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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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1 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

4 Unit measure abbreviations

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

5
6

7 **Notes**

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

10

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21 **multidrug-resistant *Escherichia coli* ST69 from poultry and humans in Lusaka,**
22 **Zambia**. *FEMS Microbiology Letters*. (Under review).

23

24

25 Preface

26 Antimicrobials have significantly improved the treatment of many life-threatening
27 bacterial diseases, leading to increased life expectancy [1]. However, as Fleming warned in
28 1945 [2], misuse and overuse of antimicrobials have accelerated the emergence and evolution
29 of antimicrobial resistance (AMR). The clinical, social, and economic impacts of AMR are
30 profound and enormously worrisome. Over 2.8 million AMR-related cases are reported every
31 year in the United States alone, with over 35,000 resulting in mortality [3]. The year-on-year
32 rise in AMR incidence is attributable to modern anthropogenic elements linked to social,
33 cultural, economic, and political factors. For instance, inaccurate or delayed diagnosis
34 resulting from limited laboratory capacity may compel clinicians to make treatment decisions
35 on a presumptive basis [4], leading to antibiotic overuse and AMR surge. Also, behavioral
36 factors such as self-medication, entwined with a lack of political will to regulate access to
37 antibiotics, have worsened the AMR burden [5]. Additionally, the growing demand for
38 animal-source food due to the steady expansion of the world population promotes
39 antimicrobial usage in livestock feed [6], further exacerbating the problem.

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

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

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

66 Chapter I describes the prevalence and diversity of *bla*_{CTX-M} genes and *bla*_{CTX-M}-
67 carrying chromosomal insertions among clinical strains in three *Enterobacteriaceae* species.
68 The strains were resistant to multiple antibiotics of clinical importance, and the MDR
69 insertions seemed to have been mobilized by the insertion sequence *ISEcp1*. To verify the
70 epidemiological linkage between poultry and humans in Lusaka, Zambia, Chapter II provides
71 evidence suggesting the clonal transmission of MDR *Escherichia coli* between the two
72 niches.

73

74 **CHAPTER I:**

75 **Novel chromosomal insertions of *ISEcp1-bla_{CTX-M-15}* and diverse**
76 **antimicrobial resistance genes in *Zambian clinical isolates of *Enterobacter****
77 ***cloacae* and *Escherichia coli***

78 **Summary**

79 Forty-six cefotaxime-resistant *Enterobacteriaceae* isolates from Zambian hospital
80 patients were sequenced on MiSeq and MinION platforms and reconstructed to nearly
81 complete genomes. Phylogenetic analysis and hierarchical clustering suggested the clonal
82 spread of the strains among patients. *In silico* genotyping detected four alleles of the *bla_{CTX-M}*
83 gene and 54 other antimicrobial resistance (AMR) genes across 45/46 (97.8%) isolates. The
84 *bla_{CTX-M}* gene existed on plasmids in 38/45 (84.4%) strains and on chromosomes in the
85 remaining 7/45 (15.6%). In one *Enterobacter cloacae* and three *Escherichia coli* strains, the
86 *bla_{CTX-M-15}* gene was found on large (> 10 kb) chromosomal insertions bordered by the
87 *ISEcp1* insertion sequence at one end. The nucleotide sequences of these insertions
88 resembled previously reported plasmids and harbored multiple AMR genes that correlated
89 with the observed phenotypic AMR profiles. These results revealed the coexistence of
90 *ISEcp1-bla_{CTX-M-15}* with multidrug resistance (MDR) determinants on the chromosomes of *E.*
91 *cloacae* and *E. coli*, signifying that *ISEcp1*-mediated transposition may be essential for the
92 mobilization of various AMR genes from plasmids to chromosomes. Retaining such
93 insertions in the chromosome may enhance the persistence and dissemination of MDR
94 clones, regardless of selection pressure.

95

96

97 Introduction

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

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

119 Although ESBLs only emerged in 1985 [30], there were over 150 documented ESBL
120 variants by the beginning of the 21st century [31], most of which were derivatives of
121 Temoneira types 1 and 2 (TEM-1 and TEM-2), and Sulphydryl variable type 1 (SHV-1) [32,
122 33]. In addition, non-TEM and non-SHV ESBLs have also emerged, including Cefotaximase-
123 Munich (CTX-M-type), Oxacillinases (OXA-type), Vietnam ESBLs (VEB-type), Brazilian
124 ESBLs (BES-type), and Pseudomonas Extended Resistance (PER-type) β -lactamases.
125 Notably, the frequency of CTX-M-type ESBLs has increased in contemporary times, with
126 over 170 variants [34], making them the most abundant type worldwide [7]. The observed
127 predominance of CTX-M-type ESBLs has been attributed to the success of the *Escherichia*
128 *coli* O25b:H4-ST131 pandemic clone, which usually carries the plasmid-borne *bla*_{CTX-M-15}
129 gene [35]. Furthermore, the dissemination of *bla*_{CTX-M} genes is also propelled by horizontal

130 gene transfer (HGT) via mobile genetic elements such as insertion sequences (IS) (e.g.,
131 *ISEcp1*, IS1, IS5, and IS26) [36]. In most studies, the *ISEcp1* insertion sequence has been
132 detected upstream of *bla*_{CTX-M} genes [37-39], highlighting its role in mobilizing the *bla*_{CTX-M}
133 genes. As transposons require two inverted repeats (IR) for recognition by the transposase, it
134 is thought that the *ISEcp1* transposase can use one IR and another sequence related to the
135 other IR to effect the transposition of large genetic elements [40]. Moreover, the *ISEcp1* has
136 also been shown to be a strong promoter for expressing the *bla*_{CTX-M} genes [41].

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

147 By analyzing the genomes and AMR profiles of *Enterobacteriaceae* isolates from
148 Zambian hospital patients, clonal expansion was identified as the primary mode of *bla*_{CTX-M}
149 spread. Additionally, the nearly complete genome sequences allowed identifying
150 chromosomal insertions of plasmid origin harboring *ISEcp1-bla*_{CTX-M-15} and various AMR
151 genes among *Enterobacter cloacae* and *E. coli*. Furthermore, the strains carrying these
152 insertions displayed MDR phenotypes consistent with the AMR genes in the insertions.
153 These findings suggest that *ISEcp1*-mediated transposition facilitates the spread of MDR
154 determinants among *Enterobacteriaceae*. Furthermore, the stable maintenance of these
155 chromosomal insertions may enhance the persistence of MDR *Enterobacteriaceae* species.

156

157

158 **Materials and methods**

159 **Strain selection**

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

167

168 **Determination of minimum inhibitory concentration (MIC)**

169 MICs of nine antimicrobials were determined by subjecting the strains to broth
170 microdilution using breakpoints specified in Table 1. To this end, the strains were cultured
171 overnight in cefotaxime (1 µg/ml) supplemented LB. The cultures were diluted 10⁴-fold,
172 added in triplicate to different antibiotic concentrations in a 96-well plate, and incubated
173 aerobically at 37°C for 18 hours while shaking at 1,600 rpm. Bacterial growth was
174 determined by measuring optical densities at 595 nm (OD₅₉₅) using the Multiskan FC
175 Microplate Photometer (Thermo Scientific, USA). Positive bacterial growth was considered
176 when the OD₅₉₅ value was at least 0.1, while MIC was defined as the least antibiotic
177 concentration for which the OD₅₉₅ value was less than 0.1. Quality control was performed
178 using the two reference strains, *E. coli* MG1655 and *E. coli* 10-β (NEB, USA).

179

180 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	Nitrofurin	DMF	≤ 32	64	≥ 128

181

182 NB; All antimicrobials were purchased from Sigma-Aldrich®. LB broth (Difco™) was used.

183 S; susceptible. I; intermediate. R; resistant.

184 DW; distilled water. DMF; dimethyl formamide. MOPS; 3-(N-morpholino)propanesulfonic
185 acid, pH 6.8.

186 Breakpoints are expressed in $\mu\text{g/ml}$.

187

188

189 **Growth rate determination**

190 Growth monitoring was performed by preparing bacterial cultures in antibiotic-free
191 LB and measuring the OD₆₀₀ continuously for 16 hours. Briefly, OD monitoring was
192 performed on duplicates of 10³-fold-diluted overnight cultures in a 96-well plate at 37°C
193 using the Varioskan LUX Multimode Microplate Reader (Thermo Scientific, USA) while
194 shaking at 600 rpm. The obtained data were used to fit parametric models using the R
195 package grofit version 1.1.1 [49], and the gradients of the fitted lines represented growth
196 rates.

197

198 **Whole-genome sequencing**

199 The strains were cultured overnight in LB containing 1 µg/ml cefotaxime, and
200 genomic DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden,
201 Germany). Libraries were prepared using NexteraXT (Illumina, USA) and subjected to
202 sequencing using MiSeq (Illumina), resulting in paired-end reads (2 x 300 bp). Poor-quality
203 reads and adapter sequences were trimmed using Trim Galore version 0.4.2 with options of "-
204 -paired --nextera" (<https://github.com/FelixKrueger/TrimGalore>). The aforesaid genomic
205 DNA was also sequenced with MinION (Oxford Nanopore Technologies, United Kingdom)
206 using Rapid Barcoding Kit (SQK-RBK004) and flowcell R9.5 (FLO-MIN107) to obtain long
207 reads. The long reads were basecalled using Guppy Basecalling Software version 3.4.5 and
208 then assembled using Canu version 1.8 [50], specifying "corOutCoverage = 1000
209 genomeSize = 6m". Gepard version 1.40 [51] was then used to identify and trim redundant
210 repeats at terminal ends of contigs, followed by base-error correction with trimmed Illumina
211 reads using Pilon version 1.23 [52]. Chromosomal sequences were defined as contigs longer
212 than 2 Mb, while plasmids were identified by the presence of plasmid replicons on contigs
213 smaller than 500 kb. The rest of the sequences were screened for redundancy through
214 BLASTn searches against a local database created for each strain using known chromosomal
215 and plasmid contigs. Query sequences matching the database with ≥ 70% identity were
216 defined as redundant and eliminated from the data pool. Nonredundant query sequences were
217 screened further using NCBI BLASTn searches against the nt database. A contig was
218 considered chromosomal if ≥ 7 of the top 10 hits were chromosomes and plasmid-based if ≥
219 7 of the top 10 were plasmids.

220

221 **Phylogenetic analysis**

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

227

228 **Detection of plasmid replicons, strain serotypes, and AMR genes**

229 Plasmid replicons and O:H serotypes were determined by interrogating contigs with
230 PlasmidFinder [56] and EcOH [57] databases, respectively, using ABRicate software version
231 0.8.10 (<https://github.com/tseemann/abricate>) with options "--mincov 90" and "--minid 90"
232 specified. AMR genes were identified using the AMRFinderPlus tool [58] with the "-i 0.7"
233 option engaged.

234

235 **Determination of clustering patterns among strains**

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

239

240 **Sequence alignment and identification of chromosomal insertions of *bla*_{CTX-M}**

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

251

252 **PCR and Sanger sequencing**

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

267

268 **Ethics approval and consent to participate**

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

274

275

276

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	GTTTCGTCAGGCTTTTTCTGGTG
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

278

279

280 Table 3. Primers used for the verification of the *bla*_{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	CGGAAACTATCCGTACAAGGG	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	

281

282

283 Results

284 Genetic diversity among *E. coli* strains

285 This study examined 46 cefotaxime-resistant *Enterobacteriaceae* isolates collected
286 from diverse clinical sources among inpatients at the UTH, Zambia (Table 4). To characterize
287 the genetic diversity among these strains, WGS was performed, and nearly complete genome
288 sequences were reconstructed. The average size of the draft genomes ranged from 4,848,171
289 to 5,773,466 bp, and mean read depths ranged from 88x to 271x. The number of contigs
290 varied from 1 to 17, with N_{50} values ranging from 788,916 to 5,322,171 bp. An MLST
291 analysis performed *in silico* identified three *Enterobacteriaceae* species, namely, *E. cloacae*
292 (1/46, 2.2%), *K. pneumoniae* (9/46, 19.6%), and *E. coli* (36/46, 78.3%) (Table 4).

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

303

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

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

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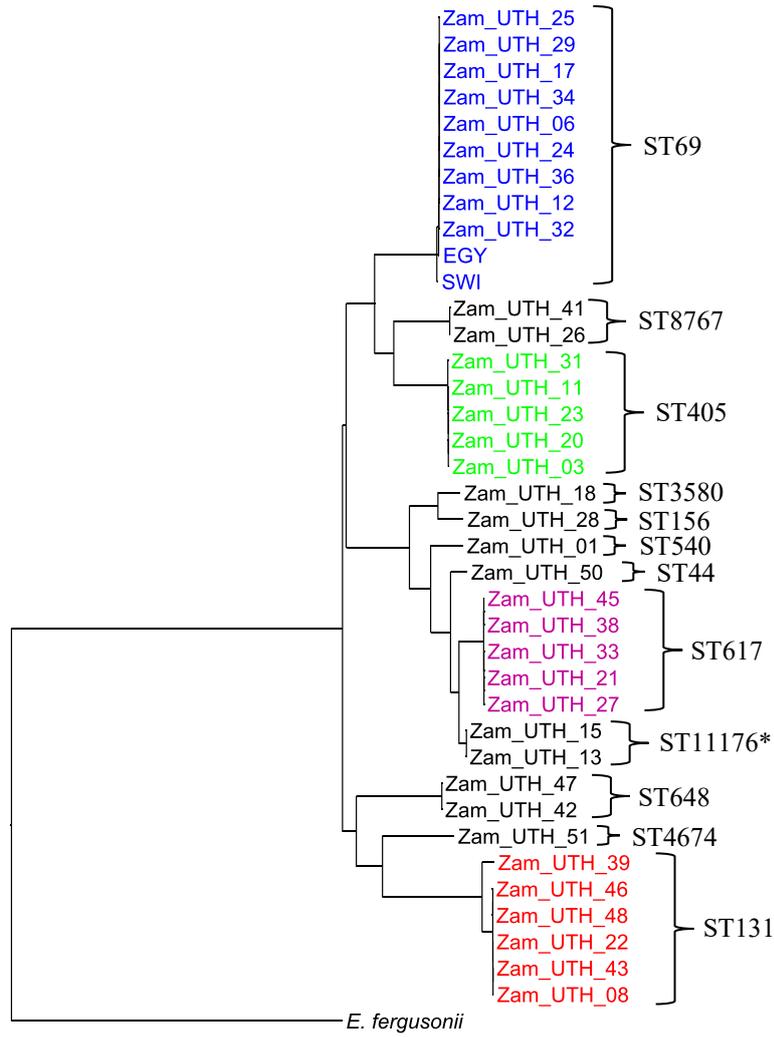
306 ^aCTX MIC is expressed in µg/ml

307 ^bN/A = not available

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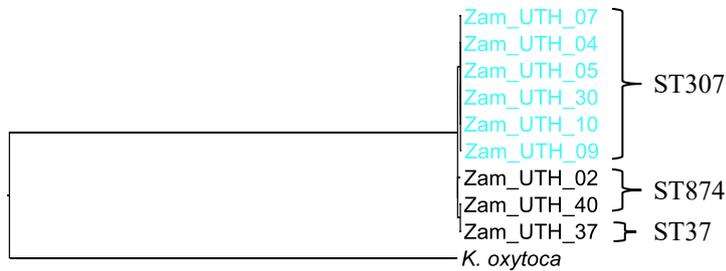


Figure 1. Phylogenetic analysis. Whole genome-based phylogenetic trees for 36 *E. coli* and 9 *K. pneumoniae* strains from Zambia.

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

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

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Location of *bla*_{CTX-M} genes

To characterize the genotypic AMR patterns of the strains, *in silico* prediction was performed on the assembled genomes. Fifty-eight AMR genes were detected across a total of 12 AMR classes (Table 5). The *bla*_{CTX-M} family, observed in 45/46 (97.8%) strains, was the most abundant β -lactamase gene class. Thirty-eight out of 45 (38/45, 84.4%) strains carried this gene family exclusively on plasmids, but seven isolates (7/45, 15.6%; one *E. cloacae* and 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., *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, 16.7%) carried this gene (Fig 2), while the other five *E. coli* ST131 strains contained plasmid-borne *bla*_{CTX-M-27}. *In silico* prediction of O:H serotypes revealed that none of the six *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 belonged to O107:H5.

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

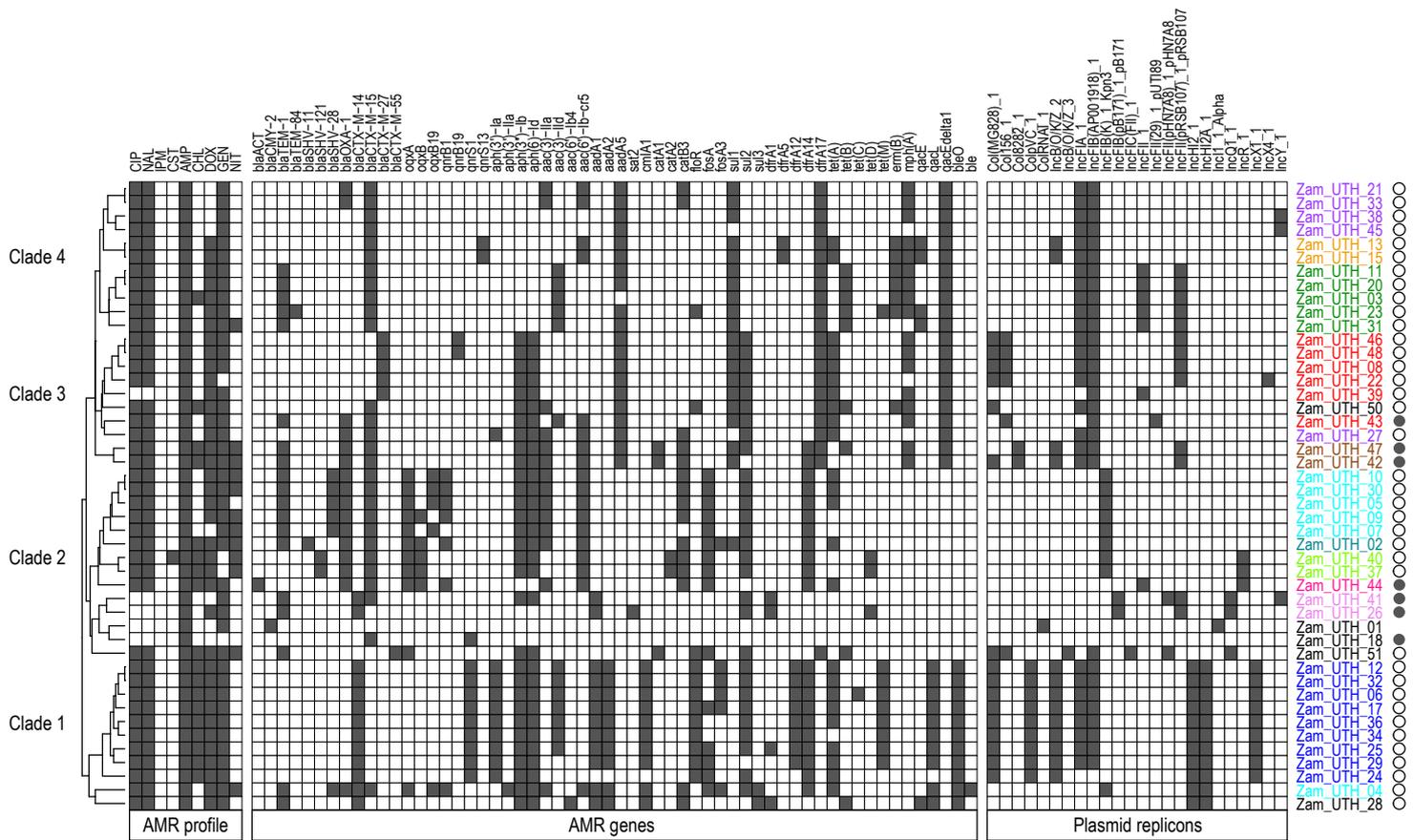
397 Table 5. AMR genes detected

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

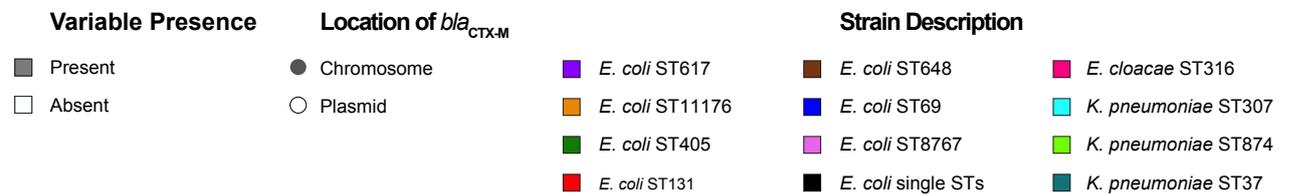
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Figure 2. AMR phenotypes, AMR genes and plasmid replicons.

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

413 AMP; ampicillin. CHL; chloramphenicol. CIP; ciprofloxacin. CST; colistin. DOX;
414 doxycycline. GEN; gentamicin. IPM; imipenem. NAL; nalidixic acid. NIT; nitrofurantoin.
415
416

417 Table 6. Prediction of phenotype from AMR genes

GEN	<i>aph(3)-Ia</i>	<i>aph(3)-IIa</i>	<i>aph(3'')-Ib</i>	<i>aph(6)-Id</i>	<i>aac(3)-IIa</i>	<i>aac(3)-IId</i>
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

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GEN	<i>aac(6')-Ib4</i>	<i>aac(6')-Ib-cr5</i>	<i>aadA1</i>	<i>aadA2</i>	<i>aadA5</i>
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

420

421

CIP	<i>oqxA</i>	<i>oqxB</i>	<i>oqxB19</i>	<i>qnrB1</i>	<i>qnrB19</i>	<i>qnrS1</i>	<i>qnrS13</i>
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

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NAL	<i>oqxA</i>	<i>oqxB</i>	<i>oqxB19</i>	<i>qnrB1</i>	<i>qnrB19</i>	<i>qnrS1</i>	<i>qnrS13</i>
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

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

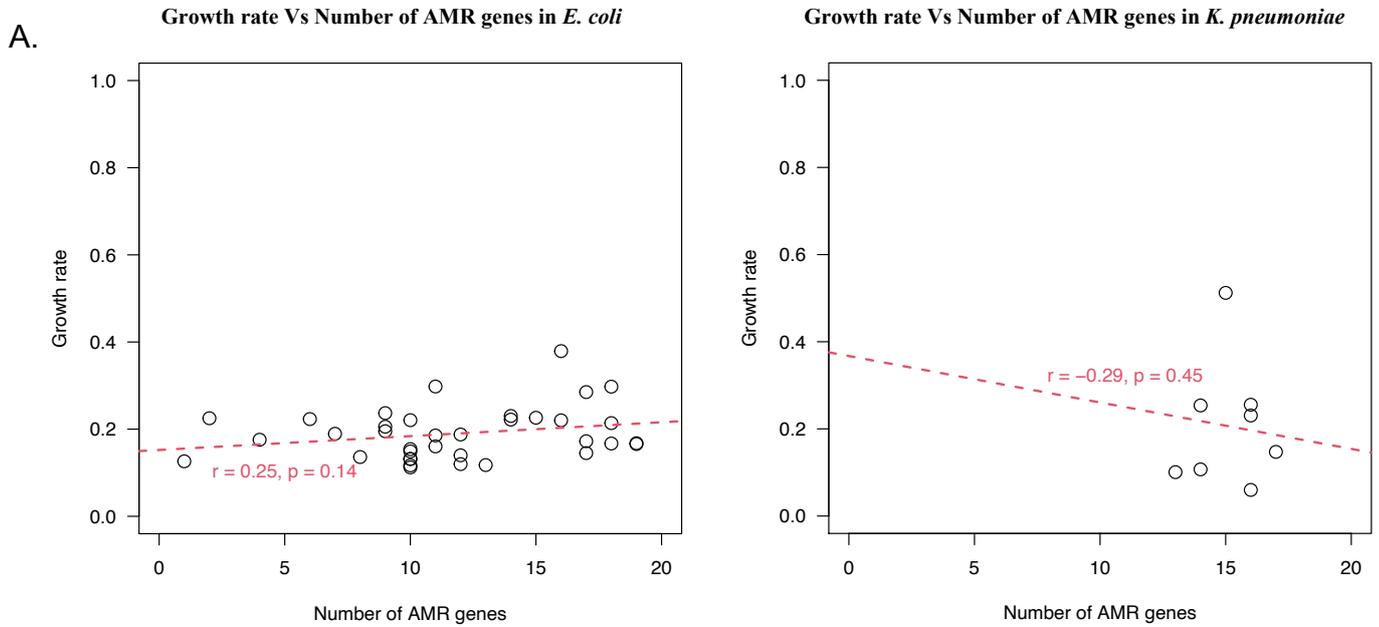
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CHL	<i>cmlA1</i>	<i>catA1</i>	<i>catA2</i>	<i>catB3</i>
R	8	3	2	6
S	2	0	0	9
Total	10	3	2	15
PPV (%)	80	100	100	42.9

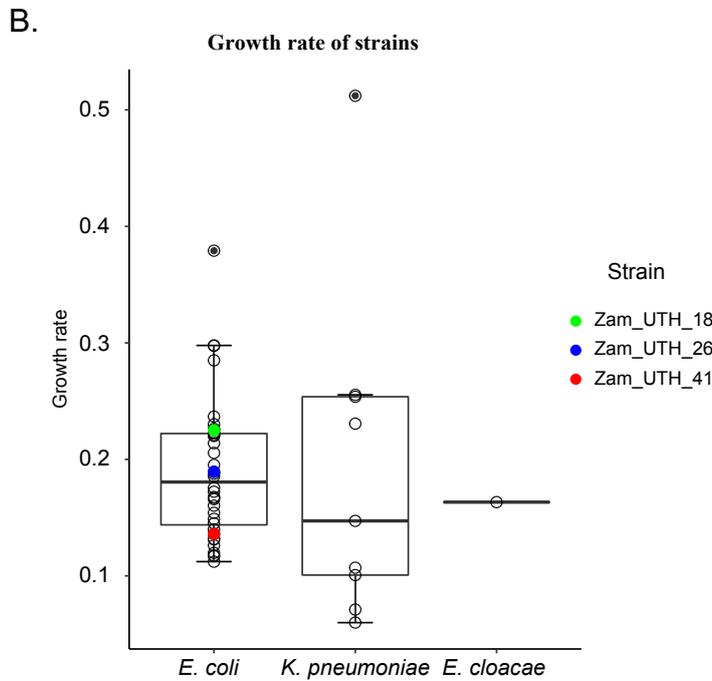
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Figure 3. Assessment of growth rate among strains.

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.

453 B. Zam_UTH_18 did not possess any plasmid and had a growth rate above the 75th
454 percentile for *E. coli* strains. Zam_UTH_26 grew at a rate above the median growth rate
455 for *E. coli*; however, a closely related strain, Zam_UTH_41, harbored an additional
456 *bla*_{CTX-M-15}-carrying plasmid and had a growth rate lower than the 25th percentile.
457

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*_{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*_{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*_{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*_{CTX-M} gene propagation.

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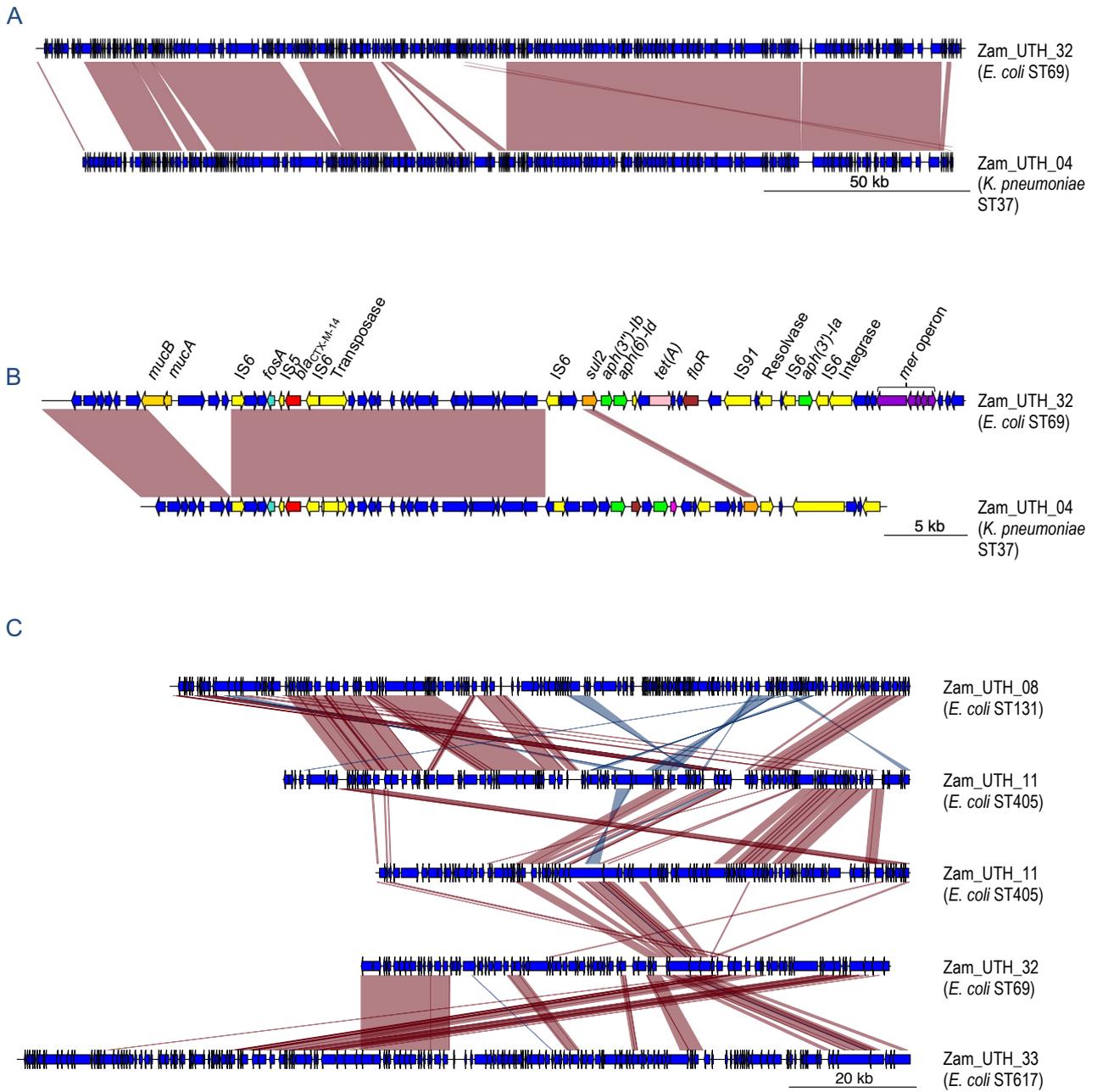


Figure 4. Comparison of plasmid sequences among strains.

- 484 A. IncHI plasmid in *E. coli* ST69 shared over 80% nucleotide sequence homology with
 485 IncHI plasmid in *K. pneumoniae* ST37.
- 486 B. IncHI plasmid from *E. coli* ST69 and *K. pneumoniae* ST37 carried the *fosA*/IS5/*bla*_{CTX-M-14}/IS6
 487 unit. In addition, the IncHI plasmid from *E. coli* ST69 also contained the *mucAB*
 488 and *mer* operons.

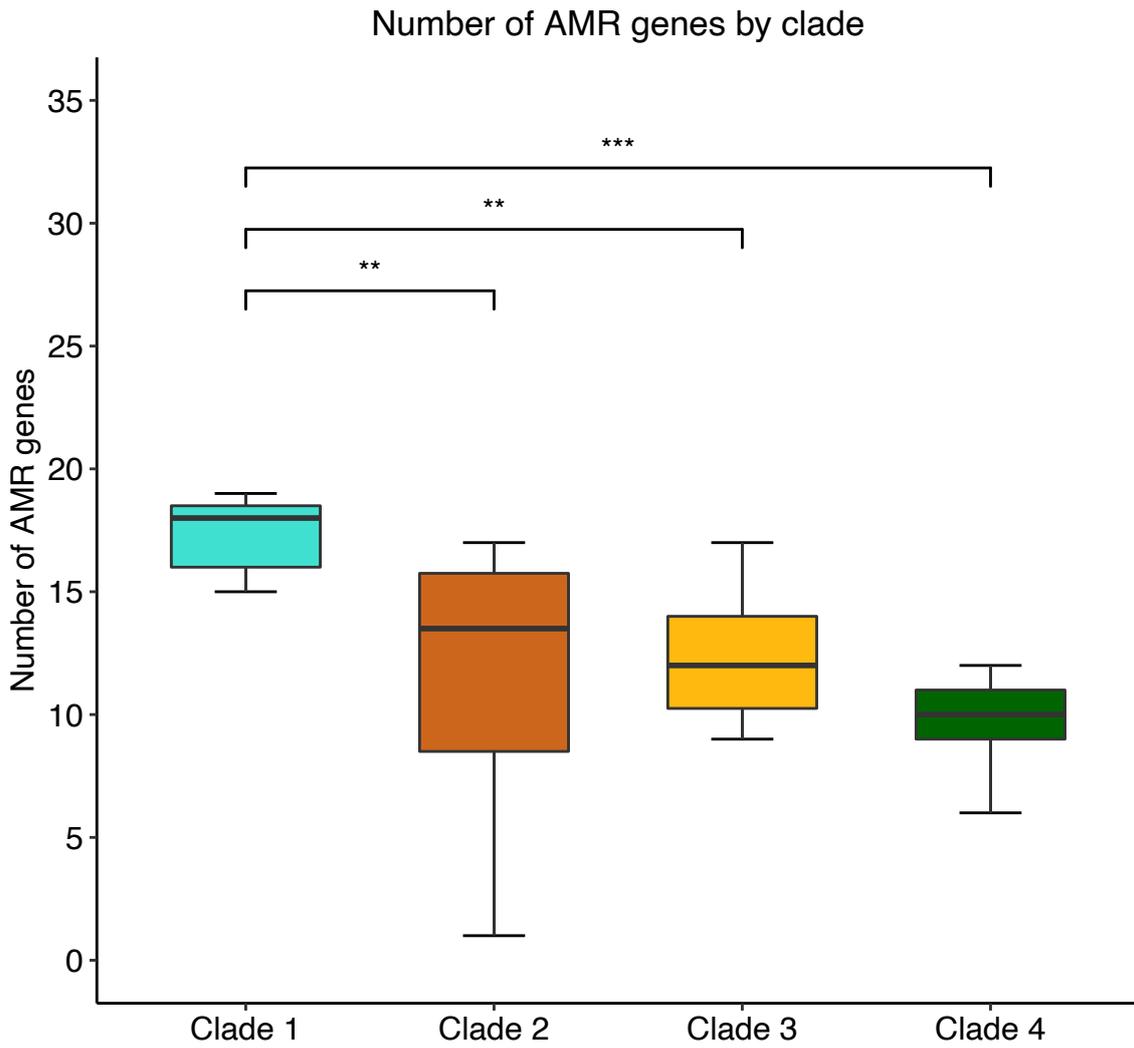
489 C. There was low nucleotide sequence similarity between IncFI plasmids from *E. coli* strains
490 belonging to different STs. In addition, strains belonging to *E. coli* ST405 carried two
491 IncF plasmids, namely IncFI and IncFII.
492 Red; β -lactam resistance. Green; aminoglycoside and/or quinolone resistance. Brown;
493 chloramphenicol resistance. Purple; mercury resistance. Turquoise; fosfomycin resistance.
494 Orange; sulfonamide resistance. Yellow; mobile genetic elements.
495

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

497 To determine the mechanism of *bla*_{CTX-M} spread among hospital patients, hierarchical
498 clustering was performed using phenotypic and genotypic AMR profiles, as well as plasmid
499 replicons. The results showed that strains could be clustered into four distinct clades (here
500 referred to as Clade 1, Clade 2, Clade 3, and Clade 4) (Fig 2). The largest clade, Clade 1, was
501 mainly composed of *E. coli* ST69 and defined by IncHI replicon and *bla*_{CTX-M-14} gene.
502 Furthermore, this clade also contained one *K. pneumoniae* ST307 and one *E. coli* ST156,
503 which carried the IncHI replicon and shared several AMR genes with *E. coli* ST69. Notably,
504 the number of AMR genes in Clade 1 was more than what was noted in Clade 2, Clade 3, and
505 Clade 4 ($P < 0.01$) (Fig 5). Clade 3 showed a close relationship to Clade 4, with both clades
506 having *qacEdelta1*, *mph(A)*, and *aad5* genes that were lacking in Clade 1 and Clade 2. Clade
507 3 was dominated by *E. coli* ST131, along with two *E. coli* ST648, one *E. coli* ST44, and one
508 *E. coli* ST617. Meanwhile, Clade 3 was represented by three *E. coli* STs, namely ST405,
509 ST617, and ST11176. Finally, the main subclade of Clade 2 was composed of *K. pneumoniae*
510 and *E. cloacae* and contained four plasmid-mediated quinolone resistance (PMQR) genes,
511 namely *oqxA*, *oqxB*, *oqxB19*, and *qnrB1*. In addition, a minor subclade of Clade 2 comprised
512 *E. coli* STs such as ST8767, ST540, ST3580, and ST4674. Altogether, the analysis revealed
513 that phylogenetically related lineages aggregated together. Consistent with the MLST
514 analysis, these findings suggest that clonal dissemination of specific lineages was responsible
515 for the spread of *bla*_{CTX-M} instead of a plasmid-driven transmission.

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

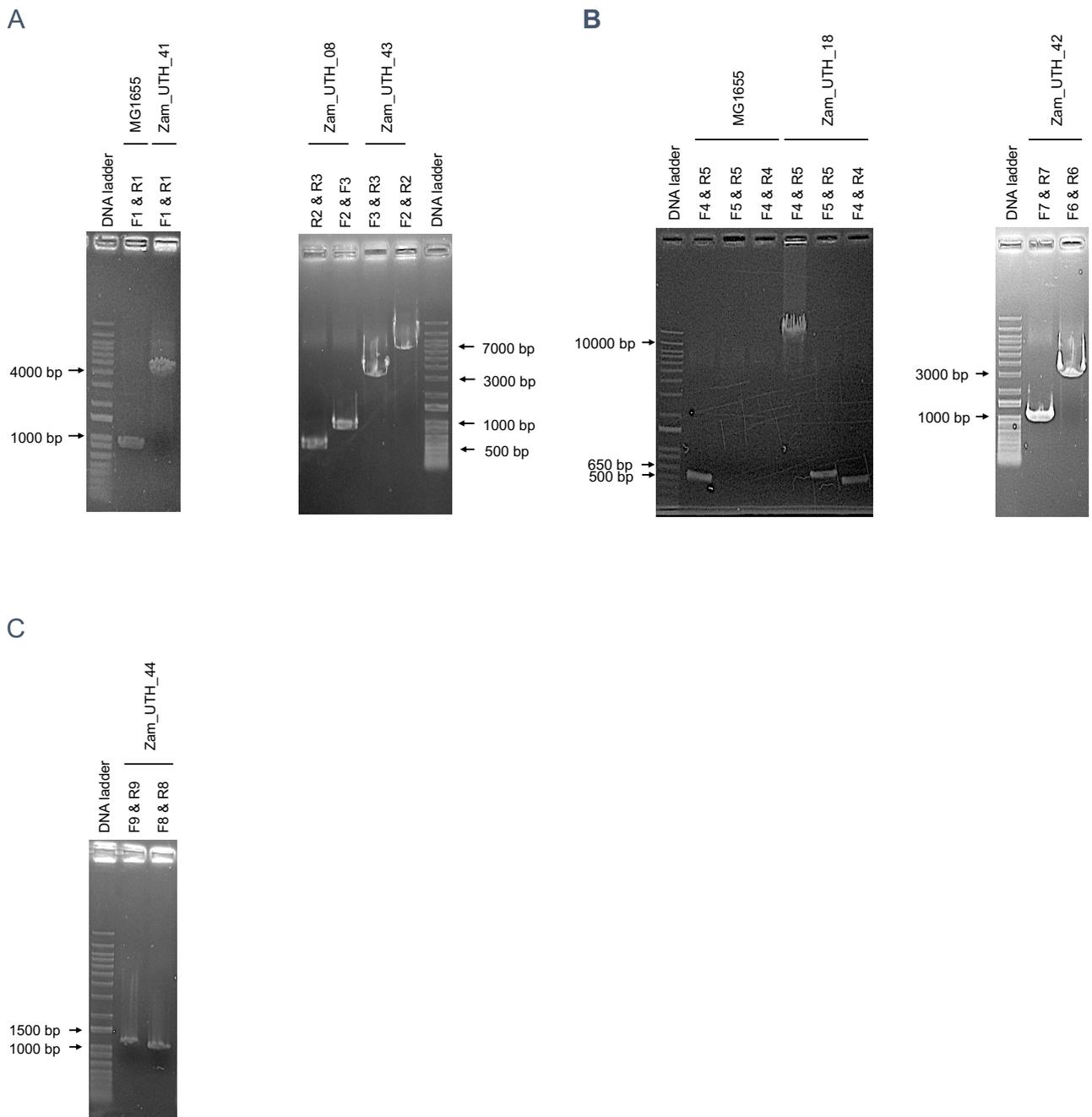
521 There were significantly more AMR genes in Clade 1 compared to other clades. The analysis
522 was performed by One-way ANOVA with Tukey's HSD comparison of means.

523 **P ≤ 0.01, ***P ≤ 0.001.

524

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

526 To describe the chromosomal locations of *bla*_{CTX-M} in seven strains (one *E. cloacae*
527 and six *E. coli*), contigs were aligned against reference strains using Mauve [61]. In all seven
528 strains, the chromosomal insertions were bordered by *ISEcp1* at one end, highlighting the
529 possible involvement of this element in the mobilization of the insertions. Nonetheless, some
530 insertions also included several other IS elements (e.g., *IS1*, *IS6*) and transposons, indicating
531 that other complex mechanisms may be involved. The chromosomal insertions, verified by
532 PCR (Fig 6), were unique for distinct clones and ranged in size from ~ 3 kb to ~ 41 kb (Fig 7-
533 9). These inserted segments resembled plasmid sequences retrieved from the NCBI GenBank,
534 suggesting the transposon-mediated transfer from plasmids to chromosomes. Interestingly,
535 four strains (one *E. cloacae* and three *E. coli*) carried *bla*_{CTX-M}-harboring insertions longer
536 than 10 kb and contained various AMR genes. Consistent with the AMR genes on the
537 insertions, these strains displayed resistance to several clinically relevant antibiotics (Fig 8
538 and 9).
539



540

Figure 6. Confirmation of chromosomal insertions.

541 A. Small chromosomal insertions among *E. coli* strains. Left. PCR performed with the same
 542 primers produced bands of different sizes in Zam_UTH_41 (~ 4 kb) and *E. coli* MG1655
 543 (~ 1 kb). Right. In Zam_UTH_43, PCR using the primer pairs F2/R2 and F3/R3 produced
 544 amplicons of sizes ~ 7 kb and ~ 3 kb, respectively. However, the primer sets F2/F3, and
 545 R2/R3 yielded products of about 850 bp and 500 bp, respectively, in a control strain
 546 (Zam_UTH_08).

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

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

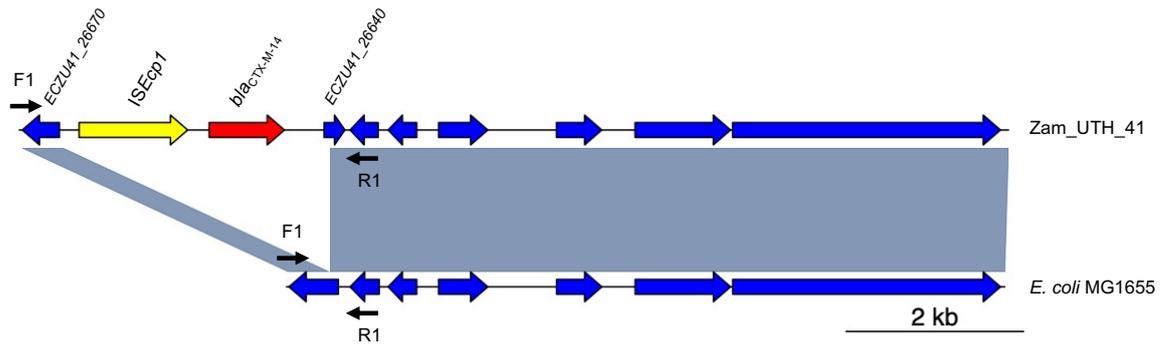
555 C. Large (> 10 kb) chromosomal insertion in one *E. cloacae* strain (Zam_UTH_44). The
556 insertion was confirmed by PCR targeting junctions between plasmid and chromosome
557 regions, but no control strain was available for comparison.

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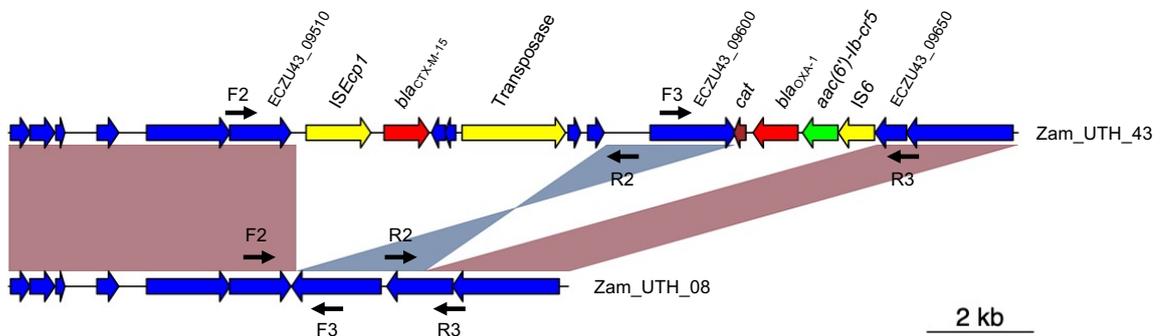
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Figure 7. *bla*_{CTX-M} genes present on short chromosomal insertions in *E. coli*.

564 A. Zam_UTH_41. This *E. coli* strain, belonging to ST8767, harbored a 3,095 bp
565 chromosomal insertion with *bla*_{CTX-M-14} located 249 bp downstream of *ISEcp1*.
566 Zam_UTH_26 also had a similar insertion and genetic architecture.

567 B. Zam_UTH_43. This *E. coli* strain, belonging to O107:H5-ST131, harbored a 6,036 bp
568 chromosomal insertion with *bla*_{CTX-M-15} located 255 bp downstream of *ISEcp1*. In
569 addition, about 2.5 kb downstream of this insertion, a smaller insertion was identified,
570 carrying genes targeting aminoglycosides/quinolones (*aac(6')-Ib-cr5*), β -lactams (*bla*_{OXA-1}),
571 and chloramphenicol (*cat*).

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

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575 In all six *E. coli* strains, chromosomal insertions were flanked by 5-bp direct repeats,
576 a characteristic feature of *ISEcpI*-mediated transposition. Nevertheless, two strains
577 (Zam_UTH_42 and Zam_UTH_47) (Fig 8B) possessed a truncated *ISEcpI*, indicating that
578 this interrupted element is unlikely to be functional; thus, the interrupting *IS1* and/or
579 transposase may have mobilized *bla*_{CTX-M-15} in these strains. Depending on the allele (i.e.,
580 *bla*_{CTX-M-14} or *bla*_{CTX-M-15}), the *bla*_{CTX-M} gene existed at a fixed position downstream of
581 *ISEcpI*, suggesting common ancestral origins among similar alleles. Specifically, in two *E.*
582 *coli* ST8767 strains (Zam_UTH_26 and Zam_UTH_41), the *bla*_{CTX-M-14} gene existed 249 bp
583 downstream of *ISEcpI* on a 3,095 bp segment. Likewise, the *bla*_{CTX-M-15} allele was detected
584 255 bp away from *ISEcpI* in the downstream region of five strains with chromosomal
585 insertions of diverse lengths. More precisely, one *E. coli* ST131 (Zam_UTH_43) (Fig 7B)
586 and one *E. coli* ST3580 (Zam_UTH_18) (Fig 8A) possessed the *bla*_{CTX-M-15} allele on 6,036
587 bp and 11,383 bp segments, respectively, while two *E. coli* ST648 (Zam_UTH_42 and
588 Zam_UTH_47) (Fig 8B) harbored a 14,328 bp *bla*_{CTX-M-15}-containing insertion. Finally, an *E.*
589 *cloacae* ST316 strain (Zam_UTH_44) possessed a large (> 41 kb) *bla*_{CTX-M-15}-carrying
590 chromosomal insertion (Fig 9).

591 Out of seven strains with chromosomally-located *bla*_{CTX-M}, four of them (i.e.,
592 Zam_UTH_18, Zam_UTH_42, Zam_UTH_44, and Zam_UTH_47) had the gene on large (>
593 10 kb) insertions that resembled plasmid or chromosome sequences obtained from the NCBI
594 GenBank (Fig 8 and 9). Particularly, Zam_UTH_18 carried an insertion observed in the
595 chromosomes of *Salmonella enterica* (GenBank accession no. CP045038) and two *E. coli*
596 ST38 isolates (GenBank accession no. CP010116 and CP018976). Also, the four large
597 insertions (> 10 kb) possessed other AMR genes in the downstream region of *bla*_{CTX-M-15}. For
598 example, the insertion in Zam_UTH_18 carried the *qnrS1* gene, which is associated with
599 reduced quinolone susceptibility. At the same time, Zam_UTH_42 and Zam_UTH_47 had
600 several genes that encode resistance to aminoglycosides (*aac(3)-IIa*),
601 aminoglycosides/quinolones (*aac(6')-Ib-cr5*), β -lactams (*bla*_{OXA-1}, *bla*_{TEM-1}), and
602 chloramphenicol (*catB3*). Likewise, the insertion in Zam_UTH_44 had seven genes
603 associated with resistance to aminoglycosides (*aac(3)-IIa*), quinolones (*qnrB1*),
604 aminoglycosides/quinolones (*aac(6')-Ib-cr5*), β -lactams (*bla*_{OXA-1}), trimethoprim (*dfrA14*),
605 chloramphenicol (*catB3*), and tetracyclines (*tet(A)*).

606 Apart from Zam_UTH_18, which was susceptible to quinolones (ciprofloxacin MIC
607 = 0.25 μ g/ml) despite possessing the *qnrS1* gene, the AMR phenotypes of the strains

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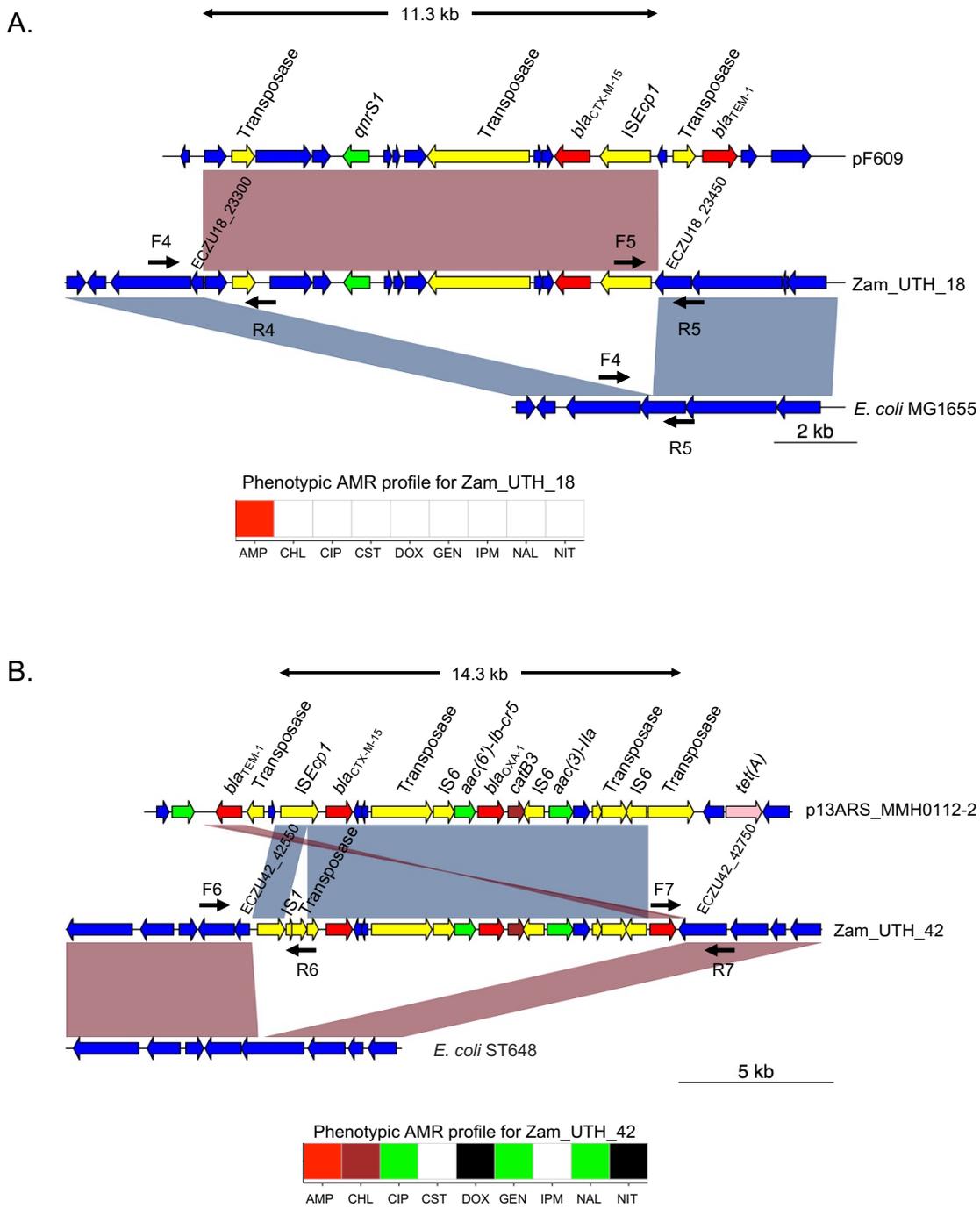


Figure 8. *bla*_{CTX-M} genes present on large chromosomal insertions in *E. coli*.

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 *ISEcp1*.

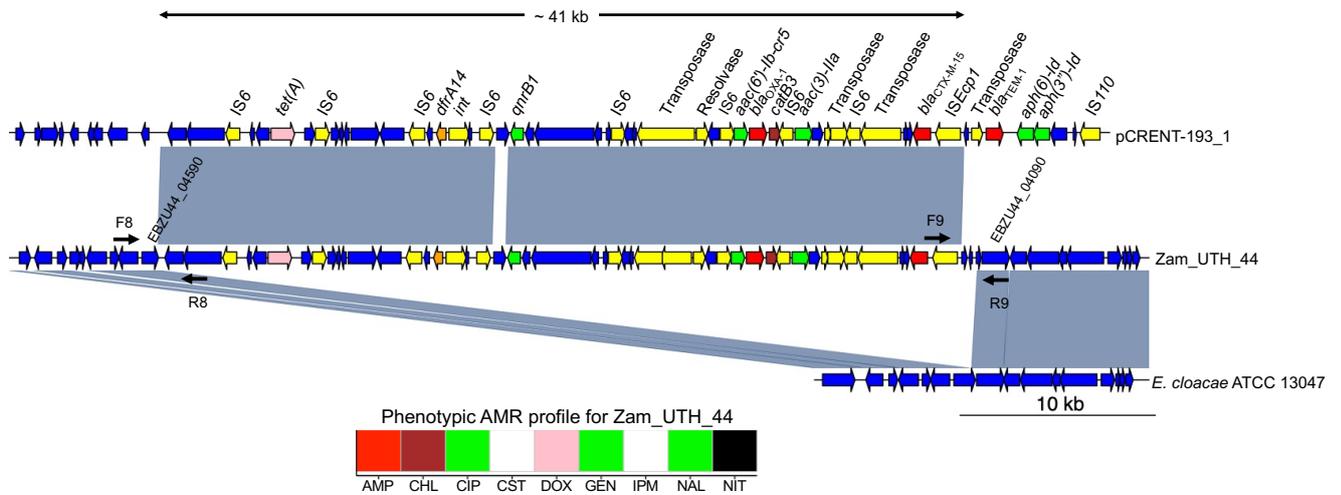
651 The *qnrS1* gene was located 4639 bp downstream of *bla*_{CTX-M-15}. Phenotypically, this
652 strain was resistant to ampicillin but susceptible to quinolones.

653 B. Zam_UTH_42. This *E. coli* strain belonging to ST648 harbored a 14,328 bp
654 chromosomal insertion carrying *bla*_{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*_{OXA-1}, *bla*_{TEM-1}),
657 and aminoglycosides/quinolones (*aac(6')-Ib-cr5*). The *bla*_{CTX-M-15} gene was located 255
658 bp downstream of *ISEcp1*, which was truncated by *IS1* 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*_{CTX-M} gene on a large chromosomal insertion in *E. cloacae*.

671 Zam_UTH_44. This *E. cloacae* strain, belonging to ST316, possessed the *bla*_{CTX-M-15} gene on
 672 a ~ 41 kb chromosomal insertion similar in nucleotide sequence to plasmid pCRENT-193_1
 673 (GenBank accession no. CP024813.1). The *bla*_{CTX-M-15} gene was located 255 bp downstream
 674 of *ISEcp1*. The insertion also harbored various AMR genes targeting aminoglycosides
 675 (*aac(3)-IIa*), chloramphenicol (*catB3*), β-lactams (*bla*_{OXA-1}), aminoglycosides/quinolones
 676 (*aac(6')-Ib-cr5*), quinolones (*qnrB1*), trimethoprim (*dfrA14*), and tetracyclines (*tet(A)*). The
 677 phenotypic resistance profile of this strain coincided with the AMR genes on the insertion.

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

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683

684 Discussion

685 This current study focused on the phenotypic and genotypic characterization of
686 *Enterobacteriaceae* strains collected from inpatients at the UTH, Zambia. Phylogenetic
687 analysis (Fig 1) and hierarchical clustering (Fig 2) suggested that the spread of *bla*_{CTX-M} has
688 been facilitated primarily by clonal expansion. The *bla*_{CTX-M} gene was located on plasmids in
689 most isolates, but seven strains carried this gene on chromosomes. While the *ISEcpI*-
690 mediated chromosomal location of the *bla*_{CTX-M} gene is a well-studied phenomenon [43, 44,
691 46-48], there are no reports of *ISEcpI*-*bla*_{CTX-M} with other AMR genes in *E. cloacae* or *E.*
692 *coli* chromosomes. Despite advances in sequencing methods allowing the characterization of
693 the regions immediately upstream and downstream of chromosomal *bla*_{CTX-M}, such
694 approaches are limited when the chromosomal insertions are large. By reconstructing nearly
695 complete genomes, the identification of large *bla*_{CTX-M}-harboring chromosomal insertions was
696 improved relative to previous analyses. The results showed that one *E. cloacae* and three *E.*
697 *coli* strains carried large *bla*_{CTX-M-15}-possessing chromosomal insertions that were very
698 similar in nucleotide sequence to plasmids in the NCBI GenBank. Furthermore, these
699 insertions were all bordered by *ISEcpI* at one end and contained various other AMR genes.
700 The AMR phenotypes of the strains correlated with the observed AMR genes on the large
701 chromosomal insertions. These findings reveal the role of *ISEcpI* in propagating *bla*_{CTX-M-15}-
702 related MDR determinants among *E. cloacae* and *E. coli*.

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

711 In most studies, *E. coli* ST131 is dominated by *bla*_{CTX-M-15}-harboring strains
712 belonging to the O25b:H4 pandemic clone [63, 74]. However, none of the six *E. coli* ST131
713 strains studied here were members of this group, but five belonged to Onovel31:H4, and one
714 had an O107:H5 serotype. Other serotypes of *E. coli* ST131 have been sporadically reported
715 [75], including Onovel31:H4 [76] identified in this study. To the best of the author's
716 knowledge, this is the first account of *E. coli* ST131 belonging to serotype O107:H5.

717 The extraintestinal pathogenic *E. coli* ST69 was overrepresented in this study and
718 belonged to a clade that contained more AMR genes than other clades (Fig 5). *E. coli* ST69
719 strains rarely carry ESBL genes [77], are more common in community-acquired infections
720 [78], and possess SXT resistance-encoding plasmids of the IncF incompatibility type [68].
721 Intriguingly, the ST69 strains studied here possessed the *bla*_{CTX-M-14} gene on a plasmid of the
722 IncHI incompatibility type and dominated the hospital isolates. This unusual occurrence of
723 IncHI plasmids in *E. coli* ST69 was also observed in a raw milk cheese isolate from Egypt
724 [79]. However, there was a difference in the AMR gene composition between the Egyptian
725 IncHI plasmid (GenBank accession no. CP023143) and the ones in this study.

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

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

747 Meanwhile, the UTH has a strict policy on carbapenem use, with prescriptions
748 supported by laboratory evidence. Promisingly, no phenotypic or genotypic carbapenem
749 resistance was detected in this study. While this is encouraging, it is worth noting that
750 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*_{CTX-M} in
758 chromosomes disclosed a relationship with *ISEcp1*, as reported earlier [90]. Interestingly,
759 three *E. coli* and one *E. cloacae* exhibited co-occurrence of *ISEcp1-bla*_{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*_{CTX-M-15} and four other AMR genes in an *E. coli* strain; however,
763 in their study, IS26 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 *ISEcp1-bla*_{CTX-M-15} and other AMR genes [91]. However, all the *K. pneumoniae* strains in the
766 current study carried plasmid-borne *bla*_{CTX-M} genes, possibly due to geographic differences
767 between Zambia and South Korea. This is the first report of chromosomal insertions
768 harboring *ISEcp1-bla*_{CTX-M-15} and multiple AMR genes in *E. cloacae* and *E. coli*. Based on
769 the identified *ISEcp1* at one end of each MDR insertion, it is likely that the *ISEcp1* element
770 mobilized these insertions. Nevertheless, the other mobile elements on the insertions, and the
771 truncated *ISEcp1* 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 *ISEcp1-bla*_{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 *ISEcp1-bla_{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_{CTX-M-15}* and *qnrSI*. 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_{CTX-M-15}* and *qnrSI*, were integrated into the chromosome. Verifying this
797 observation will require growth and competitive performance studies using carefully selected
798 control strains.
799

800 **CHAPTER II:**

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

803 **Summary**

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

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

825

826

827 **Introduction**

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

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

848 As a consequence, there has been an increase in the incidence of MDR *E. coli* in
849 food-producing animals like cattle [113], pigs [114, 115], and poultry [116], with the
850 occurrence of zoonotic transmission through clonal expansion or horizontal gene transfer
851 [117]. Chicken meat is among the most typical risks of MDR *E. coli* transmission to humans
852 [118, 119]; therefore, poultry surveillance is essential in controlling MDR dissemination.
853 Such surveillance activities have revealed a high prevalence of MDR *E. coli* in poultry strains
854 from Asia [120-122], and reports in Africa have increased [116, 123]. In Zambia, MDR *E.*
855 *coli* strains were isolated from chicken meat [17], but no information regarding their clonal
856 relationship with human isolates was available. Although reports on MDR *E. coli* have
857 increased among Zambian human patients [15, 124], the proportion originating from poultry
858 remains unknown.

859 In this study, the One Health approach revealed the MDR patterns and clonal
860 relationships between poultry and human *E. coli* isolates from Lusaka, Zambia. Analyzing
861 the samples by whole-genome sequencing (WGS) showed that 4/20 (20%) poultry strains
862 were closely related to 9/36 (25%) clinical isolates. Furthermore, the four poultry and nine
863 human isolates belonged to O17:H18-ST69 and carried 14 identical AMR genes on two
864 plasmids, suggesting clonal spread. Further comparison analysis also revealed that these
865 isolates harbored *bla*_{CTX-M}-carrying plasmids that were niche-specific. These results
866 demonstrate a clinically important link between MDR *E. coli* associated with poultry and
867 humans in Lusaka, highlighting the role of the food chain in disseminating MDR strains.
868

869 **Materials and methods**

870 **Strain collection from poultry**

871 In December 2019, cloacal swabs were collected by randomly sampling 1,000
872 disease-free laying hens at a large commercial farm in Lusaka, Zambia. The sampled farm is
873 a major supplier of poultry and poultry products in Lusaka and surrounding districts.
874 Therefore, the farm was selected based on the large catchment area it serves. The swabs were
875 pooled into 200 batches, each containing samples from five birds. The 200 pooled samples
876 were then transported in buffered peptone water (Oxoid Ltd, UK) and plated on cefotaxime-
877 supplemented (1 µg/ml) MacConkey agar, followed by 18 hours of incubation at 37 °C. One
878 colony suspected to be *E. coli* was picked from each plate and confirmed by amplifying the
879 16S rRNA gene using PCR, followed by sequencing on the 3130 Genetic Analyzer (Applied
880 Biosystems, USA) with primers described previously [125].

881

882 **Screening for MDR**

883 Phenotypic AMR profiles of cefotaxime-resistant *E. coli* were ascertained based on
884 the Minimum Inhibitory Concentrations (MICs) measured for nine different antimicrobials.
885 The drugs chosen were ampicillin, chloramphenicol, ciprofloxacin, colistin, doxycycline,
886 gentamicin, imipenem, nalidixic acid, and nitrofurantoin; the breakpoints used are shown in
887 Table 1. The choice of these antimicrobial drugs was guided by the local prescription patterns
888 [87] and research-based recommendations [126]. Nonetheless, the safety issues associated
889 with ciprofloxacin use in pediatric populations [127] prompted the addition of relatively safer
890 nalidixic acid [128], hence the two quinolones.

891 The strains were grown in cefotaxime-supplemented (1 µg/ml) LB at 37°C for 18
892 hours, shaking at 155 rpm. The cultures were then diluted 10⁴-fold and added to triplicates of
893 2-fold serial dilutions of antibiotics in a 96-well plate. Next, the 96-well plates were
894 incubated at 37°C for 18 hours while shaking at 1,600 rpm. Using the Multiskan FC
895 Microplate Photometer (Thermo Scientific, USA) to measure optical densities at 595 nm
896 (OD₅₉₅), positive bacterial growth was considered OD₅₉₅ values of at least 0.1. Therefore, the
897 MIC was defined as the lowest antibiotic concentration giving an OD₅₉₅ smaller than 0.1. The
898 reference strains, *E. coli* MG1655 and *E. coli* 10-β (NEB, USA), were used for quality
899 control.

900

901 **Growth rate determination**

902 The growth of bacterial cultures in antibiotic-free LB was monitored in real-time for
903 16 hours in a 96-well plate. This was achieved by observing the OD₆₀₀ of 10³-fold-diluted
904 overnight cultures in duplicate at 37°C using the Varioskan LUX Multimode Microplate
905 Reader (Thermo Scientific, USA) while shaking at 600 rpm. By fitting growth curves in R
906 using the package grofit version 1.1.1 [49], the obtained slopes were used as estimates of the
907 growth rates.

908

909 **Whole-genome sequencing**

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

923

924 **Phylogenetic analysis**

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

930

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

932 To predict O:H serotypes and plasmid replicons, contigs were screened against the
933 EcOH [57] and PlasmidFinder [56] databases, respectively, accessed under ABRicate version
934 0.8.10 (<https://github.com/tseemann/abricate>) with options "--mincov 90 --minid 90". Next,
935 acquired AMR genes were detected by employing the AMRFinderPlus tool [58] using the "-i
936 0.7" option. Finally, quinolone resistance mutations were called against the genes *gyrA*, *gyrB*,
937 *parC*, and *parE* from the reference strain *E. coli* MG1655 (GenBank accession no.
938 NC_000913) using snippy version 4.6.0 [129].

939

940 **Comparison of poultry isolates with human strains**

941 To investigate possible MDR *E. coli* transmission between poultry and humans, 20
942 poultry-associated cefotaxime-resistant *E. coli* strains were compared to 36 cefotaxime-
943 resistant *E. coli* isolates previously collected from human patients at the University Teaching
944 Hospital (UTH) in Lusaka, Zambia [130]. Using the R package ComplexHeatmap [59], the
945 AMR phenotype, AMR genes, and plasmid replicons were compared across the two niches.
946 In addition, plasmids with common replicons were annotated using DFAST version 1.2.4
947 [60] and subjected to comparison analysis using Mauve [61]. Finally, tandem repeats were
948 identified by comparing plasmid sequences using NCBI BLAST
949 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and reading the hit table by genoPlotR [62].

950

951

952 **Results**

953 **MDR prevalence among poultry *E. coli* strains**

954 A total of 20 cefotaxime-resistant *E. coli* strains were obtained after screening 200
955 pooled cloacal samples collected from 1,000 laying hens at a commercial poultry farm in
956 Lusaka, Zambia. The 20 strains, whose selection criteria was a cefotaxime MIC ≥ 2 $\mu\text{g/ml}$
957 (Table 7), were subjected to susceptibility tests against nine other antimicrobials representing
958 eight classes. All (20/20, 100%) strains were resistant to three or more antimicrobial
959 categories, thus meeting the MDR definition [66]. In addition, all (20/20, 100%) strains
960 exhibited resistance towards ampicillin, chloramphenicol, and doxycycline, while 18/20
961 (90%) were resistant to ciprofloxacin and nalidixic acid. Only 13/20 (65%) strains showed
962 resistance to gentamicin (13/20, 65%), and none (0/20, 0%) of the strains were resistant to
963 colistin, imipenem, or nitrofurantoin (Table 8).

964

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 ^aMICs are in µg/ml

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971

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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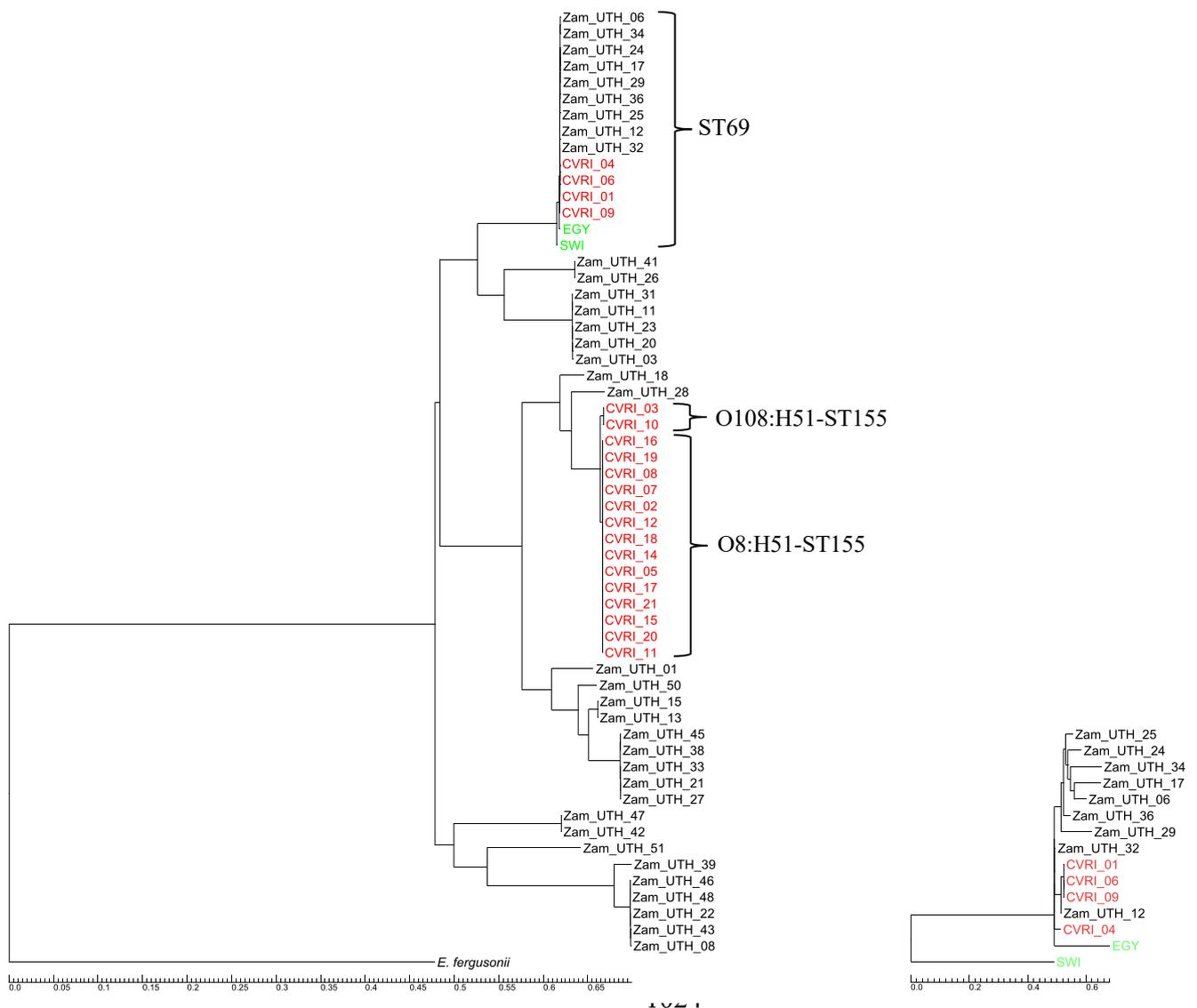
975 **Genetic diversity among poultry strains**

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

981 The strains were classified by *in silico* MLST into ST69 (4/20, 20%) and ST155
982 (16/20, 80%) (Table 7). While all ST69 isolates were classified as O17:H18 (4/4, 100%),
983 ST155 strains were categorized as either O8:H51 (14/16, 87.5%) or O108:H51 (2/16, 12.5%)
984 (Table 7). Despite ST155 strains having closely related genomes, the genes *wzx* and *wzy*,
985 which encode the O-antigen processing proteins responsible for translocating and
986 polymerizing oligosaccharides to synthesize lipopolysaccharides, were identified in two
987 strains. Specifically, strains CVRI_03 and CVRI_10 carried the *wzx*-O108var1 and *wzy*-
988 O108var1 genes, suggesting the utilization of the Wzx/Wzy-dependent pathway. In contrast,
989 14 ST155 strains harbored the *wzm*-O8 and *wzt*-O8 genes, suggesting O-antigen translocation
990 via the ABC transporter pathway [131].

991 To investigate the possibility of MDR dissemination across niches, the
992 abovementioned 20 poultry isolates were compared to 36 *E. coli* strains isolated from human
993 patients hospitalized at the UTH. A WGS-based phylogenetic analysis of 56 (20 poultry and
994 36 human) strains showed clustering of poultry isolates into two ST-specific clades, with
995 ST155 strains further separating into two subclades based on O:H serotype (i.e., O8:H51 and
996 O108:H51). Notably, the constructed phylogenetic tree showed clustering of four (4/20, 20%)
997 poultry and nine (9/36, 25%) human strains belonging to O17:H18-ST69, implying clonality
998 (Fig 10A). Furthermore, a detailed phylogenetic analysis of the chromosomes of ST69 strains
999 revealed that poultry and human strains from Zambia did not cluster based on the host but
1000 were distributed within the tree. Moreover, the Zambian poultry and human strains were
1001 closely related but distant from international sequences from Switzerland (SWI) and Egypt
1002 (EGY) (Fig 10B).

1003



1025

B.

A.

1026

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

1027A. Poultry strains formed two clusters based on two STs; one cluster had four ST69 strains,
 1028 while the other had 16 ST155 strains. The ST155 group was further divided into two
 1029 subgroups based on O:H serotypes; 14 strains belonged to O8:H51, and the remaining two
 1030 were O108:H51. In addition, the genome of *E. fergusonii* (GenBank accession number
 1031 NZ_CP057659.1) was included as an outgroup. Furthermore, *E. coli* strain CFSAN061770
 1032 (GenBank accession number NZ_CP023142.1), belonging to ST69 and isolated from raw
 1033 milk cheese in Egypt (here abbreviated as EGY), and *E. coli* strain 3347558 (also ST69)
 1034 (GenBank accession number CP071073.1) isolated from a patient in Switzerland (here

1035 abbreviated as SWI) were also included in the analysis. Red; poultry (Zambia). Black; human
1036 (Zambia). Green; International.

1037**B.** Detailed phylogenic tree focusing on ST69 chromosomes only. The Zambian poultry and
1038 human strains were closely related and did not cluster based on the host.

1039

1040 **Location of *bla*_{CTX-M} genes among poultry strains**

1041 To detect the AMR genes present in poultry strains, the AMRFinderPlus tool [58] was
1042 used. A total of 27 AMR genes targeting 11 antibiotic classes were identified, dominated by
1043 targets of aminoglycosides (7/27, 25.9%), β -lactams (3/27, 11.1%), quinolones (3/27,
1044 11.1%), and trimethoprim (3/27, 11.1%) (Table 9). These AMR genes existed on plasmids of
1045 various incompatibility (Inc) groups.

1046 The primary AMR mechanism in *E. coli* involves CTX-M type ESBLs, encoded by
1047 *bla*_{CTX-M} genes [132]. All the 20 (100%) strains carried an allele of the *bla*_{CTX-M} gene; 18/20
1048 (90%) strains harbored *bla*_{CTX-M-55}, while the other 2/20 (10%) isolates carried *bla*_{CTX-M-14}.
1049 The *bla*_{CTX-M-55} and *bla*_{CTX-M-14} genes existed 421 bp and 326 bp, respectively, downstream of
1050 the insertion sequence IS26.

1051 Previous studies show that OqxAB-encoding genes seldom correlate with
1052 ciprofloxacin and nalidixic acid resistance [133]. Furthermore, aminoglycoside modifying
1053 enzymes exhibit divergent substrate specificities, with APH(6)-Id-like and APH(3'')-Ib-like
1054 types ineffective against gentamicin [134]. Consistently, the current study showed low
1055 positive predictive values (PPVs) for genes encoding OqxAB (PPV = 0% for both *oqxA2* and
1056 *oqxB*), APH(6)-Id (PPV = 56.25 % for *aph(3'')-Ib*), and APH(3'')-Ib (PPV = 65% for *aph(6)-*
1057 *Id*). In contrast, there was a perfect correlation between other detected AMR genes and the
1058 observed phenotypes (PPV = 100%) (Table 10).

1059

1060 Table 9. Diversity of AMR genes among the 20 poultry strains

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

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1062
1063

1064 Table 10. Prediction of phenotype from AMR genes.

GEN	<i>aac(3)-IIa</i>	<i>aac(3)-IIId</i>	<i>aadA1</i>	<i>aadA2</i>	<i>aadA5</i>	<i>aph(3'')-Ib</i>	<i>aph(6)-Id</i>
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

1065

1066

CIP	<i>oqxA2</i>	<i>oqxB</i>	<i>qnrS1</i>
R	0	0	4
S	2	2	0
Total	0	0	4
PPV (%)	0	0	100

1067

1068

NAL	<i>oqxA2</i>	<i>oqxB</i>	<i>qnrS1</i>
R	0	0	4
S	2	2	0
Total	0	0	4
PPV (%)	0	0	100

1069

1070

DOX	<i>tet(A)</i>	<i>tet(M)</i>
R	20	4
S	0	0
Total	20	4
PPV (%)	100	100

1071

1072

CHL	<i>cmlA1</i>
R	4
S	0
Total	4
PPV (%)	100

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1074 GEN = gentamicin, CIP = ciprofloxacin, NAL = nalidixic acid, DOX = doxycycline, CHL =

1075 chloramphenicol

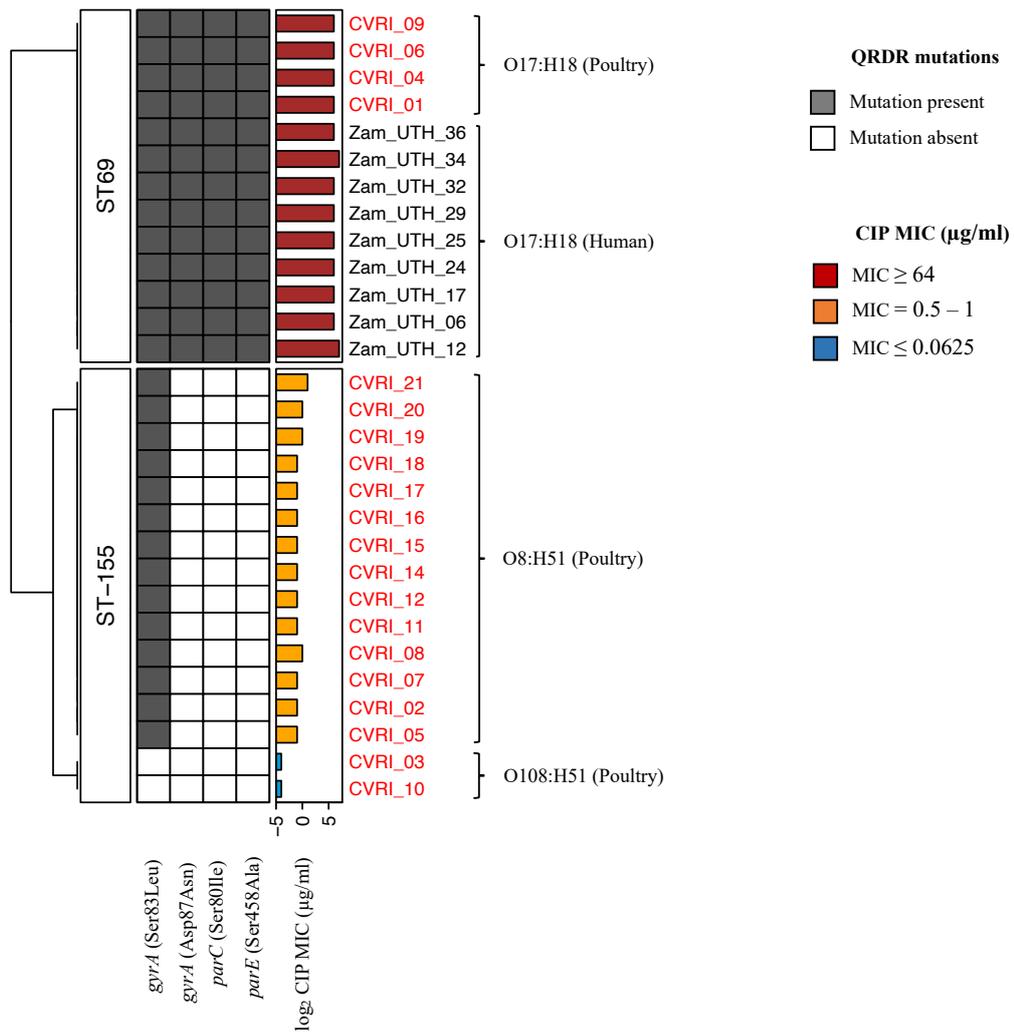
1076 R = resistant, S = susceptible

1077

1078 **Quinolone resistance mutations in *gyrA*, *parC*, and *parE***

1079 The high quinolone resistance rate (90%) observed in this study could not be
1080 explained by mobile quinolone resistance genes (PPV = 0% for *oqxA2* and *oqxB*) (Table 10).
1081 Furthermore, only 4/18 (22.2%) quinolone-resistant strains carried the *qnrS1* gene that
1082 encodes the QnrS1 protein to protect topoisomerases from quinolones. Moreover, the
1083 reduction in quinolone susceptibility by this mechanism is only complementary and not
1084 sufficient to meet the clinical breakpoint prescribed by the CLSI (0.5 µg/ml and 32 µg/ml for
1085 ciprofloxacin and nalidixic acid, respectively) [92]. To understand the observed resistance,
1086 mutations in the quinolone resistance determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and
1087 *parE* were explored. In agreement with previous reports [135-137], missense mutations were
1088 detected in three genes: *gyrA* (Ser83Leu and Asp87Asn), *parC* (Ser80Ile), and *parE*
1089 (Ser458Ala). Two O108:H51-ST155 strains that did not harbor any mutations in the
1090 abovementioned genes were susceptible to both ciprofloxacin (MIC ≤ 0.0625 µg/ml) and
1091 nalidixic acid (MIC = 16 µg/ml). However, 14 O8:H51-ST155 strains with the Ser83Leu
1092 mutation in the *gyrA* gene exhibited resistance to ciprofloxacin (MIC range; 0.5–1 µg/ml)
1093 (Fig 11) and nalidixic acid (MIC range; 512–1024 µg/ml) (Fig 12). Lastly, the four O17:H18-
1094 ST69 strains possessed mutations at all the four positions mentioned above and showed high
1095 resistance levels to ciprofloxacin (MIC ≥ 64 µg/ml) and nalidixic acid (MIC ≥ 2048 µg/ml).
1096 The same four mutations were observed in nine human-associated O17:H18-ST69 strains that
1097 also exhibited high-level quinolone resistance (Fig 11 and 12). While quinolone resistance
1098 has also been linked to amino acid substitutions at positions 426 and 447 of *gyrB* [138], none
1099 of these mutations were detected in the present study.

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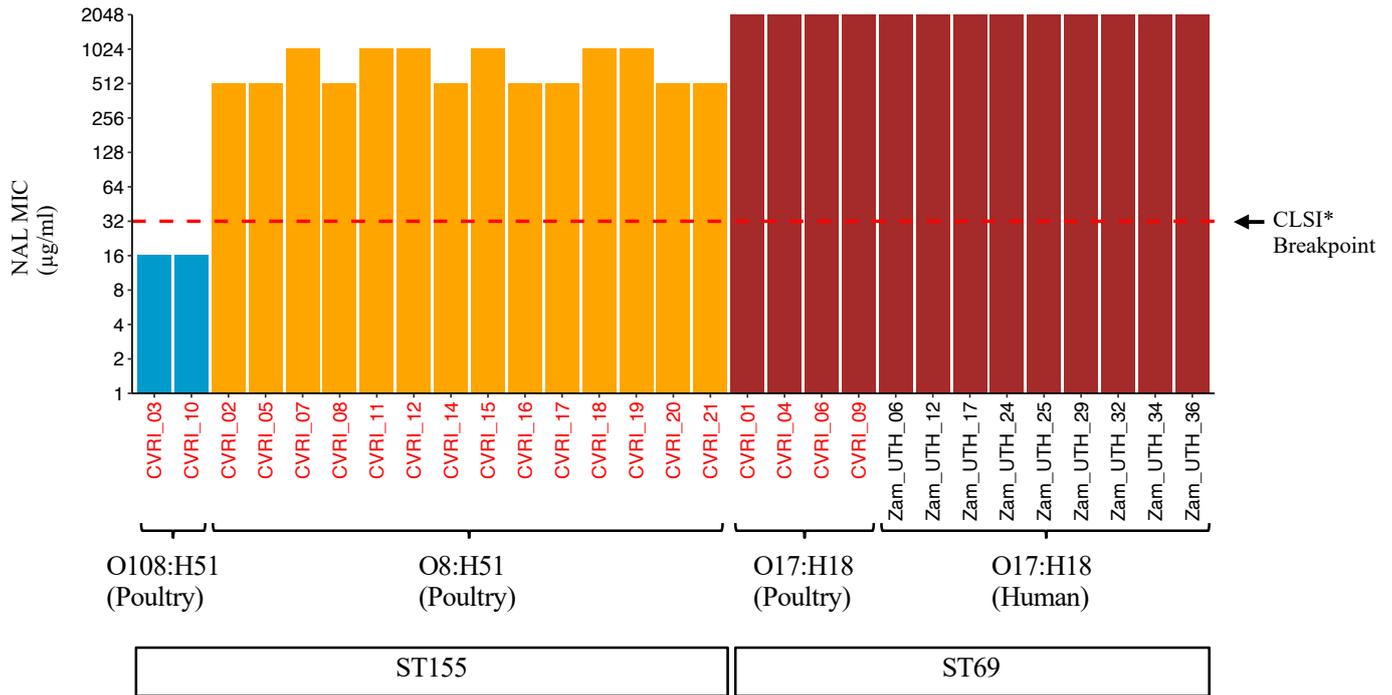
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Figure 11. Ciprofloxacin resistance. Missense mutations in the QRDR of *gyrA*, *parC* and *parE*.

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.

1112 The lack of QRDR mutations in two O108:H51-ST155 strains (blue) was associated with
 1113 susceptibility to nalidixic acid. In contrast, 14 O8:H51-ST155 strains (orange) possessing a
 1114 single *gyrA* gene mutation (Ser83Leu) exhibited nalidixic acid resistance with MICs
 1115 exceeding the clinical breakpoint by several folds. Furthermore, the four poultry and nine
 1116 human O17:H18-ST69 strains (brown), harboring quinolone resistance mutations in *gyrA*,
 1117 *parC*, and *parE*, displayed high-level resistance to nalidixic acid (MIC \geq 2048 μ g/ml).

1118 *Clinical and Laboratory Standards Institute [73]

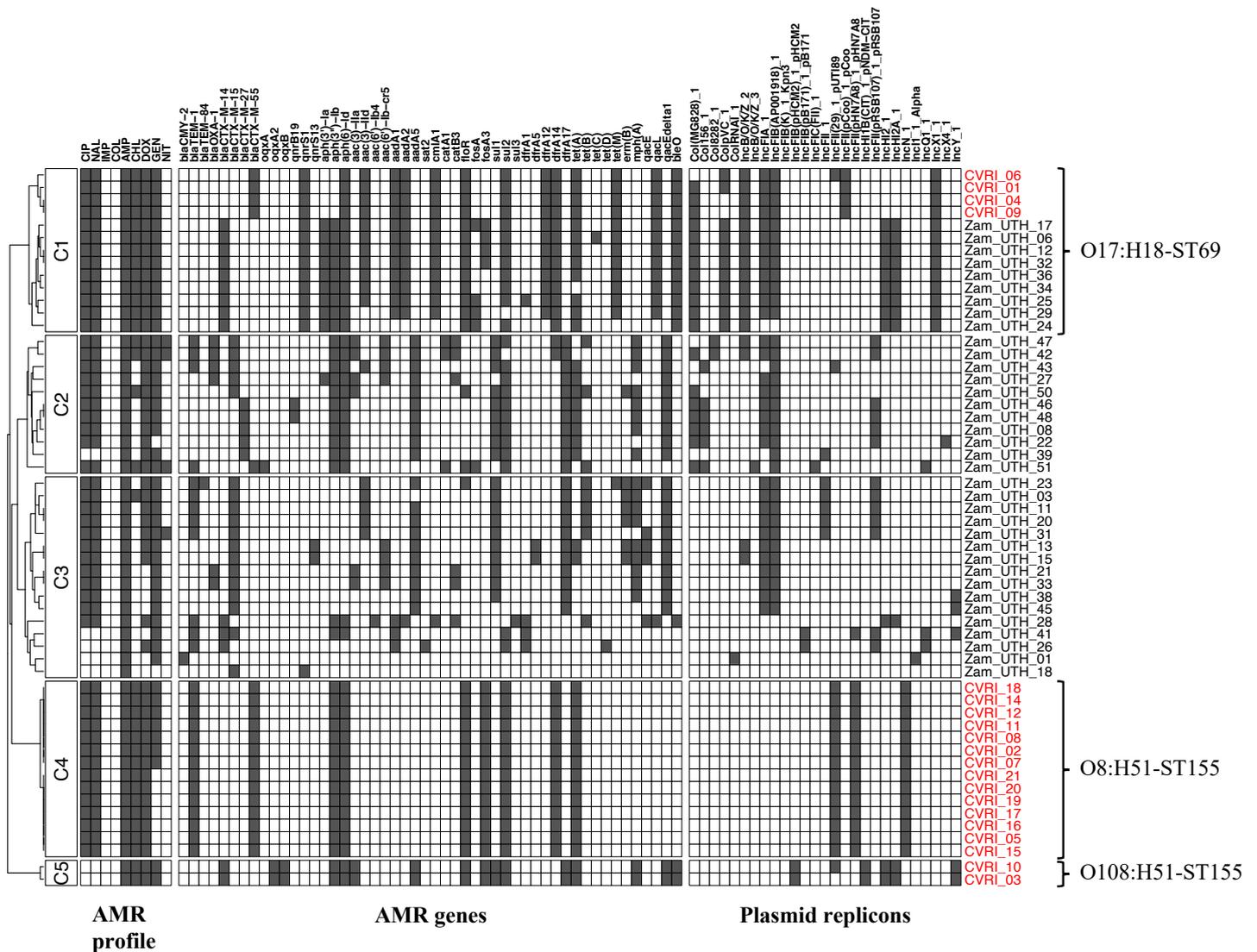
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1121 **Hierarchical clustering of ST69 strains from poultry and humans**

1122 To determine the mechanism of MDR propagation among poultry and human strains,
1123 clustering patterns of isolates were explored by examining various phenotypic and genotypic
1124 characteristics. Fifteen plasmid replicons were identified across the 20 poultry isolates, the
1125 commonest being IncFII(29)_1_pUTI89 (16/20, 80%), followed by
1126 IncFII(pHN7A8)_1_pHN7A8 (14/20, 70%), and IncN_1 (14/20, 70%). A comparison of
1127 poultry and human strains based on AMR phenotype, AMR genes, and plasmid replicons
1128 showed clustering into five main clades (referred to as C1, C2, C3, C4, and C5) (Fig 13).
1129 Most human isolates occupied separate clusters from poultry strains; however, C1 comprised
1130 four poultry and nine human isolates belonging to O17:H18-ST69. These strains displayed
1131 similar AMR patterns and had similar AMR genes and plasmid replicons, suggesting clonal
1132 spread (Fig 13). Poultry and human strains from C1 shared the plasmid replicons
1133 Col(MG828)_1, ColpVC_1, IncB/O/K/Z_2, IncFIA_1, IncFIB(AP001918)_1, and IncX1_1.
1134 However, the IncHI2A_1 and IncHI2_1 replicons were only present in human-associated
1135 strains but not in poultry strains. On the contrary, the human isolates lacked the
1136 IncFII(pCoo)_1_pCoo replicon present in poultry strains. Other overlapping replicons
1137 included two O108:H51 poultry strains in clade C5 sharing IncHI2_1 and IncHI2A_1
1138 replicons with one human isolate in C3 (Zam_UTH_28, Onovel28:H5-ST156) and nine
1139 human strains in C1 (O17:H18-ST69).

1140



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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*_{CTX-M} alleles were detected across the 20
 1145 poultry strains, with *bla*_{CTX-M-55} occurring in 18/20 (90%) strains and the remaining 2/20
 1146 (10%) isolates harboring the *bla*_{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

1148 human isolates. On hierarchical clustering, five distinct clades were formed across the 20
1149 poultry and 36 human isolates; C1 was composed of four poultry and nine human O17:H18-
1150 ST69 strains, C2 and C3 comprised human strains of various STs, C4 consisted of 14
1151 O8:H51-ST155 strains, while C5 constituted two O108:H51-ST155 strains. Only a few
1152 plasmid replicons were common between poultry and human strains.

1153 Red; poultry. Black; human.

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1157 **Comparison of AMR plasmids among poultry and human *E. coli* ST69 strains**

1158 A comparison analysis was carried out on plasmids carrying identical AMR genes and
1159 sharing plasmid replicons to understand the spread patterns of MDR among poultry and
1160 human strains. Two distinct plasmids were identified in four O17:H18-ST69 poultry isolates
1161 and nine human-associated O17:H18-ST69 strains. One of the plasmids belonged to
1162 incompatibility group IncFI, was 83 kb in size, and carried nine AMR genes (i.e., *aac(3)-IIa*,
1163 *aadA1*, *aadA2*, *cmlA1*, *dfrA12*, *floR*, *qacL*, *sul2*, and *tet(M)*). The other one belonged to IncI-
1164 complex, was 124 kb long, and carried five AMR genes (*aph(6)-Id*, *bleO*, *dfrA14*, *qnrS1*, and
1165 *tet(A)*) (Table 11). Comparison of contigs across niches revealed almost identical
1166 corresponding plasmids (Fig 14A and 14B). However, the isolates also contained other AMR
1167 plasmids specific for each niche, probably signifying additional independent mechanisms of
1168 AMR acquisition. Despite the difference in plasmid content between poultry and human
1169 O17:H18-ST69 strains, growth rates measured in antibiotic-free LB were comparable across
1170 niches (Fig 15), suggesting similar fitness.

1171 Precisely, poultry-associated O17:H18-ST69 strains harbored a 71 kb IncFII(pCoo)
1172 plasmid carrying the *bla*_{CTX-M-55} gene (Table 11). This plasmid shared over 90% nucleotide
1173 sequence similarity with pL37-4 (GenBank accession no. CP034592.1), a *bla*_{CTX-M-55}-
1174 carrying plasmid also belonging to IncFII(pCoo), reported in an *E. coli* O9:H37-ST48 isolate
1175 originating from a goose farm in Jiangsu, China (Fig 16). In contrast, O17:H18-ST69 strains
1176 from humans contained a 225 kb IncHI plasmid harboring *bla*_{CTX-M-14} and several other AMR
1177 genes (Table 11). Additionally, this IncHI plasmid exhibited over 85% nucleotide sequence
1178 similarity with another *bla*_{CTX-M-14}-carrying IncHI plasmid in two O108:H51-ST155 poultry
1179 strains, signifying origin from a common plasmid ancestor (Fig 14C). In both cases, the *fosA3*
1180 gene existed 611 bp downstream of the *bla*_{CTX-M-14} gene on a 4.8 kb unit flanked by a pair of
1181 IS26 copies (Fig 17).

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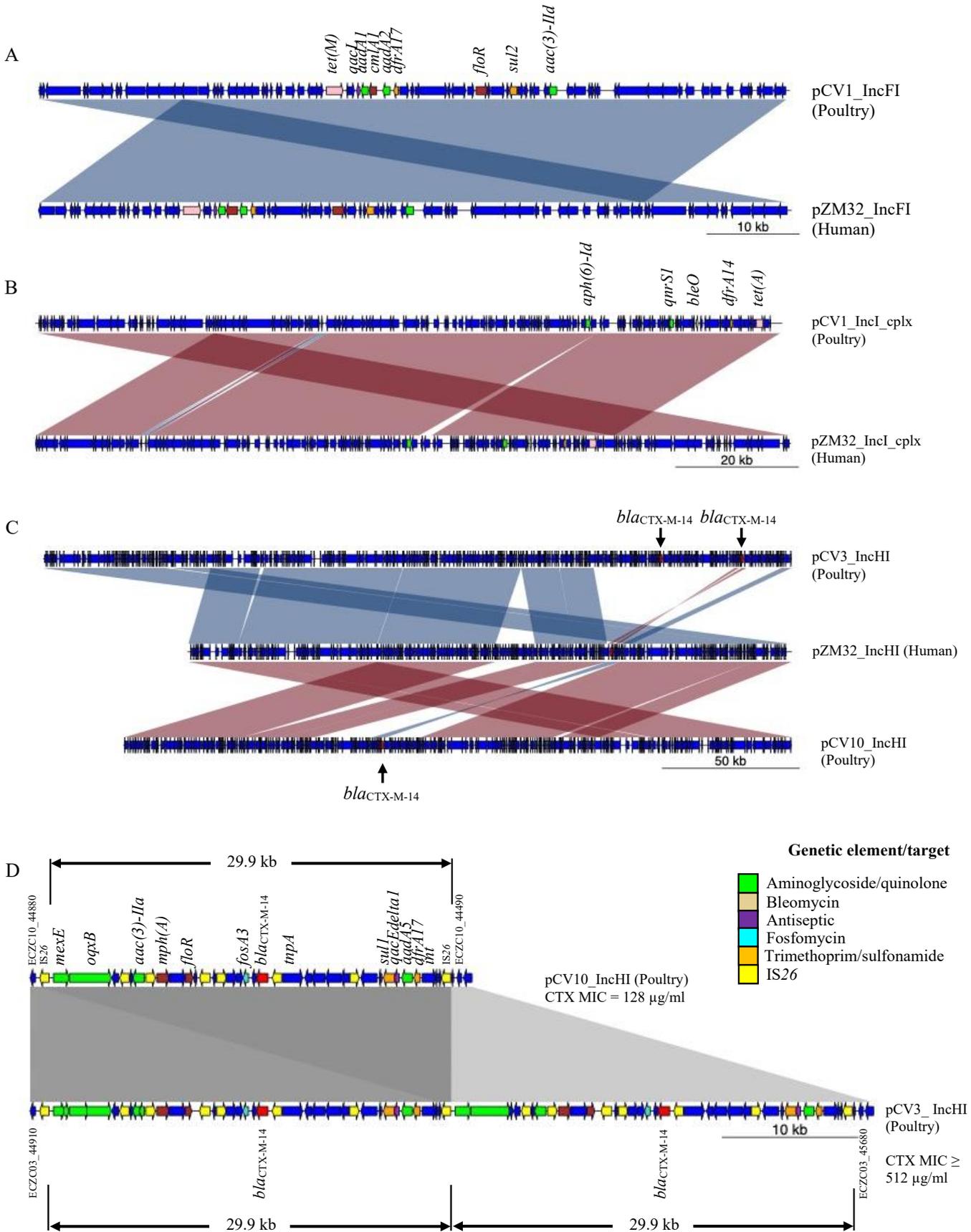
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1184 Table 11. Plasmids in *E. coli* O17:H18-ST69 from humans and poultry.

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

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

1189 Two plasmids, (A) IncFI and (B) IncI-complex, were shared across human- and poultry-
1190 associated *E. coli* O17:H18-ST69 strains. (C) In contrast, a *bla*_{CTX-M-14}-harboring IncHI
1191 plasmid was present in human-associated *E. coli* O17H18-ST69 strains but not in poultry-
1192 associated *E. coli* O17H18-ST69. A related IncHI plasmid with over 85% nucleotide
1193 sequence homology was detected in two poultry *E. coli* O108:H51-ST155 strains. (D) IncHI
1194 plasmid in the two O108:H51-ST155 poultry strains possessed multiple AMR genes on a
1195 putative 29.9 kb IS26-flanked TU. This suspected TU existed in tandem duplication in strain
1196 CVRI_03 and was associated with a higher cefotaxime resistance (CTX MIC \geq 512 μ g/ml)
1197 relative to CVRI_10 (CTX MIC = 128 μ g/ml).

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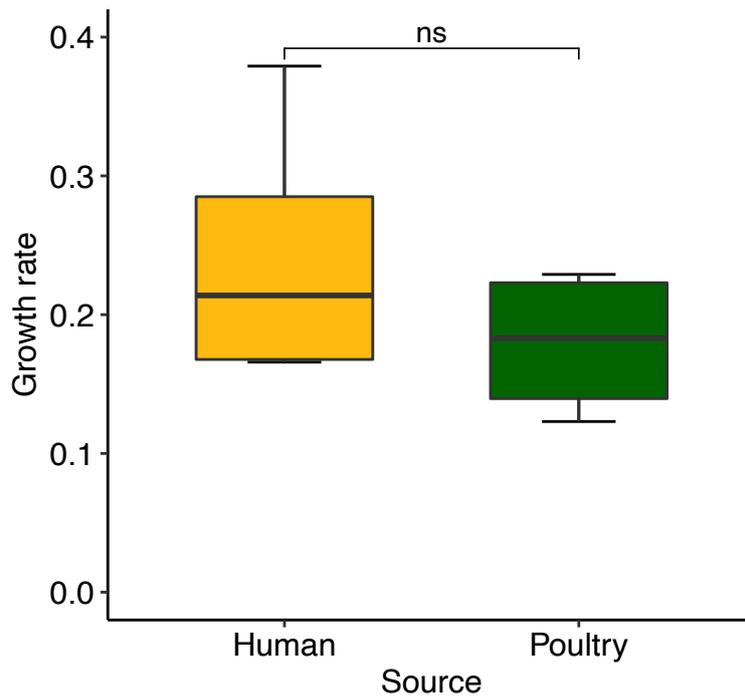


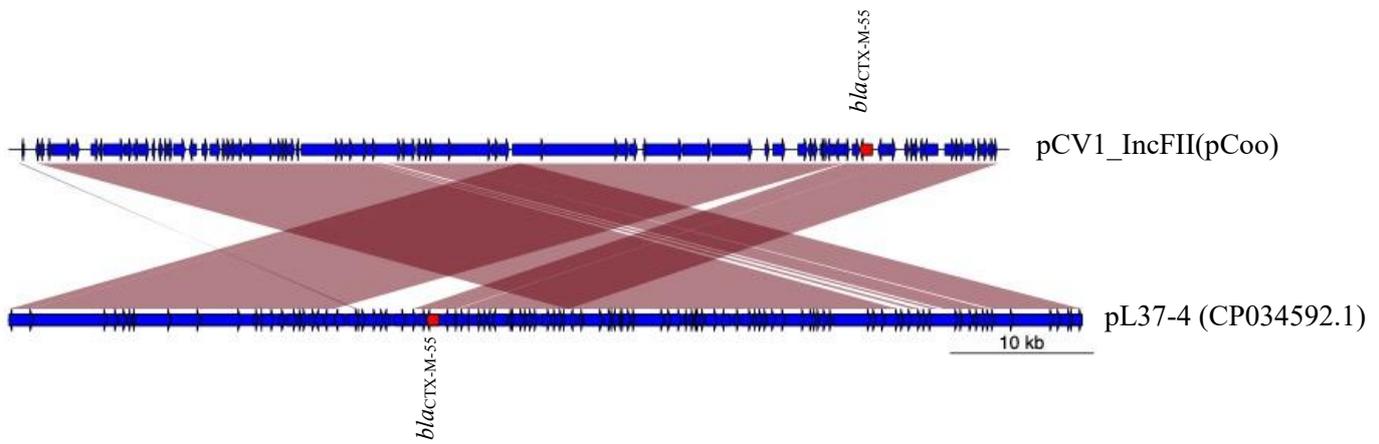
Figure 15. Comparison of growth rates between poultry and human *E. coli* O17:H18-ST69.

1215 No significant difference was found between the growth rate of poultry (n = 4) and human (n
1216 = 9) isolates (Mann-Whitney U test; P = 0.414).

1217
1218
1219

1220 Several studies have reported the coexistence of *bla*_{CTX-M-14} and *fosA3* [139, 140]
1221 among *Enterobacteriaceae* strains. One frequently observed gene arrangement includes
1222 IS26–*bla*_{CTX-M-14}–611 bp–*fosA3*–1222 bp–IS26, with directly oriented flanking IS26 copies
1223 [141]. However, a slightly different genetic architecture was identified in this study (IS26–
1224 *bla*_{CTX-M-14}–611 bp–*fosA3*–1274 bp–IS26), where IS26 existed 1274 bp downstream *fosA3*.
1225 Furthermore, the flanking IS26 copies in this study were inverted with respect to each other,
1226 thus not fulfilling the architecture required to form a cointegrate or a translocatable unit (TU)
1227 [142] during IS26-mediated co-transfer of *bla*_{CTX-M-14} and *fosA3*. Further analysis of the two
1228 poultry-associated O108:H51-ST155 strains uncovered that the abovementioned 4.8 kb unit
1229 was part of a larger segment bracketed by two IS26 copies in direct orientation, resulting in a
1230 potential 29.9 kb TU. This unit harbored several mobility-associated elements, including
1231 seven copies of IS26, an integrase gene (*int*), and a transposase gene (*tnpA*). Pseudo-
1232 compound transposons flanked by IS26 may facilitate gene amplification by excising a TU
1233 that reinserts to create an array of two or more TUs [143]. Notably, the suspected 29.9 kb TU
1234 occurred in tandem duplication in one O108:H51-ST155 strain (CVRI_03), increasing the
1235 copy number of the *bla*_{CTX-M-14} and several other AMR genes (Fig 14D). Nanopore reads
1236 traversing the junctions of this segment ruled out the possibility of erroneous assembly.
1237 Moreover, the observed duplication, which included the *bla*_{CTX-M-14} gene, coincided with a
1238 higher cefotaxime MIC in CVRI_03 (≥ 512 $\mu\text{g/ml}$) compared to CVRI_10 (128 $\mu\text{g/ml}$)
1239 (Table 7). While the two O108:H51-ST155 strains were very similar, CVRI_10 carried an
1240 extra 198 kb IncFII(29)_1_pUTI89 plasmid, lacking in CVRI_03. This difference in the
1241 plasmid content was accompanied by a discrepancy in growth rate between CVRI_03 ($\mu =$
1242 0.200) and CVRI_10 ($\mu = 0.081$), complicating the assessment of the amplified TU's effect
1243 on the fitness of CVRI_03.

1244



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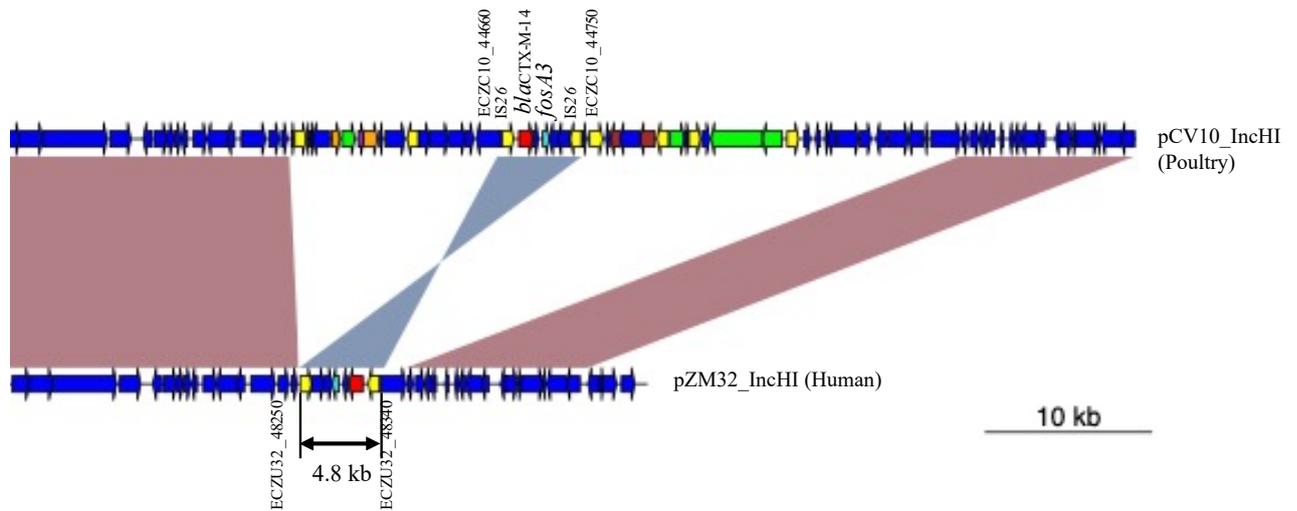
1246

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*_{CTX-M-55} gene (red).

1249

1250



1251

Figure 17. Genetic architecture around the *bla*_{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*_{CTX-M-14} and *fosA3* genes, flanked by IS26 copies
 1254 in inverted orientation.

1255

1256

1257 **Discussion**

1258 In this study, 20 cefotaxime-resistant *E. coli* isolates obtained from poultry in Lusaka,
1259 Zambia, were characterized by WGS. These isolates were tested for MDR and compared to
1260 36 cefotaxime-resistant *E. coli* strains isolated from hospital patients in the same city. Four
1261 out of 20 (4/20, 20%) poultry isolates shared clonality with 9/36 (25%) human strains. The
1262 four poultry and nine human strains had the same ST and O:H serotype and exhibited
1263 identical MDR profiles and QRDR mutations. The said strains also possessed two nearly
1264 identical plasmids carrying the same 14 AMR genes, suggesting clonal origin. Interestingly,
1265 the strains also had *bla*_{CTX-M}-harboring plasmids different for each niche, implying separate
1266 ESBL acquisition modes. These results indicate a clinically important link between MDR *E.*
1267 *coli* strains in poultry and humans, underscoring that a multisectoral approach is required to
1268 mitigate the threat of MDR.

1269 ST69, which is among the most challenging lineages known, is the second most
1270 frequent extraintestinal pathogenic *E. coli* [144]. In Zambia, 25% of ESBL-producing *E. coli*
1271 at the UTH belonged to the MDR clone *E. coli* ST69 [130]. Because of this predominance,
1272 there is a need to reveal the potential reservoirs and acquisition routes of the *E. coli* ST69
1273 clone. Notably, the present study reports a linkage between this clone and *E. coli* ST69
1274 isolates obtained from poultry in the same city. Apart from two plasmids (i.e., IncFII(pCoo)
1275 present only in poultry strains and IncHI found only in human isolates), the poultry- and
1276 human-associated ST69 lineages were nearly identical, with the same O:H serotypes and
1277 resistance plasmids (IncFI and IncI-complex). Furthermore, the ST69 isolates from poultry
1278 and humans also shared several AMR genes and QRDR mutations. The close phylogenetic
1279 relationship between poultry and human strains without host-specific clustering could imply
1280 clonality. These results suggest that MDR propagation between poultry and humans likely
1281 occurred through the direct transmission of the ST69 clone, including the two plasmids
1282 belonging to IncFI and IncI-complex. The clones could have subsequently diverged along
1283 separate evolutionary trajectories in response to various selection pressures associated with
1284 the host environment, culminating in the acquisition of distinct ESBL-encoding plasmids (Fig
1285 18).

1286 One crucial trait among bacteria is the ability to resist multiple antimicrobials, leading
1287 to their persistence in challenging ecological habitats, such as patients and animals on
1288 antibiotic treatment. The 20 poultry strains in the present study were all resistant to various
1289 antimicrobials of clinical significance, such as ampicillin, chloramphenicol, and doxycycline.

1290 The observed MDR pattern could result from the current practices and antimicrobial use in
1291 Zambian poultry, where amoxicillin, chloramphenicol, and doxycycline are among the
1292 frequently used drugs, according to the Zambia National Public Health Institute (ZNPFI)
1293 [111]. However, the observed MDR prevalence (100%) among the poultry samples was
1294 likely overestimated since the selection criterion, cefotaxime resistance, is often associated
1295 with MDR [101].

1296 Of particular concern was the observation that 90% of the isolates exhibited resistance
1297 to ciprofloxacin and nalidixic acid, clinically significant quinolones commonly used as first-
1298 and second-line drugs to treat a wide range of bacterial infections. The high quinolone
1299 resistance observed in this study was similar to the prevalence among clinical strains from the
1300 UTH, where close to 90% of the MDR *Enterobacteriaceae* displayed resistance to
1301 ciprofloxacin and nalidixic acid [130]. Again, the actual quinolone resistance burden might
1302 be overstated by these results since this study only included cefotaxime-resistant strains,
1303 potentially associated with quinolone resistance [145, 146]. Despite most poultry strains
1304 showing resistance to the quinolones, MICs were much higher in the four *E. coli* ST69
1305 isolates carrying mutations in the QRDRs of *gyrA*, *parC*, and *parE*. Similarly, the nine
1306 human *E. coli* ST69 isolates mentioned above showed equally high MICs for ciprofloxacin
1307 and nalidixic acid and harbored the same quinolone resistance mutations, thus supporting the
1308 proposed clonal expansion hypothesis.

1309 Studies elsewhere show that *E. coli* ST155 is common among poultry samples from
1310 other African countries [147]. In the present study, ST155 predominated the poultry samples
1311 at 80% (16/20), but it was absent in the clinical strains. However, other studies have reported
1312 this ST in humans [116], making it a potential MDR threat in Zambia. Besides, the MDR-
1313 encoding IS26-flanked TU identified on an IncHI plasmid in a pair of ST155 strains
1314 (CVRI_03 and CVRI_10) is a call for concern. Research on IS26-associated TUs carrying
1315 various β -lactamase genes (*bla*_{TEM-1B} [143], *bla*_{OXA-1}, and *bla*_{CTX-M-15} [148]) shows an
1316 associated increase in resistance to β -lactam antibiotics. Consistently, the *bla*_{CTX-M-14}-carrying
1317 TU in CVRI_03 seemed to have increased the cefotaxime MIC of this strain. However, no
1318 apparent treatment implications are expected from this change since it takes only a single
1319 copy of *bla*_{CTX-M} to cause cefotaxime resistance. Also, the said ST155 strains (CVRI_03 and
1320 CVRI_10) exhibited identical phenotypic AMR profiles. Still, this finding is clinically
1321 relevant because IS26 can transfer AMR genes among various *Enterobacteriaceae* clones and
1322 species [149], highlighting that ST155 is a potential MDR reservoir for human pathogens in
1323 Zambia.

1324 Nevertheless, it is unclear whether the high prevalence observed for ST155 was
1325 expected since previous reports in Zambia [17, 150] did not characterize MDR *E. coli* in
1326 terms of MLST. Moreover, all the samples included in this study were collected from the
1327 same farm, making it difficult to generalize and carefully interpret the findings. Hence, more
1328 extensive studies, including more isolates and sampling points, will be required to answer
1329 many questions definitively. Nevertheless, the results presented here still raise public health
1330 concerns since the farm sampled is commercial and supplies poultry products to several
1331 communities in Lusaka and nearby districts.

1332 Furthermore, the clonal relationship between *E. coli* ST69 strains from poultry and
1333 humans suggests that MDR could have spread between these two niches. While there is a
1334 possibility that poultry and humans independently acquired the *E. coli* ST69 clone from
1335 another source, the model (Fig 18) in the present study seems plausible considering how
1336 common poultry products are and the associated antibiotic use during production. In Zambia,
1337 poultry accounts for 50% of the country's meat consumption, and the industry generates 5%
1338 of the national GDP [151]. As the national population expands, there is an increase in the
1339 demand for animal-source protein, including poultry and poultry products. To meet this
1340 demand, poultry companies heighten production by raising large numbers of birds through
1341 intensive farming methods that often require antibiotic usage for prophylaxis and sometimes
1342 for growth enhancement. While this is crucial to alleviating food shortage problems, the
1343 associated MDR escalation is incredibly problematic.

1344 To address this dilemma, the Ministry of Health and the Ministry of Livestock and
1345 Fisheries (MLF) should make tradeoffs that limit MDR spread while adequate poultry
1346 production is maintained. The University of Zambia has been a critical stakeholder in
1347 fostering One Health [152], leading to a rise in the discourse on the concept. These efforts
1348 have attracted crucial partners such as the ZNPHI, who recently launched a One Health
1349 Surveillance Platform for AMR in Zambia [153]. Moving forward, there is a considerable
1350 need to build on the progress made so far by strengthening the main pillars of AMR
1351 prevention using a multisectoral approach. Achieving the desirable outcomes demands the
1352 MLF to educate poultry farmers about the dangers of non-rational antibiotic use and the
1353 benefits of improving animal conditions and hygiene. In addition, there should be more
1354 restrictions on the sale of antibiotics for prophylaxis. At the same time, AGP use must be
1355 guided by recommendations from thorough risk analysis and solely limited to nonmedically
1356 important antibiotics. Finally, routine food inspection must be strengthened by including
1357 antibiotic residue monitoring to increase the quality of the data available for policymaking.

1358 Moreover, extending these tests to include other food animals would help identify various
1359 potential MDR reservoirs.
1360

1369 **General conclusion**

1370 Therapeutic challenges faced by clinicians during the management of patients
1371 infected with drug-resistant *Enterobacteriaceae* raise considerable concern. The problem is
1372 further exacerbated by the rise of animal-related AMR following antibiotic use for
1373 prophylaxis and growth promotion. Developing practical control strategies requires
1374 epidemiologic research linking AMR and patient outcomes. However, the lack of detailed
1375 molecular data in many developing countries limits the successful implementation of
1376 effective AMR control policies. In Zambia, for example, previous studies on ESBL-mediated
1377 AMR in humans and poultry have relied on limited technologies such as PCR. Therefore,
1378 building on previous efforts, this work provides a WGS-based analysis of 46 human- (one *E.*
1379 *cloacae*, nine *K. pneumoniae*, and 36 *E. coli*) and 20 poultry-associated (*E. coli*) ESBL-
1380 producing strains.

1381 In Chapter I, the study characterized susceptibility profiles and AMR genes among 46
1382 cefotaxime-resistant hospital *Enterobacteriaceae*. The results revealed that the spread of
1383 *bla*_{CTX-M} genes is perpetuated by the persistence of a few resilient STs, with *E. coli* ST69
1384 dominating. In addition, one *E. cloacae* and three *E. coli* strains carried large chromosomal
1385 insertions co-harboring the *ISEcp1-bla*_{CTX-M-15} transposition unit and various AMR genes
1386 originating from plasmids. The findings imply that *ISEcp1* mobilizes large *bla*_{CTX-M-15}-
1387 containing MDR segments in diverse *Enterobacteriaceae* species. The stable maintenance of
1388 these MDR segments on chromosomes may facilitate the spread and persistence of MDR
1389 clones and lead to treatment failure and poor patient outcomes.

1390 In Chapter II, the phylogenetic relationship of MDR *E. coli* from poultry and humans
1391 in Zambia was inferred to establish the possibility of MDR spread between the two niches.
1392 The results revealed a close relationship between MDR *E. coli* ST69 from poultry and
1393 humans, suggesting possible transmission between the niches. The observation highlights the
1394 need for a closer multisectoral collaboration between human and animal health experts under
1395 the One Health umbrella. In addition, risk analysis and genome-based surveillance should
1396 guide policy formulation on AGP use in food animals.

1397 The results presented here provide an important benchmark for further studies to
1398 forecast and limit MDR spread from poultry to humans in Zambia.

1399
1400

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