [53] and the generated trees were visualized and edited in TreeGraph 2 [54]. In addition, species and sequence type (ST) identification were performed *in silico* by uploading raw Illumina reads to a public multilocus sequence typing (MLST) platform [55].

Detection of plasmid replicons, strain serotypes, and AMR genes

Plasmid replicons and O:H serotypes were determined by interrogating contigs with PlasmidFinder [56] and EcOH [57] databases, respectively, using ABRicate software version 0.8.10 (https://github.com/tseemann/abricate) with options "--mincov 90" and "--minid 90" specified. AMR genes were identified using the AMRFinderPlus tool [58] with the "-i 0.7" option engaged.

Determination of clustering patterns among strains

To identify the mechanisms propelling the dissemination of *bla*$_{\text{CTX-M}}$ at the UTH, strains were compared based on AMR phenotype, AMR genes, and plasmid replicons using the ComplexHeatmap package [59].

Sequence alignment and identification of chromosomal insertions of *bla*$_{\text{CTX-M}}$

Strains harboring the *bla*$_{\text{CTX-M}}$ gene in the chromosome were annotated using DFAST version 1.2.4 [60] and compared to reference sequences using Mauve [61]. In three *E. coli* strains (i.e., Zam_UTH_18, Zam_UTH_26, and Zam_UTH_41), chromosomal insertions were identified by comparison to the reference strain *E. coli* MG1655 (GenBank accession no. NC_000913.2). For Zam_UTH_44, an *E. cloacae* strain, the alignment was done against *E. cloacae* ATCC 13047 (GenBank accession no. NC_014121.1). Comparison analyses for the two *E. coli* strains, Zam_UTH_42 and Zam_UTH_47, were performed using *E. coli* ST648 (GenBank accession no. CP008697.1) as a reference. Finally, Zam_UTH_43, an *E. coli* strain, was aligned to another *E. coli* strain (Zam_UTH_08) in the data from this study. The comparisons were visualized and further explored using the R package genoPlotR [62].

PCR and Sanger sequencing

To confirm chromosomal insertions and rule out erroneous assembly, junctions between chromosomes and plasmids were subjected to PCR amplification using primers shown in Table 2. When an appropriate control strain was available, primers were designed to amplify the entire insertion, and the product size was compared to what was obtained in the control strain. Furthermore, when the *bla*$_{\text{CTX-M}}$ gene had a poor Illumina read coverage, the exact *bla*$_{\text{CTX-M}}$ allele was determined by PCR and Sanger sequencing using primers shown in Table 3. Briefly, PCR was carried out using KOD One Master Mix (TOYOBO, Japan), and purification of amplification products was achieved using a MinElute PCR Purification Kit (Qiagen). The purified PCR amplicons were subjected to sequencing PCR using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and subsequently sequenced using a 3130 Genetic Analyzer (Applied Biosystems, USA). The sequences were processed and assembled using SnapGene software (Insightful Science, available at snapgene.com), and the obtained contigs were subjected to the AMRFinderPlus tool [58] with the option "-i 0.7".

Ethics approval and consent to participate

The study received ethical approval from the Excellence in Research Ethics and Science Converge with reference number 2015-Feb-018, while the National Health Research Ethics Board approved the Biological Transfer permit. All isolates were collected at the UTH during routine clinical investigations and selected based on resistance to cefotaxime. Patient personal data were anonymized and unlinked to patient identifiers.

277 Table 2. Primers used for the amplification of chromosome-plasmid junctions

Name	Abbreviation	Sequence
Zam_UTH_41_jnc_1_inner_For	F1	GGCAGCCAATGAATCCGC
Zam_UTH_41_jnc_2_inner_Rev	R1	CGCAACAGGTCTTCTATCGACG
Zam_UTH_43_jnc_1_For	F2	CCAGGGATTTTCTACGGCAGG
Zam_UTH_43_jnc_1_Rev	R2	CGGACTCATTCCTCTCAGGATC
Zam_UTH_43_jnc_2_For	F3	GCTGCTGTGCAAAAAACAAGAC
Zam_UTH_43_jnc_2_Rev	R3	CGATGCTAAGCCATTTGCCTG
Str_18_jnc1_innermost_For	F4	CTGCCAGTCCCAGCACTTTG
Str_18_jnc1_inner_Rev	R4	GGCCTCAACACGAATGTCAT
Str_18_jnc2_inner_For	F5	TGCATTCTCAAGGAGCAGAA
Str_18_jnc2_innermost_Rev_1	R5	GTTCGTCAGGCTTTTTCTGGTG
Zam_UTH_42_jnc_1_For	F6	GCAAGAGGATAAACCGTCGGG
Zam_UTH_42_jnc_1_outer_Rev	R6	CCACACCCAGTCTGCCTCC
Zam_UTH_42_jnc_2_For	F7	GCACAACATGGGGGATCATG
Zam_UTH_42_jnc_2_Rev	R7	GGCATATTGCTTTGTGGTGGTG
Zam_UTH_44_jnc_1_For	F8	GTTACCCCGGCGTAGAGG
Zam_UTH_44_jnc_1_Rev	R8	GACCTGGACGTTGTGCTGAAG
Zam_UTH_44_jnc_2_For	F9	GGGCACTATTCATGCGTCAG
Zam_UTH_44_jnc_2_Rev	R9	GACGTTGTGCGCCAGTTC

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280 Table 3. Primers used for the verification of the *bla*$_{\text{CTX-M}}$ allele in seven strains

Strain	Primer	Sequence	Size (bp)
Zam_UTH_03	Zam_UTH_03_For	CGTATCAGCGCTGCATGC	1,730
	Zam_UTH_03_Rev	CTTTGCAACAGTGCCCCG	
Zam_UTH_06	Zam_UTH_06_For	GAGTGTTGCTCTGTGGATAAC	1,324
	Zam_UTH_06_Rev	GTCTGCCTCGTGAAGAAGGTG	
Zam_UTH_22	Zam_UTH_22_For	GGAGCCACGGTTGATGAGG	1,230
	Zam_UTH_22_Rev	GCTCTGTGGATAACTTGCAGAG	
Zam_UTH_25	Zam_UTH_25_For	CAGCGTAGCGGAACGTTC	1,248
	Zam_UTH_25_Rev	GGATTGACCGTATTGGGAGTTTG	
Zam_UTH_26	Zam_UTH_26_For	CGGAAAACTATCCGTACAAGGG	1,456
	Zam_UTH_26_Rev	CCGGCGGAAACAATGAGAAAAC	
Zam_UTH_28	Zam_UTH_28_For	CACCGACATTACACCGGGC	1,458
	Zam_UTH_28_Rev	GCTCTGTGGATAACTTGCAGAG	
Zam_UTH_34	Zam_UTH_34_For	CGCCTCGCCACACTAATC	1,397
	Zam_UTH_34_Rev	CTGATGTAACACGGATTGACC	

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Results

Genetic diversity among *E. coli* strains

This study examined 46 cefotaxime-resistant *Enterobacteriaceae* isolates collected from diverse clinical sources among inpatients at the UTH, Zambia (Table 4). To characterize the genetic diversity among these strains, WGS was performed, and nearly complete genome sequences were reconstructed. The average size of the draft genomes ranged from 4,848,171 to 5,773,466 bp, and mean read depths ranged from 88x to 271x. The number of contigs varied from 1 to 17, with N_{50} values ranging from 788,916 to 5,322,171 bp. An MLST analysis performed *in silico* identified three *Enterobacteriaceae* species, namely, *E. cloacae* (1/46, 2.2%), *K. pneumoniae* (9/46, 19.6%), and *E. coli* (36/46, 78.3%) (Table 4).

Overall, 12 unique *E. coli* sequence types (STs) were identified, of which one was novel and subsequently registered as ST11176. Despite this high heterogeneity of *E. coli* STs, the majority (25/36 69.4%) of the strains were assigned to four STs (i.e., ST69, ST131, ST617, and ST405) (Fig 1A), suggesting that spread is driven by the expansion of a few prominent clones. ST131 disseminates rapidly because of its diverse virulence factors and AMR mechanisms [63], but its frequency here was lower than expected, at 6/36 (16.7%). The most predominant ST was ST69, which accounted for 9/36 (25.0%) strains, highlighting that its circulation in the local hospital significantly contributes to the ESBL burden. The nine *K. pneumoniae* isolates in this study were assigned to three distinct STs, dominated by ST307, representing 6/9 (66.7%) strains (Fig 1B).

304 Table 4. Description of 46 strains used in this study

Strain ID	Source	Species	CTX MIC[a]	Growth rate	Patient Age[b]	Gender
Zam_UTH_01	Stool	*E. coli*	16	0.126	54	M
Zam_UTH_02	Stool	*K. pneumoniae*	≥ 512	0.147	21	M
Zam_UTH_03	Urine	*E. coli*	≥ 512	0.206	25	F
Zam_UTH_04	Stool	*K. pneumoniae*	≥ 512	0.071	28	M
Zam_UTH_05	Stool	*K. pneumoniae*	≥ 512	0.255	52	M
Zam_UTH_06	Urine	*E. coli*	≥ 512	0.166	8	M
Zam_UTH_07	Stool	*K. pneumoniae*	≥ 512	0.101	1	F
Zam_UTH_08	Stool	*E. coli*	≥ 512	0.117	46	F
Zam_UTH_09	Stool	*K. pneumoniae*	128	0.512	36	M
Zam_UTH_10	Stool	*K. pneumoniae*	≥ 512	0.231	65	F
Zam_UTH_11	Stool	*E. coli*	≥ 512	0.149	54	M
Zam_UTH_12	HVS	*E. coli*	256	0.213	23	F
Zam_UTH_13	Stool	*E. coli*	256	0.140	43	F
Zam_UTH_15	Urine	*E. coli*	256	0.120	40	M
Zam_UTH_17	Stool	*E. coli*	256	0.168	3	M
Zam_UTH_18	Pus	*E. coli*	256	0.225	25	F
Zam_UTH_20	Stool	*E. coli*	≥ 512	0.132	92	F
Zam_UTH_21	Stool	*E. coli*	256	0.112	11	M
Zam_UTH_22	Stool	*E. coli*	256	0.195	13	F
Zam_UTH_23	Pus	*E. coli*	≥ 512	0.188	5	M
Zam_UTH_24	Stool	*E. coli*	128	0.185	8	M
Zam_UTH_25	Stool	*E. coli*	128	0.167	73	M
Zam_UTH_26	Stool	*E. coli*	256	0.189	1	F
Zam_UTH_27	Stool	*E. coli*	256	0.118	36	M
Zam_UTH_28	Stool	*E. coli*	64	0.226	36	M
Zam_UTH_29	Urine	*E. coli*	128	0.379	44	F
Zam_UTH_30	Stool	*K. pneumoniae*	≥ 512	0.060	52	F
Zam_UTH_31	Stool	*E. coli*	≥ 512	0.237	25	M
Zam_UTH_32	Stool	*E. coli*	128	0.297	6	F
Zam_UTH_33	Stool	*E. coli*	≥ 512	0.154	70	M

Zam_UTH_34	Stool	*E. coli*	128	0.220	11	F
Zam_UTH_36	Stool	*E. coli*	128	0.285	73	M
Zam_UTH_37	Stool	*K. pneumoniae*	≥ 512	0.254	1	M
Zam_UTH_38	Sputum	*E. coli*	256	0.223	32	M
Zam_UTH_39	Pus	*E. coli*	≥ 512	0.131	64	F
Zam_UTH_40	CSF	*K. pneumoniae*	≥ 512	0.107	N/A	F
Zam_UTH_41	Stool	*E. coli*	≥ 512	0.136	N/A	F
Zam_UTH_42	Pus	*E. coli*	≥ 512	0.145	N/A	M
Zam_UTH_43	Urine	*E. coli*	256	0.230	N/A	F
Zam_UTH_44	Stool	*E. cloacae*	128	0.163	1	M
Zam_UTH_45	Sputum	*E. coli*	256	0.176	32	M
Zam_UTH_46	Sputum	*E. coli*	128	0.160	1	M
Zam_UTH_47	Stool	*E. coli*	≥ 512	0.172	1	M
Zam_UTH_48	Sputum	*E. coli*	128	0.298	27	F
Zam_UTH_50	CSF	*E. coli*	128	0.222	7	M
Zam_UTH_51	Blood	*E. coli*	128	0.221	2	F

[a]CTX MIC is expressed in µg/ml
[b]N/A = not available

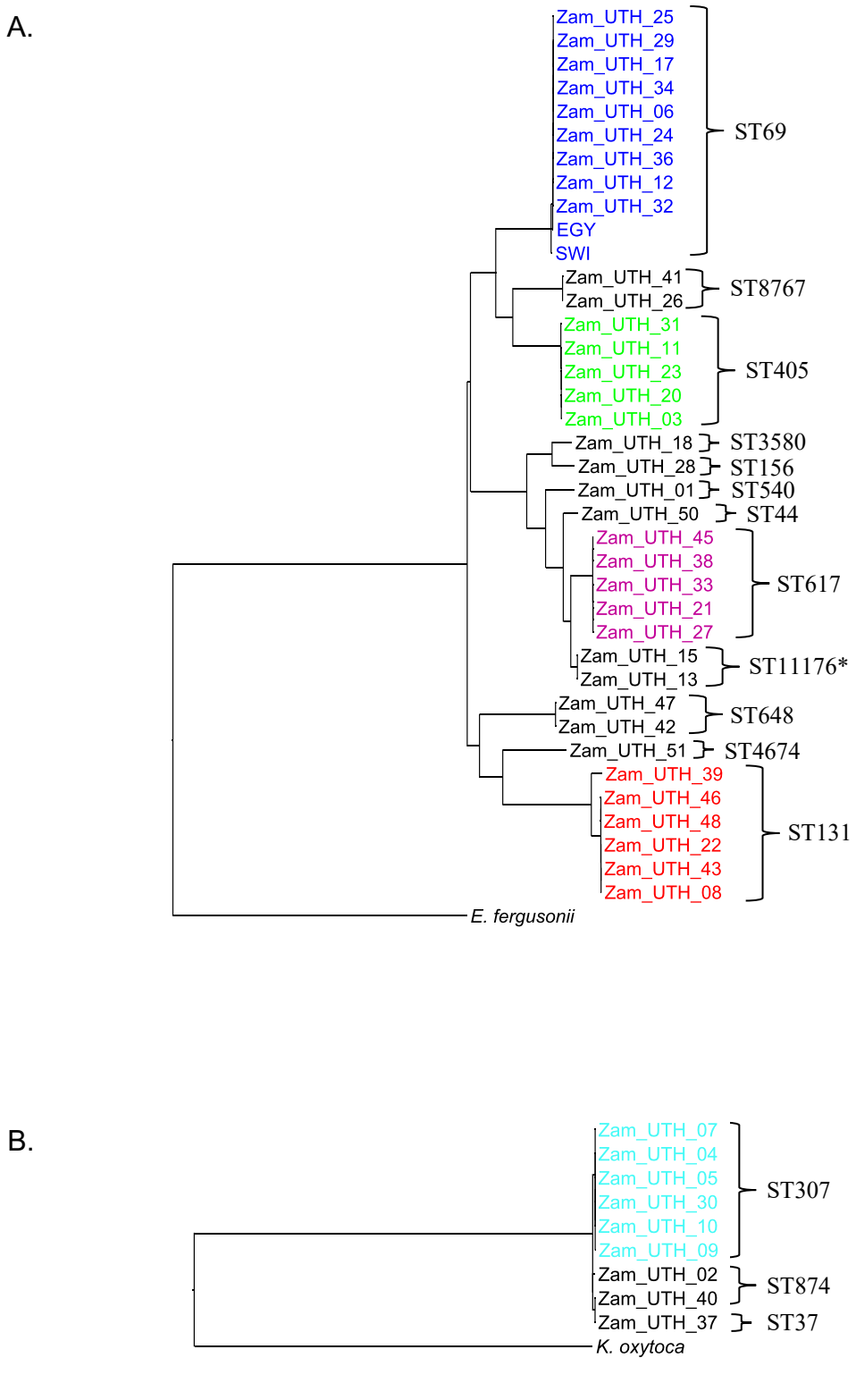

Figure 1. Phylogenetic analysis. Whole genome-based phylogenetic trees for 36 *E. coli* and 9 *K. pneumoniae* strains from Zambia.

A. *E. coli*: Twelve STs were identified, including a novel type (marked with *). Four STs formed 25/36 (69.4%) of *E. coli* strains, dominated by ST69 (n = 9) and ST131 (n = 6). The genome of *E. fergusonii* (GenBank accession number NZ_CP057659.1) was included as an outgroup. In addition, *E. coli* strain CFSAN061770 (GenBank accession number NZ_CP023142.1), belonging to ST69 and isolated from raw milk cheese in Egypt (here abbreviated as EGY), and *E. coli* strain 3347558 (ST69) (GenBank accession number CP071073.1) isolated from a patient in Switzerland (here abbreviated as SWI) were also included in the analysis.

B. *K. pneumoniae*: Of the three STs identified, ST307 alone represented 6/9 (66.7%) strains. The genome of *K. oxytoca* (GenBank accession number NZ_CP027426.1) was included as an outgroup.

368
369 **Location of *bla*$_{CTX-M}$ genes**

370 To characterize the genotypic AMR patterns of the strains, *in silico* prediction was
371 performed on the assembled genomes. Fifty-eight AMR genes were detected across a total of
372 12 AMR classes (Table 5). The *bla*$_{CTX-M}$ family, observed in 45/46 (97.8%) strains, was the
373 most abundant β-lactamase gene class. Thirty-eight out of 45 (38/45, 84.4%) strains carried
374 this gene family exclusively on plasmids, but seven isolates (7/45, 15.6%; one *E. cloacae* and
375 six *E. coli*) harbored chromosomally-located *bla*$_{CTX-M}$. As previously reported [64], *bla*$_{CTX-M-15}$ was the most prevalent (28/45, 62.2%) among the four alleles of the *bla*$_{CTX-M}$ gene (i.e.,
377 *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-27}$, and *bla*$_{CTX-M-55}$) identified (Fig 2). Although the *bla*$_{CTX-M-15}$ gene has often been associated with *E. coli* ST131 [65], only one *E. coli* ST131 (1/6,
379 16.7%) carried this gene (Fig 2), while the other five *E. coli* ST131 strains contained
380 plasmid-borne *bla*$_{CTX-M-27}$. *In silico* prediction of O:H serotypes revealed that none of the six
381 *E. coli* ST131 strains belonged to the pandemic clone O25b:H4-ST131, but the five *bla*$_{CTX-M-27}$-harboring strains belonged to Onovel31:H4 while the *bla*$_{CTX-M-15}$-possessing strain
383 belonged to O107:H5.

384 To determine the phenotypic AMR profiles, MICs for nine antimicrobial agents were
385 determined against each strain. Of the 46 strains tested, 45 (45/46, 97.8%) exhibited MDR
386 patterns, described as resistance to one or more antimicrobial agents from at least three
387 antimicrobial drug classes [66]. Resistance was highest to ampicillin (46/46, 100%) and
388 gentamicin (43/46, 93.5%), followed by ciprofloxacin (41/46, 89.1%) and nalidixic acid
389 (41/46, 89.1%). Although there was no carbapenem resistance detected, one *K. pneumoniae*
390 strain (1/46, 2.2%) exhibited borderline resistance (MIC = 4 μg/ml) to a crucial last-resort
391 drug, colistin (Fig 2). WGS-based prediction of AMR genes was highly concordant with
392 phenotypic resistance, with positive predictive values greater than 80% in most cases (Table
393 6). While earlier reports have shown that a high resistance range could result in a fitness cost
394 [67], the number of AMR genes in these strains did not correlate with fitness (expressed as
395 bacterial growth rate) (Fig 3).

396

397 Table 5. AMR genes detected

Target drug class	AMR genes detected
β-lactam	*bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-84}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{SHV-121}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-27}}$, *bla*$_{\text{CTX-M-55}}$, *bla*$_{\text{ACT}}$, *bla*$_{\text{CMY-2}}$
Aminoglycoside	*aph(3')-Ia, aph(3')-IIa, aph(3'')-Ib, aph(6)-Id, aac(3)-IIa, aac(3)-IId, aac(6')-Ib4, aac(6')-Ib-cr5b, aadA1, aadA2, aadA5,*
Streptothricin	*sat2*
Trimethoprim	*dfrA1, dfrA5, dfrA12, dfrA14, dfrA17*
Tetracycline	*tet(A), tet(B), tet(C), tet(D), tet(M)*
Chloramphenicol	*cmlA1, catA1, catA2, catB3, floR*
Sulfonamide	*sul1, sul2, sul3*
Fosfomycin	*fosA, fosA3*
Macrolide	*erm(B), mph(A)*
Quinolone	*oqxA, oqxB, oqxB19, qnrB1, qnrB19, qnrS1, qnrS13,*
Disinfectant	*qacE, qacL, qacEdelta1*
Bleomycin	*bleO, ble*

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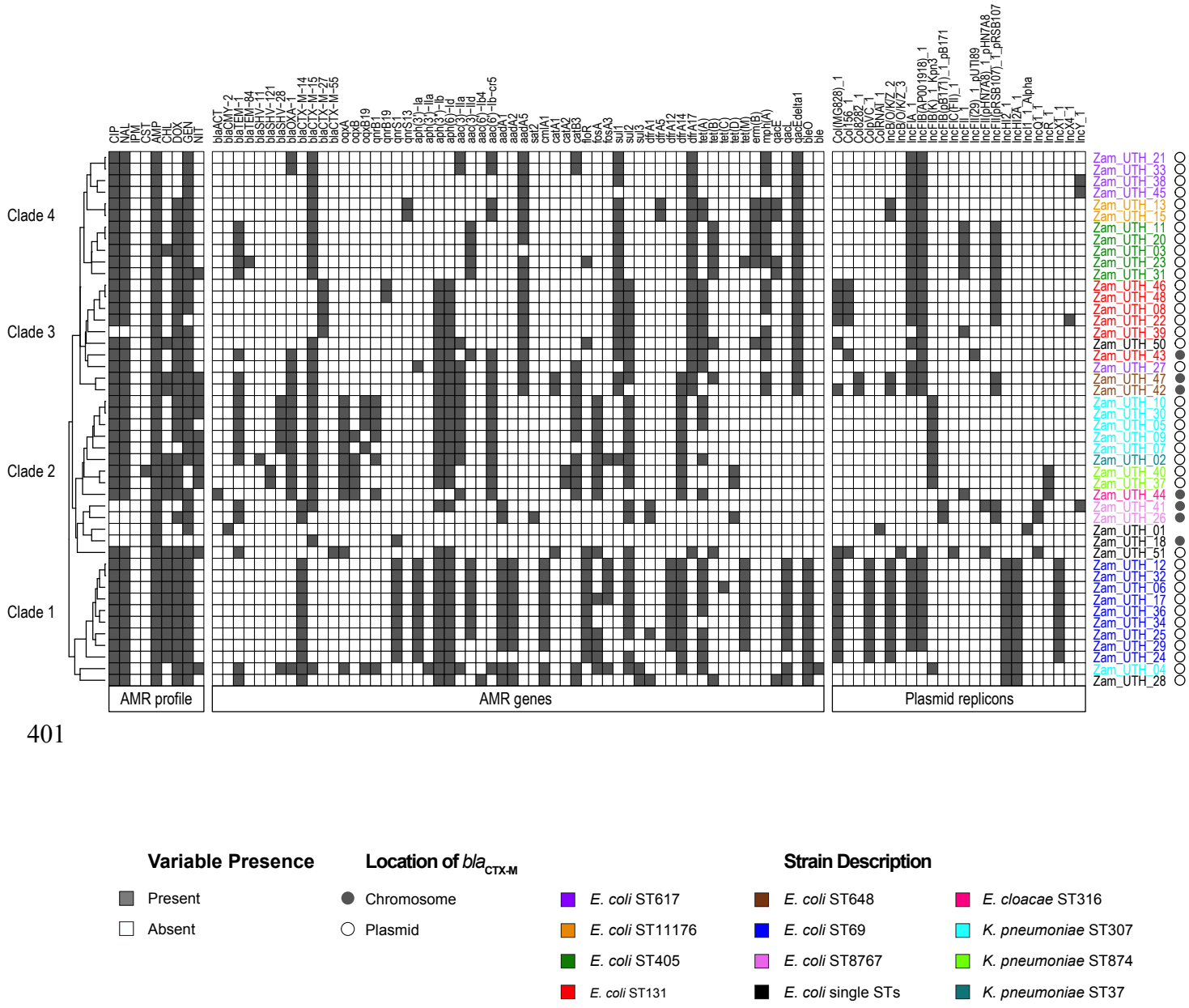

Figure 2. AMR phenotypes, AMR genes and plasmid replicons.

All but one strain displayed resistance to at least three antimicrobial classes. None of the isolates was resistant to imipenem, but one strain (Zam_UTH_40) was phenotypically resistant to colistin. Overall, 12 AMR gene classes were detected. The *bla*$_{\text{CTX-M}}$ family, dominated by the *bla*$_{\text{CTX-M-15}}$ variant, was the most diverse β-lactamase gene. Most *bla*$_{\text{CTX-M}}$ genes were identified on plasmids; however, these genes were chromosomal in 7/45 (15.6%) strains. A total of 24 plasmid replicons were detected, the commonest being the IncFIB(AP001918)_1 replicon with a prevalence of 30/46 (65.2%). Hierarchical clustering showed aggregation of strains with the same ST. Since cefotaxime (CTX) resistance was the selection criterion, CTX was not included in this analysis.

AMP; ampicillin. CHL; chloramphenicol. CIP; ciprofloxacin. CST; colistin. DOX; doxycycline. GEN; gentamicin. IPM; imipenem. NAL; nalidixic acid. NIT; nitrofurantoin.

Table 6. Prediction of phenotype from AMR genes

GEN	*aph(3)-Ia*	*aph(3)-IIa*	*aph(3'')-Ib*	*aph(6)-Id*	*aac(3)-IIa*	*aac(3)-IId*
R	10	1	28	28	14	13
S	0	0	3	3	0	0
Total	10	1	31	31	14	13
PPV (%)	**100**	**100**	**90.3**	**90.3**	**100**	**100**

GEN	*aac(6')-Ib4*	*aac(6')-Ib-cr5*	*aadA1*	*aadA2*	*aadA5*
R	1	16	12	10	19
S	0	2	0	0	1
Total	1	18	12	10	20
PPV (%)	**100**	**88.9**	**100**	**100**	**95**

CIP	*oqxA*	*oqxB*	*oqxB19*	*qnrB1*	*qnrB19*	*qnrS1*	*qnrS13*
R	11	5	5	7	2	9	2
S	0	0	0	0	0	1	0
Total	11	5	5	7	2	10	2
PPV (%)	**100**	**100**	**100**	**100**	**100**	**90**	**100**

NAL	*oqxA*	*oqxB*	*oqxB19*	*qnrB1*	*qnrB19*	*qnrS1*	*qnrS13*
R	11	5	5	7	2	9	2
S	0	0	0	0	0	1	0
Total	11	5	5	7	2	10	2
PPV (%)	**100**	**100**	**100**	**100**	**100**	**90**	**100**

DOX	*tet(A)*	*tet(B)*	*tet(C)*	*tet(D)*	*tet(M)*
R	24	9	1	3	9
S	0	0	0	0	0
Total	24	9	1	3	9
PPV (%)	**100**	**100**	**100**	**100**	**100**

CHL	*cmlA1*	*catA1*	*catA2*	*catB3*
R	8	3	2	6
S	2	0	0	9
Total	10	3	2	15
PPV (%)	**80**	**100**	**100**	**42.9**

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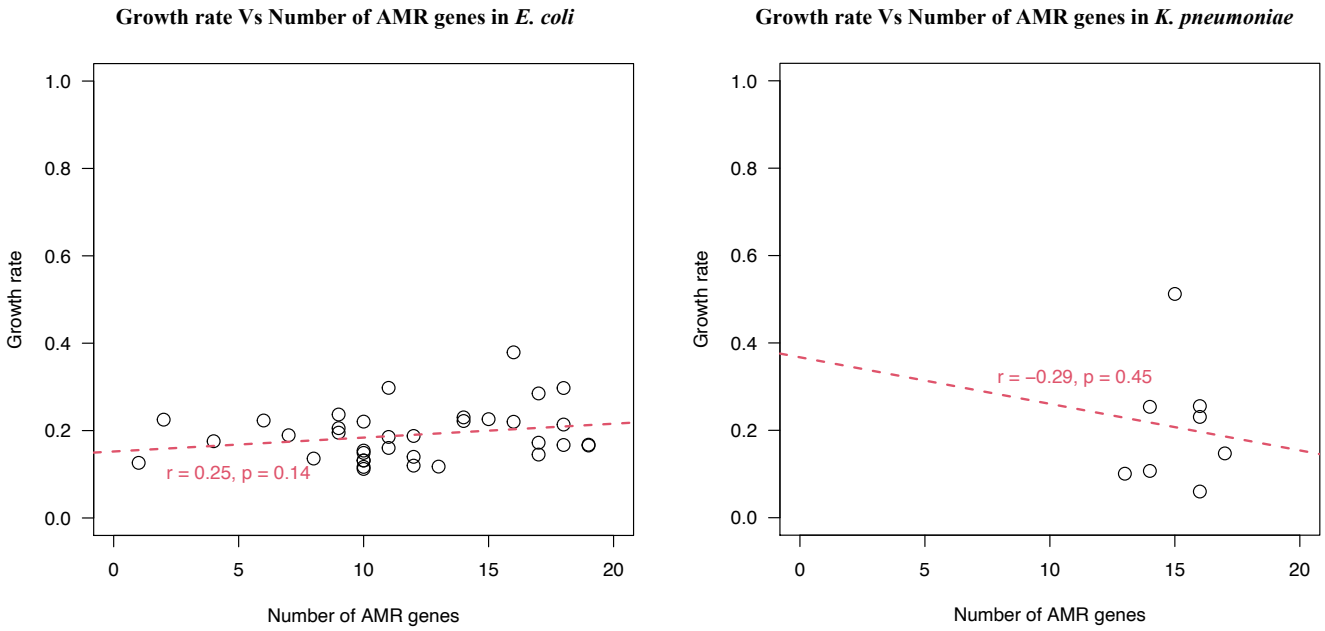

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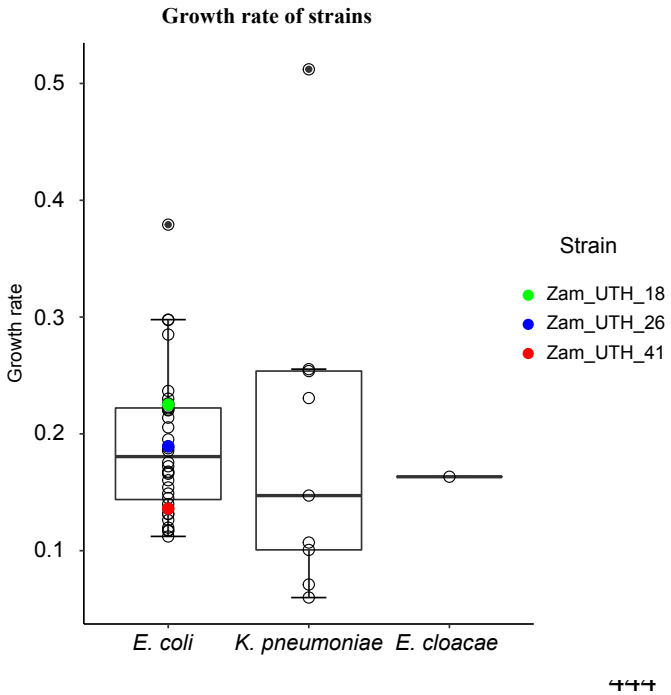

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Figure 3. Assessment of growth rate among strains.

449 A. There was no significant association between growth rate and the number of AMR genes.
450 Left; *E. coli*. Right; *K. pneumoniae*. Red lines represent the linear regression models with
451 growth rate as the outcome variable and the number of AMR genes as the predictor. At
452 the same time, r represents Pearson's correlation coefficient.

B. Zam_UTH_18 did not possess any plasmid and had a growth rate above the 75th percentile for *E. coli* strains. Zam_UTH_26 grew at a rate above the median growth rate for *E. coli*; however, a closely related strain, Zam_UTH_41, harbored an additional *bla*$_{\text{CTX-M-15}}$-carrying plasmid and had a growth rate lower than the 25th percentile.

458 **Plasmid composition among *E. coli* ST69 strains**

459 To describe the plasmid composition of the strains, contigs were screened for plasmid
460 replicons using the PlasmidFinder database [56]. A total of 24 replicon types were identified,
461 the most frequent being IncFIB(AP001918)_1 (30/46, 65.2%), followed by IncFIA_1 (27/46,
462 58.7%), and Col(MG828)_1 (16/46, 34.8%) (Fig 2). Previous studies have reported the
463 dominance of IncF plasmids encoding trimethoprim-sulfamethoxazole (SXT) resistance
464 among *E. coli* ST69 strains [68]. In concordance with these reports, the *E. coli* ST69 strains
465 analyzed here harbored an IncF (particularly IncFI) plasmid carrying *dfrA12* and *sul2* genes,
466 associated with trimethoprim and sulfamethoxazole resistance, respectively. In contrast to
467 past reports, the *E. coli* ST69 strains studied here also had two additional MDR plasmids,
468 including a large IncHI plasmid, uncommon in this ST [68, 69]. This 225 kb IncHI plasmid
469 possessed the $bla_{\text{CTX-M-14}}$ gene and shared over 80% nucleotide sequence identity with an
470 IncHI plasmid from a *K. pneumoniae* ST37 isolate (Zam_UTH_04) (Fig 4A), suggesting that
471 these plasmids could have arisen from a common ancestor. Furthermore, the two plasmids
472 displayed similar genetic architectures around the $bla_{\text{CTX-M-14}}$ gene, although both possessed
473 distinct large insertions harboring several AMR genes and other elements associated with
474 survival (Fig 4B). For example, the *mucAB* operon identified on the IncHI plasmid from *E.*
475 *coli* ST69 offers resistance against genotoxic agents such as ultraviolet light [70]. Also, the
476 *mer* operon on this plasmid can confer resistance to organomercury compounds [71].

477 In line with previous reports [72], the other three primary *E. coli* STs (i.e., ST131,
478 ST617, and ST405) harbored $bla_{\text{CTX-M}}$ genes on IncFI plasmids. However, the IncFI plasmids
479 in strains of different STs had unrelated plasmid backbones (Fig 4C), suggesting that HGT
480 was unlikely to be the mode of $bla_{\text{CTX-M}}$ gene propagation.

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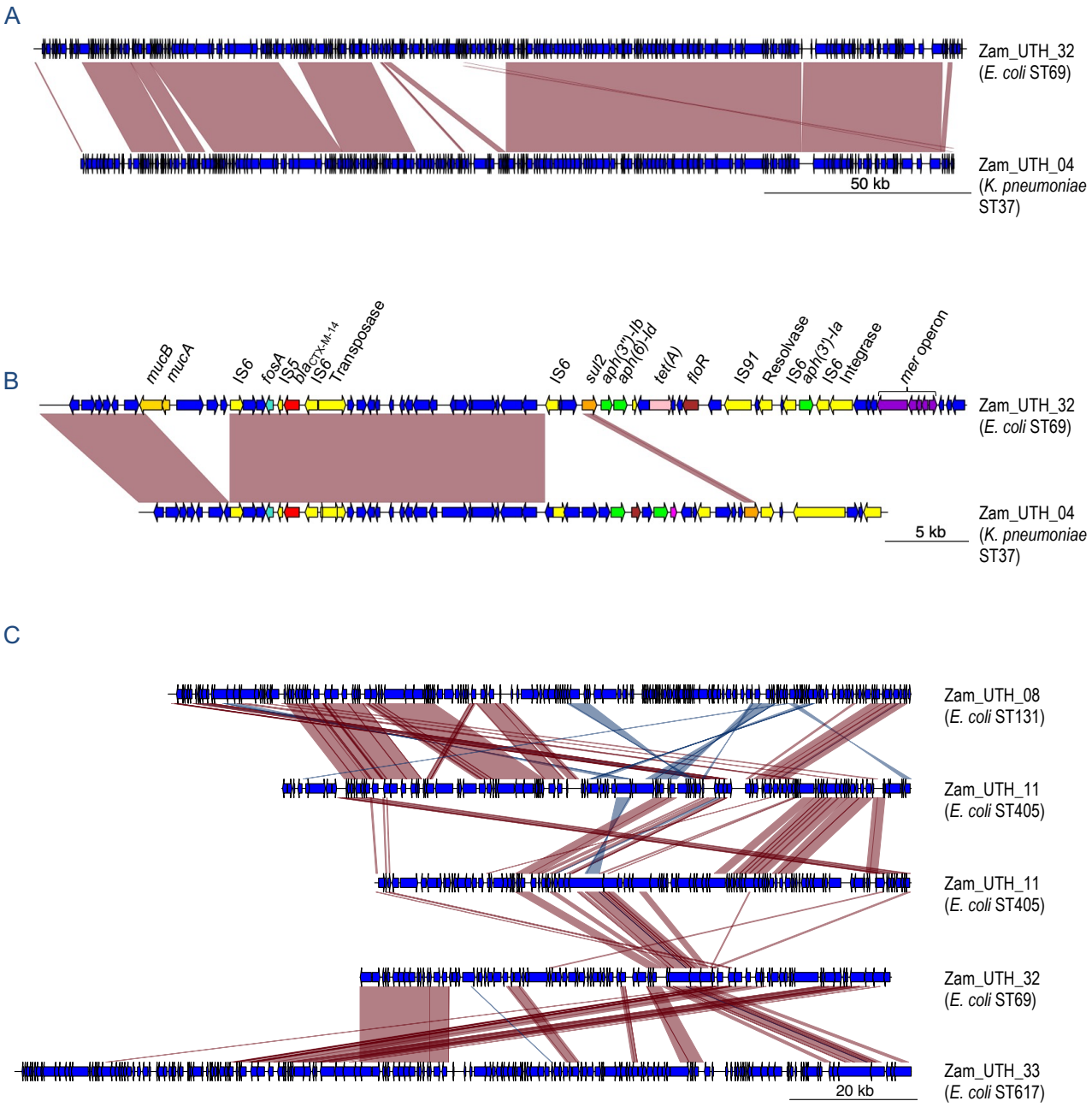

Figure 4. Comparison of plasmid sequences among strains.

A. IncHI plasmid in *E. coli* ST69 shared over 80% nucleotide sequence homology with IncHI plasmid in *K. pneumoniae* ST37.

B. IncHI plasmid from *E. coli* ST69 and *K. pneumoniae* ST37 carried the *fosA*/IS*5*/*bla*$_{\text{CTX-M-14}}$/IS*6* unit. In addition, the IncHI plasmid from *E. coli* ST69 also contained the *mucAB* and *mer* operons.

C. There was low nucleotide sequence similarity between IncFI plasmids from *E. coli* strains belonging to different STs. In addition, strains belonging to *E. coli* ST405 carried two IncF plasmids, namely IncFI and IncFII.

Red; β-lactam resistance. Green; aminoglycoside and/or quinolone resistance. Brown; chloramphenicol resistance. Purple; mercury resistance. Turquoise; fosfomycin resistance. Orange; sulfonamide resistance. Yellow; mobile genetic elements.

Spread of *bla*$_{\text{CTX-M}}$ genes by clonal expansion of specific lineages

To determine the mechanism of *bla*$_{\text{CTX-M}}$ spread among hospital patients, hierarchical clustering was performed using phenotypic and genotypic AMR profiles, as well as plasmid replicons. The results showed that strains could be clustered into four distinct clades (here referred to as Clade 1, Clade 2, Clade 3, and Clade 4) (Fig 2). The largest clade, Clade 1, was mainly composed of *E. coli* ST69 and defined by IncHI replicon and *bla*$_{\text{CTX-M-14}}$ gene. Furthermore, this clade also contained one *K. pneumoniae* ST307 and one *E. coli* ST156, which carried the IncHI replicon and shared several AMR genes with *E. coli* ST69. Notably, the number of AMR genes in Clade 1 was more than what was noted in Clade 2, Clade 3, and Clade 4 ($P < 0.01$) (Fig 5). Clade 3 showed a close relationship to Clade 4, with both clades having *qacEdelta1*, *mph(A)*, and *aad5* genes that were lacking in Clade 1 and Clade 2. Clade 3 was dominated by *E. coli* ST131, along with two *E. coli* ST648, one *E. coli* ST44, and one *E. coli* ST617. Meanwhile, Clade 3 was represented by three *E. coli* STs, namely ST405, ST617, and ST11176. Finally, the main subclade of Clade 2 was composed of *K. pneumoniae* and *E. cloacae* and contained four plasmid-mediated quinolone resistance (PMQR) genes, namely *oqxA*, *oqxB*, *oqxB19*, and *qnrB1*. In addition, a minor subclade of Clade 2 comprised *E. coli* STs such as ST8767, ST540, ST3580, and ST4674. Altogether, the analysis revealed that phylogenetically related lineages aggregated together. Consistent with the MLST analysis, these findings suggest that clonal dissemination of specific lineages was responsible for the spread of *bla*$_{\text{CTX-M}}$ instead of a plasmid-driven transmission.

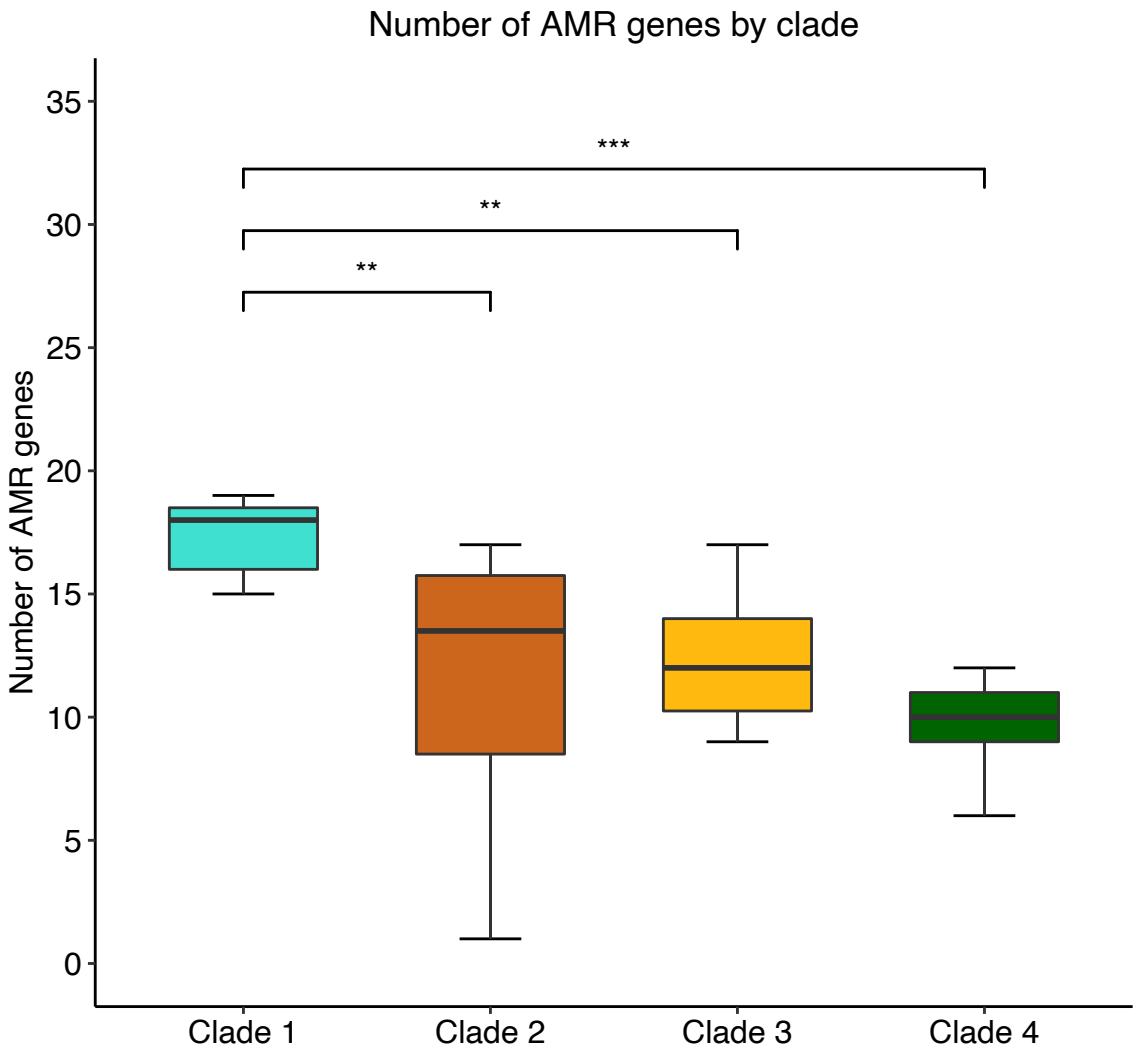

Figure 5. Comparison of number of AMR genes among clades.

There were significantly more AMR genes in Clade 1 compared to other clades. The analysis was performed by One-way ANOVA with Tukey's HSD comparison of means.

**P ≤ 0.01, ***P ≤ 0.001.

Co-occurrence of *bla*$_{\text{CTX-M-15}}$ and other AMR genes on large chromosomal insertions

To describe the chromosomal locations of *bla*$_{\text{CTX-M}}$ in seven strains (one *E. cloacae* and six *E. coli*), contigs were aligned against reference strains using Mauve [61]. In all seven strains, the chromosomal insertions were bordered by IS*Ecp1* at one end, highlighting the possible involvement of this element in the mobilization of the insertions. Nonetheless, some insertions also included several other IS elements (e.g., IS*1*, IS*6*) and transposons, indicating that other complex mechanisms may be involved. The chromosomal insertions, verified by PCR (Fig 6), were unique for distinct clones and ranged in size from ~ 3 kb to ~ 41 kb (Fig 7-9). These inserted segments resembled plasmid sequences retrieved from the NCBI GenBank, suggesting the transposon-mediated transfer from plasmids to chromosomes. Interestingly, four strains (one *E. cloacae* and three *E. coli*) carried *bla*$_{\text{CTX-M}}$-harboring insertions longer than 10 kb and contained various AMR genes. Consistent with the AMR genes on the insertions, these strains displayed resistance to several clinically relevant antibiotics (Fig 8 and 9).

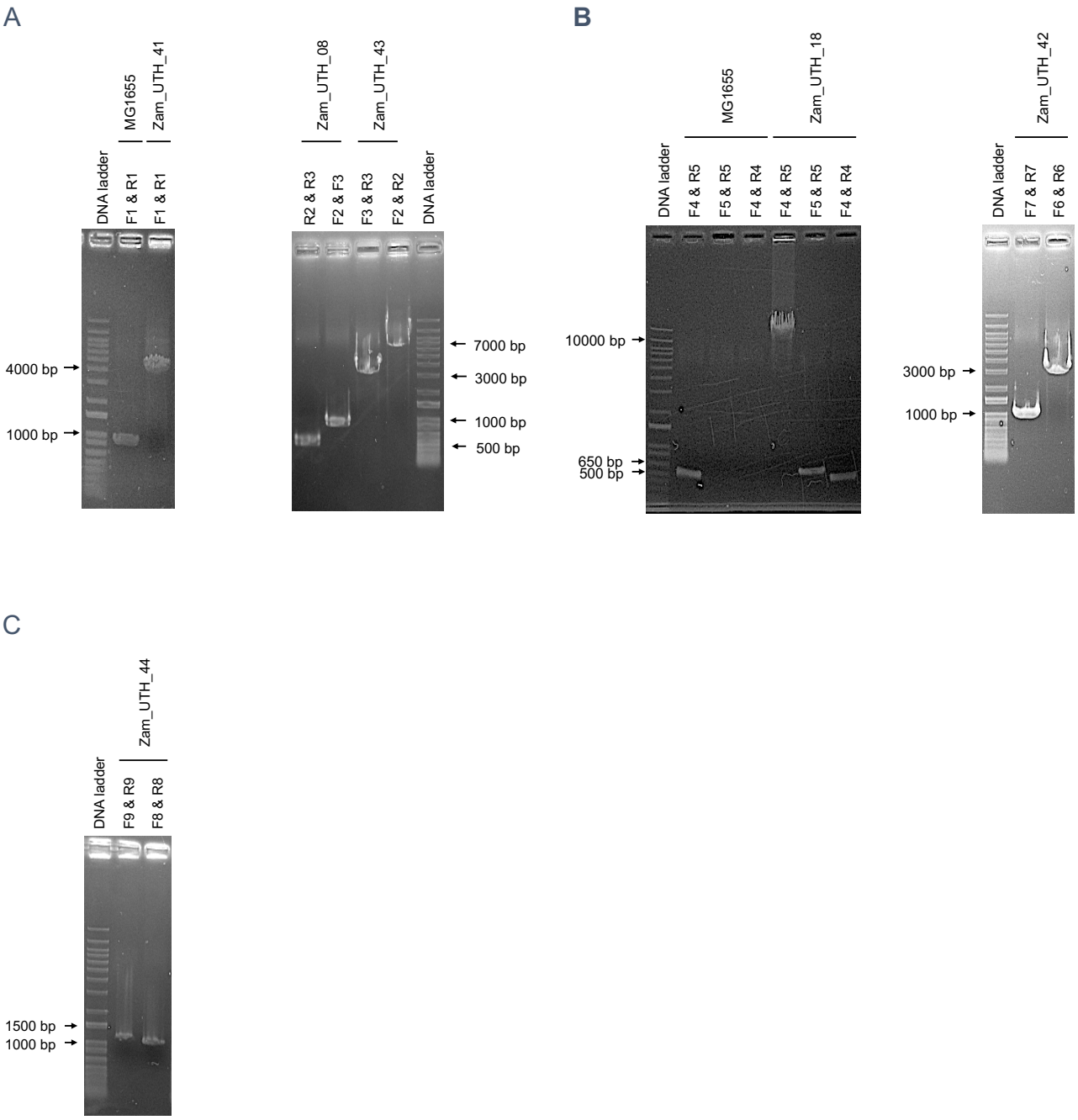

Figure 6. Confirmation of chromosomal insertions.

A. Small chromosomal insertions among *E. coli* strains. Left. PCR performed with the same primers produced bands of different sizes in Zam_UTH_41 (~ 4 kb) and *E. coli* MG1655 (~ 1 kb). Right. In Zam_UTH_43, PCR using the primer pairs F2/R2 and F3/R3 produced amplicons of sizes ~ 7 kb and ~ 3 kb, respectively. However, the primer sets F2/F3, and R2/R3 yielded products of about 850 bp and 500 bp, respectively, in a control strain (Zam_UTH_08).

B. Large (> 10 kb) chromosomal insertions among *E. coli* strains. Left. PCR targeting

junctions between plasmid and chromosome regions yielded products between 500 bp and 650 bp in Zam_UTH_18 but using the same primers on the reference strain *E. coli* MG1655 gave no products. Moreover, using primers outside the insertion produced a band larger than 10 kb in Zam_UTH_18 but the amplicon size in the control strain *E. coli* MG1655 was between 500 bp and 650 bp. Right. In Zam_UTH_42, PCR performed with primers targeting junctions between plasmid and chromosome regions produced bands of the expected size. However, no control strain was available for comparison.

C. Large (> 10 kb) chromosomal insertion in one *E. cloacae* strain (Zam_UTH_44). The insertion was confirmed by PCR targeting junctions between plasmid and chromosome regions, but no control strain was available for comparison.

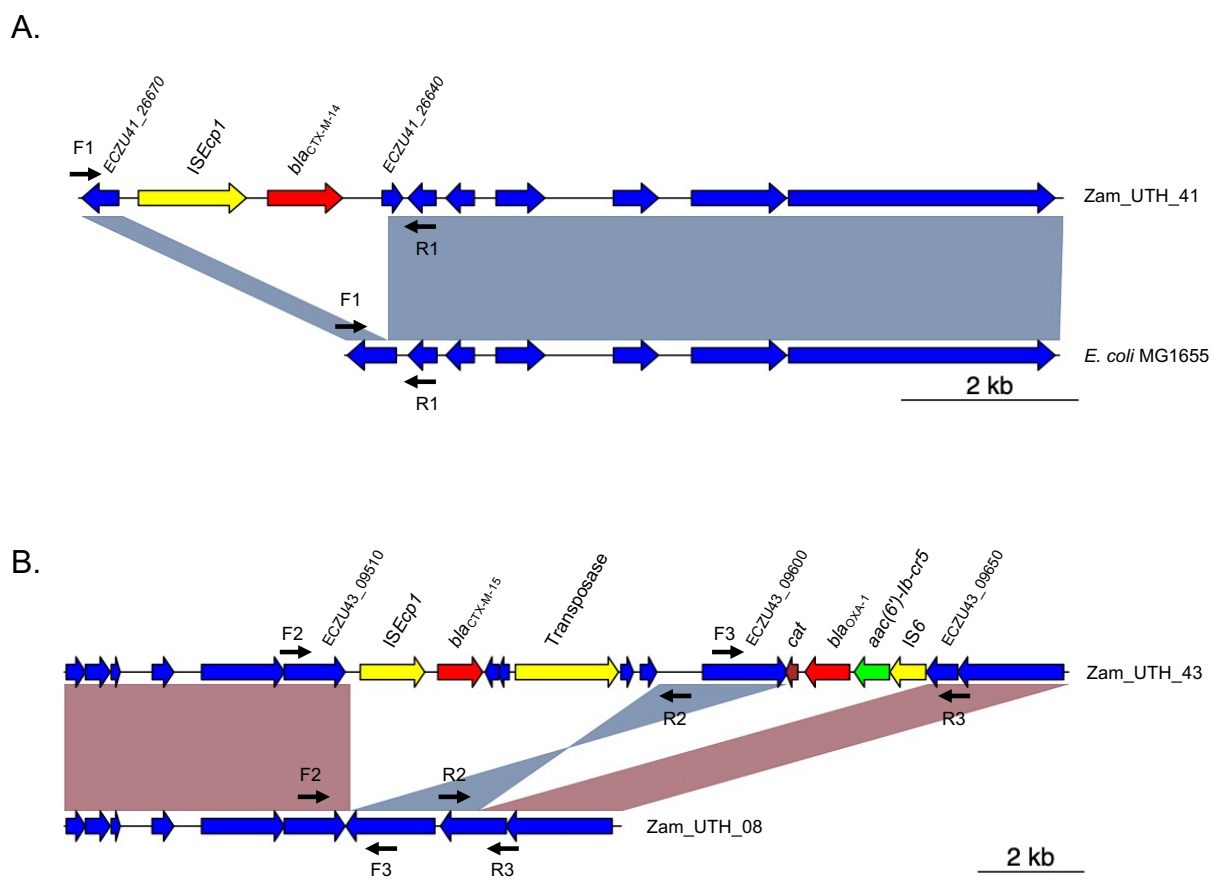

Figure 7. *bla*$_{CTX-M}$ genes present on short chromosomal insertions in *E. coli*.

A. Zam_UTH_41. This *E. coli* strain, belonging to ST8767, harbored a 3,095 bp chromosomal insertion with *bla*$_{CTX-M-14}$ located 249 bp downstream of IS*Ecp1*. Zam_UTH_26 also had a similar insertion and genetic architecture.

B. Zam_UTH_43. This *E. coli* strain, belonging to O107:H5-ST131, harbored a 6,036 bp chromosomal insertion with *bla*$_{CTX-M-15}$ located 255 bp downstream of IS*Ecp1*. In addition, about 2.5 kb downstream of this insertion, a smaller insertion was identified, carrying genes targeting aminoglycosides/quinolones (*aac(6')-Ib-cr5*), β-lactams (*bla*$_{OXA-1}$), and chloramphenicol (*cat*).

F1, F2, F3, R1, R2, R3; primers used to confirm insertions.

In all six *E. coli* strains, chromosomal insertions were flanked by 5-bp direct repeats, a characteristic feature of IS*Ecp1*-mediated transposition. Nevertheless, two strains (Zam_UTH_42 and Zam_UTH_47) (Fig 8B) possessed a truncated IS*Ecp1*, indicating that this interrupted element is unlikely to be functional; thus, the interrupting IS*1* and/or transposase may have mobilized $bla_{\text{CTX-M-15}}$ in these strains. Depending on the allele (i.e., $bla_{\text{CTX-M-14}}$ or $bla_{\text{CTX-M-15}}$), the $bla_{\text{CTX-M}}$ gene existed at a fixed position downstream of IS*Ecp1*, suggesting common ancestral origins among similar alleles. Specifically, in two *E. coli* ST8767 strains (Zam_UTH_26 and Zam_UTH_41), the $bla_{\text{CTX-M-14}}$ gene existed 249 bp downstream of IS*Ecp1* on a 3,095 bp segment. Likewise, the $bla_{\text{CTX-M-15}}$ allele was detected 255 bp away from IS*Ecp1* in the downstream region of five strains with chromosomal insertions of diverse lengths. More precisely, one *E. coli* ST131 (Zam_UTH_43) (Fig 7B) and one *E. coli* ST3580 (Zam_UTH_18) (Fig 8A) possessed the $bla_{\text{CTX-M-15}}$ allele on 6,036 bp and 11,383 bp segments, respectively, while two *E. coli* ST648 (Zam_UTH_42 and Zam_UTH_47) (Fig 8B) harbored a 14,328 bp $bla_{\text{CTX-M-15}}$-containing insertion. Finally, an *E. cloacae* ST316 strain (Zam_UTH_44) possessed a large (> 41 kb) $bla_{\text{CTX-M-15}}$-carrying chromosomal insertion (Fig 9).

Out of seven strains with chromosomally-located $bla_{\text{CTX-M}}$, four of them (i.e., Zam_UTH_18, Zam_UTH_42, Zam_UTH_44, and Zam_UTH_47) had the gene on large (> 10 kb) insertions that resembled plasmid or chromosome sequences obtained from the NCBI GenBank (Fig 8 and 9). Particularly, Zam_UTH_18 carried an insertion observed in the chromosomes of *Salmonella enterica* (GenBank accession no. CP045038) and two *E. coli* ST38 isolates (GenBank accession no. CP010116 and CP018976). Also, the four large insertions (> 10 kb) possessed other AMR genes in the downstream region of $bla_{\text{CTX-M-15}}$. For example, the insertion in Zam_UTH_18 carried the *qnrS1* gene, which is associated with reduced quinolone susceptibility. At the same time, Zam_UTH_42 and Zam_UTH_47 had several genes that encode resistance to aminoglycosides (*aac(3)-IIa*), aminoglycosides/quinolones (*aac(6')-Ib-cr5*), β-lactams ($bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$), and chloramphenicol (*catB3*). Likewise, the insertion in Zam_UTH_44 had seven genes associated with resistance to aminoglycosides (*aac(3)-IIa*), quinolones (*qnrB1*), aminoglycosides/quinolones (*aac(6')-Ib-cr5*), β-lactams ($bla_{\text{OXA-1}}$), trimethoprim (*dfrA14*), chloramphenicol (*catB3*), and tetracyclines (*tet(A)*).

Apart from Zam_UTH_18, which was susceptible to quinolones (ciprofloxacin MIC = 0.25 μg/ml) despite possessing the *qnrS1* gene, the AMR phenotypes of the strains

possessing chromosomal insertions correlated with the AMR genes on the insertions. Furthermore, these strains displayed cefotaxime MICs that were several folds higher than the MIC breakpoint of 2 μg/ml recommended by the Clinical and Laboratory Standards Institute (CLSI) [73]. Additionally, two closely related strains (Zam_UTH_26 and Zam_UTH_41) (Fig 1A) exhibited cefotaxime MICs that differed by more than two-fold, probably due to the extra plasmid-borne *bla*$_{\text{CTX-M-15}}$ gene in Zam_UTH_41, in addition to chromosomal *bla*$_{\text{CTX-M-14}}$. This variation was also highlighted by the lower fitness of Zam_UTH_41 ($\mu = 0.136$) relative to Zam_UTH_26 ($\mu = 0.189$), signifying the costly effect of the additional *bla*$_{\text{CTX-M-15}}$-carrying plasmid. Equally, the lack of plasmids in Zam_UTH_18 was demonstrated by its high growth rate, more than the 75$^{\text{th}}$ percentile of the rates among *E. coli* strains (Fig 3B).

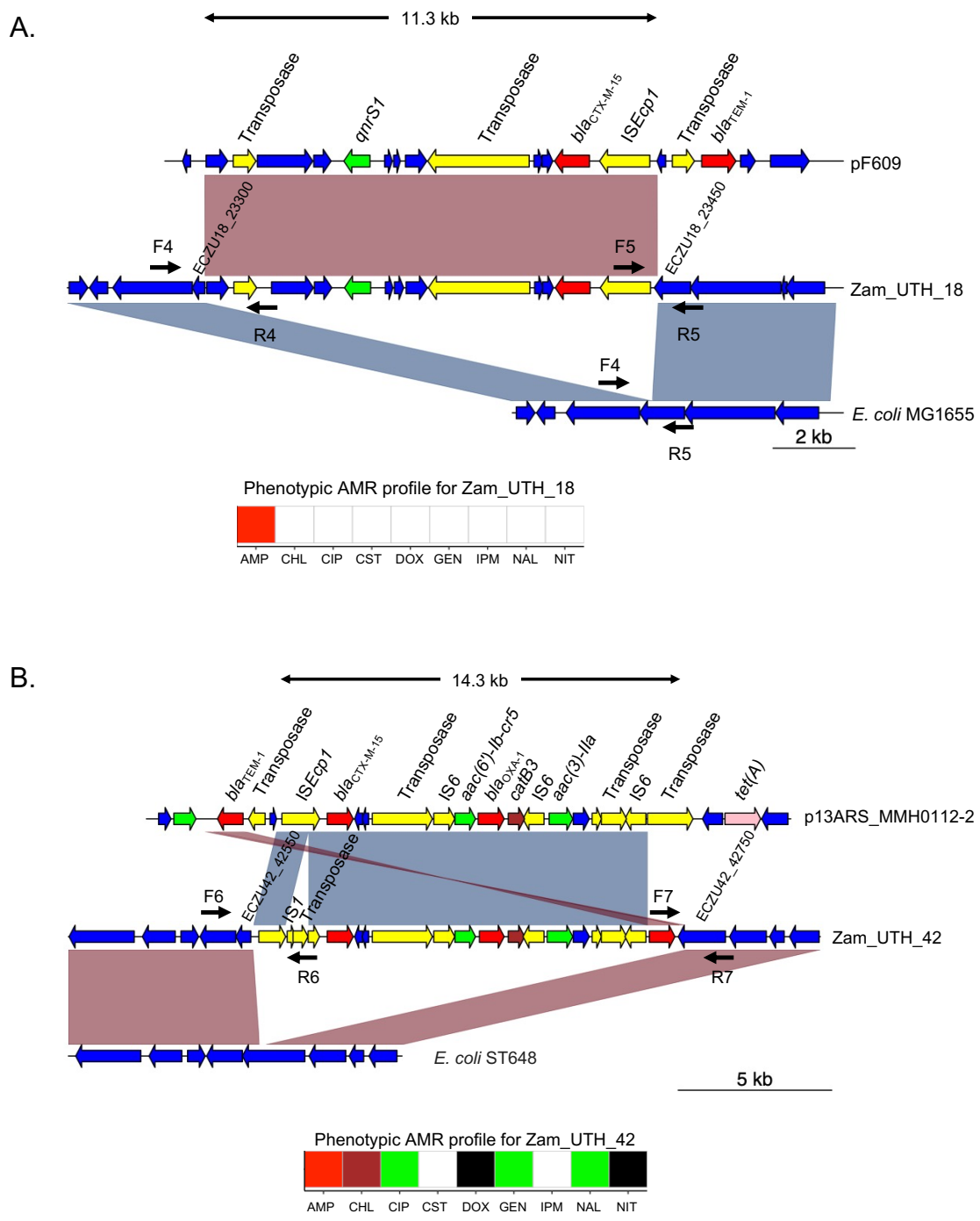

Figure 8. *bla*$_{CTX-M}$ genes present on large chromosomal insertions in *E. coli*.

A. Zam_UTH_18. This *E. coli* strain, belonging to ST3580, contained an 11,383 bp *bla*$_{CTX-M-15}$-carrying chromosomal insertion, which resembled plasmid pF609 (GenBank accession no. MK965545.1). The *bla*$_{CTX-M-15}$ gene existed 255 bp downstream IS*Ecp1*.

651 The *qnrS1* gene was located 4639 bp downstream of *bla*$_{\text{CTX-M-15}}$. Phenotypically, this
652 strain was resistant to ampicillin but susceptible to quinolones.
653 B. Zam_UTH_42. This *E. coli* strain belonging to ST648 harbored a 14,328 bp
654 chromosomal insertion carrying *bla*$_{\text{CTX-M-15}}$ resembling plasmid p13ARS_MMH0112-2
655 (GenBank accession no. LR697123.1). This insertion possessed genes that target
656 aminoglycosides (*aac(3)-IIa*), chloramphenicol (*catB3*), β-lactams (*bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1}}$),
657 and aminoglycosides/quinolones (*aac(6')-Ib-cr5*). The *bla*$_{\text{CTX-M-15}}$ gene was located 255
658 bp downstream of IS*Ecp1*, which was truncated by IS*1* and transposase. The phenotypic
659 resistance profile of this strain coincided with the AMR genotype of the insertion. The
660 same genetic context and phenotypic profile were observed in a closely related strain,
661 Zam_UTH_47.
662 F4, F5, F6, F7, R4, R5, R6, R7; primers used to confirm the insertions. White; susceptible.
663 Black; resistance phenotype not explained by AMR genes. Red; β-lactam resistance. Green;
664 aminoglycoside and/or quinolone resistance. Brown; chloramphenicol resistance.
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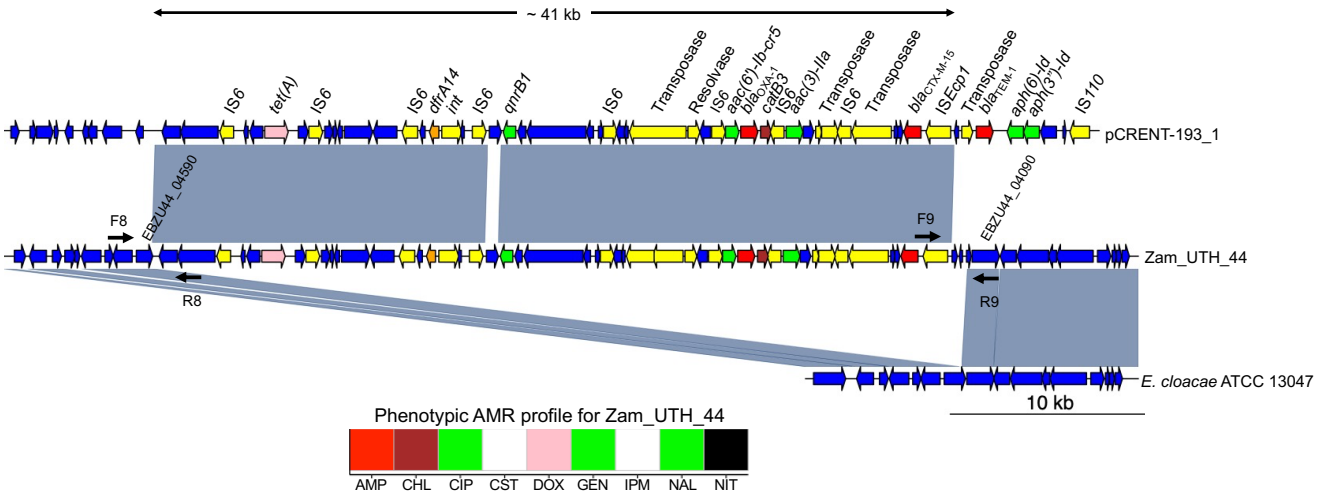

Figure 9. *bla*$_{\text{CTX-M}}$ gene on a large chromosomal insertion in *E. cloacae*.

Zam_UTH_44. This *E. cloacae* strain, belonging to ST316, possessed the *bla*$_{\text{CTX-M-15}}$ gene on a ~ 41 kb chromosomal insertion similar in nucleotide sequence to plasmid pCRENT-193_1 (GenBank accession no. CP024813.1). The *bla*$_{\text{CTX-M-15}}$ gene was located 255 bp downstream of IS*Ecp1*. The insertion also harbored various AMR genes targeting aminoglycosides (*aac(3)-IIa*), chloramphenicol (*catB3*), β-lactams (*bla*$_{\text{OXA-1}}$), aminoglycosides/quinolones (*aac(6')-Ib-cr5*), quinolones (*qnrB1*), trimethoprim (*dfrA14*), and tetracyclines (*tet(A)*). The phenotypic resistance profile of this strain coincided with the AMR genes on the insertion.

F8, F9, R8, R9; primers used to confirm the insertions. White; susceptible. Black; resistance phenotype not explained by AMR genes. Red; β-lactam resistance. Green; aminoglycoside and/or quinolone resistance. Pink; tetracycline resistance. Brown; chloramphenicol resistance.

Discussion

This current study focused on the phenotypic and genotypic characterization of *Enterobacteriaceae* strains collected from inpatients at the UTH, Zambia. Phylogenetic analysis (Fig 1) and hierarchical clustering (Fig 2) suggested that the spread of $bla_{\text{CTX-M}}$ has been facilitated primarily by clonal expansion. The $bla_{\text{CTX-M}}$ gene was located on plasmids in most isolates, but seven strains carried this gene on chromosomes. While the IS*Ecp1*-mediated chromosomal location of the $bla_{\text{CTX-M}}$ gene is a well-studied phenomenon [43, 44, 46-48], there are no reports of IS*Ecp1*-$bla_{\text{CTX-M}}$ with other AMR genes in *E. cloacae* or *E. coli* chromosomes. Despite advances in sequencing methods allowing the characterization of the regions immediately upstream and downstream of chromosomal $bla_{\text{CTX-M}}$, such approaches are limited when the chromosomal insertions are large. By reconstructing nearly complete genomes, the identification of large $bla_{\text{CTX-M}}$-harboring chromosomal insertions was improved relative to previous analyses. The results showed that one *E. cloacae* and three *E. coli* strains carried large $bla_{\text{CTX-M-15}}$-possessing chromosomal insertions that were very similar in nucleotide sequence to plasmids in the NCBI GenBank. Furthermore, these insertions were all bordered by IS*Ecp1* at one end and contained various other AMR genes. The AMR phenotypes of the strains correlated with the observed AMR genes on the large chromosomal insertions. These findings reveal the role of IS*Ecp1* in propagating $bla_{\text{CTX-M-15}}$-related MDR determinants among *E. cloacae* and *E. coli*.

When the strains were analyzed for phylogenetic grouping (Fig 1), the results showed four predominant *E. coli* STs (ST69, ST131, ST617, and ST405) and one major *K. pneumoniae* ST (ST307). Furthermore, hierarchical clustering based on plasmid replicons and phenotypic and genotypic AMR (Fig 2) showed strain aggregation according to ST. While these results suggest clonal transmission of the strains within the hospital, robust geo-temporal clonal spread modeling is required to confirm the hypothesis. However, the lack of patient information such as contact history and admission dates could not allow such detailed analysis.

In most studies, *E. coli* ST131 is dominated by $bla_{\text{CTX-M-15}}$-harboring strains belonging to the O25b:H4 pandemic clone [63, 74]. However, none of the six *E. coli* ST131 strains studied here were members of this group, but five belonged to Onovel31:H4, and one had an O107:H5 serotype. Other serotypes of *E. coli* ST131 have been sporadically reported [75], including Onovel31:H4 [76] identified in this study. To the best of the author's knowledge, this is the first account of *E. coli* ST131 belonging to serotype O107:H5.

The extraintestinal pathogenic *E. coli* ST69 was overrepresented in this study and belonged to a clade that contained more AMR genes than other clades (Fig 5). *E. coli* ST69 strains rarely carry ESBL genes [77], are more common in community-acquired infections [78], and possess SXT resistance-encoding plasmids of the IncF incompatibility type [68]. Intriguingly, the ST69 strains studied here possessed the $bla_{\text{CTX-M-14}}$ gene on a plasmid of the IncHI incompatibility type and dominated the hospital isolates. This unusual occurrence of IncHI plasmids in *E. coli* ST69 was also observed in a raw milk cheese isolate from Egypt [79]. However, there was a difference in the AMR gene composition between the Egyptian IncHI plasmid (GenBank accession no. CP023143) and the ones in this study.

Previous studies have associated *E. coli* ST69 with SXT-resistant community-acquired infections [68, 78]. Accordingly, the ST69 clone reported here could have originated from the community, where SXT is frequently used as a preventive intervention against *Pneumocystis jirovecii* pneumonia (PCP). Based on specified laboratory and clinical parameters, HIV-infected or exposed individuals at risk of succumbing to PCP are commenced on SXT prophylaxis until they are no longer at risk [80]. While empiric SXT is clinically beneficial, this practice imposes selection pressure for SXT-resistant species, including *E. coli* [81-83]. However, the acquisition of the abovementioned IncHI plasmid could have changed the ecology and adaptation of the ST69 clone. The resulting MDR phenotype, coupled with various adaptation and survival mechanisms such as the error-prone SOS repair encoded by *mucAB* operon, perhaps made ST69 a successful clone dominating the hospital.

The high MDR rate observed in this study may be a consequence of antimicrobial overuse and misuse. In Zambia, policies on antimicrobial use are usually not enforced, and many clients can access most antibiotics without a prescription [84]. As an additional challenge, clinicians at the UTH prescribe antibiotics 53.7% of the time [85], which is more than the 30% threshold set by the World Health Organization [86]. Furthermore, most UTH inpatients with serious bacterial infections are managed with third-generation cephalosporins (e.g., cefotaxime) [87], whereas quinolones (e.g., ciprofloxacin) are frequently used in the outpatients' department [85]. Worryingly, this study showed that about 90% of cefotaxime-resistant strains also exhibited resistance to quinolones.

Meanwhile, the UTH has a strict policy on carbapenem use, with prescriptions supported by laboratory evidence. Promisingly, no phenotypic or genotypic carbapenem resistance was detected in this study. While this is encouraging, it is worth noting that carbapenem resistance has been on the rise globally, including in Africa [88], necessitating

751 the re-introduction of colistin as a last-resort drug [89]. Despite many countries' renewed
752 interest in colistin, the drug is yet to be made available for clinical use in Zambia. However,
753 one CSF *K. pneumoniae* isolate (Zam_UTH_40) already displayed low-level colistin
754 resistance. While this finding might have significant clinical implications, there is a need for
755 additional studies to verify this observation.
756
757 Analysis of the diverse genetic environments associated with *bla*$_{\text{CTX-M}}$ in
758 chromosomes disclosed a relationship with IS*Ecp1,* as reported earlier [90]. Interestingly,
759 three *E. coli* and one *E. cloacae* exhibited co-occurrence of IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ and various
760 AMR genes on chromosomes. The said genes were detected on large insertions similar to
761 plasmid sequences in a public database. Goswami *et al*. recently reported a chromosomal
762 insertion containing the *bla*$_{\text{CTX-M-15}}$ and four other AMR genes in an *E. coli* strain; however,
763 in their study, IS*26* was the mobilizing element [68]. In another report from South Korea,
764 Yoon *et al*. characterized *K. pneumoniae* strains with chromosomal insertions harboring the
765 IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ and other AMR genes [91]. However, all the *K. pneumoniae* strains in the
766 current study carried plasmid-borne *bla*$_{\text{CTX-M}}$ genes, possibly due to geographic differences
767 between Zambia and South Korea. This is the first report of chromosomal insertions
768 harboring IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ and multiple AMR genes in *E. cloacae* and *E. coli*. Based on
769 the identified IS*Ecp1* at one end of each MDR insertion, it is likely that the IS*Ecp1* element
770 mobilized these insertions. Nevertheless, the other mobile elements on the insertions, and the
771 truncated IS*Ecp1* in two strains, may imply other intricate genetic events.
772 Phenotypic AMR profiles suggested that chromosomal insertions carrying multiple
773 AMR genes conferred resistance to several antimicrobial drugs. For example, the *E. cloacae*
774 strain (Zam_UTH_44) had all its AMR genes on the chromosome and exhibited resistance to
775 ampicillin, ciprofloxacin, gentamicin, and chloramphenicol. However, Zam_UTH_18 had a
776 ciprofloxacin MIC below the clinical breakpoint despite carrying chromosomal *qnrS1*.
777 Although *qnrS1* gene expression studies were not conducted, the observed result is probably
778 because PMQR determinants (such as *qnrS1*) only cause slight changes in quinolone
779 susceptibility [92]. Nevertheless, these small susceptibility reductions often result in
780 treatment failure, thus prompting some researchers to foster a movement to revise quinolone
781 breakpoints [93].
782 Although the advantages of chromosomal insertions carrying IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ and
783 other AMR genes are not well understood, a possible benefit is the acceleration of MDR
784 spread through an intermediary reservoir. It is generally known that plasmids from the same

785 incompatibility class cannot stably reside in the same host [94]. As a result, direct gene
786 transfer is unlikely between such plasmids; thus, the chromosome may be an intermediate for
787 gene sharing. Also, the insertion of IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ and other AMR genes into the
788 chromosomes ensures the stable propagation of MDR strains even in the absence of antibiotic
789 selection pressure [91]. Since bacterial plasmids are likely to be lost when associated with a
790 fitness cost [95, 96], insertion of crucial AMR genes into the chromosome may warrant
791 survival and further AMR spread. This assumption is supported by the result on
792 Zam_UTH_18 (*E. coli*), which did not contain any plasmids but had an 11 kb chromosomal
793 insertion possessing *bla*$_{\text{CTX-M-15}}$ and *qnrS1*. This strain displayed a growth rate higher than
794 most *E. coli* isolates, suggesting a fitness advantage possibly conferred by the lack of
795 plasmids. It can be speculated that Zam_UTH_18 once carried a plasmid but lost it after the
796 crucial genes, *bla*$_{\text{CTX-M-15}}$ and *qnrS1*, were integrated into the chromosome. Verifying this
797 observation will require growth and competitive performance studies using carefully selected
798 control strains.
799

CHAPTER II:

Clonal relationship between multidrug-resistant *Escherichia coli* ST69 from poultry and humans in Lusaka, Zambia

Summary

The emergence of multidrug-resistant (MDR) *Escherichia coli* among chickens in Zambia poses a threat to human health. Twenty MDR *E. coli* strains collected from poultry in Lusaka, Zambia, were sequenced on MiSeq and MinION platforms, and the genomes were reconstructed *de novo*. Next, the poultry strains were compared to 36 MDR *E. coli* isolates previously obtained from inpatients at a teaching hospital in the same city. All the 20 poultry strains were resistant to ampicillin, chloramphenicol, and doxycycline, while 18/20 (90%) also exhibited resistance to the quinolones. Twenty-seven antimicrobial resistance (AMR) genes belonging to 11 classes were detected, with aminoglycoside resistance genes dominating (7/27, 25.9%), followed by β-lactamase genes (3/27, 11.1%). One *E. coli* clone, belonging to O17:H18-ST69, was identified in 4/20 (20%) poultry- and 9/36 (25%) human-associated strains. In both niches, the O17:H18-ST69 clone possessed an IncFI plasmid with nine AMR genes and an IncI-complex plasmid with five AMR genes. Furthermore, the poultry and human O17:H18-ST69 strains formed one clade on phylogenetic analysis and hierarchical clustering, suggesting clonality.

Further investigation showed that the strains also had distinct AMR plasmids specific for each niche; for instance, all the four poultry strains contained an IncFII(pCoo) plasmid harboring the $bla_{\text{CTX-M-55}}$ gene, while the nine human isolates possessed the $bla_{\text{CTX-M-14}}$ gene on an IncHI plasmid. These results were suggestive of clonal dissemination of MDR *E. coli* O17:H18-ST69 between poultry and humans, with the independent acquisition of $bla_{\text{CTX-M}}$ genes. Thus, there is a need for strategic and concerted efforts from human and animal health sectors to prevent and control foodborne MDR.

Introduction

The importance of antimicrobial resistance (AMR) has been growing at the national, regional, and global levels, posing a threat to both "health and wealth." Future projections characterize the plight of AMR as a potential catastrophe for humanity that will kill 10 million people every year by 2050, coupled with a loss of up to 100 trillion USD [18]. Despite the urgent need for new therapeutic agents, the antibiotic drug discovery industry is economically unattractive, and only a few compounds are currently in phase II or III clinical development [97]. The diminishing stock of novel antibiotics in the pipeline suggests that we are heading for a "post-antibiotic era" from which recovery is uncertain.

As an added challenge, multidrug resistance (MDR), described as resistance to at least one antimicrobial drug from at least three antimicrobial groups [66], has become an increasing global concern due to its alarming mortality rates [98]. In Gram-negative bacteria, MDR is generally attributed to extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, which are usually resistant to cefotaxime [99-101]. MDR *E. coli* frequently occurs among hospital patients [102, 103], but animals also serve as reservoirs [104, 105], perhaps because of antimicrobial growth promoters (AGPs). The debate around the nonrational use of antimicrobials in animals prompted the progressive ban of AGPs [106], though they are still being used in over 35 countries [107]. Moreover, the ban's effect may not be substantial as antimicrobials are still used for prophylaxis in many regions, including the USA [108]. This controversial practice is not restricted to Western countries, as shown by reports in Africa [109], with AGPs used in South Africa [110] and Zambia [111, 112].

As a consequence, there has been an increase in the incidence of MDR *E. coli* in food-producing animals like cattle [113], pigs [114, 115], and poultry [116], with the occurrence of zoonotic transmission through clonal expansion or horizontal gene transfer [117]. Chicken meat is among the most typical risks of MDR *E. coli* transmission to humans [118, 119]; therefore, poultry surveillance is essential in controlling MDR dissemination. Such surveillance activities have revealed a high prevalence of MDR *E. coli* in poultry strains from Asia [120-122], and reports in Africa have increased [116, 123]. In Zambia, MDR *E. coli* strains were isolated from chicken meat [17], but no information regarding their clonal relationship with human isolates was available. Although reports on MDR *E. coli* have increased among Zambian human patients [15, 124], the proportion originating from poultry remains unknown.

In this study, the One Health approach revealed the MDR patterns and clonal relationships between poultry and human *E. coli* isolates from Lusaka, Zambia. Analyzing the samples by whole-genome sequencing (WGS) showed that 4/20 (20%) poultry strains were closely related to 9/36 (25%) clinical isolates. Furthermore, the four poultry and nine human isolates belonged to O17:H18-ST69 and carried 14 identical AMR genes on two plasmids, suggesting clonal spread. Further comparison analysis also revealed that these isolates harbored *bla*$_{\text{CTX-M}}$-carrying plasmids that were niche-specific. These results demonstrate a clinically important link between MDR *E. coli* associated with poultry and humans in Lusaka, highlighting the role of the food chain in disseminating MDR strains.

Materials and methods

Strain collection from poultry

In December 2019, cloacal swabs were collected by randomly sampling 1,000 disease-free laying hens at a large commercial farm in Lusaka, Zambia. The sampled farm is a major supplier of poultry and poultry products in Lusaka and surrounding districts. Therefore, the farm was selected based on the large catchment area it serves. The swabs were pooled into 200 batches, each containing samples from five birds. The 200 pooled samples were then transported in buffered peptone water (Oxoid Ltd, UK) and plated on cefotaxime-supplemented (1 μg/ml) MacConkey agar, followed by 18 hours of incubation at 37 ºC. One colony suspected to be *E. coli* was picked from each plate and confirmed by amplifying the 16S rRNA gene using PCR, followed by sequencing on the 3130 Genetic Analyzer (Applied Biosystems, USA) with primers described previously [125].

Screening for MDR

Phenotypic AMR profiles of cefotaxime-resistant *E. coli* were ascertained based on the Minimum Inhibitory Concentrations (MICs) measured for nine different antimicrobials. The drugs chosen were ampicillin, chloramphenicol, ciprofloxacin, colistin, doxycycline, gentamicin, imipenem, nalidixic acid, and nitrofurantoin; the breakpoints used are shown in Table 1. The choice of these antimicrobial drugs was guided by the local prescription patterns [87] and research-based recommendations [126]. Nonetheless, the safety issues associated with ciprofloxacin use in pediatric populations [127] prompted the addition of relatively safer nalidixic acid [128], hence the two quinolones.

The strains were grown in cefotaxime-supplemented (1 μg/ml) LB at 37°C for 18 hours, shaking at 155 rpm. The cultures were then diluted 10^4-fold and added to triplicates of 2-fold serial dilutions of antibiotics in a 96-well plate. Next, the 96-well plates were incubated at 37°C for 18 hours while shaking at 1,600 rpm. Using the Multiskan FC Microplate Photometer (Thermo Scientific, USA) to measure optical densities at 595 nm (OD_{595}), positive bacterial growth was considered OD_{595} values of at least 0.1. Therefore, the MIC was defined as the lowest antibiotic concentration giving an OD_{595} smaller than 0.1. The reference strains, *E. coli* MG1655 and *E. coli* 10-β (NEB, USA), were used for quality control.

Growth rate determination

The growth of bacterial cultures in antibiotic-free LB was monitored in real-time for 16 hours in a 96-well plate. This was achieved by observing the OD_{600} of 10^3-fold-diluted overnight cultures in duplicate at 37°C using the Varioskan LUX Multimode Microplate Reader (Thermo Scientific, USA) while shaking at 600 rpm. By fitting growth curves in R using the package grofit version 1.1.1 [49], the obtained slopes were used as estimates of the growth rates.

Whole-genome sequencing

Genomic DNA was extracted from overnight cultures prepared in LB supplemented with 1 μg/ml cefotaxime using a QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). A Ligation Sequencing Kit (SQK-LSK109) and an R9 flowcell (FLO-MIN106D) were used on the MinION (Oxford Nanopore Technologies, United Kingdom) for long-read sequencing. Furthermore, short-read 2 x 300 bp sequencing was conducted on libraries prepared with NexteraXT (Illumina, USA) using MiSeq (Illumina). Trim Galore version 0.4.2 (https://github.com/FelixKrueger/TrimGalore) was used to process the short reads by trimming adapters and poor-quality sequences with options of "--paired --nextera". Nanopore FAST5 reads were basecalled with Guppy version 4.5.2, followed by *de novo* assembly using Canu version 2.1.1 [50], with the "corOutCoverage = 1000 genomeSize = 6m" options. Gepard version 1.40 [51] was used to identify and fix false duplications at terminal ends of contigs. Also, a base-error correction of contigs was achieved with Illumina reads using Pilon version 1.23 [52].

Phylogenetic analysis

To elucidate the evolutionary relatedness of poultry and human strains, whole genome-based phylogenetic trees were constructed with Parsnp version 1.2 [53] and TreeGraph 2 [54] was used for visualization. Multilocus sequence typing (MLST) was done *in silico* by uploading raw Illumina short reads to an MLST web server (www.cbs.dtu.dk/services/MLST) [55].

Detection of strain serotypes, plasmid replicons, AMR genes, and AMR mutations

To predict O:H serotypes and plasmid replicons, contigs were screened against the EcOH [57] and PlasmidFinder [56] databases, respectively, accessed under ABRicate version 0.8.10 (https://github.com/tseemann/abricate) with options "--mincov 90 --minid 90". Next, acquired AMR genes were detected by employing the AMRFinderPlus tool [58] using the "-i 0.7" option. Finally, quinolone resistance mutations were called against the genes *gyrA, gyrB, parC,* and *parE* from the reference strain *E. coli* MG1655 (GenBank accession no. NC_000913) using snippy version 4.6.0 [129].

Comparison of poultry isolates with human strains

To investigate possible MDR *E. coli* transmission between poultry and humans, 20 poultry-associated cefotaxime-resistant *E. coli* strains were compared to 36 cefotaxime-resistant *E. coli* isolates previously collected from human patients at the University Teaching Hospital (UTH) in Lusaka, Zambia [130]. Using the R package ComplexHeatmap [59], the AMR phenotype, AMR genes, and plasmid replicons were compared across the two niches. In addition, plasmids with common replicons were annotated using DFAST version 1.2.4 [60] and subjected to comparison analysis using Mauve [61]. Finally, tandem repeats were identified by comparing plasmid sequences using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and reading the hit table by genoPlotR [62].

Results

MDR prevalence among poultry *E. coli* strains

A total of 20 cefotaxime-resistant *E. coli* strains were obtained after screening 200 pooled cloacal samples collected from 1,000 laying hens at a commercial poultry farm in Lusaka, Zambia. The 20 strains, whose selection criteria was a cefotaxime MIC ≥ 2 µg/ml (Table 7), were subjected to susceptibility tests against nine other antimicrobials representing eight classes. All (20/20, 100%) strains were resistant to three or more antimicrobial categories, thus meeting the MDR definition [66]. In addition, all (20/20, 100%) strains exhibited resistance towards ampicillin, chloramphenicol, and doxycycline, while 18/20 (90%) were resistant to ciprofloxacin and nalidixic acid. Only 13/20 (65%) strains showed resistance to gentamicin (13/20, 65%), and none (0/20, 0%) of the strains were resistant to colistin, imipenem, or nitrofurantoin (Table 8).

965 Table 7. Characteristics of 20 *E. coli* poultry strains, collected in December 2019 in Lusaka.

Strain ID	Sequence type	Serotype	CTX MIC[a]	Growth rate
CVRI_01	69	O17H18	≥ 512	0.123
CVRI_02	155	O8H51	128	0.133
CVRI_03	155	O108:H51	≥ 512	0.200
CVRI_04	69	O17H18	≥ 512	0.221
CVRI_05	155	O8H51	256	0.197
CVRI_06	69	O17H18	≥ 512	0.145
CVRI_07	155	O8H51	256	0.143
CVRI_08	155	O8H51	256	0.128
CVRI_09	69	O17H18	≥ 512	0.229
CVRI_10	155	O108:H51	128	0.081
CVRI_11	155	O8H51	≥ 512	0.118
CVRI_12	155	O8H51	256	0.136
CVRI_14	155	O8H51	≥ 512	0.114
CVRI_15	155	O8H51	≥ 512	0.087
CVRI_16	155	O8H51	256	0.154
CVRI_17	155	O8H51	256	0.139
CVRI_18	155	O8H51	256	0.148
CVRI_19	155	O8H51	256	0.161
CVRI_20	155	O8H51	256	0.170
CVRI_21	155	O8H51	256	0.216

966
967 [a]MICs are in µg/ml
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972 Table 8. Summary of antimicrobial susceptibility test results

Antimicrobial	Number of resistant isolates	Percentage resistance
Ampicillin	20/20	100
Chloramphenicol	20/20	100
Ciprofloxacin	18/20	90
Colistin	0/20	0
Doxycycline	20/20	100
Gentamicin	13/20	65
Imipenem	0/20	0
Nalidixic acid	18/20	90
Nitrofurantoin	0/20	0

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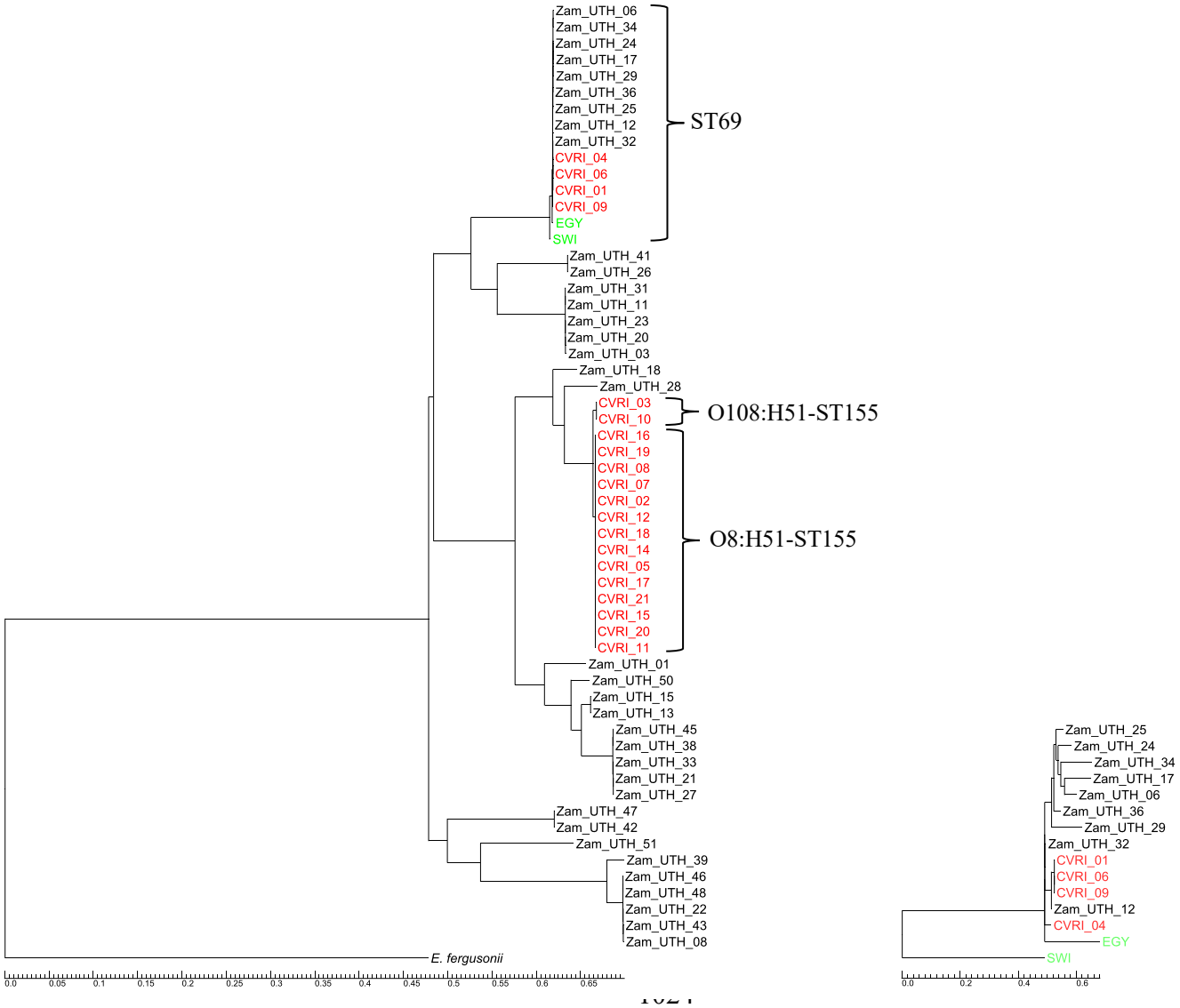
Genetic diversity among poultry strains

To comprehensively characterize the isolates, WGS was performed, and nearly complete genome sequences were reconstructed. The average size of the draft genomes ranged from 5,112,451 to 5,855,691 bp, and mean read depths ranged from 178x to 333x. In addition, the number of contigs varied from 5 to 18, with N_{50} values ranging from 4,599,588 to 5,263,002 bp.

The strains were classified by *in silico* MLST into ST69 (4/20, 20%) and ST155 (16/20, 80%) (Table 7). While all ST69 isolates were classified as O17:H18 (4/4, 100%), ST155 strains were categorized as either O8:H51 (14/16, 87.5%) or O108:H51 (2/16, 12.5%) (Table 7). Despite ST155 strains having closely related genomes, the genes *wzx* and *wzy*, which encode the O-antigen processing proteins responsible for translocating and polymerizing oligosaccharides to synthesize lipopolysaccharides, were identified in two strains. Specifically, strains CVRI_03 and CVRI_10 carried the *wzx*-O108var1 and *wzy*-O108var1 genes, suggesting the utilization of the Wzx/Wzy-dependent pathway. In contrast, 14 ST155 strains harbored the *wzm*-O8 and *wzt*-O8 genes, suggesting O-antigen translocation via the ABC transporter pathway [131].

To investigate the possibility of MDR dissemination across niches, the abovementioned 20 poultry isolates were compared to 36 *E. coli* strains isolated from human patients hospitalized at the UTH. A WGS-based phylogenetic analysis of 56 (20 poultry and 36 human) strains showed clustering of poultry isolates into two ST-specific clades, with ST155 strains further separating into two subclades based on O:H serotype (i.e., O8:H51 and O108:H51). Notably, the constructed phylogenic tree showed clustering of four (4/20, 20%) poultry and nine (9/36, 25%) human strains belonging to O17:H18-ST69, implying clonality (Fig 10A). Furthermore, a detailed phylogenic analysis of the chromosomes of ST69 strains revealed that poultry and human strains from Zambia did not cluster based on the host but were distributed within the tree. Moreover, the Zambian poultry and human strains were closely related but distant from international sequences from Switzerland (SWI) and Egypt (EGY) (Fig 10B).



B. A.

Figure 10. Phylogenetic analysis. Whole genome-based phylogenetic tree for 20 poultry and 36 human *E. coli* strains.

A. Poultry strains formed two clusters based on two STs; one cluster had four ST69 strains, while the other had 16 ST155 strains. The ST155 group was further divided into two subgroups based on O:H serotypes; 14 strains belonged to O8:H51, and the remaining two were O108:H51. In addition, the genome of *E. fergusonii* (GenBank accession number NZ_CP057659.1) was included as an outgroup. Furthermore, *E. coli* strain CFSAN061770 (GenBank accession number NZ_CP023142.1), belonging to ST69 and isolated from raw milk cheese in Egypt (here abbreviated as EGY), and *E. coli* strain 3347558 (also ST69) (GenBank accession number CP071073.1) isolated from a patient in Switzerland (here

abbreviated as SWI) were also included in the analysis. Red; poultry (Zambia). Black; human (Zambia). Green; International.

B. Detailed phylogenic tree focusing on ST69 chromosomes only. The Zambian poultry and human strains were closely related and did not cluster based on the host.

Location of *bla*$_{\text{CTX-M}}$ genes among poultry strains

To detect the AMR genes present in poultry strains, the AMRFinderPlus tool [58] was used. A total of 27 AMR genes targeting 11 antibiotic classes were identified, dominated by targets of aminoglycosides (7/27, 25.9%), β-lactams (3/27, 11.1%), quinolones (3/27, 11.1%), and trimethoprim (3/27, 11.1%) (Table 9). These AMR genes existed on plasmids of various incompatibility (Inc) groups.

The primary AMR mechanism in *E. coli* involves CTX-M type ESBLs, encoded by *bla*$_{\text{CTX-M}}$ genes [132]. All the 20 (100%) strains carried an allele of the *bla*$_{\text{CTX-M}}$ gene; 18/20 (90%) strains harbored *bla*$_{\text{CTX-M-55}}$, while the other 2/20 (10%) isolates carried *bla*$_{\text{CTX-M-14}}$. The *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CTX-M-14}}$ genes existed 421 bp and 326 bp, respectively, downstream of the insertion sequence IS*26*.

Previous studies show that OqxAB-encoding genes seldom correlate with ciprofloxacin and nalidixic acid resistance [133]. Furthermore, aminoglycoside modifying enzymes exhibit divergent substrate specificities, with APH(6)-Id-like and APH(3")-Ib-like types ineffective against gentamicin [134]. Consistently, the current study showed low positive predictive values (PPVs) for genes encoding OqxAB (PPV = 0% for both *oqxA2* and *oqxB*), APH(6)-Id (PPV = 56.25 % for *aph(3")-Ib*), and APH(3")-Ib (PPV = 65% for *aph(6)-Id*). In contrast, there was a perfect correlation between other detected AMR genes and the observed phenotypes (PPV = 100%) (Table 10).

Table 9. Diversity of AMR genes among the 20 poultry strains

Target drug class	AMR genes detected
β-lactam	*bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-55}}$
Aminoglycoside	*aac(3)-IIa, aac(3)-IId, aadA1, aadA2, aadA5, aph(3'')-Ib, aph(6)-Id*
Trimethoprim	*dfrA12, dfrA14, dfrA17*
Tetracycline	*tet(A), tet(M)*
Chloramphenicol	*cmlA1, floR*
Sulfonamide	*sul1, sul2*
Fosfomycin	*fosA3*
Macrolide	*mph(A)*
Quinolone	*oqxA2, oqxB, qnrS1*
Disinfectant	*qacL, qacEdelta1*
Bleomycin	*bleO*

Table 10. Prediction of phenotype from AMR genes.

GEN	*aac(3)-IIa*	*aac(3)-IId*	*aadA1*	*aadA2*	*aadA5*	*aph(3'')-Ib*	*aph(6)-Id*
R	2	4	4	4	2	9	13
S	0	0	0	0	0	7	7
Total	2	4	4	4	2	16	20
PPV (%)	**100**	**100**	**100**	**100**	**100**	**56.25**	**65**

CIP	*oqxA2*	*oqxB*	*qnrS1*
R	0	0	4
S	2	2	0
Total	0	0	4
PPV (%)	**0**	**0**	**100**

NAL	*oqxA2*	*oqxB*	*qnrS1*
R	0	0	4
S	2	2	0
Total	0	0	4
PPV (%)	**0**	**0**	**100**

DOX	*tet(A)*	*tet(M)*
R	20	4
S	0	0
Total	20	4
PPV (%)	**100**	**100**

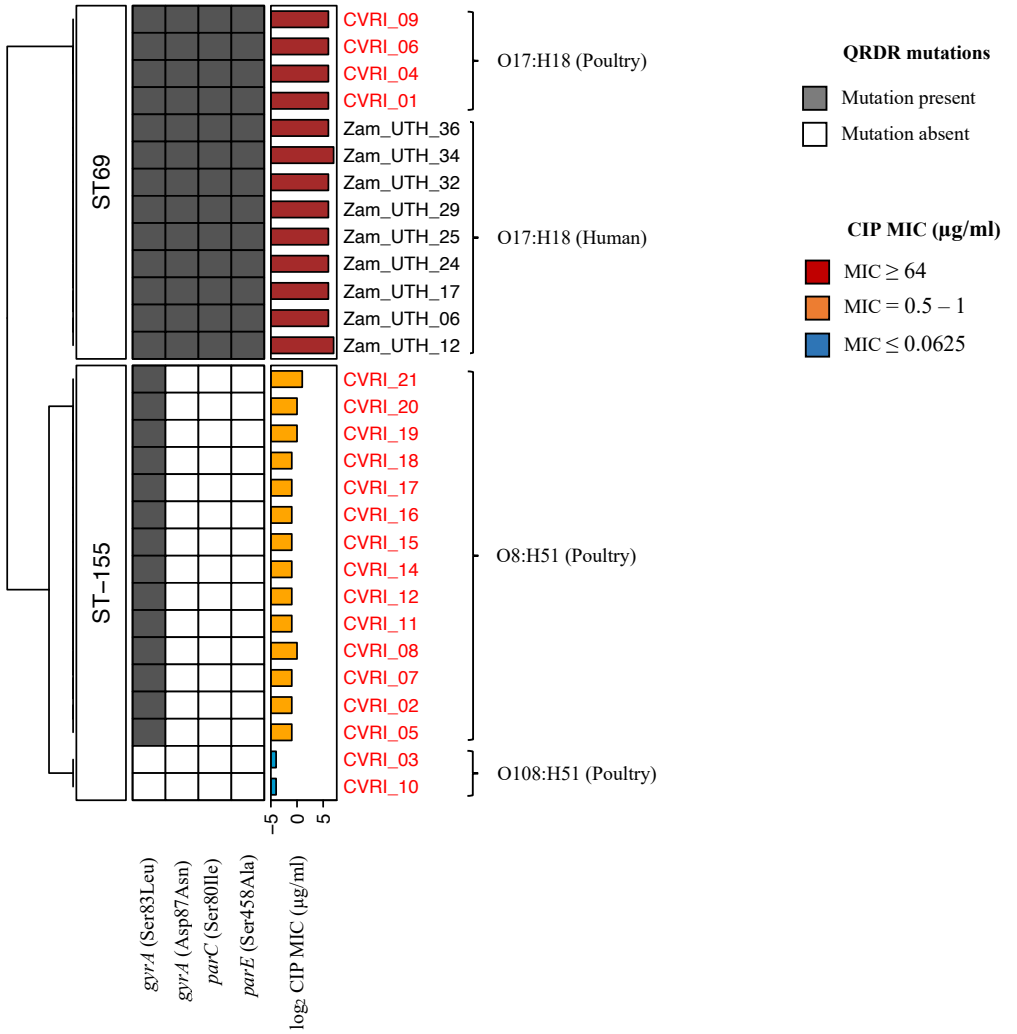
CHL	*cmlA1*
R	4
S	0
Total	4
PPV (%)	**100**

GEN = gentamicin, CIP = ciprofloxacin, NAL = nalidixic acid, DOX = doxycycline, CHL = chloramphenicol

R = resistant, S = susceptible

Quinolone resistance mutations in *gyrA*, *parC,* and *parE*

The high quinolone resistance rate (90%) observed in this study could not be explained by mobile quinolone resistance genes (PPV = 0% for *oqxA2* and *oqxB*) (Table 10). Furthermore, only 4/18 (22.2%) quinolone-resistant strains carried the *qnrS1* gene that encodes the QnrS1 protein to protect topoisomerases from quinolones. Moreover, the reduction in quinolone susceptibility by this mechanism is only complementary and not sufficient to meet the clinical breakpoint prescribed by the CLSI (0.5 μg/ml and 32 μg/ml for ciprofloxacin and nalidixic acid, respectively) [92]. To understand the observed resistance, mutations in the quinolone resistance determining regions (QRDRs) of *gyrA, gyrB, parC,* and *parE* were explored. In agreement with previous reports [135-137], missense mutations were detected in three genes: *gyrA* (Ser83Leu and Asp87Asn), *parC* (Ser80Ile), and *parE* (Ser458Ala). Two O108:H51-ST155 strains that did not harbor any mutations in the abovementioned genes were susceptible to both ciprofloxacin (MIC ≤ 0.0625 μg/ml) and nalidixic acid (MIC = 16 μg/ml). However, 14 O8:H51-ST155 strains with the Ser83Leu mutation in the *gyrA* gene exhibited resistance to ciprofloxacin (MIC range; 0.5–1 μg/ml) (Fig 11) and nalidixic acid (MIC range; 512–1024 μg/ml) (Fig 12). Lastly, the four O17:H18-ST69 strains possessed mutations at all the four positions mentioned above and showed high resistance levels to ciprofloxacin (MIC ≥ 64 μg/ml) and nalidixic acid (MIC ≥ 2048 μg/ml). The same four mutations were observed in nine human-associated O17:H18-ST69 strains that also exhibited high-level quinolone resistance (Fig 11 and 12). While quinolone resistance has also been linked to amino acid substitutions at positions 426 and 447 of *gyrB* [138], none of these mutations were detected in the present study.



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1102

Figure 11. Ciprofloxacin resistance. Missense mutations in the QRDR of *gyrA*, *parC* and *parE*.

1103 The lack of mutations in the QRDR of *gyrA*, *parC,* and *parE* was associated with very low
1104 ciprofloxacin MIC (blue bars), but strains with a single mutation at position 83 of the *gyrA*
1105 gene had ciprofloxacin MIC above the CLSI breakpoint of 0.5 µg/ml. Furthermore, co-
1106 occurrence of mutations at all four positions coincided with high-level ciprofloxacin
1107 resistance (MIC ≥ 64 µg/ml).
1108 CIP; ciprofloxacin.

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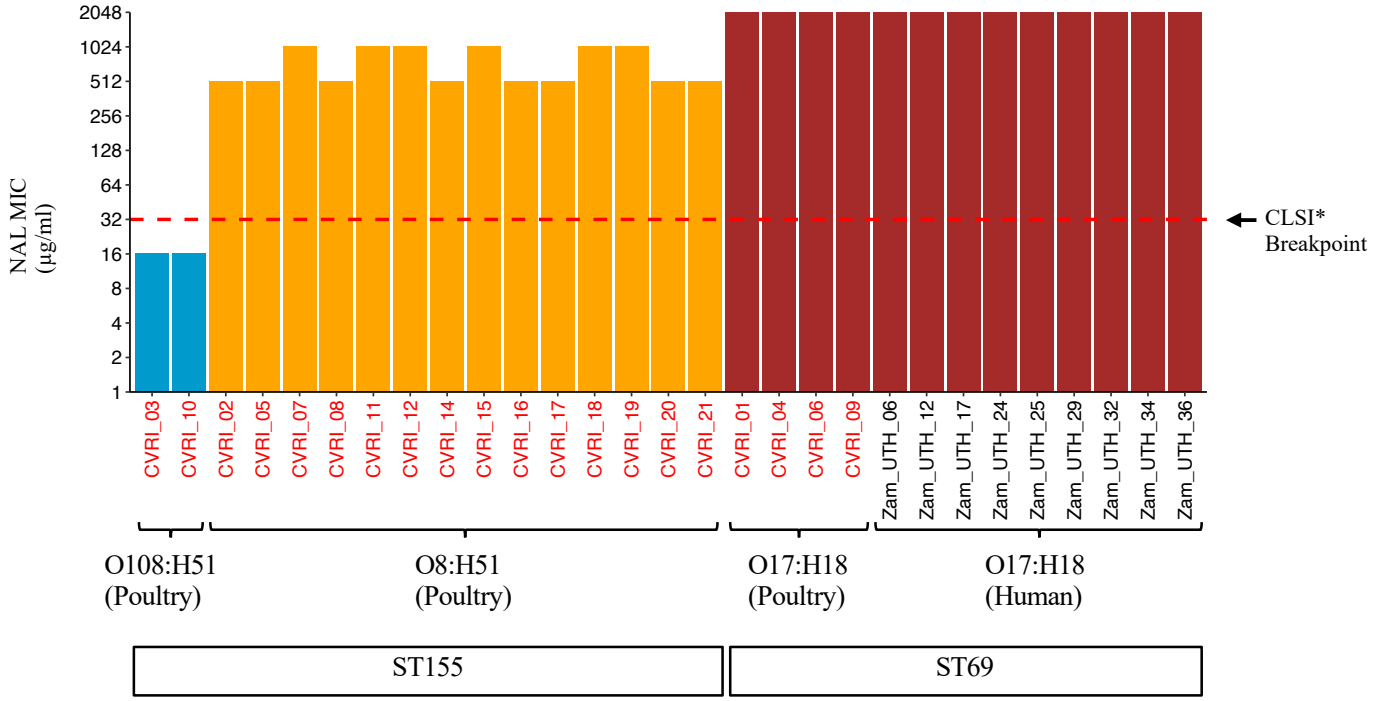

Figure 12. Effect of topoisomerase mutations on nalidixic acid MIC.

The lack of QRDR mutations in two O108:H51-ST155 strains (blue) was associated with susceptibility to nalidixic acid. In contrast, 14 O8:H51-ST155 strains (orange) possessing a single *gyrA* gene mutation (Ser83Leu) exhibited nalidixic acid resistance with MICs exceeding the clinical breakpoint by several folds. Furthermore, the four poultry and nine human O17:H18-ST69 strains (brown), harboring quinolone resistance mutations in *gyrA*, *parC,* and *parE*, displayed high-level resistance to nalidixic acid (MIC ≥ 2048 µg/ml).

*Clinical and Laboratory Standards Institute [73]

Hierarchical clustering of ST69 strains from poultry and humans

To determine the mechanism of MDR propagation among poultry and human strains, clustering patterns of isolates were explored by examining various phenotypic and genotypic characteristics. Fifteen plasmid replicons were identified across the 20 poultry isolates, the commonest being IncFII(29)_1_pUTI89 (16/20, 80%), followed by IncFII(pHN7A8)_1_pHN7A8 (14/20, 70%), and IncN_1 (14/20, 70%). A comparison of poultry and human strains based on AMR phenotype, AMR genes, and plasmid replicons showed clustering into five main clades (referred to as C1, C2, C3, C4, and C5) (Fig 13). Most human isolates occupied separate clusters from poultry strains; however, C1 comprised four poultry and nine human isolates belonging to O17:H18-ST69. These strains displayed similar AMR patterns and had similar AMR genes and plasmid replicons, suggesting clonal spread (Fig 13). Poultry and human strains from C1 shared the plasmid replicons Col(MG828)_1, ColpVC_1, IncB/O/K/Z_2, IncFIA_1, IncFIB(AP001918)_1, and IncX1_1. However, the IncHI2A_1 and IncHI2_1 replicons were only present in human-associated strains but not in poultry strains. On the contrary, the human isolates lacked the IncFII(pCoo)_1_pCoo replicon present in poultry strains. Other overlapping replicons included two O108:H51 poultry strains in clade C5 sharing IncHI2_1 and IncHI2A_1 replicons with one human isolate in C3 (Zam_UTH_28, Onovel28:H5-ST156) and nine human strains in C1 (O17:H18-ST69).

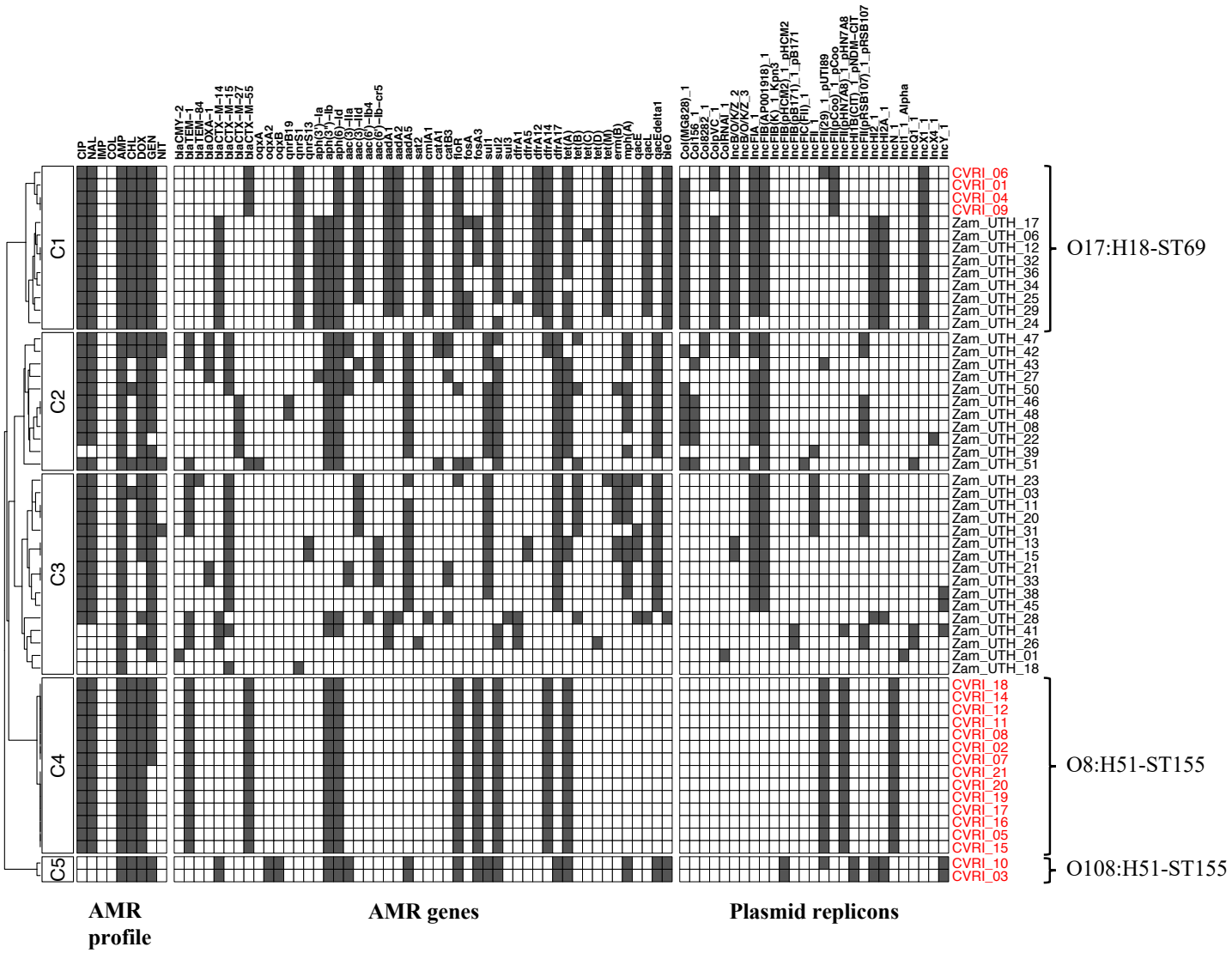

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Figure 13. AMR phenotypes, AMR genes, and plasmid replicons among poultry and human isolates.

1143 All (20/20, 100%) poultry strains exhibited MDR phenotypes, but none of them was resistant
1144 to colistin, imipenem, or nitrofurantoin. Only two *bla*$_{\text{CTX-M}}$ alleles were detected across the 20
1145 poultry strains, with *bla*$_{\text{CTX-M-55}}$ occurring in 18/20 (90%) strains and the remaining 2/20
1146 (10%) isolates harboring the *bla*$_{\text{CTX-M-14}}$ gene. Similarly, there was a narrow plasmid replicon
1147 diversity among poultry strains, with only 15 types detected compared to 24 types among

human isolates. On hierarchical clustering, five distinct clades were formed across the 20 poultry and 36 human isolates; C1 was composed of four poultry and nine human O17:H18-ST69 strains, C2 and C3 comprised human strains of various STs, C4 consisted of 14 O8:H51-ST155 strains, while C5 constituted two O108:H51-ST155 strains. Only a few plasmid replicons were common between poultry and human strains.

Red; poultry. Black; human.

Comparison of AMR plasmids among poultry and human *E. coli* ST69 strains

A comparison analysis was carried out on plasmids carrying identical AMR genes and sharing plasmid replicons to understand the spread patterns of MDR among poultry and human strains. Two distinct plasmids were identified in four O17:H18-ST69 poultry isolates and nine human-associated O17:H18-ST69 strains. One of the plasmids belonged to incompatibility group IncFI, was 83 kb in size, and carried nine AMR genes (i.e., *aac(3)-IId*, *aadA1*, *aadA2*, *cmlA1*, *dfrA12*, *floR*, *qacL*, *sul2*, and *tet(M)*). The other one belonged to IncI-complex, was 124 kb long, and carried five AMR genes (*aph(6)-Id*, *bleO*, *dfrA14*, *qnrS1*, and *tet(A)*) (Table 11). Comparison of contigs across niches revealed almost identical corresponding plasmids (Fig 14A and 14B). However, the isolates also contained other AMR plasmids specific for each niche, probably signifying additional independent mechanisms of AMR acquisition. Despite the difference in plasmid content between poultry and human O17:H18-ST69 strains, growth rates measured in antibiotic-free LB were comparable across niches (Fig 15), suggesting similar fitness.

Precisely, poultry-associated O17:H18-ST69 strains harbored a 71 kb IncFII(pCoo) plasmid carrying the $bla_{\text{CTX-M-55}}$ gene (Table 11). This plasmid shared over 90% nucleotide sequence similarity with pL37-4 (GenBank accession no. CP034592.1), a $bla_{\text{CTX-M-55}}$-carrying plasmid also belonging to IncFII(pCoo), reported in an *E. coli* O9:H37-ST48 isolate originating from a goose farm in Jiangsu, China (Fig 16). In contrast, O17:H18-ST69 strains from humans contained a 225 kb IncHI plasmid harboring $bla_{\text{CTX-M-14}}$ and several other AMR genes (Table 11). Additionally, this IncHI plasmid exhibited over 85% nucleotide sequence similarity with another $bla_{\text{CTX-M-14}}$-carrying IncHI plasmid in two O108:H51-ST155 poultry strains, signifying origin from a common plasmid ancestor (Fig 14C). In both cases, the *fosA3* gene existed 611 bp downstream of the $bla_{\text{CTX-M-14}}$ gene on a 4.8 kb unit flanked by a pair of IS*26* copies (Fig 17).

1183

1184 Table 11. Plasmids in *E. coli* O17:H18-ST69 from humans and poultry.

Strain ID	Plasmid Inc. group	Plasmid size (bp)	Plasmid replicons	AMR genes
CVRI_01 (Poultry)	IncFI	83,168	IncFIA_1, IncFIB(AP001918)_1	*tet(M), qacL, aadA1, cmlA1, aadA2, dfrA12, floR, sul2, aac(3)-IId*
	IncI-complex	124,080	IncB/O/K/Z_2, IncX1_1	*aph(6)-Id, qnrS1, bleO, dfrA14, tet(A)*
	IncFII(pCoo)	70,832	IncFII(pCoo)_1_pCoo	*bla*$_{\text{CTX-M-55}}$
Zam_UTH_32 (Human)	IncFI	82,946	IncFIA_1, IncFIB(AP001918)_1	*tet(M), qacL, aadA1, cmlA1, aadA2, dfrA12, floR, sul2, aac(3)-IId*
	IncI-complex	125,522	IncB/O/K/Z_2, IncX1_1	*aph(6)-Id, qnrS1, bleO, dfrA14, tet(A)*
	IncHI	224,891	IncHI2A_1, IncHI2_1	*fosA3, bla*$_{\text{CTX-M-14}}$*, sul2, aph(3'')-Ib, aph(6)-Id, tet(A), floR, aph(3')-I*

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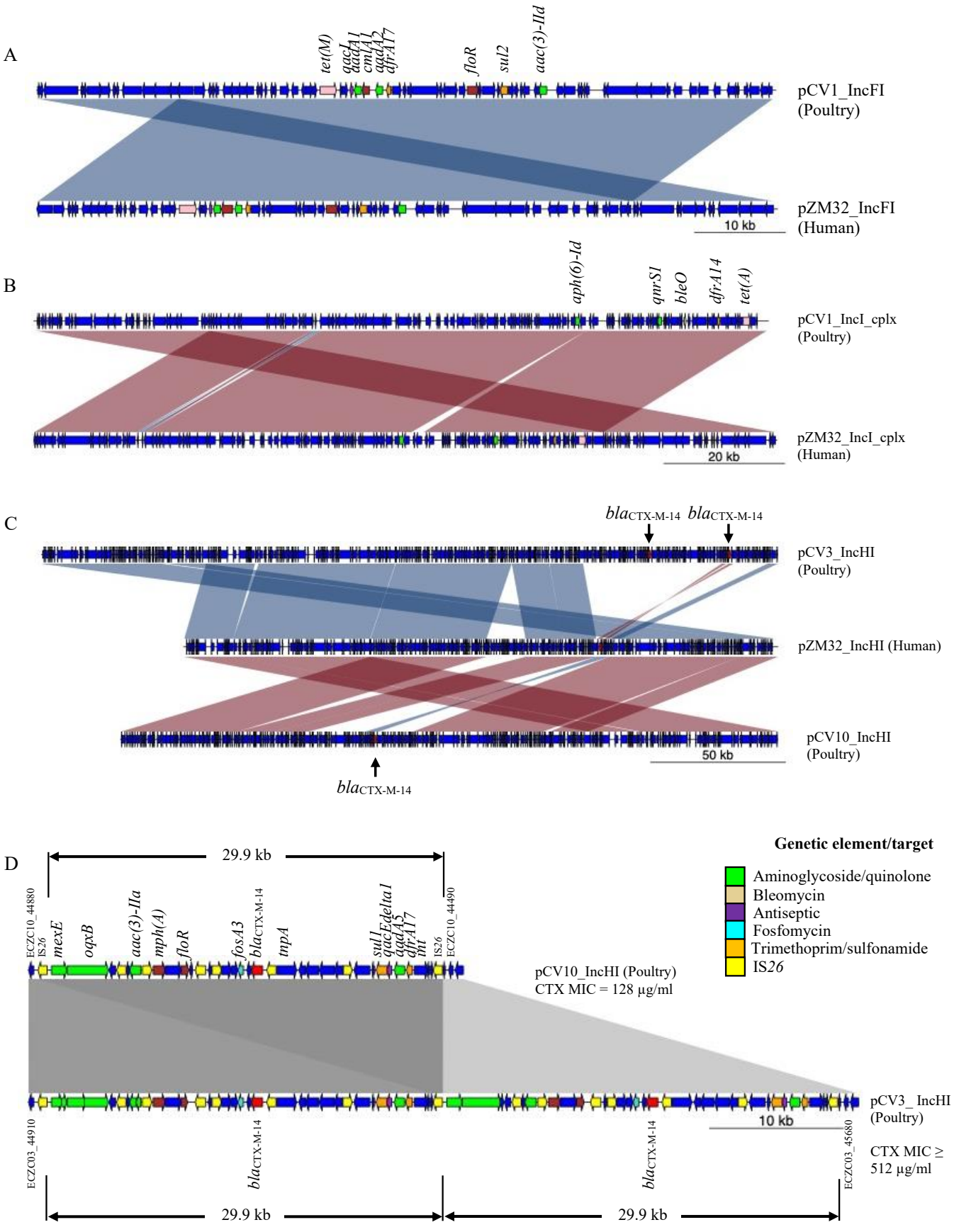
A
tet(M)
gacI
dadA1
cmlA1
qadA2
dfrA17
floR
sul2
aac(3)-IId
pCV1_IncFI (Poultry)
pZM32_IncFI (Human)
10 kb
B
aph(6)-Id
qnrS1
bleO
dfrA14
tet(A)
pCV1_IncI_cplx (Poultry)
pZM32_IncI_cplx (Human)
20 kb
C
blaCTX-M-14
blaCTX-M-14
pCV3_IncHI (Poultry)
pZM32_IncHI (Human)
pCV10_IncHI (Poultry)
50 kb
blaCTX-M-14
D
29.9 kb
Genetic element/target
Aminoglycoside/quinolone
Bleomycin
Antiseptic
Fosfomycin
Trimethoprim/sulfonamide
IS26
ECZC10_44880
IS26
mexE
oqxB
aac(3)-IIa
mph(A)
floR
fosA3
blaCTX-M-14
tnpA
sul1
qacEdelta1
qadA5
dfrA17
int
IS26
ECZC10_44490
pCV10_IncHI (Poultry)
CTX MIC = 128 µg/ml
pCV3_ IncHI (Poultry)
CTX MIC ≥ 512 µg/ml
10 kb
ECZC03_44910
blaCTX-M-14
blaCTX-M-14
ECZC03_45680
29.9 kb
29.9 kb

1187

Figure 14. Comparison of plasmids among *E. coli* strains from humans and poultry.

Two plasmids, (A) IncFI and (B) IncI-complex, were shared across human- and poultry-associated *E. coli* O17:H18-ST69 strains. (C) In contrast, a *bla*$_{\text{CTX-M-14}}$-harboring IncHI plasmid was present in human-associated *E. coli* O17H18-ST69 strains but not in poultry-associated *E. coli* O17H18-ST69. A related IncHI plasmid with over 85% nucleotide sequence homology was detected in two poultry *E. coli* O108:H51-ST155 strains. (D) IncHI plasmid in the two O108:H51-ST155 poultry strains possessed multiple AMR genes on a putative 29.9 kb IS*26*-flanked TU. This suspected TU existed in tandem duplication in strain CVRI_03 and was associated with a higher cefotaxime resistance (CTX MIC ≥ 512 μg/ml) relative to CVRI_10 (CTX MIC = 128 μg/ml).

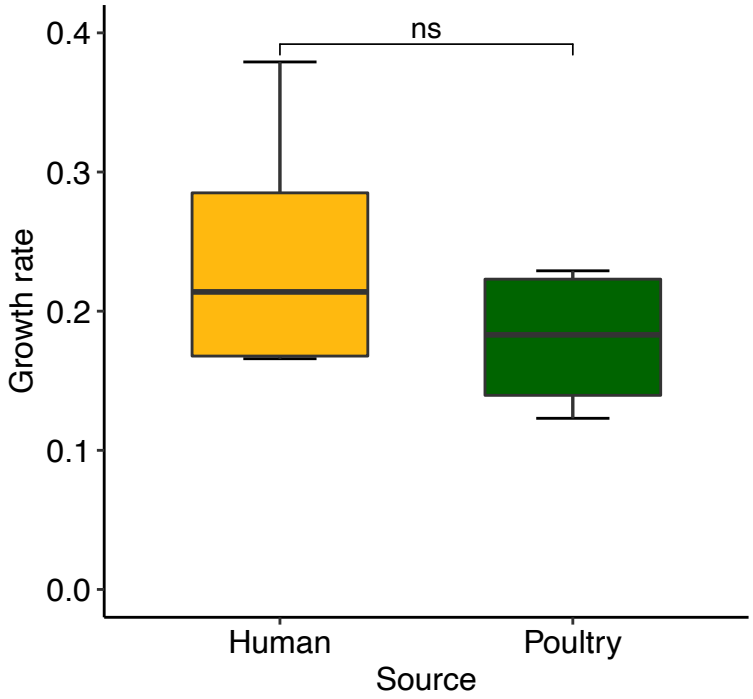

Figure 15. Comparison of growth rates between poultry and human *E. coli* O17:H18-ST69.

No significant difference was found between the growth rate of poultry (n = 4) and human (n = 9) isolates (Mann-Whitney U test; P = 0.414).

Several studies have reported the coexistence of *bla*$_{\text{CTX-M-14}}$ and *fosA3* [139, 140] among *Enterobacteriaceae* strains. One frequently observed gene arrangement includes IS*26*–*bla*$_{\text{CTX-M-14}}$–611 bp–*fosA3*–1222 bp–IS*26*, with directly oriented flanking IS*26* copies [141]. However, a slightly different genetic architecture was identified in this study (IS*26*–*bla*$_{\text{CTX-M-14}}$–611 bp–*fosA3*–1274 bp–IS*26*), where IS*26* existed 1274 bp downstream *fosA3*. Furthermore, the flanking IS*26* copies in this study were inverted with respect to each other, thus not fulfilling the architecture required to form a cointegrate or a translocatable unit (TU) [142] during IS*26*-mediated co-transfer of *bla*$_{\text{CTX-M-14}}$ and *fosA3*. Further analysis of the two poultry-associated O108:H51-ST155 strains uncovered that the abovementioned 4.8 kb unit was part of a larger segment bracketed by two IS*26* copies in direct orientation, resulting in a potential 29.9 kb TU. This unit harbored several mobility-associated elements, including seven copies of IS*26*, an integrase gene (*int*), and a transposase gene (*tnpA*). Pseudo-compound transposons flanked by IS*26* may facilitate gene amplification by excising a TU that reinserts to create an array of two or more TUs [143]. Notably, the suspected 29.9 kb TU occurred in tandem duplication in one O108:H51-ST155 strain (CVRI_03), increasing the copy number of the *bla*$_{\text{CTX-M-14}}$ and several other AMR genes (Fig 14D). Nanopore reads traversing the junctions of this segment ruled out the possibility of erroneous assembly. Moreover, the observed duplication, which included the *bla*$_{\text{CTX-M-14}}$ gene, coincided with a higher cefotaxime MIC in CVRI_03 (≥ 512 μg/ml) compared to CVRI_10 (128 μg/ml) (Table 7). While the two O108:H51-ST155 strains were very similar, CVRI_10 carried an extra 198 kb IncFII(29)_1_pUTI89 plasmid, lacking in CVRI_03. This difference in the plasmid content was accompanied by a discrepancy in growth rate between CVRI_03 (μ = 0.200) and CVRI_10 (μ = 0.081), complicating the assessment of the amplified TU's effect on the fitness of CVRI_03.

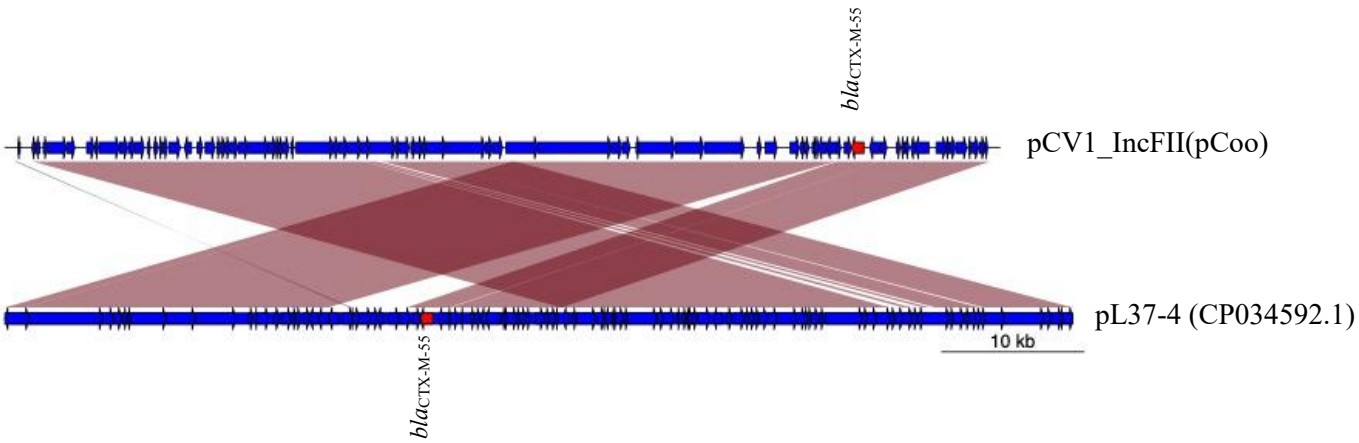

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Figure 16. Similarity between plasmids pCV1_IncFII(pCoo) and pL37-4.

1247 Both plasmids belonged to IncFII(pCoo) incompatibility group, with over 90% nucleotide
1248 sequence similarity and harboring the *bla*$_{\text{CTX-M-55}}$ gene (red).

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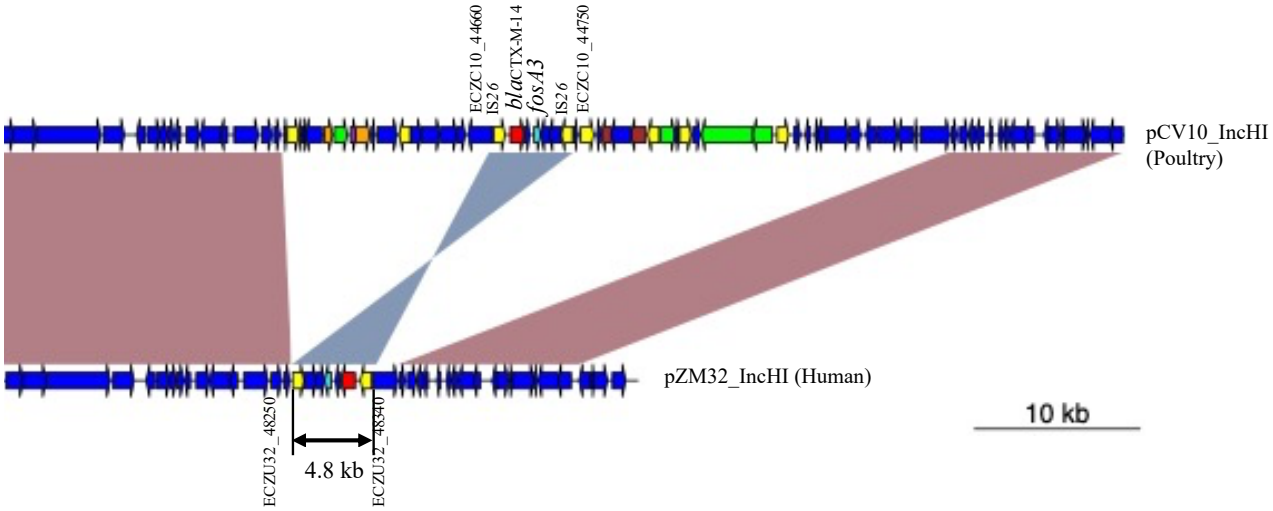

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Figure 17. Genetic architecture around the *bla*$_{\text{CTX-M-14}}$ gene in IncHI plasmid.

1252 The IncHI plasmid in poultry-associated ST155 strains shared a 4.8 kb unit with ST69
1253 isolates from humans. This unit carried *bla*$_{\text{CTX-M-14}}$ and *fosA3* genes, flanked by IS*26* copies
1254 in inverted orientation.

1255

1256

Discussion

In this study, 20 cefotaxime-resistant *E. coli* isolates obtained from poultry in Lusaka, Zambia, were characterized by WGS. These isolates were tested for MDR and compared to 36 cefotaxime-resistant *E. coli* strains isolated from hospital patients in the same city. Four out of 20 (4/20, 20%) poultry isolates shared clonality with 9/36 (25%) human strains. The four poultry and nine human strains had the same ST and O:H serotype and exhibited identical MDR profiles and QRDR mutations. The said strains also possessed two nearly identical plasmids carrying the same 14 AMR genes, suggesting clonal origin. Interestingly, the strains also had $bla_{\text{CTX-M}}$-harboring plasmids different for each niche, implying separate ESBL acquisition modes. These results indicate a clinically important link between MDR *E. coli* strains in poultry and humans, underscoring that a multisectoral approach is required to mitigate the threat of MDR.

ST69, which is among the most challenging lineages known, is the second most frequent extraintestinal pathogenic *E. coli* [144]. In Zambia, 25% of ESBL-producing *E. coli* at the UTH belonged to the MDR clone *E. coli* ST69 [130]. Because of this predominance, there is a need to reveal the potential reservoirs and acquisition routes of the *E. coli* ST69 clone. Notably, the present study reports a linkage between this clone and *E. coli* ST69 isolates obtained from poultry in the same city. Apart from two plasmids (i.e., IncFII(pCoo) present only in poultry strains and IncHI found only in human isolates), the poultry- and human-associated ST69 lineages were nearly identical, with the same O:H serotypes and resistance plasmids (IncFI and IncI-complex). Furthermore, the ST69 isolates from poultry and humans also shared several AMR genes and QRDR mutations. The close phylogenic relationship between poultry and human strains without host-specific clustering could imply clonality. These results suggest that MDR propagation between poultry and humans likely occurred through the direct transmission of the ST69 clone, including the two plasmids belonging to IncFI and IncI-complex. The clones could have subsequently diverged along separate evolutionary trajectories in response to various selection pressures associated with the host environment, culminating in the acquisition of distinct ESBL-encoding plasmids (Fig 18).

One crucial trait among bacteria is the ability to resist multiple antimicrobials, leading to their persistence in challenging ecological habitats, such as patients and animals on antibiotic treatment. The 20 poultry strains in the present study were all resistant to various antimicrobials of clinical significance, such as ampicillin, chloramphenicol, and doxycycline.

The observed MDR pattern could result from the current practices and antimicrobial use in Zambian poultry, where amoxicillin, chloramphenicol, and doxycycline are among the frequently used drugs, according to the Zambia National Public Health Institute (ZNPHI) [111]. However, the observed MDR prevalence (100%) among the poultry samples was likely overestimated since the selection criterion, cefotaxime resistance, is often associated with MDR [101].

Of particular concern was the observation that 90% of the isolates exhibited resistance to ciprofloxacin and nalidixic acid, clinically significant quinolones commonly used as first- and second-line drugs to treat a wide range of bacterial infections. The high quinolone resistance observed in this study was similar to the prevalence among clinical strains from the UTH, where close to 90% of the MDR *Enterobacteriaceae* displayed resistance to ciprofloxacin and nalidixic acid [130]. Again, the actual quinolone resistance burden might be overstated by these results since this study only included cefotaxime-resistant strains, potentially associated with quinolone resistance [145, 146]. Despite most poultry strains showing resistance to the quinolones, MICs were much higher in the four *E. coli* ST69 isolates carrying mutations in the QRDRs of *gyrA*, *parC*, and *parE*. Similarly, the nine human *E. coli* ST69 isolates mentioned above showed equally high MICs for ciprofloxacin and nalidixic acid and harbored the same quinolone resistance mutations, thus supporting the proposed clonal expansion hypothesis.

Studies elsewhere show that *E. coli* ST155 is common among poultry samples from other African countries [147]. In the present study, ST155 predominated the poultry samples at 80% (16/20), but it was absent in the clinical strains. However, other studies have reported this ST in humans [116], making it a potential MDR threat in Zambia. Besides, the MDR-encoding IS*26*-flanked TU identified on an IncHI plasmid in a pair of ST155 strains (CVRI_03 and CVRI_10) is a call for concern. Research on IS*26*-associated TUs carrying various β-lactamase genes ($bla_{\text{TEM-1B}}$ [143], $bla_{\text{OXA-1}}$, and $bla_{\text{CTX-M-15}}$ [148]) shows an associated increase in resistance to β-lactam antibiotics. Consistently, the $bla_{\text{CTX-M-14}}$-carrying TU in CVRI_03 seemed to have increased the cefotaxime MIC of this strain. However, no apparent treatment implications are expected from this change since it takes only a single copy of $bla_{\text{CTX-M}}$ to cause cefotaxime resistance. Also, the said ST155 strains (CVRI_03 and CVRI_10) exhibited identical phenotypic AMR profiles. Still, this finding is clinically relevant because IS*26* can transfer AMR genes among various *Enterobacteriaceae* clones and species [149], highlighting that ST155 is a potential MDR reservoir for human pathogens in Zambia.

Nevertheless, it is unclear whether the high prevalence observed for ST155 was expected since previous reports in Zambia [17, 150] did not characterize MDR *E. coli* in terms of MLST. Moreover, all the samples included in this study were collected from the same farm, making it difficult to generalize and carefully interpret the findings. Hence, more extensive studies, including more isolates and sampling points, will be required to answer many questions definitively. Nevertheless, the results presented here still raise public health concerns since the farm sampled is commercial and supplies poultry products to several communities in Lusaka and nearby districts.

Furthermore, the clonal relationship between *E. coli* ST69 strains from poultry and humans suggests that MDR could have spread between these two niches. While there is a possibility that poultry and humans independently acquired the *E. coli* ST69 clone from another source, the model (Fig 18) in the present study seems plausible considering how common poultry products are and the associated antibiotic use during production. In Zambia, poultry accounts for 50% of the country's meat consumption, and the industry generates 5% of the national GDP [151]. As the national population expands, there is an increase in the demand for animal-source protein, including poultry and poultry products. To meet this demand, poultry companies heighten production by raising large numbers of birds through intensive farming methods that often require antibiotic usage for prophylaxis and sometimes for growth enhancement. While this is crucial to alleviating food shortage problems, the associated MDR escalation is incredibly problematic.

To address this dilemma, the Ministry of Health and the Ministry of Livestock and Fisheries (MLF) should make tradeoffs that limit MDR spread while adequate poultry production is maintained. The University of Zambia has been a critical stakeholder in fostering One Health [152], leading to a rise in the discourse on the concept. These efforts have attracted crucial partners such as the ZNPHI, who recently launched a One Health Surveillance Platform for AMR in Zambia [153]. Moving forward, there is a considerable need to build on the progress made so far by strengthening the main pillars of AMR prevention using a multisectoral approach. Achieving the desirable outcomes demands the MLF to educate poultry farmers about the dangers of non-rational antibiotic use and the benefits of improving animal conditions and hygiene. In addition, there should be more restrictions on the sale of antibiotics for prophylaxis. At the same time, AGP use must be guided by recommendations from thorough risk analysis and solely limited to nonmedically important antibiotics. Finally, routine food inspection must be strengthened by including antibiotic residue monitoring to increase the quality of the data available for policymaking.

Moreover, extending these tests to include other food animals would help identify various potential MDR reservoirs.

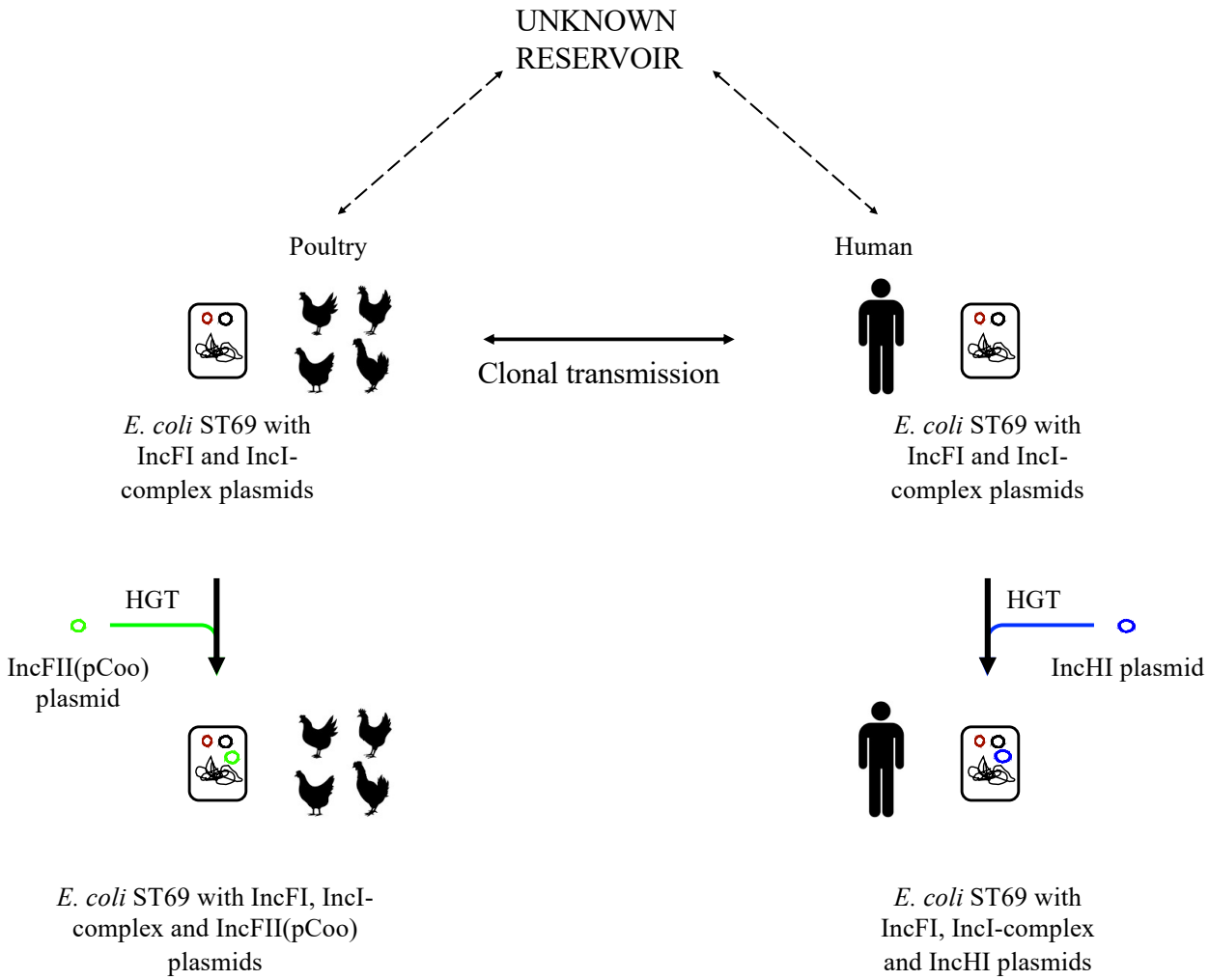

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Figure 18. Schematic proposal for the transmission of *E. coli* O17:H18-ST69 between poultry and humans.

1362 The model proposes that the *E. coli* O17:H18-ST69 strain, along with the two AMR plasmids
1363 (IncFI and IncI-complex), was likely acquired by poultry or humans from an unknown
1364 source. This could have been followed by direct transmission between poultry and humans;
1365 the strain probably underwent different evolutionary trajectories in the two hosts where the
1366 poultry lineage acquired a *bla*$_{\text{CTX-M-55}}$-carrying IncFII(pCoo) plasmid, while the human
1367 lineage gained a *bla*$_{\text{CTXM-14}}$-carrying IncHI plasmid.

1368

General conclusion

Therapeutic challenges faced by clinicians during the management of patients infected with drug-resistant *Enterobacteriaceae* raise considerable concern. The problem is further exacerbated by the rise of animal-related AMR following antibiotic use for prophylaxis and growth promotion. Developing practical control strategies requires epidemiologic research linking AMR and patient outcomes. However, the lack of detailed molecular data in many developing countries limits the successful implementation of effective AMR control policies. In Zambia, for example, previous studies on ESBL-mediated AMR in humans and poultry have relied on limited technologies such as PCR. Therefore, building on previous efforts, this work provides a WGS-based analysis of 46 human- (one *E. cloacae*, nine *K. pneumoniae*, and 36 *E. coli*) and 20 poultry-associated (*E. coli*) ESBL-producing strains.

In Chapter I, the study characterized susceptibility profiles and AMR genes among 46 cefotaxime-resistant hospital *Enterobacteriaceae*. The results revealed that the spread of $bla_{\text{CTX-M}}$ genes is perpetuated by the persistence of a few resilient STs, with *E. coli* ST69 dominating. In addition, one *E. cloacae* and three *E. coli* strains carried large chromosomal insertions co-harboring the IS*Ecp1*-$bla_{\text{CTX-M-15}}$ transposition unit and various AMR genes originating from plasmids. The findings imply that IS*Ecp1* mobilizes large $bla_{\text{CTX-M-15}}$-containing MDR segments in diverse *Enterobacteriaceae* species. The stable maintenance of these MDR segments on chromosomes may facilitate the spread and persistence of MDR clones and lead to treatment failure and poor patient outcomes.

In Chapter II, the phylogenetic relationship of MDR *E. coli* from poultry and humans in Zambia was inferred to establish the possibility of MDR spread between the two niches. The results revealed a close relationship between MDR *E. coli* ST69 from poultry and humans, suggesting possible transmission between the niches. The observation highlights the need for a closer multisectoral collaboration between human and animal health experts under the One Health umbrella. In addition, risk analysis and genome-based surveillance should guide policy formulation on AGP use in food animals.

The results presented here provide an important benchmark for further studies to forecast and limit MDR spread from poultry to humans in Zambia.

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