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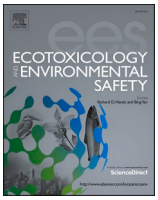

Unexpected role of pig nostrils in the clonal and plasmidic dissemination of extended-spectrum beta-lactamase-producing *Escherichia coli* at farm level

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ABSTRACT

The presence of methicillin-resistant or -susceptible *S. aureus* in pig nostrils has been known for a long time, but the occurrence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* has hardly been investigated. Here, we collected 25 *E. coli* recovered from nasal samples of 40 pigs/10 farmers of four farms. Nine ESBL-producing isolates belonging to ST48, ST117, ST847, ST5440, ST14914 and ST10 were retrieved from seven pigs. All bla_{ESBL} genes ($bla_{CTX\text{-}M\text{-}32}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}65}$, and $bla_{SHV\text{-}12}$) were horizontally transferable by conjugation through plasmids belonging to IncI1 (n=3), IncX1 (n=3) and IncHI2 (n=1) types. IncI1-plasmids displayed different genetic environments: i) IS*26*-$bla_{SHV\text{-}12}$-*deoR*-IS*26*, ii) *wbuC*-$bla_{CTX\text{-}M\text{-}32}$-IS*Kpn26* (IS*5*), and iii) IS*930*-$bla_{CTX\text{-}M\text{-}14}$-IS*26*. The IncHI2-plasmid contained the genetic environment IS*903*-$bla_{CTX\text{-}M\text{-}65}$-*fipA* with multiple resistance genes associated either to: a) Tn*21*-like transposon harbouring genes conferring aminoglycosides/beta-lactams/chloramphenicol/macrolides resistance located on two atypical class 1 integrons with an embedded ΔTn*5393*; or b) Tn*1721*-derived transposon displaying an atypical class 1 integron harbouring *aadA2*-*arr3*-*cmlA5*-$bla_{OXA\text{-}10}$-*aadA24*-*dfrA14*, preceding the genetic platform IS*26*-$bla_{TEM\text{-}95}$-*tet*(A)-*lysR*-*floR*-*virD2*-IS*Vsa3*-IS*3075*-IS*26*-*qnrS1*, as well as the tellurite resistance module. Other plasmids harbouring clinically relevant genes were detected, such as a *ColE*-type plasmid carrying the *mcr-4.5* gene. Chromosomally encoded genes (*fosA7*) or integrons (*intI1*-*dfrA1*-*aadA1*-*qacE*-*sul1*/*intI1*-IS*15*-*dfrA1*-*aadA2*) were also identified. Finally, an IncY plasmid harbouring a class 2 integron (*intI2*-*dfrA1*-*sat2*-*aadA1*-*qacL*-IS*406*-*sul3*) was detected but not associated with a bla_{ESBL} gene. Our results evidence that pig nostrils might favour the spread of ESBL-*E. coli* and *mcr*-mediated colistin-resistance. Therefore, enhanced monitoring should be considered, especially in a sector where close contact between animals in intensive farming increases the risk of spreading antimicrobial resistance.

1. Introduction

Pigs are frequent nasal carriers of methicillin-resistant *S. aureus* (MRSA), mainly of the CC398 lineage, and it has been associated with a higher prevalence of nasal carriage among healthy humans professionally in contact with these animals (farmers, slaughterhouse workers, veterinarians.). In addition, MRSA-CC398 prevalence at the hospital level correlates significantly with the density of swine herds where healthcare facilities are located. Consequently, the pigs' nostrils are a privileged sampling site to estimate the carriage rate of *S. aureus* or MRSA. While livestock-associated MRSA has been extensively disclosed in pig nostrils (Abdullahi et al., 2023a), some studies have concomitantly pointed out the occurrence of plasmidic AmpC-producing *E. coli* (Endimiani et al., 2012) or linezolid-resistant enterococci (LRE) (Abdullahi et al., 2023b).

The risk of *E. coli* colonization leading to neonatal diarrhoea in piglets is notably soaring, and prophylaxis/metaphylaxis therapies have been used as preventive measures. This rampant use promoted the dissemination of numerous antimicrobial resistance (AMR) genes in animals, such as the extended-spectrum beta-lactamases (ESBLs) conferring resistance to last-generation cephalosporins, the plasmid-borne *mcr-1* gene conferring colistin resistance and the *fosA7* gene encoding fosfomycin resistance. Strict measures were implemented worldwide to limit the spread of AMR genes, starting with Spain

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arranging the "Reduce Colistin" programme, which had commendable results and served as a model of best practice in Europe. Recent regulations (28 January 2022, Regulation (EU) 2019/6) now banned routine prophylaxis, only allowing metaphylaxis exceptionally.

Owing to the limited awareness concerning the role of pigs' nostrils as reservoirs of *E. coli* carrying important AMR genes, we aimed to investigate the presence of these critical AMR genes from pigs/farmers linked to four Spanish farms based on chromosomal or plasmid location.

2. Materials and methods

2.1. Sampling and E. coli isolation

Nasal samples of 40 pigs from four farms (A-D, 10 pigs/farm) as well as nasal samples from 10 farmers (2, 3, 2 and 3 humans in farms A, B, C and D, respectively) were collected in Aragón (Spain) and tested for *E. coli* recovery. This study was conducted in a general groundwork analysis concerning the nasal microbiota of healthy pigs assessing the staphylococci (Abdullahi et al., 2023a), and enterococci (Abdullahi et al., 2023b) occurrence. Samples were inoculated on non-selective blood agar and suspected *E. coli* were identified using MALDI-TOF mass spectrometry (MALDI-Biotyper, Bruker). All procedures were revised and endorsed by the ethical committees of the Universities of Zaragoza and La Rioja (Spain) and were conducted following all national and/or international guidelines applicable to human experiments.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted by disk-diffusion method according to the Clinical Laboratory Standards Institute (CLSI, 2022). A total of 12 antibiotics were tested: ampicillin, amoxicillin/clavulanate, ceftazidime, cefotaxime, cefoxitin, imipenem, ciprofloxacin, gentamicin, amikacin, chloramphenicol, trimethoprim/sulfamethoxazole, and tetracycline. ESBL production was screened using the double-disk synergy test with cefotaxime, ceftazidime and amoxicillin/clavulanate disks (CLSI, 2022). *E. coli* ATCC 25922 was used as a control strain.

2.3. Characterization of non-ESBL E. coli

Genes conferring resistance to β-lactams (bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$), quinolones (*qnrA, qnrB, qnrS, aac(6′)-Ib-cr, oqxAB, qepA*, *gyrA* and *parC*), aminoglycosides (*aac(3)-I, aac(3)-II, aac(3)-III, aac(3)-IV*), tetracycline (*tet*(A), *tet*(B)), sulfonamides (*sul1, sul2, sul3*) and phenicols (*catA, floR, cmlA*) were examined by PCR and subsequent sequencing (Martínez-Álvarez et al., 2022; Sáenz et al., 2004). Class 1 and class 2 integrons were determined by PCR (*intI1* and *intI2)* (Sáenz et al., 2004), and the full-length sequences of their variable regions were investigated employing the PCR primer-walking strategy.

2.4. Conjugation experiments

Conjugation experiments were carried out on ESBL-producing *E. coli* and conducted in liquid medium using the rifampicin-resistant *E. coli* strain J53 as the recipient strain. Transconjugants (TC) were spotted on BHI agar plates containing rifampicin (100 μg/ml) and cefotaxime (2 μg/ml) and controlled by phenotypic screening and targeted PCR for the detection of bla_{ESBL} genes and replicons using the PBRT method (Carattoli et al., 2005).

2.5. Short-read sequencing of ESBL-producing E. coli

The genomic DNA of the ESBL producers was extracted with the NucleoSpin® Microbial DNA Kit (Macherey-Nagel) according to the manufacturer's instructions. The concentration and purity of DNA were determined with Qubit R 3.0 fluorometer (Thermo Fisher Scientific) and NanoDrop One (Ozyme). Sequencing (2×150 paired-end) was performed with Illumina NovaSeq 6000 technology. Illumina adapter sequences were stripped, and reads were trimmed with trimmomatic version 0.38.1 (Bolger et al., 2014). *De novo* assemblies were performed with Shovill v.1.1.0 (SPAdes v.0.5.0) and the quality of the assemblies was scored with QUAST v.5.2.0 (Gurevich et al., 2013) (Table S1). The STs, pSTs, plasmid replicon content and resistance genes were identified using the CGE online tools MLSTFinder v.2.0.9, pMLSTFinder v.2.0, PlasmidFinder v.2.1 and ResFinder v.4.1 (http://www.genomicepidemiology.org/).

2.6. Clonal relationships

A core-genome Multilocus-Sequence-Typing scheme (cgMLST) phylogenetic analysis was performed based on the alignment of 2513 core genes. ESBL-producing isolates gathered under this study, along with genomes from the National Center for Biotechnology Information (NCBI) based on STs closely related to swine (ST117, ST10/ST14914 and ST101) were included. The obtained cgMLST target gene variants were used to visualize phylogenetic distances by calculating a neighbour-joining tree (parameters: pairwise ignoring missing values; % column difference) based on the distance matrix of the core genome differences. The tree was visualized using iTOL (v.6).

2.7. Analysis of plasmids based on Southern blots and long-read sequencing

Plasmids encoding bla_{ESBL} genes were identified, and their respective sizes were determined by Southern blots on S1-PFGE gels using adequate DIG-labelled probes (Roche Applied Science, Meylan, France) according to the manufacturer's protocol. Long-read sequencing was performed on all ESBL-producing *E. coli* isolates. Genomic DNA was extracted with the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre). MinION libraries were generated following the manufacturer's guidelines (Oxford Nanopore Technologies, UK) using a native barcode expansion kit (EXP-NBD104; Oxford Nanopore Technologies) and a ligation sequencing kit (SQK-LSK109). Sequencing was performed using a MinION sequencer (SpotON Mk 1 R9) with a flow cell (FLO-MIN106D). Hybrid assemblies of Illumina short-reads and Nanopore long-reads were accomplished using Unicycler (Wick et al., 2017). Output files were corrected for individual base errors, indels and local assembly errors using Pilon (Walker et al., 2014).

2.8. Genome data analysis

Plasmid functional annotations were done with the RAST prokaryotic genome annotation server (Aziz et al., 2008). Manually analyzed data were curated using Artemis (Rutherford et al., 2000), IS finder software (www-is.biotoul.fr) and Swiss-Prot databases (http://www.uniprot.org). Sequence comparisons were assessed using the EMBOSS Needle alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) and the NCBI Basic Local Alignment Search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). DNAPlotter was employed to attain circular maps of bla_{ESBL}- and *mcr*-carrying plasmids (Carver et al., 2009).

2.9. Data accession

Raw sequence reads obtained by short-read Illumina sequencing were deposited under NCBI BioProject PRJNA1077871. Full-length plasmid sequences of pUR5084, pUR5093, pUR5159, pUR5388, pUR5726, pURmcr5726 (carried by *E. coli* X5084, X5093, X5159, X5388 and X5726) and the entire structure of the Tn*21*-like transposon displayed in X5093 were deposited in the EMBL database under the accession numbers OR790946, OR764763, OR790945, OR767240 OR805764, OR805765 and OR790947, respectively.

3. Results

3.1. E. coli nasal carriage in swine and livestock farmers

Out of 50 nasal samples taken from individual pigs (n=40) and farmers (n=10), 17/40 (42.5%) pigs and 3/10 (30%) farmers presented growth of at least one *E. coli* on non-selective plates. One to two presumptive *E. coli* colonies were picked out per plate leading to a collection of 28 *E. coli.* Based on antibiotic susceptibility testing and phylogroups, only 25 non-duplicate *E. coli* isolates (22 recovered from pigs and 3 from farmers) were kept for further analysis. Phenotypic characterization (Table S2) revealed the presence of nine ESBL producers in the nostrils of seven pigs (7/40, 17.5 %) originating from three out of the four farms, while no ESBL producers were detected in the farmers' nares.

3.2. Resistance phenotypes and genotypes in non-ESBL-producing E. coli

All 16 non-ESBL-producing *E. coli* showed resistance to at least one antibiotic, with 14 out of 16 (86.7%) displaying a multidrug-resistance (MDR) phenotype (Table S3). A wide diversity of resistance support was detected (Table S3), with several genes potentially responsible for identical resistance phenotype (i.e., *tet*(A) and/or *tet*(B) genes conferring resistance to tetracycline, or *sul1*, *sul2* and/or *sul3* genes conferring resistance to sulfonamides). Ciprofloxacin resistance (n=3) was mediated by point mutations in the QRDR of the chromosomal genes *gyrA* and *parC* (S80I, D87N/ S83I). Two integrons were found and characterized by PCR and sequencing: i) a class 1 integron carrying the *intI1-aadA1-qacE1-sul1* gene cassette (GC) was identified in one pig (farm D), and ii) a class 2 integron sheltering *intI2-dfrA1-sat2-aadA1* whose GC was found shared by one farmer and one pig (farm C).

3.3. Genetic diversity of bla_{ESBL}-carrying isolates

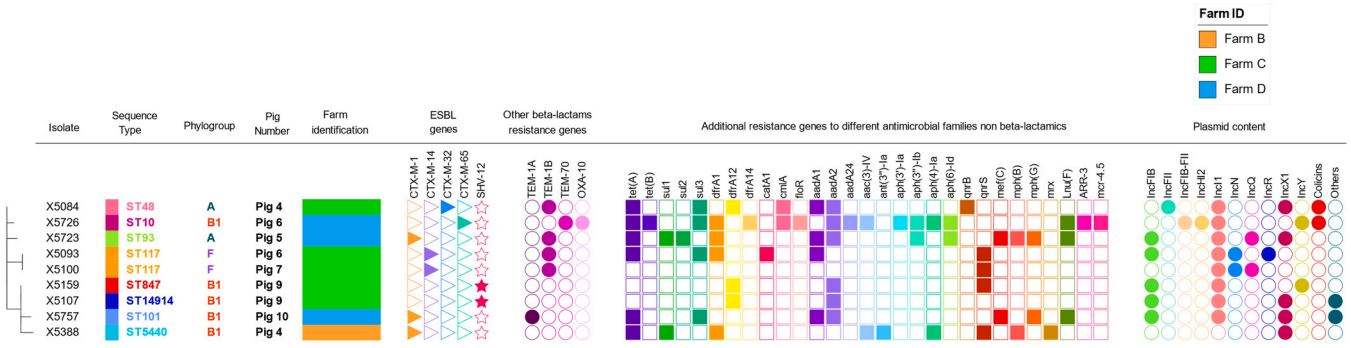
Nine ESBL-positive *E. coli* were retrieved: a) $bla_{CTX\text{-}M\text{-}1}$/ST5440-B1 (n=1) in farm B; b) $bla_{CTX\text{-}M\text{-}32}$/ST48-A (n=1), $bla_{CTX\text{-}M\text{-}14}$/ST117-F (n=2), $bla_{SHV\text{-}12}$/ST847-B1 (n=1) and the newly assigned ST14914-B1 linked to $bla_{SHV\text{-}12}$ (n=1) were identified in farm C; c) $bla_{CTX\text{-}M\text{-}1}$/ST93-A/ST101-B1 (n=2), $bla_{CTX\text{-}M\text{-}65}$/ST10-B1 (n=1) in farm D exhibiting MDR genotypes at all levels (Table 1).

Southern blot hybridizations and long-read sequencing proved that $bla_{SHV\text{-}12}$, $bla_{CTX\text{-}M\text{-}14}$, and $bla_{CTX\text{-}M\text{-}32}$ genes were located on IncI1 plasmids, three $bla_{CTX\text{-}M\text{-}1}$ genes were based on IncX1 plasmids, and the $bla_{CTX\text{-}M\text{-}65}$ gene was placed on an IncHI2 plasmid (Fig. 1). Conjugal transfer of the bla_{ESBL}-carrying plasmids, whose sizes varied between 40,000 and 260,000 bp, was evidenced in all isolates with conjugation frequencies ranging from 10^{-2} to 10^{-6} (Table 1).

3.3.1. Inc I1 plasmids

Three ESBL-conferring genes were carried by different IncI1 plasmids where the replication, transfer, and leading regions shared 100.0%−95% identity/coverage (Figure S1) and 99%−100% identity/coverage with pCE-R2–11–0435_92 (CP016520), pDD02341–3 (CP087632) and pESBL-117 (CP094200) plasmids from *Salmonella enterica*, *Klebsiella pneumoniae* and *E. coli* with some insertions/deletions suggesting recombination between related plasmids. Contrarily, their adaptability modules were more variable (Figure S1). The pUR5159 presented a large adaptability module (11,748 bp) located between the replication and the *relE-parE* toxin-antitoxin system (leading region) (Fig. 1c), while pUR5084 (8196 bp) and pUR5093 (4144 bp), had much smaller accessory modules located in the transfer module between the leading region and the replication zone (Figs. 1a, 1b). The adaptability modules of the different IncI1 plasmids showed a 74% similarity in the homologous regions and were accompanied by a set of proteins of known and unknown functions that were entirely differentiated.

Table 1
Characteristics of the nine bla_{ESBL}-carrying *E. coli* isolates and carrying plasmids.



ID	ST –PhG[1]	bla_{ESBL}-carrying plasmid: ESBL genetic environment	Replicon type/ pMLST (Size)	Conjugation frequency	Other co-located resistance genes
X5084	48-A	*wbuC-bla*$_{CTX\text{-}M\text{-}32}$ *-ISKpn26*	I1/ST36 (CC5) (79 kb)	6.1×10^{-2}	-
X5726	10-B1	IS*930-bla*$_{CTX\text{-}M\text{-}65}$*-ISEcp1*	HI2/ST3 (260 kb)	3.6×10^{-4}	$bla_{TEM\text{-}1B}$, *aac(3)-IV, aph(3'')-Ib, aph(3')-Ia, aph(4)-Ia, aph(6)-Id, tet*(A), $bla_{TEM\text{-}95}$, *arsR, floR,* $bla_{OXA\text{-}10}$, *aadA24, dfrA14, qnrS1, cmlA5, arR3, aadA2*
X5723	93-A	IS*930-bla*$_{CTX\text{-}M\text{-}1}$-IS*26*	X1 (50 kb)	5.1×10^{-3}	-
X5093	117-F	IS930-*bla*$_{CTX\text{-}M\text{-}14}$-IS*26*	I1/ST80 (CC31) (98 kb)	2.2×10^{-6}	-
X5100	117-F	IS*930-bla*$_{CTX\text{-}M\text{-}14}$.IS*26*	I1/ST80 (CC31) (100 kb)	2.4×10^{-6}	-
X5159	847-B1	IS*26-bla*$_{SHV\text{-}12}$ *-deoR*	I1/ST3 (CC3) (100 kb)	2.8×10^{-6}	-
X5107	14914-B1	IS*26-bla*$_{SHV\text{-}12}$*-deoR*	I1/ST3 (CC3) (100 kb)	2.5×10^{-6}	-
X5757	101-B1	IS*26-bla*$_{CTX\text{-}M\text{-}1}$*-mrx*	X1 (50 kb)	3.3×10^{-5}	-
X5388	5440-B1	IS*26-bla*$_{CTX\text{-}M\text{-}1}$*-mrx*	X1 (43 Kb)	2.7×10^{-6}	$bla_{TEM\text{-}1A}$, *mrx,*

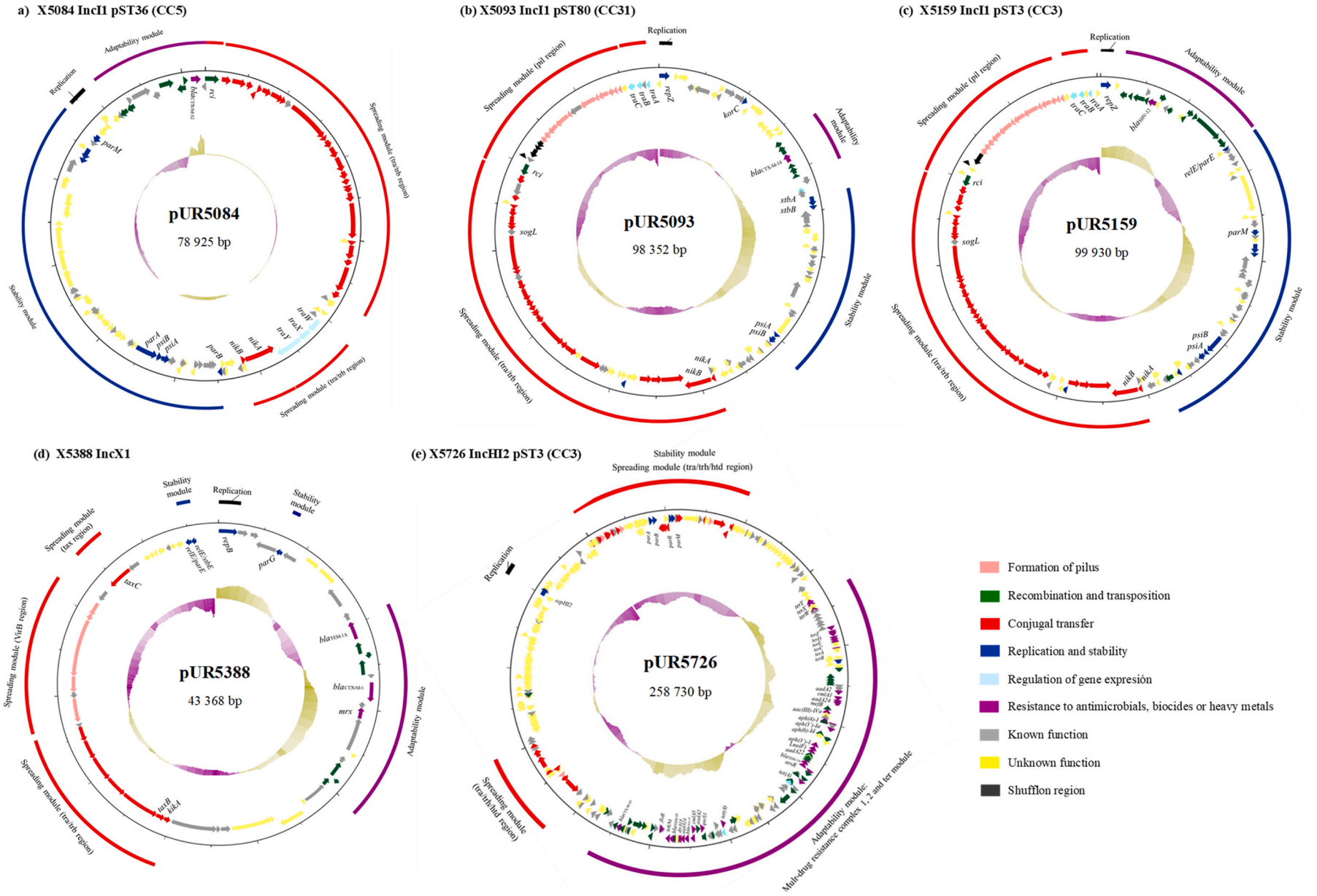


Fig. 1. Circular map of the five bla_{ESBL}-carrying plasmids a) pUR5084 (accession number ORF790946), b) pUR5093 (accession number OR764763), c) pUR5159 (accession number OR790945), d) pUR5388 (accession number OR767240) and, e) pUR5726 (accession number OR805764),.

Subsequently, according to their genetic environments, we detected i) IS*26*-bla_{SHV-12}-*deoR*-IS*26* carried by pST3-CC3 plasmids in two isolates, ii) *wbuC*-$bla_{CTX-M-32}$-IS*Kpn26* (IS*5*) carried by pST36-CC5 in one isolate, and finally iii) IS*930*-$bla_{CTX-M-14}$-IS*26* carried by pST80-CC31 plasmids in two isolates (Table 1, Fig. 1a-c, Figure S2a-c).

3.3.2. IncX1 plasmids

The three IncX1 plasmids reported were carriers of $bla_{CTX-M-1}$ in their adaptability modules (Fig. 1d). Long- and short-read sequencing of a single plasmid revealed 100%−87% identity/coverage with previously reported plasmids such as pHP2 (MH121702) detected in *E. coli* or

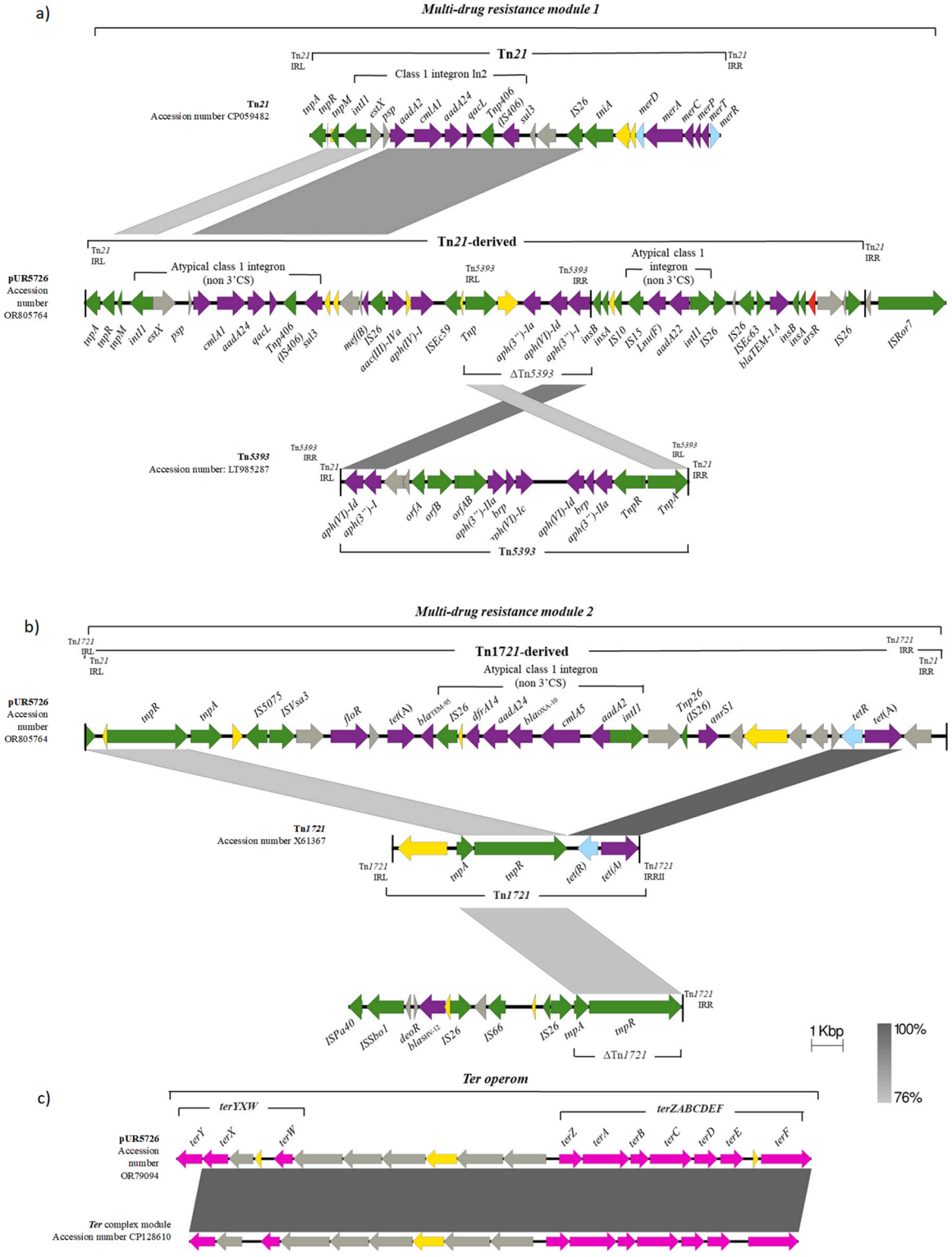


Fig. 2. Linear illustration of the resistance complexes of pUR5726 (IncHI2, pST3) and comparative mapping of this region with Tn*21* (accession number AF071413), Tn*5393* (accession number LT985287) and Tn*1721* (accession number X61367) also with pUR5159 (IncI1, pST3). A few important genes have been 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 In*2* integron.

pSF07202 (KJ201886) disclosed in *Shigella flexneri*. Our pUR5388 presented an adaptability module of 8304 bp encompassing resistance genes for beta-lactams (bla_{TEM-1A}, $bla_{CTX-M-1}$) and macrolides (*mrx*) flanked by IS*26* (Fig. 1d, Figure S2d). Other resistance genes found in our X5388 isolate were chromosomally located and inserted in a classical class 1 integron (*intI1-dfrA1-aadA1-qacE-sul1*).

3.3.3. IncHI2 plasmid

The $bla_{CTX-M-65}$ gene was carried on a large IncHI2/pST3 plasmid (258,730 bp) co-harbouring multiple AMR genes (Fig. 1e, Table 1). Specifically, the genetic environment of the $bla_{CTX-M-65}$ gene was flanked by IS*903* (35 bp downstream) and by a hypothetical protein preceding the *fipA* gene (236 bp upstream) (Figure S2e).

The complex adaptability module detected in this plasmid consisted of two AMR resistance modules and the tellurite resistance module (Fig. 2). The first one involves a Tn*21* comprising two atypical class 1 integrons and a ΔTn*5393* embedded (Fig. 2a), indicating the sequential acquisition of AMR genes (aminoglycosides, beta-lactams, chloramphenicol, and macrolides). The second one incorporates a Tn*1721*-like transposon, which displayed an atypical class 1 integron sheltering the GC array *aadA2-arr3-cmlA5-*bla_{OXA-10}*-aadA24-dfrA14* and the genetic platform IS*26-*bla_{TEM-95}*-tet*(A)-*lysR-floR-virD2*-IS*Vsa3*-IS*3075* preceded by IS*26-qnrS1* (Fig. 2b). Finally, the tellurite resistance module shared full identity/coverage with others previously described (Fig. 2c) such as pHZ13-NDM in *E. coli*, pTYL-T1 in *Klebsiella oxytoca*, pHNAH212836K in *K. pneumoniae* or pS304_1 in *S. enterica*.

3.3.4. Other non-ESBL-carrying plasmids harbouring AMR genes

Firstly, colistin resistance was co-hosted with $bla_{CTX-M-65}$ gene in our X5726 isolate carrying the *mcr-4.5* gene located on a colicin (*ColE*-type plasmid) (Fig. 3). This very small plasmid (8158 bp) bestowed the *mcr-4.5* resistance gene exclusively, flanked by IS*Kpn26* (IS*5*) and antitoxin *higA1* (Fig. 3). The upstream flanking region of *mcr-4.5* displayed 100%−99.9% identity/coverage with genetic settings reported in pIB2020_ColE_MCR (CP059482), pMUMC-1_5 (CP119978) from *Enterobacter kobei* plasmids; WUR-NRS20181408_mcr4 (CP119535), pMCR-4. 2_R4278 (MG8003389) from *E. coli* and pMCR_R3445 (MF543359) from *S. enterica*, also showing an identical structure of the downstream flanking region with those previously documented in pETEC1734 (CP122808) from *E. coli* (Fig. 3).

Secondly, the X5093 isolate additionally carried an IncFIB-FII plasmid (F18:A-:B58) presenting a ΔTn*21* transposon where the genes involved in its transposition (*tnpA*, *tnpR*, *tnpM*), the characteristic In*2* integron of Tn*21*, as well as the imperfect terminal IRi and the complete mercury resistance module (*merRTPCAD*) were conserved. In addition, a ΔTn*1721* was inserted preceding the genes involved in the transposition of Tn*21* (Figure S3).

In addition, chromosomally encoded non-classical class 1 integron was found (X5159 isolate) curiously preceded by the insertion of IS*15* before its variable region and harbouring the trimethoprim (*dfrA1*) and streptomycin-spectinomycin gene (*aadA2*) lacking the 3′CS. The usually plasmid-borne gene *fosA7* conferring resistance to fosfomycin was also inserted into the chromosome flanked by AMP-nucleosidase and a glycosyltransferase family 9 protein.

Finally, a class 2 integron was settled on an IncY plasmid (X5726 isolate) containing the AMR determinants *dfrA1-sat2-aadA1* in its variable region but lacking the five genes involved in its transposition (*tns*). Instead, the atypical 3′C-terminus *qacL-sul3* was truncated by the insertion sequence IS*406*.

3.4. Cross-sectoral phylogenetic comparisons of ST10, ST101 and ST117 E. coli

Our five *E. coli* genomes linked to ST10/ST14914, ST101 and ST117 were mapped with public genomes of the same STs reported in humans (n=32), animals (n=62), meat (n=1) and natural environments (n=5). The phylogenetic tree proved that human and animal isolates clustered into three major groups according to their ST (Fig. 4). ST10/ST14914 isolates differed by a maximum of 2402 allelic differences (ADs), ST101 isolates by 2395 ADs and ST117 by 2398 ADs (Fig. 4).

Among the animal isolates, a large group of 52 genomes joined to ST117 from Australia, Brazil, China, Norway, and the USA differed by 141–333 ADs. The smallest differences among isolates were observed between our X5100 and X5093, with seven isolates belonging to poultry production in China framed in 2007–2010 with ADs ranging from 141 to 148. In addition, the two isolates from our study also differed slightly from a poultry meat isolate reported in the USA and collected in 2017 (168–169 ADs) and those reported from three Australian livestock collected during 2008–2018 (177–200 ADs). Regarding ST10 and ST101, we found that the allelic differences clustered them in different groups, so despite being from the same ST, they showed markedly distinctive profiles (Fig. 4).

4. Discussion

Most of the AMR studies conducted in pig nostrils aimed to detect *S. aureus* isolates, and more specifically MRSA. Contrarily, *E. coli* isolates are routinely screened in faecal samples retrieved from animals and humans. In this study, 42.5% (17/40) of pigs and 7.5% (3/40) of farmers carried *E. coli*, while 17.5% (7/40) of pigs contained ESBL-producing *E. coli* isolates. Moreover, it is important to highlight that these ESBL-positive were obtained from non-selective plates, meaning that the real carriage rate among pigs would have been greater if ESBL-selective media had been used. Most ESBL-positive isolates (77.8%) belonged to phylogroups A and B1, which are mainly considered commensals, thus mitigating the infectious risk of these bacteria for pigs and humans. All the pigs included in this study were also formerly screened for MRSA (Abdullahi et al., 2023a) and LRE (Abdullahi et al., 2023b) interestingly, ESBL-positive pigs from both farms, B and D, were also carriers of MRSA and/or LRE isolates. Considering the multiplicity of resistant pathogens that may not only persist in live animals but also on surfaces in close contact with humans and even in the dust, the establishment of control points to limit their transmission "from stable to fork" is thus of utmost importance (Schmithausen et al., 2015).

According to the Veterinary Antibiotic Prescription Database (EMA, 2019), antibiotic usage in the Spanish swine industry has almost halved in two years, with 47% fewer consumed in 2022 compared to 2020. In any case, tetracyclines and aminopenicillins remain the most used in swine, matching the resistance profiles reported in our study (TET-^{R}-AMP^{R} >50%). Despite the new regulatory framework, quinolone resistance rates are still high, so further follow-up monitoring will be required.

The former use of polymyxin most probably promoted the spread of *mcr* genes in *E. coli* recovered from pigs, as has occurred worldwide, especially in livestock. Their presence in the nostrils might be related to their natural affinity for digging in the soil to forage food, frequently bringing faecal matter into contact with the snout. Here, we reported the occurrence of a rare *mcr* variant, *mcr-4.5*, that was interestingly first described in an *E. coli* collected from a pig in Spain (García et al., 2018). Unfortunately, the plasmid involved was not described. In our study, the *mcr-4.5* gene was carried on a colicin (*ColE*-type) plasmid, as already observed by Caratolli *et al.* (Carattoli et al., 2017) sharing the same genetic environment *relE/parE* and IS*Kpn26* (IS*5*).

Here, given the occurrence of bla_{ESBL} genes, we hypothesised that the co-selection of different genes from other antibiotic families into the bacterial genome may have occurred. The ESBL carriers described displayed a compelling genetic diversity; eight distinct STs were identified, among which several have already been reported in pigs, amid other niches. ST10 is a well-known ubiquitous high-risk lineage that causes human extra-intestinal infections and has frequently been associated with the *mcr-1* colistin-resistance gene in Spain (García-Meniño et al., 2018). Our findings corroborated these results, linking them to the *mcr-4*

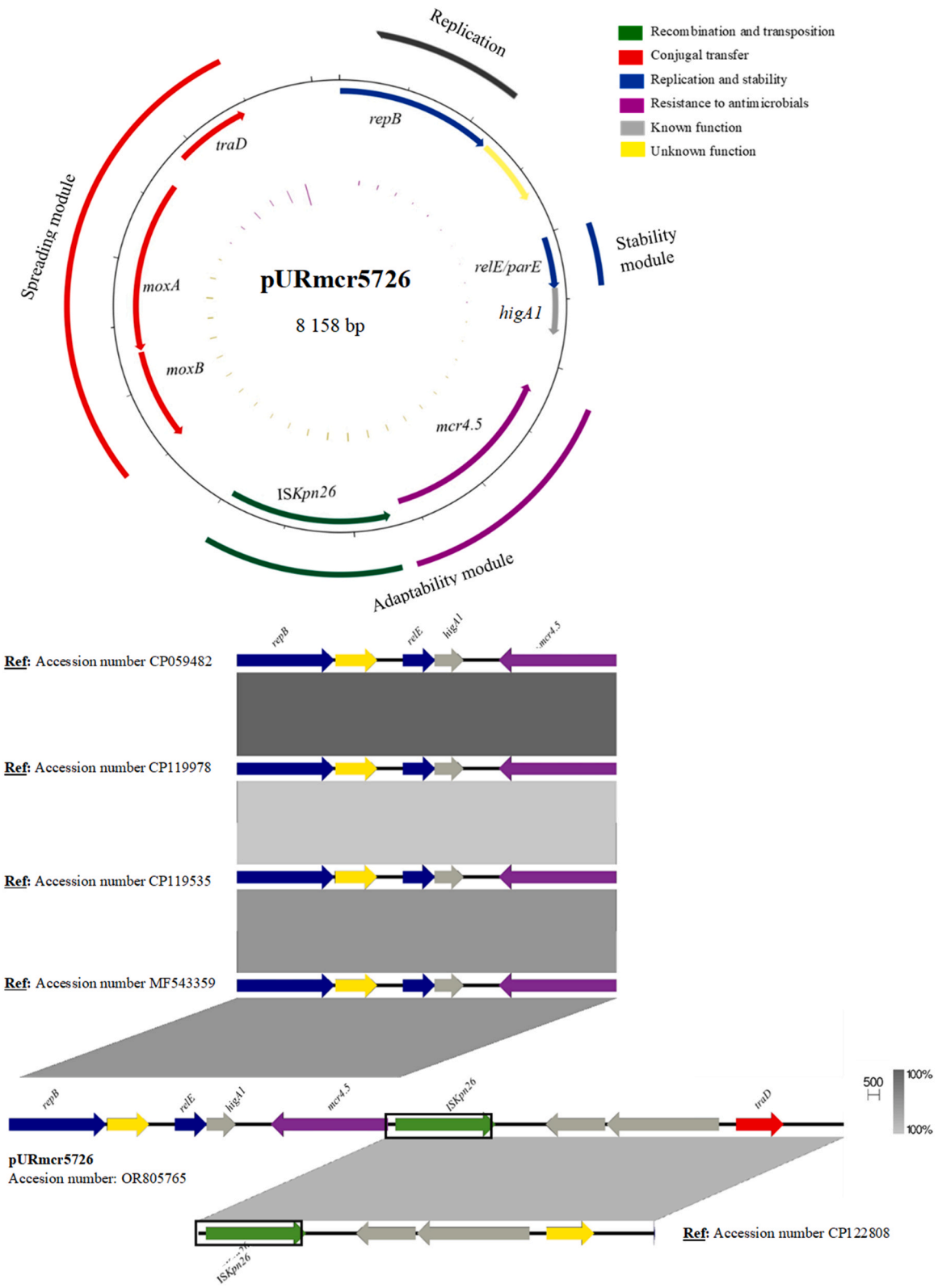


Fig. 3. Circular map of the *mcr-4.5* carrying plasmid and the comparison with genetic environments of other colicins; pIB2020_ColE_MCR (accession number CP059482), pMUMC-1_5 (accession number CP119978) from *Enterobacter kobei*, and plasmids WUR-NRS20181408_mcr4 (accession number CP119535) from *Escherichia coli* and pMCR_R3445 (accession number MF543359) from *Salmonella enterica* upstream, while the downstream region is 100% identical to pETEC1734 (accession number CP122808) from *Escherichia coli*. 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.

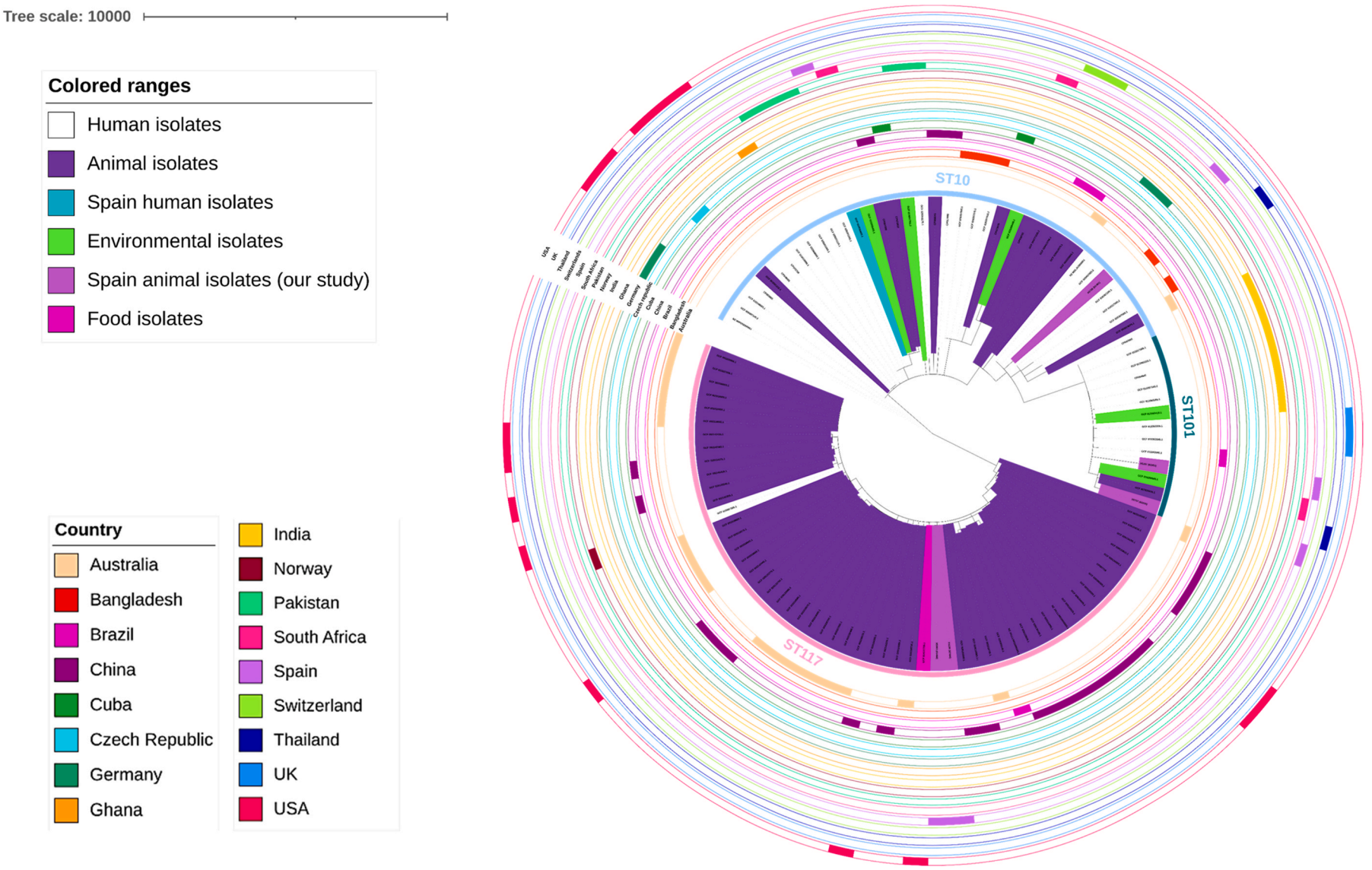


Fig. 4. Phylogenetic tree of the ST10, ST101 and ST117 *E. coli* isolates from animals, human, environment and food-derivates placed in the One Health context. The phylogenetic tree was constructed based on nucleotide sequence alignments of 2513 core genes. Sequence types are shown with coloured bars, 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 source of the isolates is indicated by coloured shading.

variant and the ESBL production ($bla_{CTX-M-65}$). ST117 has its primary reservoir in poultry, linked to enteric colibacillosis (Xia et al., 2022), but has also extensively disseminated in various environments, including humans (Novais et al., 2007), free-living animals (Cristóvão et al., 2017), and pigs (Freitag et al., 2018). This ST is associated with numerous variants of the CTX-M family but is also linked to SHV-12 in poultry production (Martínez-Álvarez et al., 2022), as observed in our study. As is commonly understood, ST93 is a ubiquitous clone among companion animals (Rocha-Gracia et al., 2015) and poultry (Ćwiek et al., 2021) worldwide and started covering relevance due to the uncovered role in ESBL production in pigs, along with ST101. In Australia, ST5440 and ST48 have also been previously detected in pigs, besides ST48 being a widely prevalent clone in cattle and poultry.

Our isolates recovered from pig nostrils also displayed a variety of bla_{ESBL} genes, including the bla_{SHV-12}, $bla_{CTX-M-1}$, $bla_{CTX-M-14}$, $bla_{CTX-M-32}$ and $bla_{CTX-M-65}$ genes, which were encoded by different plasmids. The bla_{CTX-M} genes were the most prevalent ones, coinciding with genes previously described in other studies on *E. coli* in pigs at the European level (Hayer et al., 2022; Ewers et al., 2012). The $bla_{CTX-M-1}$ genes (n=3) were consistently carried by IncX1 plasmids, which are known vectors of *qnrS1* and bla_{TEM-52} genes, but not as preferential carriers of bla_{ESBL} (Dobiasova et al., 2016; Aguirre et al., 2020; Liu et al., 2016). However, IncX1 plasmids have already been reported as $bla_{CTX-M-32}$ bearers in the swine trade in the Netherlands (Ceccarelli et al., 2019) and, more recently, in Cuba (Hernández-Fillor et al., 2021). In our study, we detected this gene located in an IS*26*-mediated composite transposon (IS*26*-IS*Ecp1*-$bla_{CTX-M-32}$-*wbuC*-Tn*2*-IS*26*) previously described with chromosomal location in Iran at hospital level (Pajand et al., 2021).

IncI1-type plasmids have been conclusively shown harbouring the $bla_{CTX-M-14}$, bla_{CTX-32}, and bla_{SHV-12} genes, which have been previously reported in livestock (Alonso et al., 2017; Liao et al., 2015), free-living animals (Alonso et al., 2017), and humans (Novais et al., 2007). The flanking regions of these genes were found to be IS*26* preceding the transposon containing bla_{SHV-12}, and IS*26*, IS*903*, or IS*15* for $bla_{CTX-M-14}$ and $bla_{CTX-M-32}$ (Fernández et al., 2007). The IncI1 plasmids are also preferential vectors of these genes, especially IncI1/pST3, that has been responsible for the wide dissemination of the $bla_{CTX-M-1}$ gene between farms.

The $bla_{CTX-M-65}$ gene was placed in an IncHI2/pST3 previously noted on that in Korea (Lü et al., 2020). This gene has been increasingly important in swine in China since 2007 and has now spread to hospitals, becoming the most encountered enzyme. IncHI2 plasmids, originally found in the environment, are larger and have a colossal potential to spread multiple AMR genes. Our plasmid indeed contained two multidrug resistance regions that were like those found in pGD80–2 (KY075659). Through our research, we identified a ΔTn*21* transposon with a ΔTn*5393* embedded, displaying the same structure as the one described by Lü et al. (Lü et al., 2020). A second structure, more convoluted than the original Tn*1721*, was formed and contained a new non-classical type 1 integron (*aadA2-arr3-cmlA5-*bla_{OXA-10}*-aadA24-dfrA14*). Preceding those was a genetic platform consisting of i) IS*26*-bla_{TEM-95} and ii) *tet*(A)-*lysR-floR-virD2*-IS*Vsa3*-IS*3075*, which resembled the one found in the pGD80–2 plasmid.

The MDR phenotype observed in the IncF/F18:A-:B58 of our X5093 isolate could be partly attributed to the presence of an atypical class 1 integron embedded in Tn*21*, specifically containing the class 1 integron In*2*, but lacking the *cmlA1* gene (*intI1-estX-psp-aadA2-aadA24-qacL*-tnp*406-sul3*). This transposon strongly resembled those found in other IncI1 plasmids carrying bla_{SHV-12} (Alonso et al., 2017) and in IncF plasmids (Martínez-Álvarez et al., 2023). Based on the widespread distribution of these transposons in Enterobacterales, it is possible to speculate that those found in our X5093 isolate may have been mobilised from IncI1 plasmids.

Even though class 2 integrons are commonly found in *E. coli* and usually located in Tn*7* (Alonso et al., 2018), this study presents the first description of a new class 2 integron structure (*intI2-dfrA1-sat2-aadA1-qacL*-IS*406-sul3*) in an IncY plasmid. These plasmids have gained significant epidemiological importance in recent years, making this discovery noteworthy.

One limitation of our study is the relatively small number of samples included, which prevents us from drawing statistically solid conclusions. Nevertheless, our results showing a high rate and wide genetic diversity of colistin-resistant and ESBL-producing isolates in the pig nostrils are opening the doors to future studies.

In conclusion, pig nostrils are another niche to consider that promotes the diffusion of ESBL-producing *E. coli* in the swine sector, and close contact between animals in intensive farming further increases the risk of their dissemination. Our study, performed without selective media, showed an important proportion of ESBL-producing *E. coli* contamination of pig nostrils with a diversity of STs and resistance genes. Our results also suggested vertical (i.e. clonal) and horizontal (plasmidic) transmission events. Although the presence of similar plasmids and/or clones on farms is not an argument in favour of direct transmission, we cannot exclude the possibility that different companies in the pig sector share a common supplier. Finally, our results raised questions on the proportion of pigs that are carriers of ESBL-producing *E. coli*. Since the EFSA will launch a second baseline survey on MRSA in pig production in 2025, this might be an opportunity to implement the detection of ESBL-producing *E. coli* to have large-scale data over several European countries.

Ethical approval

This study was approved by the ethical committees of the University of La Rioja and the University of Zaragoza (Ref. PI58/21). Concerning the ethical use of animals, this study complied with the specific directives: 2010/63/EU and the Spanish laws 9/2003 and 32/2007, RD 178/2004 and RD 1201/2005.

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CRediT authorship contribution statement

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

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116145.

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