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Comparative phylogenomics of extended-spectrum beta-lactamase-producing *Escherichia coli* revealed a wide diversity of clones and plasmids in Spanish chicken meat

Sandra Martínez-Álvarez^a, Pierre Châtre^b, Pauline François^b, Myriam Zarazaga^a, Jean-Yves Madec^b, Marisa Haenni^b, Carmen Torres^{a,*}

^a Area of Biochemistry and Molecular Biology, One Health-UR Research Group, University of La Rioja – Logroño, Spain

^b ANSES – Université de Lyon, Unité Antibiorésistance et Virulence Bactériennes – Lyon, France

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ABSTRACT

Animal food products are important sources of zoonotic agents, increasing the risk of exposure to antibiotic-resistant bacteria from farm to fork. Therefore, we aimed to detect and fully characterise Extended-Spectrum Beta-Lactamase (ESBL)-producing *E. coli* from the poultry sector in a One Health approach. From December 2021 to March 2022, 48 chicken meat samples were collected from 16 establishments in La Rioja (Northern Spain). Antibiotic susceptibility testing was assessed by the disk-diffusion method. Forty *E. coli* isolates were recovered from 33 of the 48 chicken meat samples tested (68.8%) when plated on MacConkey-agar. In addition, six ESBL-*E. coli* (6/48, 12.5%) were obtained on cefotaxime-supplemented MacConkey-agar, which were Whole-Genome Sequenced. A large diversity of clones and ESBL genes was observed, namely ST1140-E/*bla*_{CTX-M-32} ($n = 1$), ST752-A/*bla*_{TEM-52} ($n = 1$), ST117-B2/*bla*_{CTX-M-1}/*bla*_{SHV-12} ($n = 2$), ST10-A/*bla*_{SHV-12} ($n = 1$) and ST223-B1/*bla*_{SHV-12} ($n = 1$). Three IncI1-plasmids (pST3-CC3) were found carrying the *bla*_{SHV-12}/*bla*_{CTX-M-1}/*bla*_{CTX-M-32} genes in two genetic environments: i) IS26-*smc-glpR-bla*_{SHV-12}-IS26; and ii) *wbuC-bla*_{CTX-M-32}/*bla*_{CTX-M-1}-ISEcp1. The *bla*_{TEM-52} gene was carried on a P1-like phage-plasmid flanked by an IS4-mediated composite transposon. An IncHI2 plasmid harboured a *bla*_{SHV-12} gene flanked by an IS26-mediated composite transposon but also additional genes conferring resistance to aminoglycosides, chloramphenicol, and sulphonamides. To analyse the cross-sectoral relatedness of our ESBL-*E. coli* isolates, our six genomes were mapped with publicly available genomes ($n = 2588$) related to the STs detected, revealing that one of our genomes (X3078-ST117) displayed strong similarities (34–40 allelic differences) with few genomes belonging to ST117 from the poultry sector from Germany and USA. This study demonstrated that the proportion of ESBL-*E. coli* is still high in chicken meat in Spain. In addition, the ST117 clone and the IncI1-*bla*_{CTX-M-1-32}/*bla*_{SHV-12} plasmids might represent successful clones and plasmids adapted to the chicken host.

1. Introduction

Since 2010, poultry meat production in the European Union (EU) has grown notably, owing mainly to a less expensive source of protein and to the perception of a healthier product, with Spain ranked 2nd in the EU poultry meat production (Eurostat Agricultural Production - livestock and meat 2023). Poultry-derived products are important sources of zoonotic agents for humans, increasing the risk of antimicrobial-resistant bacteria (ARB) exposure from farm to fork (European Food

Safety Authority EFSA and European Centre for Disease Prevention and Control ECDC, 2023; Vitas et al., 2018; Díaz-Jiménez et al., 2020). Therefore, controlling and preventing the transmission of these bacteria along the food chain is a key challenge facing the poultry industry (de Mesquita Souza Saraiva et al., 2022) and requires continuous monitoring.

Antibiotics have been used on poultry farms as treatments, prophylactic measures, and growth promotor agents (Manyi-Loh et al., 2018), although the latter use was banned in 2006 in the EU and in other

* Corresponding author at: Universidad de La Rioja, Faculty of Science and Technology, Department of Agriculture and Food, Madre de Dios 53, 26006 Logroño, Spain.

E-mail address: carmen.torres@unirioja.es (C. Torres).

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countries but is still allowed in many others (OIE, 2022). Antibiotic misuse led to selective pressure on the chicken microbiota, favouring the emergence and spread of ARB and creating reservoirs of antimicrobial resistance genes (ARGs) (Vidovic and Vidovic, 2020). Importantly, several zoonotic bacteria are naturally present in the chicken microbiota, such as *Campylobacter* spp., *Salmonella* spp. and *Escherichia coli*, and transfers of ARGs to these (opportunistic) pathogens might jeopardize the antimicrobials efficacy in animals, and potentially further in human medicine (Osman et al., 2018; Vidovic and Vidovic, 2020). In the poultry sector, the burden of Extended-Spectrum Beta-Lactamase (ESBL)-producing *E. coli* is a recurring issue that needs to be tackled using a One Health approach (PRAN, 2019).

Previous studies have been performed on poultry meat sampled in Spain in which the prevalence of ESBL-producing *E. coli* has been analysed (Egea et al., 2012; Ojer-Usoz et al., 2013; Vitas et al., 2018; Díaz-Jiménez et al., 2020; Martínez-Laorden et al., 2023). However, none of them performed a full genomic analysis including comparison with other public genomes from human, animal, and environmental settings at a global scale. To fill in this gap, we performed a comparative phylogenetic analysis and plasmid characterisation of ESBL-producing *E. coli* isolates recovered from chicken meat samples in La Rioja region. We characterised both the clones (at the core-genome level) and the genetic determinants involved in the dissemination of critically important AMR genes at large (plasmids) and small scales (transposons, integrons) by Whole-Genome Sequencing (WGS). In addition, a holistic analysis of our ESBL-*E. coli* genomes with those publicly available in databases were carried out to explore possible linkages between them to pave the way for future One Health epidemiological studies with a wider scope.

2. Materials and methods

2.1. Sampling, bacterial isolation and identification

From December 2021 to March 2022, 48 chicken meat samples (thigh, breast, and minced meat) were collected from 16 establishments (nine local shops and seven hypermarkets) in La Rioja region (Northern Spain) and were transported at 4°C in sterile bags to the microbiology laboratory for processing. A sterile cotton swab was wiped over cut meat pieces under sterile conditions and immediately placed in Brain-Heart Infusion Broth being incubated overnight at 37°C. Different aliquots were seeded on MacConkey (MC) agar plates supplemented with cefotaxime (MC-CTX; 2µg/mL) or not supplemented (MC) for cefotaxime-resistant (CTX^R) *E. coli* and non-CTX^R *E. coli* recovery, respectively. Presumptive *E. coli* colonies were picked up arbitrarily (up to eight per sample), and identification was confirmed by MALDI-TOF (Bruker Daltonik, Bremen, Germany).

2.2. Antimicrobial susceptibility testing

Susceptibility testing was performed using the disk-diffusion method on Mueller-Hinton agar, according to the guidelines and clinical breakpoints of the Clinical Laboratory Standard Institute 2023 (Clinical and Laboratory Standards Institute CLSI, 2023). The following antibiotic disks of human and/or veterinary interest were tested: ampicillin, amoxicillin/clavulanate, ceftazidime, cefotaxime, ceftiofur, imipenem, nalidixic acid, ciprofloxacin, gentamicin, amikacin, tobramycin, chloramphenicol, trimethoprim/sulphamethoxazole, and tetracycline. ESBL screening was checked by the double disc synergy test. *E. coli* strain ATCC 25922 was included for quality control.

2.3. Characterisation of AMR genes and integrons in non-ESBL *E. coli* isolates

Based on the phenotypic resistance profiles of *E. coli* recovered from MC plates, a collection of non-duplicate isolates was stored for further

analysis, corresponding to one isolate per sample and per antibiotic resistance pattern. The following AMR genes were identified by PCR and sequencing: β-lactams (*bla*_{TEM}, *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{OXA}), quinolone (*qnrA*, *qnrB*, *qnrS*, *aac(6)-Ib-cr*, and *qepA*), gentamicin (*aac(3)-II*, *aac(3)-III*, and *aac(3)-IV*), tetracycline (*tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, and *tet(E)*), chloramphenicol (*cmlA*, *catA1*, and *floR*) and sulphonamides (*sul1*, *sul2*, and *sul3*) (Jouini et al., 2007; Alonso et al., 2017a). In addition, amino acid changes in ParC and GyrA proteins were studied by PCR-sequencing in the quinolone-resistant isolates (Alonso et al., 2017a). The presence of integrons was screened by PCR amplification of the integrase gene *intI1* (for class 1 integrons) and *intI2* (for class 2 integrons) (Mazel et al., 2000). Their genetic structure was obtained following the “primer-walking” PCR strategy to obtain the complete array (Vinué et al., 2008a, 2008b).

2.4. Short-read sequencing of ESBL *E. coli* isolates

According to the manufacturer's instructions, genomic DNA was extracted using a NucleoSpin Microbial DNA extraction kit (Macherey-Nagel, Hoerd, France). Library preparation was performed using Nextera XT technology, and sequencing was performed on a NovaSeq 6000 instrument (Illumina, San Diego, CA, USA). After sequencing, reads were quality trimmed, de novo assembled using Shovill v1.0.4 and the quality of assemblies was assessed using QUAST v5.0.2 (Table S1). Sequence types (STs) were determined using the CGE online tool MLSTFinder v2.0.4 (<http://www.genomicepidemiology.org/>), while resistance genes and replicon content were inferred using ABRicate v1.0.1 (<https://github.com/tseemann/abricate>). Virulence factors (VFs) were determined using VirulenceFinder and serotypes were determined using SeroType Finder (<http://www.genomicepidemiology.org/>).

2.5. Phylogenetic analysis

For phylogenetic analyses, a core-genome alignment based on the concatenation of 2513 core genes was obtained with the pyMLST pipeline (<https://github.com/bvalot/pyMLST>). The cut-off for closely related strains was divided into 0–10 allelic differences (ADs), 11–20 ADs, 21–50 ADs, 51–100 ADs, 101–200 ADs and >200 ADs. This alignment was used to construct a maximum likelihood phylogenetic tree using IQ-TREE v1.6.12 (model GTR+F+ASC+R5), and the resulting tree was visualized using iTol v6 (<http://itol.embl.de/itol.cgi>).

2.6. Transfer, plasmid characterisation and long-read sequencing of ESBL *E. coli* isolates

Plasmids were transferred by conjugation in liquid medium using the sodium azide-resistant *E. coli* J53 strain as recipient cells. Transconjugants were selected on LB agar supplemented with cefotaxime (2mg/L) and sodium azide (200mg/L). Conjugation frequencies were calculated by dividing the number of transconjugants by the number of donors. The plasmid content was determined from the WGS data using PlasmidFinder 2.0.1 (<http://www.genomicepidemiology.org/>). Plasmids carrying the ESBL genes were assigned in silico when the ESBL gene was located on the same contig as the plasmid marker. When in silico data showed no co-occurrence on the same contig, plasmids carrying the ESBL genes were detected using S1-PFGE gels (6V/cm for 20h with an angle of 120° at 14°C with pulse times ranging from 1 to 30s) followed by Southern blot using adequate probes, as previously described (Saidani et al., 2019). In addition, long-read sequencing was performed using the native barcode expansion kit (EXP-NBD104; Oxford Nanopore Technologies) and the ligation sequencing kit (SQK-LSK109). Sequencing was performed on a MinION sequencer (SpotON Mk 1 R9) with flow cell (FLO-MIN106D), assembled using Unicycler (Wick et al., 2017) and corrected with Pilon (Walker et al., 2014) to create the hybrid assemblies.

2.7. Accession numbers

All genomic sequences were deposited in DDBJ/EMBL/GenBank under the BioProject accession number PRJNA1102028. The full-length sequence of plasmids pUR3078 (*E. coli* X3078), pUR3556 (*E. coli* X3556), pUR3561 (*E. coli* X3561) and pUR3081 (*E. coli* X3081) were deposited in the EMBL database under accession no. PP711258, PP711259, PP711261 and PP711260, respectively.

3. Results

3.1. Prevalence of *E. coli* on chicken meat

E. coli isolates were recovered on MC agar plates in 33 out of 48 chicken meat samples tested (68.8%), leading to a collection of 40 non-duplicate *E. coli* isolates (1–2 *E. coli* isolates/sample). Moreover, ESBL-producing *E. coli* isolates were retrieved from selective MC-CTX plates in six samples (12.5%) from the thigh and breast, of which five samples came from hypermarkets and one sample from local shops (Table S2).

3.2. Genetic features of non-ESBL-producing *E. coli* isolates and integron characterisation

The 40 non-repetitive *E. coli* isolates obtained on non-selective plates were all non-ESBL producers. The highest resistance rates disclosed were for ampicillin, amoxicillin/clavulanate, nalidixic acid, ciprofloxacin, and trimethoprim/sulphamethoxazole (22.5%–50%), while resistance to tetracycline, chloramphenicol, kanamycin, and gentamicin was also detected but at lower rates (3%–15%) (Table 1).

A great diversity of AMR genes was observed among the 40 non-ESBL-producing *E. coli* isolates (Table 2). Ampicillin resistance was mostly conferred by the *bla*_{TEM} gene, and ciprofloxacin resistance was mediated by amino acid changes in the quinolone resistance determining region of the topoisomerases GyrA and ParC (S80I, D87N/S83I), as well as by the presence of plasmid-mediated quinolone resistance genes *aac*(6′)-*Ib*-*cr* and *qnrA*. All gentamicin-resistant isolates carried the *aac*(3)-*II* gene. Tetracycline resistance was mediated by the *tet*(A) or *tet*(B) genes, and all chloramphenicol-resistant isolates carried the *cmlA* gene. In addition, SXT-resistant isolates harboured the *sul1*, *sul2*, and/or *sul3* genes as well as the *dfrA1*/*dfrA12* genes (Table 2A). *E. coli* isolates belonged to the following phylogroups: E (*n* = 14; 27.5%), A (*n* = 13, 25.5%), B1 (*n* = 8, 15.7%), C (*n* = 6, 11.8%), B2 (*n* = 4, 7.8%) and F (*n* = 4, 7.8%) (Table S3). Two class 1 integrons were each detected in one

Table 1

Antimicrobial resistance rates of ESBL-*E. coli* isolates (obtained in CTX-supplemented MC media) and the non-ESBL-*E. coli* isolates (recovered in MC media).

Antibiotics	ESBL <i>E. coli</i> (N = 6)	non-ESBL <i>E. coli</i> (N = 40)
	N/%	N/%
Amoxicillin-clavulanate	2/33.3	14/35.0
Ampicillin	6/100	20/50.0
Cefotaxime	6/100	0/0.0
Ceftazidime	6/100	0/0.0
Ceftriaxone	6/100	0/0.0
Cefoxitin	0/0.0	0/0.0
Imipenem	0/0.0	0/0.0
Kanamycin	2/33.3	0/0.0
Streptomycin	1/16.7	0/0.0
Gentamicin	1/16.7	6/15.0
Chloramphenicol	1/16.7	3/7.5
Tetracycline	3/50.0	12/30.0
Trimethoprim/ sulphamethoxazole	2/33.3	9/22.5
Nalidixic acid	2/33.3	15/37.5
Ciprofloxacin	2/33.3	15/37.5

isolate; *intI1-dfrA1-aadA1-qacEΔ1-sul1* and *intI1-dfrA12-orfX-aadA1-qacEΔ1-sul1* (Table 2B), while the classical class 2 integron linked to Tn7 (*intI2-dfrA1-sat2-aadA1-orfX-ybfA-ybfB-ybgA-trnE-trnD-trnC-trnB-trnA*) was observed in two isolates (Table 2C).

3.3. Characterisation of *bla*_{ESBL}-carrying *E. coli* isolates

Six ESBL-producing *E. coli* isolates (one per sample) were collected on MC-CTX selective plates. Two isolates were multi-drug resistant, presenting additional genes conferring resistance either to aminoglycosides and tetracyclines (X3560) or to sulphonamides and aminoglycosides (X3556). Cefoxitin and imipenem resistance was not identified (Table 3).

The six ESBL-producing *E. coli* isolates presented the following ST-phylogroups/ESBL gene combinations: ST1140-E/*bla*_{CTX-M-32} (*n* = 1), ST752-A/*bla*_{TEM-52} (*n* = 1), ST117-B2/*bla*_{CTX-M-1} (*n* = 1), ST117-B2/*bla*_{SHV-12} (*n* = 1), ST10-A/*bla*_{SHV-12} (*n* = 1) and ST223-B1/*bla*_{SHV-12} (*n* = 1) (Table 3). The phylogenetic analysis of these STs revealed that the two isolates belonging to the lineage ST117 (obtained from two different establishments) differed by 135 ADs and were thus not clonally related (Table S4).

3.3.1. IncI plasmids

Three IncI-plasmids were found carrying an ESBL gene in the following genetic environment: *ISEcp1-bla*_{CTX-M-1}-*wbuC* (IncI1/pST3), *ISEcp1-bla*_{CTX-M-1/32}-*wbuC* (IncI2) and the new *IS26-smc-glpR-bla*_{SHV-12}-*IS26* (IncI1/pST3) (Fig. 1a-b, Fig. S1a-b). Long-read sequencing of the two IncI1/pST3 plasmids showed full identity/coverage with the replication, transfer, and leading region (Fig. S2) and 81%–97% identity with a 99.96%–100% coverage with pJ31 (accession no. CP053788.1), pEC7 (CP053679.1) and pEC1 (CP053560.1), pESBL-283 (CP008736.1) or pEC161_1 (KU932032.1) plasmids, previously reported in *E. coli* in wildlife, humans and livestock, with some insertions/deletions suggesting recombination between plasmids. The IncI2-*bla*_{CTX-M-32}-carrying plasmid displayed an estimated size of approximately 90 kb, close to sizes reported from IncI1-plasmids (100 kb–110 kb). All IncI plasmids were conjugative, displaying conjugation frequencies ranging from 2.6/3.1 × 10⁻⁶ for the IncI1-plasmids and 5.1 × 10⁻⁴ for the IncI2-plasmid.

Contrarily, their adaptability modules were more variable (Fig. 1, Fig. S2). The pUR3078 plasmid presented a large adaptability module (9,516 bp) located between the replication and the *yacA type II* toxin-antitoxin system (leading region) (Fig. 1), as well as pUR3081 (12,672 bp), between the replication region and *parA-parM* partition genes (Fig. 1a-b). The adaptability modules of the different IncI1/I2 plasmids showed a 100% similarity with the transfer, leading and replication region differing only in the genetic content of the adaptability module (AMR genes and proteins of known function).

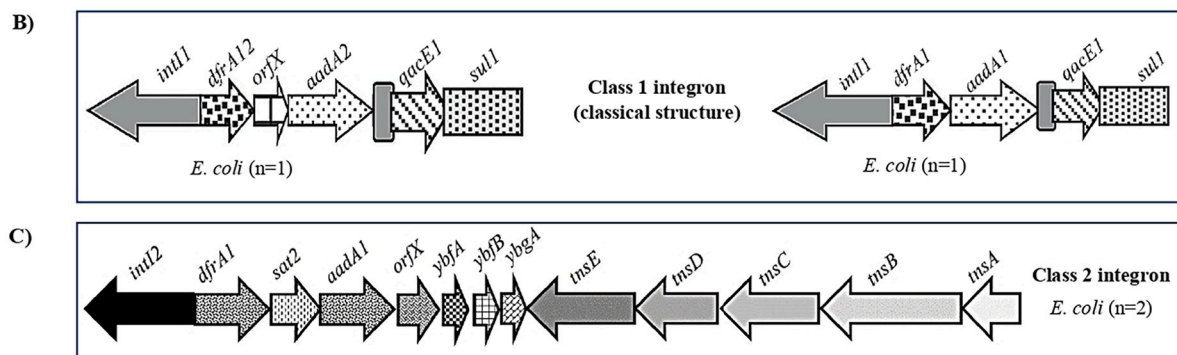
3.3.2. Phage plasmid

The *bla*_{TEM-52} gene was found in a P1-like phage-plasmid (107,992 bp), showing 83%–99.7% coverage/identity with others previously identified in *E. coli* (accession no. OY757102.1, OY754380.1, CP134391.1) belonging to broiler farms and human infections. Our ΔP1-plasmid displayed some conserved regions as the original one (accession no. AF234172 and AF234173) (Fig. 1c): the res-mod (restriction-modification protein), the ISI-associated genes (*isaA*, *insB*, *insA*, and *isaB*), the encoding holing and tail-fiber related protein (*lydC*, *cin*, *Sv*, *U*, *U*, and *S*), the super immunity-linked function (*simC*, *simB*, and *simA*), the possibly replication-linked function (*rlfA*, *rlfB*, and *pmgF*), the plasmid replication (*repA* and *upfA*), the tellurite/colicin resistance/inhibition of cell division (*tcIA*, *tcIB*, and *tcIC*), and the SOS putative morphogenetic function (*pmgT*, *pmgU*, *pmgV*, *upfM*, *upfN*, *upfO*, *hot*, *lxx*, and *humD*). The adaptability module only consisted of the *bla*_{TEM-52} and its flanking module compound by a transposon framed by IS4 (Table 3). In addition, a Tn3-derived transposon containing only the elements involved in its transposition (*tnpA*, *tnpR*), was inserted upstream of the *bla*_{TEM-52} gene (183

Table 2

A) Antimicrobial resistance phenotypes, genotypes and specific chromosomal mutations in 40 non-repetitive *E. coli* isolates of chicken meat recovered in non-supplemented MC plates. B) Schematic diagrams of the general structure of a class 1 integron and the gene cassette arrays detected. C) Schematic diagrams of the general structure of a class 2 integron and the gene cassette arrays detected.

A) Antimicrobial resistance phenotype	N. of isolates with characteristics	Antimicrobial resistance genotype												Chr mutations					
		<i>bla</i> _{TEM}	<i>cmiA</i>	<i>sul1</i>	<i>sul2</i>	<i>dfiA1</i>	<i>dfiA12</i>	<i>aadA1</i>	<i>aadA2</i>	<i>sul3</i>	<i>tef(B)</i>	<i>tef(A)</i>	<i>aac(3)-II</i>	<i>aac(6)-Ib-cr</i>	<i>qnrA</i>	<i>gyrA</i> (D87N)	<i>gyrA</i> (S83L)	<i>parC</i> (S80I)	<i>parC</i> (S80R)
AMP-AMC-GEN-TET-SXT-CIP	1	■																	
AMP-AMC-CIP-SXT-CHL	1		■																
AMP-AMC-CIP-TET-SXT	1			■															
AMP-AMC-TET-SXT ^C -CIP	1				■														
AMP-CIP-GEN-SXT ^B -TET	1	■		■															
AMP-AMC-CIP-TET	2	■																	
AMP-AMC-TET-SXT ^C	1			■															
AMP-CIP-TET-CHL	1		■																
AMP-AMC-GEN-CIP	1	■																	
AMP-AMC-GEN-TET	1	■																	
AMP-TET-SXT ^B -CIP	1	■																	
AMP-AMC-SXT	1	■																	
AMP-AMC-TET	1	■																	
AMP-CIP-GEN	1																		
AMP-AMC	3	■																	
CHL-TET-CIP	1		■																
AMP-GEN	1	■																	
SXT-CIP	1			■															
AMP	1	■																	
CIP	2																		
TET	1																		
Susceptible ¹	15																		



¹No AMR genes were examined in these 15 isolates fully susceptible to the antibiotics tested.

bp) (Fig. S1c).

3.3.3. *IncHI2* plasmid

One of the three *bla*_{SHV-12} genes was carried on a large *IncHI2*/pST3 plasmid (225,327 bp) harbouring multiple AMR genes (Fig. 1d, Table 3). Within its adaptability module (34,056 bp), a Tn21-derived transposon comprising an atypical class 1 integron associated with *sul3* (Fig. 2a) was evidenced, indicating sequential acquisition of AMR genes (aminoglycosides, chloramphenicol, and sulphonamides). Subsequently, embedded in its structure was a Δ Tn1721, preserving only the elements involved in its transposition (*tnpR*, *tnpA*) and the terminal inverted repeat IRR and IRL (Fig. 2), flanking downstream the *bla*_{SHV-12} (Fig. S2d). Interestingly, this Tn21-derived transposon was identical to one previously reported in the gastrointestinal tract of white storks' nestlings in Spain (pUR5279 plasmid, Fig. 2). Our *IncHI2* plasmid was conjugative, exhibiting a conjugation frequency of 4.2×10^{-5} (Table 3).

3.4. Cross-sectoral phylogenetic comparisons of the STs detected in ESBL *E. coli*

The two ST117 and the ST10 genomes from our collection were mapped to the public genomes endorsed in the "EU AMR monitoring in livestock and meat" project (Brouwer et al., 2023) (accession no. PRJNA885502; counting 52 genomes including cecal content of pigs ($n = 5$), broiler ($n = 2$), dairy cows ($n = 7$), veal calves ($n = 14$) as well as retail chicken ($n = 18$), retail pork ($n = 1$), retail turkey ($n = 1$) and retail veal ($n = 4$) (Fig. 3). These results revealed that three genomes linked to ST117 reported in the Netherlands from retail chicken showed allelic differences ranging from 53 to 56 with our chicken meat genomes and more distant ADs with those related to broiler/veal calf cecal content or retail veal (125–138 ADs) (Tables S5-S6). No meaningful relationships (200 ADs) between ST10-associated genomes were observed within this project (Table S6).

Secondly, we broadened the One Health analysis by mapping our genomes against all publicly available genomes at NCBI associated with ST10 ($n = 2130$), ST117 ($n = 341$), ST223 ($n = 81$), ST752 ($n = 19$), and

Table 3

Characteristics of the six *bla*_{ESBL}-harbouring *E. coli* isolates recovered in MC-CTX plates and their plasmids.

ID number	Sample/ Establishment ¹	ST -PhG ²	<i>bla</i> _{ESBL} -carrying plasmid	ESBL genetic environment	Replicon type/ pMLST (Size)	Conjugation frequency	Other co-located resistance genes
X3560*	T/H-M48	1140-E	ISEcp1- <i>bla</i> _{CTX-M-32} - <i>wbuC</i>	I2 (90 kb)	5.1×10^{-4}	<i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>tet(A)</i>	
X3561	B/H-M46	752-A	IS4-Tn3- <i>bla</i> _{TEM-52C} -IS4	P1-phage (110 kb)	-	-	
X3556*	T/H-M45	117-B2	IS26- <i>bla</i> _{SHV-12} - <i>deoR</i> -Tn1721	HI2/pST3 (CC3) (225 kb)	4.2×10^{-5}	<i>sul3</i> , <i>qacL</i> , <i>aadA1</i> , <i>aadA2</i> , <i>cmlA1</i> , <i>terABCDEFWY</i>	
X3274	T/H-M30	10-A	IS26- <i>bla</i> _{SHV-12} - <i>deoR</i> -IS26	X3 (50 kb)	3.8×10^{-6}	<i>qnrS1</i>	
X3081	B/H-M40	223-B1	IS26- <i>smc</i> - <i>gfpR</i> - <i>bla</i> _{SHV-12} -IS26	I1/pST3 (CC3) (100 kb)	2.6×10^{-6}	<i>qnrB19</i>	
X3078*	T/RS-M12	117-B2	ISEcp1- <i>bla</i> _{CTX-M-1} - <i>wbuC</i>	I1/pST3 (CC3) (100 kb)	3.1×10^{-6}	<i>sul2</i>	

*Multidrug-resistant *E. coli* isolates¹T - Thig, B - Breast, H - Hypermarket, RS - Retail Shop²Phylogroup

ST1140 ($n = 17$) (Fig. 4, Fig. S3). For ST10, we observed similarities (57–129 ADs) between genomes obtained from chicken farms in China ($n = 4$), Paraguay ($n = 1$), Italy ($n = 1$) and Spain ($n = 1$), including some associated with human infections in Argentina ($n = 1$), Saudi Arabia ($n = 1$) and China ($n = 1$). Additionally, tight links were also seen with genomes obtained from sewage in Japan ($n = 1$), pigs in China ($n = 1$) and alpacas in the USA ($n = 1$) (Table S5, Table S7, Fig. 4a-b). ST117, a widely distributed lineage in the poultry sector, displayed narrow linkages (34–40 ADs) between our genome (X3078) and the i) eight ones previously reported from German fattening broiler farms, ii) one genome recovered from chicken breast (USA), iii) one from Pakistani chickens and iv) one from a human infection in Lithuania (Table S5, Table S8, Fig. 4c-d).

For the least disseminated STs, as ST223, we only evidenced weak similarities (117–195 ADs) with genomes belonging to animal ($n = 1$) and human ($n = 1$) infections in the USA and wastewater ($n = 1$) in Brazil (Table S5, Table S9, Fig. S3a-b). Finally, for the ST752 and ST1140, no significant similarities were shown between them (84–499 ADs), although a clear association with the avian clade was noticed for the ST752 (11/19) (Table S5, Tables S10–11, Fig. S3c-f).

4. Discussion

In this study, we detected *E. coli* isolates in 68.8% of the chicken meat samples, suggesting potential contamination during slaughter, meat processing, or transport. These *E. coli* isolates exhibited high resistance rates to narrow-spectrum β -lactams, quinolones, and sulphonamides, in line with previous studies (Jammoul and El Darra, 2019; Pena et al., 2010). The occurrence of resistance to first-line treatments in the poultry sector is opening the door to second-line treatments with third/fourth-generation cephalosporins or fluoroquinolones, which are critically important for human medicine. Consequently, resistant bacteria might be transmitted to humans through the food chain.

The prevalence of retail poultry meat colonised by ESBL-producing *E. coli* isolates in Spain increased notably from 62.5% in 2007 to 93.3% in 2010 (Egea et al., 2012), before a study from 2018 reported a reduction of almost 50% (Vitas et al., 2018). Even though they should be confirmed by a larger-scale study, our results suggest that the implemented measures on the control and use of antimicrobials in poultry

farming are bearing positive results. These are encouraging results, despite the fact that the proportion of ESBL-producing *E. coli* is still high. This might be due to the wide use of ceftiofur in commercial chicken flocks to treat one-day-old chicks and to prevent early mortality associated with *E. coli* in turkeys and broilers, therefore promoting the emergence and dissemination of ESBL producers in their gastrointestinal tract (de Mesquita Souza Saraiva et al., 2022). Today, most European Union countries (including Spain) still use ceftiofur in livestock, albeit to a limited extent. These results indicate the necessity of adopting similar measures to those already used in Australia, where the restriction of ceftiofur coincided with the disappearance of ESBL-producing strains in meat samples (Abraham et al., 2019).

A first study of ESBL-producing *E. coli* in the poultry sector was performed in Spanish chicken farms (Briñas et al., 2003) and reported the presence of *bla*_{CTX-M-14} and *bla*_{SHV-12}, which were concomitantly emerging in Spanish and European hospitals (Coque et al., 2008; Diestra et al., 2008). During the last decades, many studies reported ESBL-producing *E. coli* in poultry and food-derived products, ranging from 44% to 94% in Spain, Sweden, and the Netherlands (Leverstein-van Hall et al., 2011; Börjesson et al., 2013; Martínez-Álvarez et al., 2022); being mainly encoded by *bla*_{CTX-M-1} gene in Sweden, *bla*_{SHV-12} in Spain and to *bla*_{TEM-52} in the Netherlands. Genomic analysis of our ESBL-*E. coli* isolates showed a wide heterogeneity of genetic lineages. Only ST117 was identified twice, but isolates were not clonal. ST117 has first been associated with avian hosts and the poultry sector (Mora et al., 2012) but is now also considered a high-risk clone associated with urinary tract infections or septicemia in humans. In Spain, ST117 is one of the most frequently reported clones in the poultry sector, usually carrying the *bla*_{CTX-M-1} and *bla*_{SHV-12} genes (Martínez-Álvarez et al., 2022), as reported here. Additionally, it has also been found to carry other ESBL genes such as *bla*_{CTX-M-14}/*bla*_{CTX-M-15} (Apostolakos et al., 2020) or plasmid AmpC genes as *bla*_{CMY-2} (Roedel et al., 2021) in the poultry sector in other European countries. ST10 is a ubiquitous clone previously detected in the environment, in clinical settings (involved in invasive human infections) or in animals, including in the poultry sector (Day et al., 2019).

Mobile genetic elements are essential in the spread of ARGs between human and environmental sources, especially within Enterobacterales. Our isolates recovered from chicken meat also displayed a variety of

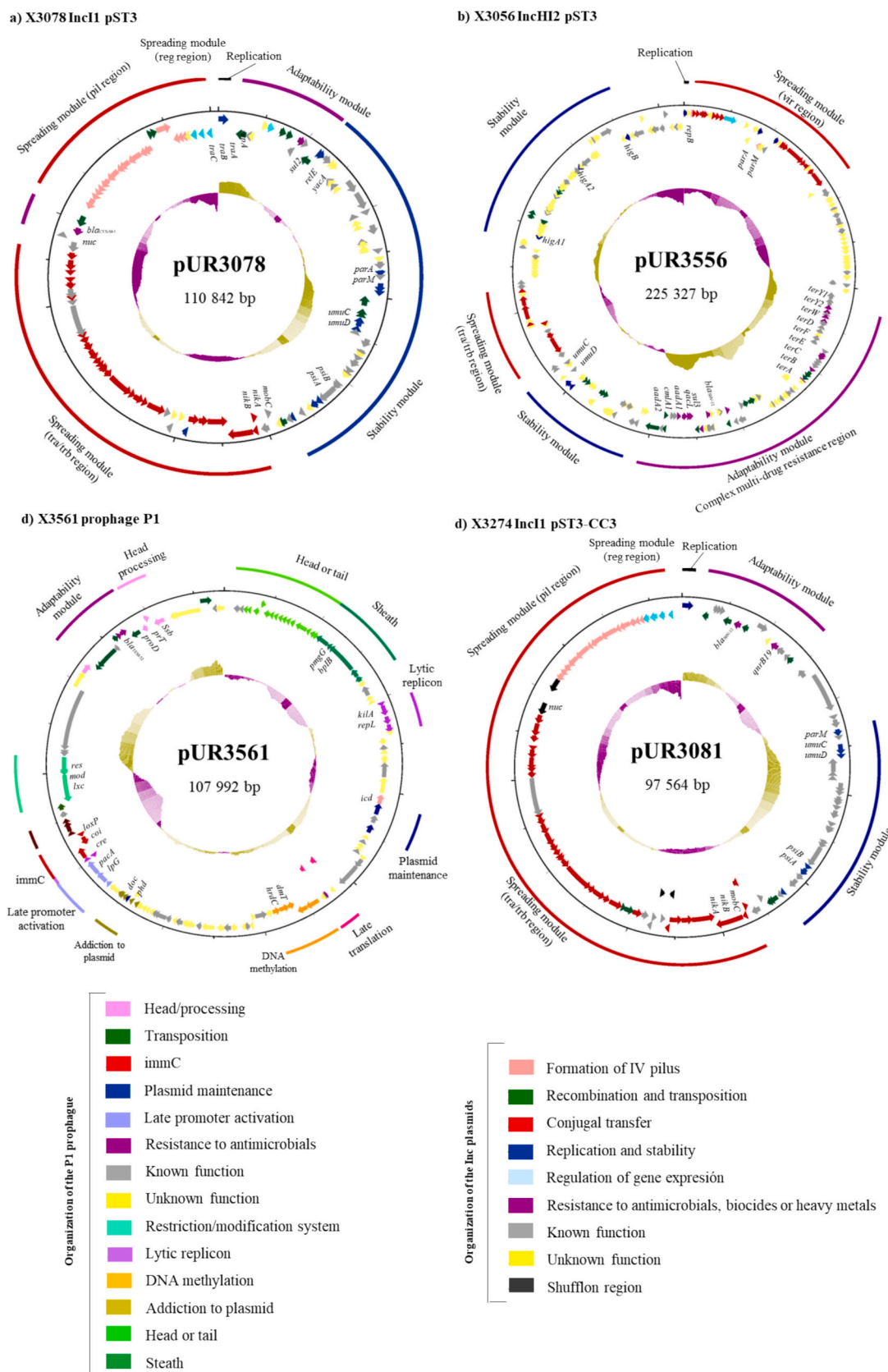


Fig. 1. Circular map of the four *bla*_{ESBL}-carrying plasmids a) pUR3078 (accession no. PP711258), b) pUR3556 (accession no. PP711259), c) pUR3561 (accession no. PP711261), d) pUR3081 (accession no. PP711260). Some relevant genes are labelled. Second inner ring shows the fragments of truncated genes and the forward and reverse coding sequences are shown in the third and fourth inner rings, respectively. They are shown as arrows (the direction of transcription is indicated by the arrowheads) and are coloured according to their function as shown in the legend. Names of functional regions are shown in the outer ring. First inner ring shows a plot of the GC skew (yellow, above average; purple, below average).

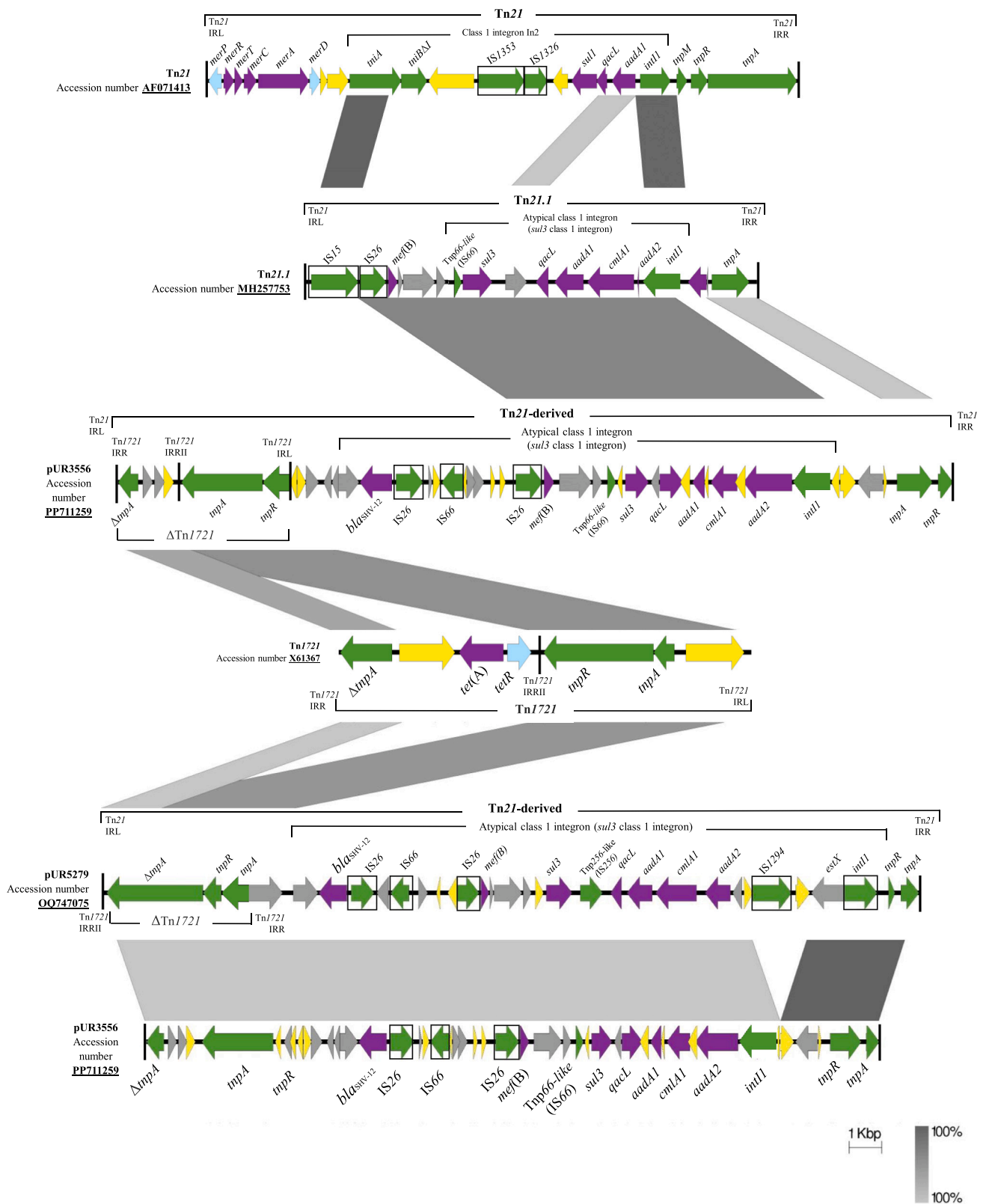


Fig. 2. Linear illustration of the resistance complexes of pUR3556 (IncHI2/pST3; accession number PP711259) and comparative mapping of this region with Tn21 (Accession no. AF071413, MH257753), Tn1721 (Accession no. X61367) as well as with pUR5279 transposon structure (Accession no. OQ747075). A few important genes have been tagged. The coding reading frames are shown as arrows (“transcription direction” is indicated by the arrowheads). IS are displayed as boxes and the arrows without boxes represent transposon genes. Vertical lines denote the IRs of the ISs, the transposons or the In2 integron.

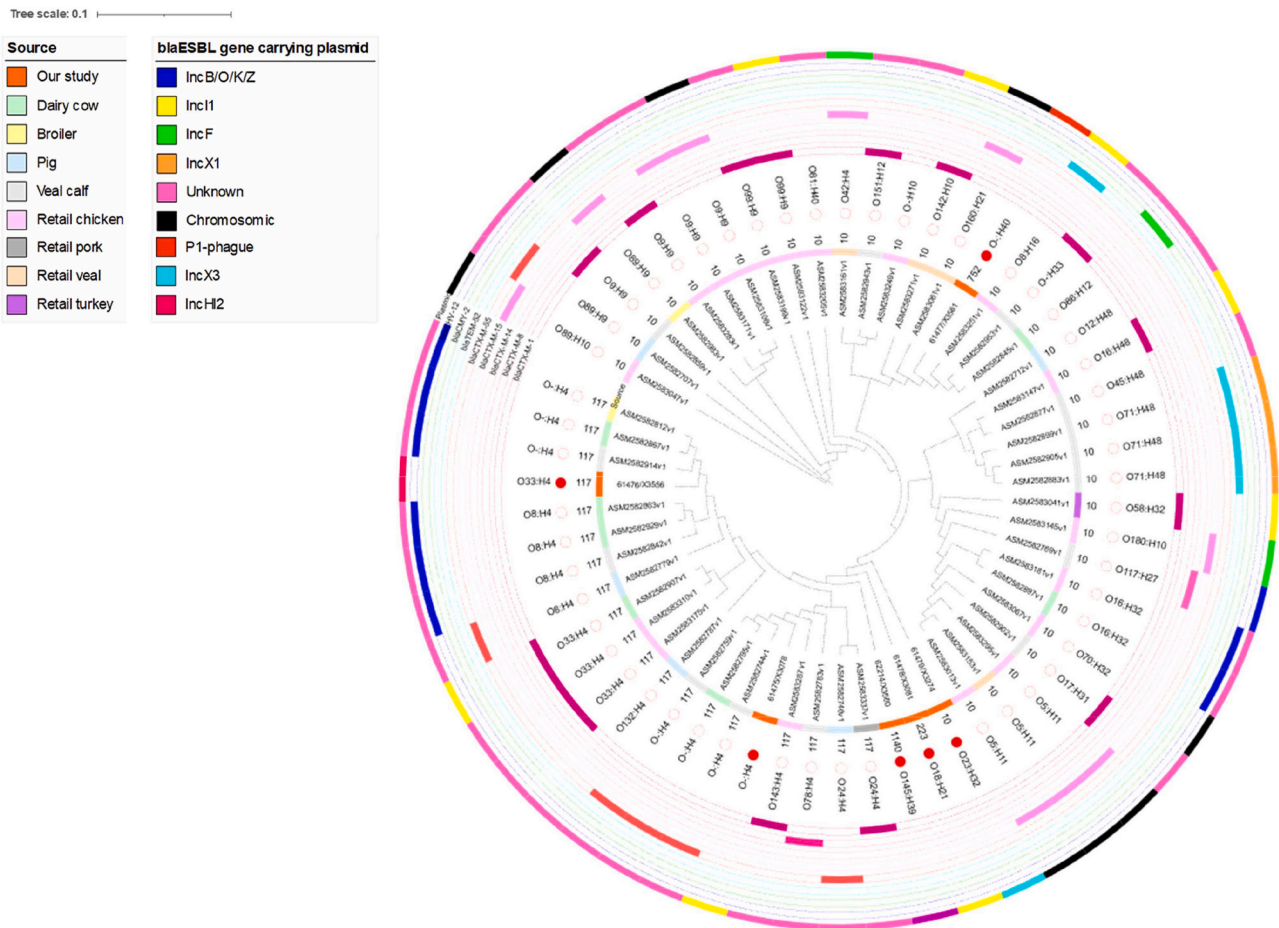


Fig. 3. Phylogenetic tree of the ST10 and ST117 *E. coli* isolates from this study and those from the “EU AMR monitoring in livestock and meat” project (Accession no. PRJNA885502; counting 39 genomes associated with ST10 and 22 genomes with ST117): cecal content pig ($n = 5$), cecal content broiler ($n = 2$), cecal content dairy cow ($n = 7$), cecal content veal calf ($n = 14$), retail chicken ($n = 18$), retail pork ($n = 1$), retail turkey ($n = 1$), retail veal ($n = 4$). The phylogenetic tree was constructed based on nucleotide sequence alignments of 2,513 core genes. Sequence types are shown with coloured bars, each colour corresponds to one ST. Countries of origin are indicated with coloured bars denoting the presence (filled bars) or absence (empty bars) of the gene. The red dot indicates our genomes recovered from chicken meat.

*bla*_{ESBL} genes, including the *bla*_{SHV-12}, *bla*_{CTX-M-1}, *bla*_{CTX-M-32}, and *bla*_{TEM-52} genes, which were carried by different plasmids. The IncI1-type plasmids described here harboured the *bla*_{SHV-12} and *bla*_{CTX-M-1} genes, as recently reported in chickens (García-Fernández et al., 2008; Bortolaia et al., 2010), in pigs/meat (Alonso et al., 2017a, 2017b; Martínez-Álvarez et al., 2024), or even in migratory birds (Martínez-Álvarez et al., 2023). The genetic environment reported for *bla*_{SHV-12} was slightly different from previously reported, as we detected it upstream of the gene flanked by the transcriptional regulator *glpR* (IS26-*smc-glpR-bla*_{SHV-12}-IS26) rather than by the common *deoR* (Alonso et al., 2017a, 2017b; Martínez-Álvarez et al., 2023, 2024). For the *bla*_{CTX-M} genes, their flanking regions (ISEcp1-*bla*_{CTX-M-1/32}-*wbuC*) were highly conserved and in line with previous reports (Coque et al., 2008; Diestra et al., 2008; Tian et al., 2011; Martínez-Álvarez et al., 2023).

Interestingly, we found the *bla*_{SHV-12} gene located on an IncHI2/pST3 plasmid. This gene has been widely documented on both IncX3 plasmids at the clinical scale (Liakopoulos et al., 2018) and on IncI1 plasmids in livestock and derivative products (Alonso et al., 2017a, 2017b; Martínez-Álvarez et al., 2024). Recently a study in Germany in livestock also reported the *bla*_{SHV-12} on an IncHI2 plasmid but co-harboured the *bla*_{VIM-1} gene. IncHI2 plasmids carrying *bla*_{SHV-12} (Pauly et al., 2021), originally encountered in the environment, are large (> 200 kbp) and have an important potential to spread multiple AMR genes, as was confirmed here. Interestingly, the adaptability module comprised a large

Δ Tn21 with a Tn1721 embedded, a genetic structure identical to the one described recently in nestling storks in Spain (Martínez-Álvarez et al., 2023).

Additionally, we reported a rare phage-plasmid carrying the *bla*_{TEM-52} gene. This ESBL gene has been previously detected in clinical isolates and/or production animals from Canada, Portugal, France, Greece, the Netherlands, Germany, Belgium, Great Britain, Croatia, and Japan with different plasmid locations but never found in *E. coli* on a phage-plasmid (Clockaert et al., 2007; Bielak et al., 2011). Overall, coinciding with the findings described, this gene may be distributed on diverse plasmid replicons, most probably due to the association with Tn3 elements. Once integrated into the plasmid backbone, the Tn3-*bla*_{TEM-52} element may have been transferred both horizontally and clonally (Bielak et al., 2011). However, in our phage plasmid, only this gene was found in its resistance module as well as certain conserved regions with maintenance, stability, and replication functions of the plasmid.

Integrations have been frequently detected in isolates of all origins, such as in food animals (Casella et al., 2015), hospitalized patients (Vinué et al., 2008b), and/or healthy humans (Vinué et al., 2008a). Some of them are globally disseminated, such as class I (*dfrA1-aadA1*; *dfrA12-orfX-aadA2*) or class 2 integrons (*dfrA1-sat2-aadA1*; *sat2-aadA1*) (Machado et al., 2008; Soufi et al., 2009, 2011), while here, integrons with different arrangements in their gene cassettes were also identified, especially located on transposons (Tn21) and/or plasmids (IncI1, IncF,

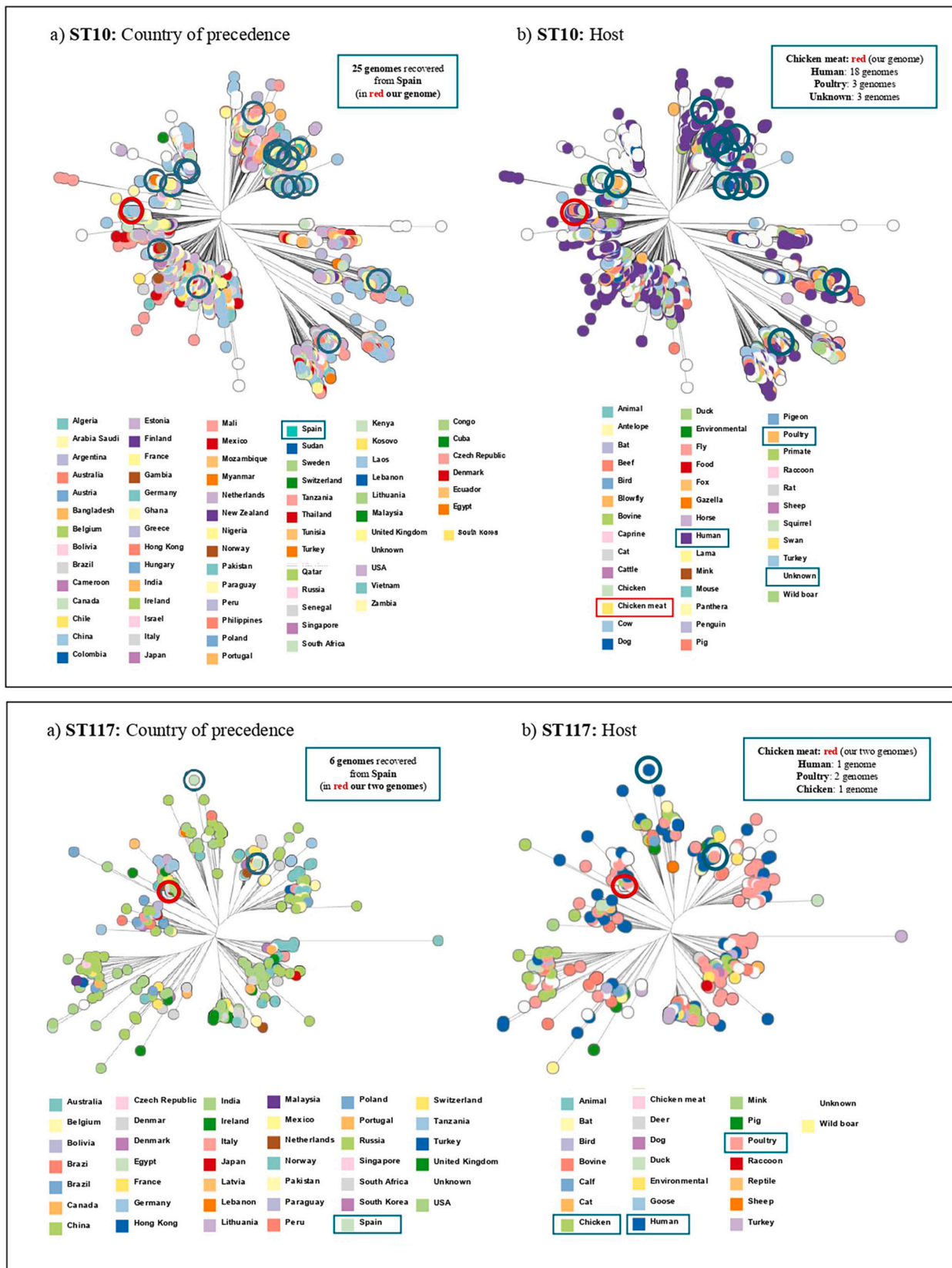


Fig. 4. Minimum spanning tree (MST) of *E. coli* isolates from this study, as well as was, all the ones included in GenBank linked to ST10/ST117 and calculated based on the cgMLST scheme available. Each node whose size is proportional to a number of isolates corresponds to a single sequence type profile based on 2,513 core genes. Nodes are colour-coded according to the country of precedence as shown in the legend. All nodes are linked (black lines) sequentially to the most closely related node with the highest number of cgMLST alleles in common.

IncHI2) coinciding with previously reports in different ecosystems (Alonso et al., 2017a, 2017b; Martínez-Álvarez et al., 2023, 2024).

The risk of transmission by consumption of chicken meat contaminated with ESBL-producing *E. coli* may be linked with the selection of different genetic backgrounds that are prone to persist and, therefore, be disseminated in this setting and/or along the food supply chain (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2021, 2022, 2024). Here, we found very closely related cluster links (between 34 and 40 ADs) with our ST117 genomes especially within ST117 public genomes linked to the poultry sector (German or Pakistani fattening farms, poultry meat in the USA) but also in one case with a human clinical isolate reported in Lithuania. These outcomes could be explained as this genetic lineage emerged in the clinical sphere and leapt into the animal scope many years ago, causing great notoriety in the livestock sector. Moreover, Spain is one of the countries with the highest percentage of meat exports in the EU, so this movement of livestock or meat products or even just the movement of human populations has favoured its widespread dissemination.

In conclusion, our findings highlight that some isolates are more adapted to the poultry host highlighting the necessity to establish containment measures throughout the food traceability chain to strongly reduce possible transmission from the “environment to the bedside”. Good practice regulations should be set up during meat processing as well as in our own households to prevent the risk of infections caused by ESBL-producing *E. coli*, but also resistant *Campylobacter* spp. or *Salmonella* spp., through raw meat. In addition, future investigations should address food traceability at a global scale to understand how processing, handling, daily cleaning, and disinfection procedures influence the persistence and transmission of ARBs and ARGs. Furthermore, this study suggested that the transmissibility of clones, ESBL genes and their carrying plasmids through chicken meat can cross barriers due to the import/export of poultry products at a European and global scale, significantly contributing to their successful dissemination.

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Ethical approval

Not required.

CRediT authorship contribution statement

Sandra Martínez-Álvarez: Writing – review & editing, Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pierre Châtre:** Writing – review & editing, Software, Methodology. **Pauline François:** Writing – review & editing, Software, Methodology. **Myriam Zarazaga:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Jean-Yves Madec:** Writing – review & editing, Supervision, Formal analysis, Project administration, Funding acquisition. **Marisa Haenni:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Carmen Torres:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2024.110900>.

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