Journal of Global Antimicrobial Resistance 34 (2023) 186–194

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Detection and genetic characterization of bla_{ESBL}-carrying plasmids of cloacal *Escherichia coli* isolates from white stork nestlings (*Ciconia ciconia*) in Spain

Sandra Martínez-Álvarez[a], Pierre Châtre[b], Teresa Cardona-Cabrera[c], Pauline François[b], Alberto Sánchez-Cano[c], Ursula Höfle[c], Myriam Zarazaga[a], Jean-Yves Madec[b], Marisa Haenni[b], Carmen Torres[a,*]

[a] Area of Biochemistry and Molecular Biology, OneHealth-UR Research Group, University of La Rioja, Logroño, Spain
[b] ANSES – Université de Lyon, Unité Antibiorésistance et Virulence Bactériennes, Lyon, France
[c] Health and Biotechnology (SaBio) Research Group, Institute for Game and Wildlife Research IREC (CSIC-UCLM), Ciudad Real, Spain

ARTICLE INFO

Article history:
Received 10 May 2023
Revised 17 July 2023
Accepted 17 July 2023
Available online 22 July 2023

Editor: Stefania Stefani

Keywords:
ESBL
White stork nestlings
Plasmids
Integrons
Transfer technique

ABSTRACT

Objectives: This study aimed to characterize *Escherichia coli* isolates from cloacal samples of white stork nestlings, with a special focus on extended-spectrum β-lactamases (ESBLs)-producing *E. coli* isolates and their plasmid content.

Methods: Cloacal samples of 88 animals were seeded on MacConkey-agar and chromogenic-ESBL plates to recover *E. coli* and ESBL-producing *E. coli*. Antimicrobial susceptibility was screened using the disc diffusion method, and the genotypic characterization was performed by polymerase chain reaction (PCR) and subsequent sequencing. S1 nuclease Pulsed-Field-Gel-Electrophoresis (PFGE), Southern blotting, and conjugation essays were performed on ESBL-producing *E. coli*, as well as whole-genome sequencing by short- and long-reads. The four bla_{ESBL}-carrying plasmids were completely sequenced.

Results: A total of 113 non-ESBL-producing *E. coli* isolates were collected on antibiotic-free MacConkey-agar, of which 27 (23.9%) showed a multidrug-resistance (MDR) phenotype, mainly associated with β-lactam-phenicol-sulfonamide resistance (*bla*$_{TEM}$/*cmlA*/*floR*/*sul1*/*sul2*/*sul3*). Moreover, four white stork nestlings carried ESBL-producing *E. coli* (4.5%) with the following characteristics: bla_{SHV-12}/ST38-D, bla_{SHV-12}/ST58-B1, $bla_{CTX-M-1}$/ST162-B1, and $bla_{CTX-M-32}$/ST155-B1. Whole-genome sequencing followed by Southern blot hybridizations on S1-PFGE gels in ESBL-positive isolates proved that the $bla_{CTX-M-1}$ gene and one of the bla_{SHV-12} genes were carried by IncI1/pST3 plasmids, while the second bla_{SHV-12} gene and the $bla_{CTX-M-32}$ gene were located on IncF plasmids. The two bla_{SHV-12} genes and the two bla_{CTX-M} genes had similar but non-identical close genetic environments, as all four genes were flanked by a variety of insertion sequences.

Conclusion: The role played by several genetic platforms in the mobility of ESBL genes allows for interchangeability on a remarkably small scale (gene-plasmid-clones), which may support the spread of ESBL genes.

1. Introduction

The World Health Organization defines third- and fourth-generation extended-spectrum cephalosporins (ESCs) as critically important antimicrobials for human health [1]. Among Gram-negative bacteria, resistance to ESCs has become a major public health challenge that is related to the high level of spread of extended-spectrum β-lactamases (ESBLs). To date, the ESBL phenotype is largely conferred by genes of the CTX-M family, although older TEM/SHV variants, including bla_{SHV-12}, currently cause nosocomial and community-acquired infections [2] in many countries in Europe and North America [3]. ESBL-producing *E. coli* have been widely detected not only in humans, but also in food [4],

* Corresponding author. Mailing address: Faculty of Science and Technology, Department of Agriculture and Food, Universidad de La Rioja, Madre de Dios 51, Logroño 26006, Spain.
E-mail address: carmen.torres@unirioja.es (C. Torres).

https://doi.org/10.1016/j.jgar.2023.07.011

Sampling area	Nestling reference	Cloacal samples analyzed	Percentage of isolates with characteristics	
			E. coli detection (n° of isolates)	ESBL *E. coli* detection (n° of isolates)
Natural-1	468-481	14	100.0 (4)	0.0 (0)
Naturtal-2	426-455	30	93.3 (28)	7.1 (2)
Landfill-1	487-490	4	100.0 (4)	0.0 (0)
Landfill-2	504-514	11	90.9 (10)	10.0 (1)
Semi-landfill	530-558	29	93.1 (27)	3.7 (1)

Fig. 1. Geographical location of the different white stork nestlings sampling areas in Ciudad Real (Castilla–La Mancha, Spain).

food-producing animals [5], companion animals [6], and even wildlife [7], hence the use of this gene/species linkage as a major marker of antimicrobial resistance (AMR) burden in the "One Health" approach.

AMR in migratory birds most likely reflects contamination of their natural habitats with genes, plasmids, or clones, since the selective pressure of antibiotics is expected to be very weak in the environment. Acquisition of ESBL-producing *E. coli* most likely occurs through feeding (eg, in areas with intensive livestock production or open rubbish dumps) and foraging behaviour such as scavenging or predation. Once the ESBL genes have been acquired in their gut, migratory birds can spread these genes over vast distances by faecal shedding, within and between continents [8,9], rendering them potential AMR vectors for the wider environment, animals, and humans.

Worldwide, cases of ESBL-producing *E. coli* have been reported in migratory birds, from gulls in Patagonia [10,11], Chile, Canada [12], and Europe [13] to rooks in Austria [14] or several avian species in Pakistan [15], as well as all over the world. In Spain, $bla_{\text{CTX-M}}$ and/or $bla_{\text{SHV-12}}$ genes have been identified from yellow-legged gulls in Barcelona [16], from a griffon vulture in Eastern Spain [17], and from diverse bird species throughout the country [18]. The first detection of ESBL-producing *E. coli* in adult white storks (*Ciconia ciconia*) in Spain was reported in 2016 [18], showing high rates of ESBL production (44.4%). However, a recent study reported much lower rates (8.8% of ESBL and/or acquired AmpC β-lactamase producers), which suggests that white stork nestlings may also be prone to carry resistant bacteria despite having only indirect contact with antibiotic-exposed environments [8]. This prompted us to study white stork nestlings, whose diet relies entirely on parental feeding. Five white stork colonies subjected to a gradient of anthropogenic pressure were sampled with the aim to (i) evaluate the presence of *E. coli* independently of their resistance phenotype, and (ii) selectively detect ESBL-producing *E. coli* isolates. These resistant isolates were fully characterized to evaluate the risk of transmission of these ESBL genes, plasmids, or clones under the One Health approach.

2. Material and methods

2.1. Ethics

Nestling handling was carried out following international, national, and/or institutional guidelines for the care and ethical use of animals, specifically directive 2010/63/EU, Spanish laws 9/2003 and 32/2007, and RD 53/2013. All procedures were approved by the ethical committee for animal experimentation of the University of Castilla-La Mancha and authorized by the regional government of Castilla–La Mancha (permit no.: VS/MLCE/avp_21_198).

2.2. Study design

Cloacal swabs (n = 88) from white stork nestlings (*C. ciconia*) were collected from five colonies located in South–Central Spain (Ciudad Real province): two from natural habitats (Natural-1, Natural-2), one from a semi-natural setting with nearby landfills (Semi-landfill) and two from anthropized habitats located near solid household waste landfill premises (Landfill-1 and Landfill-2) (Fig. 1). Satellite transmitter data collected in 2013–2020 from adult white storks tagged on four of the five colonies showed that, during the breeding season, adults foraged in the vicinity of the colony and moved to their respective nests to feed their nestlings [19]. White stork nestlings were sampled in June 2021 at 45–55 days of age (prior to fledging). They were extracted from the nest by gently wrapping them in a towel and lowering them to the floor by hand or in a large bag. Cloacal swabs were stored in AMIES transport medium without charcoal and maintained at 4°C until arrival at the laboratory, where they were frozen at -80°C.

2.3. Bacterial isolation and identification

Cloacal swabs were enriched at 37°C for 24 h in 5 mL of Brain Heart Infusion (BHI) broth. Different aliquots (10–100 μL) of this enrichment step were seeded on MacConkey agar supplemented or

not with cefotaxime (2 μg/mL) and on chromogenic selective media (Brilliance^{TM} ESBL Agar; Oxoid). After incubation at 37°C for 24 h, one to six (if possible) presumptive *E. coli* isolates were selected from each media, and identification was performed by MALDI-TOF mass spectrometry (MALDI Biotyper®, Bruker).

2.4. Antimicrobial susceptibility testing

Susceptibility testing of *E. coli* isolates was performed by the disc diffusion method using the Clinical Laboratory and Standards Institute (CLSI) methodology and breakpoints [20]. Twelve antibiotics of both human and veterinary interest were tested: ampicillin, amoxicillin/clavulanate, ceftazidime, cefotaxime, cefoxitin, imipenem, ciprofloxacin, gentamicin, amikacin, chloramphenicol, trimethoprim/sulfamethoxazole, and tetracycline. Screening for phenotypic ESBL production was performed by the double disc synergy test using cefotaxime, ceftazidime, and amoxicillin/clavulanate discs [20]. *E. coli* ATCC 25922 was used as a control strain.

2.5. Characterization of AMR genes and integrons

The presence of genes conferring resistance to β-lactams (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}), quinolones (*qnrA, qnrB, qnrS, aac(6′)-Ib-cr,* and *qepA,* as well as mutations in the *gyrA* and *parC* genes), aminoglycosides (*aac(3)-I, aac(3)-II, aac(3)-III, aac(3)-IV*) and tetracyclines (*tet*(A), *tet*(B)) were analyzed by polymerase chain reaction (PCR) and sequencing [21,22]. The presence of class 1 and class 2 integrons was detected by PCR amplification of the integrase genes *intI1* and *intI2*, and their complete sequence was obtained using the "primer-walking" PCR strategy [21].

2.6. Molecular typing of E. coli isolates

The phylogenetic groups (A, B1, B2, C, D, E, and F) of all *E. coli* isolates were determined according to Clermont et al [23]. ESBL-producing isolates were typed by multilocus sequence typing (MLST) using the Achtman scheme, obtaining the sequence types (STs) as well as the clonal complexes (ST Cplx) (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search).

2.7. Conjugal transfer

Conjugation experiments for ESBL-producing *E. coli* isolates were carried out in Luria Bertani broth using the rifampicin-resistant *E. coli* strain J53 as the recipient strain. Transconjugants were selected 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 the bla_{ESBL} genes and replicon content [24].

2.8. Short-read sequencing

Bacterial genomic DNA was extracted using the NucleoSpin® Microbial DNA Kit (Macherey-Nagel) according to the manufacturer's instructions. DNA concentration and purity were respectively determined using the Qubit R 3.0 Fluorometer (Thermo Fisher Scientific) and the NanoDrop One (Ozyme). Short-read (2 × 150 paired-end) sequencing was performed using the NovaSeq 6000 Illumina technology. Illumina adapter sequences were removed, and reads were quality trimmed using trimmomatic version 0.38.1. De novo assemblies were generated with Shovill version 1.1.0, and the quality of assemblies was assessed using QUAST 5.2.0 (Table S1). STs, pST, plasmid replicon content, and resistance genes were determined using the CGE online tools MLSTFinder 2.0.9, pMLSTFinder 2.0, PlasmidFinder 2.1, and ResFinder 4.1 (http://www.genomicepidemiology.org/).

2.9. Plasmid characterization using Southern blot and long-read sequencing

Bla_{ESBL}-carrying plasmids were identified, and their respective sizes were determined by Southern blotting on S1 and Pulsed-Field-Gel-Electrophoresis (PFGE) using adequate DIG-labelled probes (Roche Applied Science, Meylan, France) according to the manufacturer's protocol. All ESBL-producing *E. coli* isolates were additionally long-read sequenced. MinION libraries were prepared according to the manufacturer's instructions (Oxford Nanopore Technologies, UK) using the native barcoding 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) flow cell (FLO-MIN106D). Assembly of Illumina short reads and Nanopore long reads was performed using Unicycler [25]. The resulting files were used to fix individual base errors, indels, and local miss-assemblies, using Pilon [26].

2.10. Genome sequencing analysis

Using the RAST Prokaryotic Genome Annotation Server, functional annotation of the plasmids was completed. Data were manually curated using the Artemis software, IS finder (https://www-is.biotoul.fr), and Swiss-Prot database (http://www.uniprot.org). Sequence comparison was done using the EMBOSS Needle alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) and NCBI Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Using DNAPlotter, a circular map of the bla_{ESBL}-carrying plasmids was created [27–29].

2.11. Data availability

The full-length sequence of plasmids pUR5229, pUR5239, pUR5279, and pUR5854 (carried by *E. coli* X5229, X5239, X5279, and X5854, respectively) as well as the structure of Tn*21* of *E. coli* X5229 were deposited in the EMBL database under the accession numbers OQ658192, OQ747074, OQ747075, OQ747076 and OQ787092, respectively.

Raw sequence reads obtained by short-read sequencing and reported in this paper were deposited under NCBI BioProject PRJNA954768.

3. Results

3.1. Carriage of resistant E. coli in white stork nestlings

Of the 88 cloacal samples taken from individual white stork nestlings, 78 (88.6%) presented growth of at least one *E. coli* isolate on non-selective plates. Since one to six presumptive *E. coli* colonies were picked out, 194 *E. coli* isolates were collected. Based on antibiotic susceptibility tests and phylogroups, only non-duplicate isolates were kept for further characterization, leading to a collection of 117 *E. coli* isolates.

The cloacal swabs taken from four white stork nestlings (4/88, 4.5%) led to the isolation of ESBL-producing isolates on selective plates. It is noteworthy that these ESBL-positive *E. coli* grew not only on MacConkey-CTX plates but also extensively on antibiotic-free MacConkey agar (>500 CFU/plate on selective and non-selective media).

3.2. Antimicrobial susceptibility and resistance determinants in non-ESBL-producing E. coli isolates

Among the 113 non-ESBL-producing *E. coli* isolates, 48 (42.5%) showed resistance to at least one antibiotic, whereas 27 isolates (23.9%) showed a MDR phenotype mainly associated with

Table 1
Overall distribution of antimicrobial resistance in the 117 *E. coli* isolates from white stork nestling cloacal samples and based on whether they are ESBL-producing or non-ESBL-producing *E. coli*.

Antibiotics	Total *E. coli* (n = 117)		ESBL *E. coli* (n = 4) / non ESBL *E. coli* (n = 113)	
	n	%	n	%
Amoxicillin-clavulanate	4	3.4	2/2	50.0/1.8
Ampicillin	33	28.2	4/29	100.0/25.7
Cefotaxime	4	3.4	4/0	100.0/0.0
Ceftazidime	4	3.4	4/0	100.0/0.0
Cefoxitin	0	0.0	0/0	0.0/0.0
Imipenem	0	0.0	0/0	0.0/0.0
Amikacin	0	0.0	0/0	0.0/0.0
Gentamicin	4	3.4	0/4	0.0/3.5
Chloramphenicol	15	12.8	1/14	25.0/12.4
Tetracycline	27	23.1	2/25	50.0/22.1
Trimethoprim/sulfamethoxazole	22	18.8	2/20	50.0/17.7
Ciprofloxacin	22	18.8	4/18	100.0/15.9

beta-lactam-phenicol-sulphonamide resistances. Antibiotic resistance rates for non-ESBL-producing *E. coli* isolates were significantly lower than those of ESBL producers (Table 1). A wide diversity of resistance genes was detected, with resistance to ampicillin (25.7%, n = 29) and tetracycline (22.1%, n = 25) due to the bla_{TEM}, *tet*(A), and *tet*(B) genes. Resistance to ciprofloxacin (15.9%, n = 18) was averaged by the Plasmid Mediated Quinolone Resistance (PMQR) genes (*aac(6′)-Ib-cr*) and point mutations in the quinolone resistance determining region of the topoisomerases *gyrA* and *parC* (S80I, D87N/ S83I). Furthermore, chloramphenicol and/or florfenicol resistance (12.4%, n = 14) was mostly encoded by *cmlA* and *floR*, while sulphonamide resistance (SXT-R) (17.7%, n = 20) was due to the *sul1, sul2*, and/or *sul3* genes (Fig. S1). Additionally, the presence of class 1 and/or 2 integrons was detected in two isolates carrying the following gene cassette arrays: *intI1-aadA1-qacE1-sul1* and *intI2-aadA1-sat2* (Table S2).

3.3. Genetic diversity of ESBL-producing isolates

The four ESBL-producing isolates belonged to the bla_{SHV-12}/ST38-D, bla_{SHV-12}/ST58-B1, $bla_{CTX-M-1}$/ST162-B1, and $bla_{CTX-M-32}$/ST155-B1 clones, and all except one showed MDR phenotypes (Table 2). The isolate X5279 was the only carrier of a PMQR gene, namely *qnrS1*, which was chromosomally encoded. This gene was inserted in an atypical class 1 integron described for the first time in this study, with the gene cassette array *aadA1-lnu*(F) and the flanking element IS*26* located where this insertion sequence truncated the conserved segment 3′CS.

3.4. Characterization of the bla$_{ESBL}$-carrying plasmids

The $bla_{CTX-M-1}$ gene (strain X5854) and one of the two bla_{SHV-12} genes (strain X5229) were located on IncI1/pST3 (pUR5854 and pUR5229), as proved by Southern blot hybridizations and MinION sequencing (Fig. 2). These plasmids did not carry additional resistance genes or integrons (Table 2). The $bla_{CTX-M-32}$ gene (strain X5239) and the second bla_{SHV-12} gene (strain X5279) were found on IncF plasmids (pUR5239 and pUR5279) belonging to the F4:A-:B- and F24:A-:B58 formulas, respectively (Fig. 2, Table 2). Conjugal transfer of the bla_{ESBL}-carrying plasmids, whose sizes varied between 90,000 and 102,000 bp, was evidenced in all isolates, with conjugation frequencies ranging from 10^{-3} to 10^{-5} (Table 2).

3.4.1. Inc I1/pST3 plasmids

The replication, transfer, and leading regions of pUR5854 and pUR5229 were very similar to those of other IncI1 plasmids, with some insertions/deletions suggesting recombination between related plasmids (Fig. 2). The entire region involved in conjugal transfer (*tra*/*trb* genes) was closely related (99.69% identity) to the archetypal IncI1 plasmid R64 (accession number **AP005147**). The larger portions of the backbone shared high identity (99.0%) with each other (Fig. S3A) and in turn with plasmids pCFSAN000520 (**CP074613**), pC1122_2 (**CP067953**) and pEC405 (**CP094200**) from *Salmonella enterica, Klebsiella pneumoniae*, and *E. coli* strains. pUR5854 and pUR5229 presented accessory modules of different sizes. pUR5229 presented a large adaptability module (11,747 bp) associated with AMR, located between the replication and *ph-doc* toxin-antitoxin systems (leading region) (Fig. 2A). This resistance module comprised a Tn*1721*-derived transposon, the bla_{SHV-12} gene, flanking elements (IS*26-deoR*-IS*1294*), two additional insertion sequences (IS*66* and IS*26*), and a transposase IS*91*-like. In contrast, pUR5854 had a much smaller accessory module (2,824 bp), located in the transfer module between the *tra/trb* and *pil* region (Fig. 2B) and comprising the $bla_{CTX-M-1}$ gene, the flanking element IS*Ecp1*, and the metalloprotein *wbuC*.

3.4.2. IncF plasmids

Both pUR5239 and pUR5279 plasmids, despite having different FAB formulas, showed 97.13% homology and 55% coverage (Fig. S3B). Therefore, most of the transfer region and the stability module were strongly associated with each other and with plasmids pLAO22 (**OP242255**), p47EC (**CP057369**), and pF18S043 (**CP082385**) from *E. coli* and *S. enterica* strains, respectively. pUR5239 and pUR5279 had large accessory modules (between 28,000 and 30,000 bp) (Fig. 2C, 2D). Both presented a resistance module comprising a Tn*21*-derived transposon containing an atypical class 1 integron, the ESBL gene (bla_{SHV-12} or $bla_{CTX-M-32}$), and the genetic platforms IS*26*-ΔTn*1721* or IS*26*-IS*Kpn26*-IS*Ecp1*. The Tn*21*-derived region carried the left and right Tn*21* terminal IRs and the genes involving its own transposition (*tnpA, tnpR, tnpM*). In addition, the imperfect terminal IRi of the class 1 integron In*2* and the class 1 integrase gene (*intI1*) were also conserved, although the entire structure of the In*2* integron was missing in both cases. Instead of In*2*, two atypical integrons were found, whose arrays included (i) the standard 5′-CS (*intI1* gene), (ii) the gene cassettes: *aadA22/*, the new structure *estX*-IS*1294-psp-aadA2-cmlA1-aadA1-qacL*); and (iv) the genetic platforms (IS*1*-IS*26-strA-strB*-IS*26-bla*$_{TEM-1A}$-IS*66*-IS*26*/Tnp*256*-like(IS*256*)-*sul3-mef*(B)-IS*26*-IS*66*-IS*26*). These two genetic platforms presented a terminal common region consisting of IS*26*, IS*66*, and a set of proteins of known and unknown function. The *mef*(B) gene, encoding a macrolide-efflux protein, was found to be disrupted by IS*26*. The IS*26-bla*$_{SHV-12}$*-deoR* segment was followed by a ΔTn*1721* in both pUR5239 and pUR5279, without detection of the characteristic region encoding the tetracycline resistance. The ΔTn*1721* contained only the genes involved in its transposi-

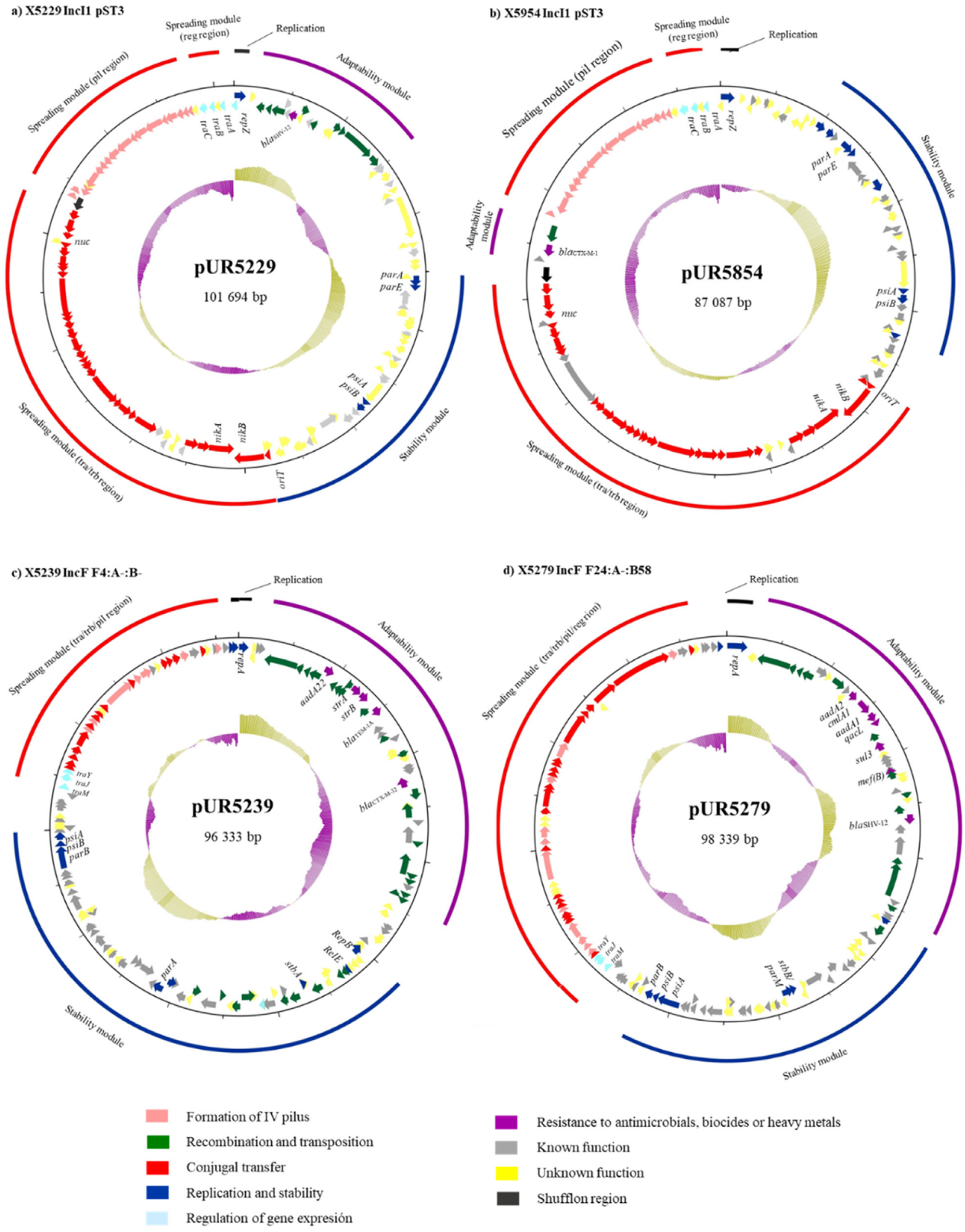

Fig. 2. Circular map of the four bla_{ESBL}-carrying plasmids (A) pUR5229 (accession number **OQ658192**), (B) pUR5854 (accession number **OQ747076**), (C) pUR5239 (accession number **OQ747074**), and (D) pUR5279 (accession number **OQ747075**).

Table 2
Characteristics of bla_{ESBL} gene-carrying plasmids in the studied *E. coli* isolates.

E. coli isolate	Sample Origin	ST/phylogroup/ clonotype/serotype	Resistance genes	Replicon content	bla_{ESBL}-carrying plasmid					Other co-located resistance genes
					Replicon type/pMLST or FAB (Size)	Conjugation frequency	Genetic environment	Class 1 integron		
								intI1/3′CS	Variable region	
X5229	Natural-2	58-B1/C4:H27/ O9:H25	*aph(3″)-Ib, aph(6)-Id, dfrA5, sitABCD, sul2*	FII, FIB, I1, Q1, Y	SHV-12, I1/pST3 (110 kb)	2.8×10^{-5}	IS*26*-bla_{SHV-12}-*deoR*	ND	ND	-
X5239	Natural-2	155-B1/C4:H32/O- :H151	*aadA22, aph(3″)-Ib, aph(6)-Id, tet*(A)	FII, FIB, X1	CTX-M-32, FII/ F4:A-:B-(100 kb)	3.3×10^{-5}	*wbuC*-$bla_{CTX-M-32}$-IS*Kpn26*-IS*Ecp1*	ND	*aadA22*	*strA, strB,* bla_{TEM-1A}
X5279	Landfill-1	38-D/C26:H65	*aadA1, aadA2b, aph(6)-Id, cmlA1, lnu*(F), *qnrS1, sul3*	ColpVC, FIB, FII, X1, Y	SHV-12, FII, FIB F24:A-:B58 (100 kb)	5.1×10^{-3}	IS*26*-bla_{SHV-12}-*deoR*	+/–	*estX*-IS*1294-aadA2-cmlA1-aadA1-qacL-sul3*	-
X5854	Semi-landfill	162-B1/C65:H32	*sitABCD, tet*(A)	FIB, I1	CTX-M-1, I1/pST3 (90 kb)	4.2×10^{-4}	*wbuC*- $bla_{CTX-M-1}$-IS*Ecp1*	ND	ND	-

tion (*tnpR, tnpA*) and the inverted repeat sequences IRR and IRL (Fig. 3).

The mercury resistance module (*merRTPCAD*), normally located in Tn*21*, was not found in the pUR5239 and pUR5279 plasmids. However, this Tn*21* transposon was found on the IncF plasmid of the X5229 isolate, which carries the bla_{SHV-12} gene on an IncI1/pST3 plasmid. Our study revealed the structure of a ΔTn*21*, in which the mercury resistance module flanked on the left end by IS*26* was inserted, as well as an atypical class 1 integron (*dfrA5*-IS*26*) different from those detected in the other Tn*21* derivatives (Fig. S4).

3.5. Genetic environment of the bla_{ESBL} genes

The bla_{SHV-12} was located on an IncF/F24:A-:B58 plasmid (pUR5279) and an IncI1/pST3 plasmid (pUR5229). In pUR5279, the bla_{SHV-12} gene was flanked by an IS*26* located 130 bp upstream and by the putative transcriptional regulatory gene *deoR* located 20 bp downstream (Fig. S2A). In pUR5229, the IS*26* and the *deoR* gene were also found upstream and downstream the of bla_{SHV-12} gene. However, IS*26* was located 268 bp upstream and truncated a protein of unknown function located 53 bp upstream, while the *deoR* gene was truncated at position 599 by the insertion of a 94 bp DNA segment preceding an IS*1294* element.

The flanking regions of the $bla_{CTX-M-1}$ and $bla_{CTX-M-32}$ genes were similar, despite the fact that they were respectively located on an IncI1/pST3 (pUR5854) and on an IncF/F4:A-:B- plasmid (pUR5239). In pUR5854, the IS*Ecp1* was located 287 bp upstream of the $bla_{CTX-M-1}$ gene, and the metalloprotein *wbuC* was 26 bp downstream. In pUR5239, *wbuC* was also located downstream of the $bla_{CTX-M-32}$ gene (46 bp), while the upstream region presented the insertion of an additional IS*Kpn26* inserted 300 bp upstream of the $bla_{CTX-M-32}$ gene and preceded by the IS*Ecp1* truncating a protein of undefined function (Fig. S2B).

4. Discussion

This study revealed that 88.6% of white stork nestlings, whose diet only relies on parents feeding, were colonized by *E. coli* isolates. Among them, 41.0% were resistant to at least one antibiotic, and 23.1% were MDR isolates, containing up to seven different AMR genes that might be potentially mobile. Additionally, four white stork nestlings (4.5%) were heavily colonized by ESBL-producing *E. coli*. Comparisons with the few previous studies on white storks in Spain are unfortunately difficult. Indeed, Alcalá et al. [18] only sampled nine injured adult white storks, of which four were ESBL carriers. As a result, the small number of isolates prevents any reliable conclusion being drawn. A previous comparative study showed that white storks sampled at rehabilitation centres reflect a different section of the population (fledged nestlings and adults that feed at rubbish dumps) than nestlings that are representative of the habitat of their colony [30]. The second study on ESBL-producing *E. coli* from white storks in Spain conducted by Höfle et al. [8] analyzed 467 samples, including 441 from white stork nestlings; the mean proportion of ESBL- and/or acquired AmpC beta-lactamase-producing *E. coli* isolates from both adult white storks and nestlings was 8.8%, but large disparities were found, with proportions of ESBL-positive nestlings ranging from 0% (in 6/11 sampling sites) to 37.2% in one specific sampling site. This indicates that proportions found in one place cannot be inferred to all situations. In any case, the presence of ESBL-producing *E. coli* in nestlings is of concern, firstly because it was most likely passed on by the parent who nurtured it, and secondly because the colonial behaviour of storks might favour the efficient transmission of ESBL-conferring genes by the multiplicity of contacts between adult and juvenile birds.

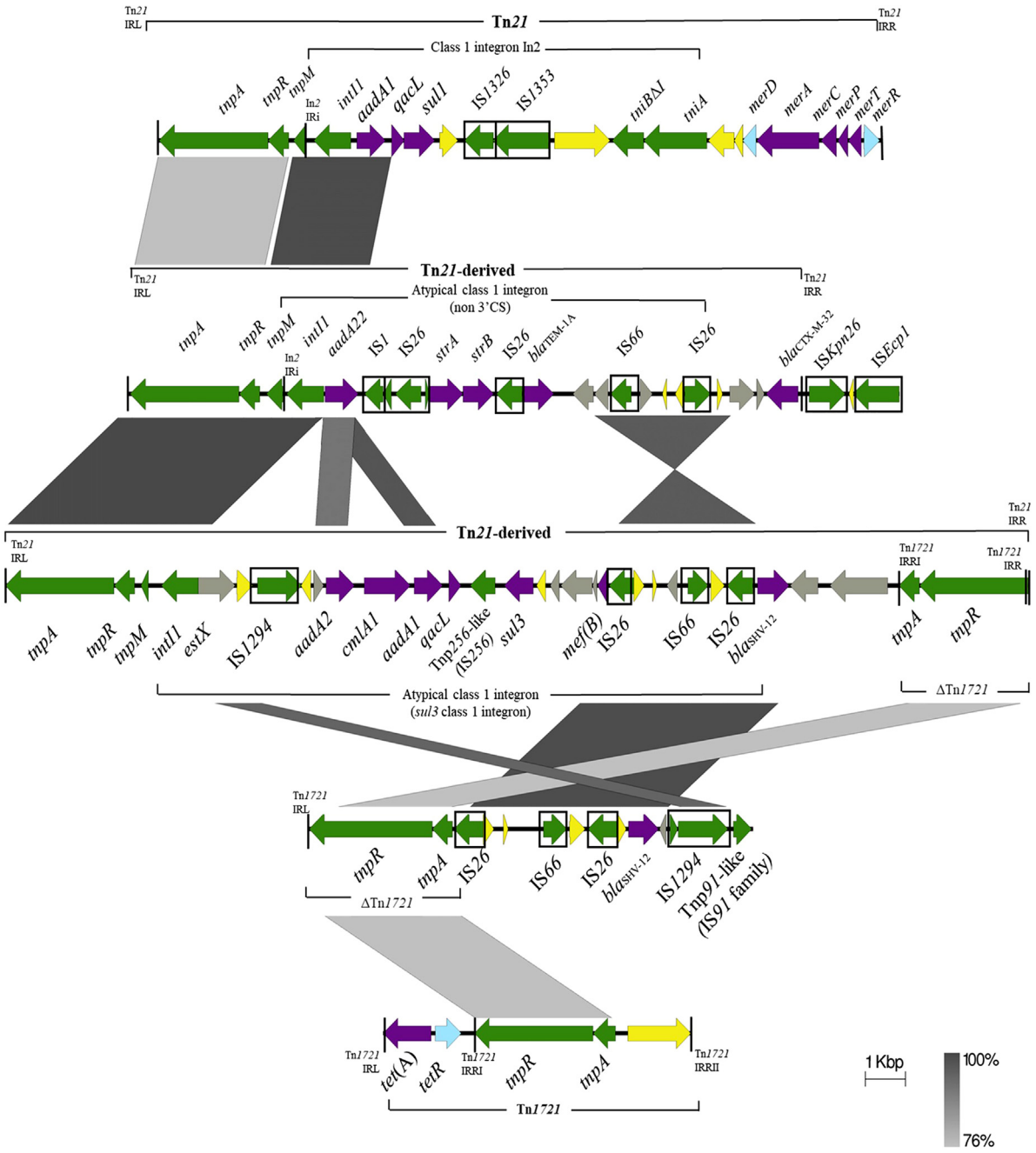

Fig. 3. Linear illustration of the resistance complexes of pUR5239 (IncF/F4:A-:B-), pUR5279 (IncF/F24:A-:B58), and pUR5229 (IncI1 pST3) and comparative mapping of this region with Tn*21* (accession number **AF071413**) and Tn*1721* (accession number **X61367**). A few important genes have been tagged. The coding reading frames are shown as arrows ("transcription direction" is indicated by the arrowheads). ISs 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.

The four ESBL-producing *E. coli* belonged to the lineages ST38, ST58, ST155, and ST162. Interestingly, ST58, ST38, and ST162 are pandemic clones reported in numerous human and non-human contexts [31–33] including wild birds [34]. ST155, which is commonly found in African poultry [35], belongs to the same clonal complex as ST58 (CC155) [36] and has been described in wild birds in France [37]. This strongly suggests that these pandemic clones with a weak host specificity can be transported over long distances and are then able to colonize numerous hosts, possibly also transmitting plasmidic resistance genes to other bacterial backgrounds.

The detected ESBL-producing *E. coli* presented a mixed combination of genes and plasmids. Indeed, the bla_{SHV-12} gene was identified in two isolates, carried in ST58 on an IncI1/pST3 plasmid and in ST38 on an IncF plasmid. The two bla_{CTX-M} genes were also alternatively carried by an IncI1/pST3 plasmid ($bla_{CTX-M-1}$ in ST162) or an IncF plasmid ($bla_{CTX-M-32}$ in ST155). IncI1/pST3 plasmids are major carriers of the $bla_{CTX-M-1}$ gene, and to a lesser extent, of bla_{SHV-12} [38]; they have recurrently been identified in the poultry production and food thereof [39,40], and in pets [6] and domestic animals [41], as well as in less antibiotic-exposed hosts such as storks [8]. Likewise, IncF are also major vectors of bla_{CTX-M} genes. As an example of the wide spread and adaptability of these plasmids, the IncF/F4:A-:B- plasmid carrying $bla_{CTX-M-1}$ in our study has also been associated with the epidemic ST131 *E. coli* clone carrying $bla_{CTX-M-14}$ in diarrhoeic patients in Japan [42]. It is notewor-

thy that our study evidenced the presence of bla_{SHV-12} on an IncF plasmid, which is a rare event [43].

The two IncI1 plasmids only carried the ESBL-conferring genes with no other associated resistance genes, which is a common feature for these plasmids. On the contrary, the two IncF plasmids showed an MDR phenotype associated with the presence of atypical class 1 integrons, embedded in both cases in Tn*21*, containing the gene cassettes *aadA22* for the $bla_{CTX-M-32}$-carrying ST155 and *aadA2-cmlA1-aadA1* for the bla_{SHV-12}-carrying ST38. Interestingly, the new structure of the atypical integron (*intI1-estX*-IS*1294-psp-aadA2-cmlA1-aadA1-qacL*-IS*1-sul3*) in the X5279 isolate presented similarities with those found integrated into Tn*21*-derived transposons in other bla_{SHV-12}-carrying IncI1 plasmids [44]. Since these transposons have extensively spread across Enterobacterales in several genetic backgrounds [39,44], we might hypothesize that the transposon in our X5279 isolate has been mobilized from IncI1 plasmids, which are also common carriers of the bla_{SHV-12} gene.

Analysis of the close genetic environment of the bla_{SHV-12} gene proved that this gene is usually flanked by an IS*26* and the putative transcriptional regulatory *deoR* gene, itself followed by a second IS*26* representing a putative composite transposon or another mobilizable element [43–45]. In our study, this organization was broadly comparable except for the left flank of the *deoR* gene, which was followed by Tn*1721*-like, located downstream of the bla_{SHV-12} gene [39]. In pUR5229, the *deoR* was truncated by a gene structure containing a DNA fragment preceded by IS*1294*, an insertion that might favour the block exchange of bla_{SHV-12}. The close genetic environment of the two bla_{CTX-M} genes was also conserved, with the presence of the *wbuC*-IS*Ecp1* element. This genetic environment has already been associated with diverse bla_{CTX-M} genes [46], including $bla_{CTX-M-15}$ and $bla_{CTX-M-55}$ [47], but, to our knowledge, this is its first description for $bla_{CTX-M-1}$. The genetic environment of $bla_{CTX-M-32}$ was identical to the one previously detected in dairy farms where IncHI2 plasmids harboured IS*Ecp1* with IS*Kpn26* in the opposite orientation [48]. The presence of these different types of gene platforms plays an important role in the mobility of ESBL genes and allows interchangeability at a very small scale. This proves multiple levels of transmissibility at the level of the genes, plasmids, or clones, reflecting diversified evolutionary processes.

In conclusion, this study revealed that ESBL-producing *E. coli* isolates were genetically diverse but carried highly similar plasmids and genes. The horizontal dissemination was primarily promoted by IncI1 and IncF plasmids, which displayed highly conserved co-located resistance genes. While this study provides significant information on the clones, genes, and plasmids that colonize white stork nestlings, additional studies involving a larger number of samples are now required to identify the sources of contamination and the pathways of transmission of these resistance determinants.

Funding: This work was financed in part by MCIN/AEI /10.13039/501100011033 of Spain and by project SBPLY/19/180501/000325 of the regional government of Castilla–La Mancha by the European Union's funds for regional development (FEDER).

Competing interests: S.M-A has a pre-doctoral fellowship from the Government of La Rioja (FPI–CAR–2021–3567).

Acknowledgements: We are grateful to the National Park Authority of Cabañeros National Park, landowners (La Puya, El Rostro), and RESIDUOS SOLIDOS URBANOS DE CASTILLA LA MANCHA (RSU) for authorisation of access to the stork nests on their premises, and to Jose Manuel Hernández for assistance in the ringing and handling of nestling storks. We acknowledge Idris N. Abdullahi, from the University of La Rioja, for the English proofreading.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.07.011.

References

[1] World Health Organization. Critically important antimicrobials for human medicine: 6th revision, https://www.who.int/publications/i/item/9789241515528; 2018 [Accessed 23 February 2023].
[2] Cantón R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, et al. Prevalence and spread of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in Europe. Clin Microbiol Infect 2008;14:144–53 Erratum in: Clin Microbiol Infect 2008;14:21–4. doi:10.1111/j.1469-0691.2007.01850.x.
[3] Zamudio R, Boerlin P, Beyrouthy R, Madec JY, Schwarz S, Mulvey MR, et al. Dynamics of extended-spectrum cephalosporin resistance genes in *Escherichia coli* from Europe and North America. Nat Commun 2022;13:7490. doi:10.1038/s41467-022-34970-7.
[4] European Food Safety AuthorityEuropean Centre for Disease Prevention and Control. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017. EFSA J 2019;17:e05598. doi:10.2903/j.efsa.2019.5598.
[5] Briñas L, Moreno MA, Teshager T, Sáenz Y, Porrero MC, Domínguez L, et al. Monitoring and characterization of extended-spectrum beta-lactamases in *Escherichia coli* strains from healthy and sick animals in Spain in 2003. Antimicrob Agents Chemother 2005;49:1262–4. doi:10.1128/AAC.49.3.1262-1264.2005.
[6] Haenni M, Saras E, Métayer V, Médaille C, Madec JY. High prevalence of $bla_{CTX-M-1}$/IncI1/ST3 and bla_{CMY-2}/IncI1/ST2 plasmids in healthy urban dogs in France. Antimicrob Agents Chemother 2014;58:5358–62. doi:10.1128/AAC.02545-14.
[7] Wang J, Ma ZB, Zeng ZL, Yang XW, Huang Y, Liu JH. The role of wildlife (wild birds) in the global transmission of antimicrobial resistance genes. Zool Res 2017;38:55–80. doi:10.24272/j.issn.2095-8137.2017.003.
[8] Höfle U, Jose Gonzalez-Lopez J, Camacho MC, Solà-Ginés M, Moreno-Mingorance A, Manuel Hernández J, et al. Foraging at solid urban waste disposal sites as risk factor for cephalosporin and colistin resistant *Escherichia coli* carriage in white storks (*Ciconia ciconia*). Front Microbiol 2020;11:1397. doi:10.3389/fmicb.2020.01397.
[9] López-Calderón C, Martín-Vélez V, Blas J, Höfle U, Sánchez MI, Flack A, et al. White stork movements reveal the ecological connectivity between landfills and different habitats. Mov Ecol 2023;11. doi:10.1186/s40462-023-00380-7.
[10] Zeballos-Gross D, Rojas-Sereno Z, Salgado-Caxito M, Poeta P, Torres C, Benavides JA. The role of gulls as reservoirs of antibiotic resistance in aquatic environments: a scoping review. Front Microbiol 2021;12. doi:10.3389/fmicb.2021.703886.
[11] Fuentes-Castillo D, Castro-Tardón D, Esposito F, Neves I, Rodrigues L, Fontana H, et al. Genomic evidences of gulls as reservoirs of critical priority CTX-M-producing *Escherichia coli* in Corcovado Gulf. Patagonia. Sci Total Environ 2023;874:162564. doi:10.1016/j.scitotenv.2023.162564.
[12] Bonnedahl J, Stedt J, Waldenström J, Svensson L, Drobni M, Olsen B. Comparison of extended-spectrum β-lactamase (ESBL) CTX-M genotypes in Franklin gulls from Canada and Chile. PLoS One 2015;10:e0141315. doi:10.1371/journal.pone.0141315.
[13] Stedt J, Bonnedahl J, Hernandez J, Waldenström J, McMahon BJ, Tolf C, et al. Carriage of CTX-M type extended spectrum β-lactamases (ESBLs) in gulls across Europe. Acta Vet Scand 2015;57:74. doi:10.1186/s13028-015-0166-3.
[14] Loncaric I, Stalder GL, Mehinagic K, Rosengarten R, Hoelzl F, Knauer F, et al. Comparison of ESBL-and AmpC producing *Enterobacteriaceae* and methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from migratory and resident population of rooks (*Corvus frugilegus*) in Austria. PLoS One 2013;8:e84048. doi:10.1371/journal.pone.0084048.
[15] Mohsin M, Raza S, Schaufler K, Roschanski N, Sarwar F, Semmler T, et al. High prevalence of CTX-M-15-Type ESBL-producing *E. coli* from migratory avian species in Pakistan. Front Microbiol 2017;8:2476. doi:10.3389/fmicb.2017.02476.
[16] Vergara A, Pitart C, Montalvo T, Roca I, Sabaté S, Hurtado JC, et al. Prevalence of extended-spectrum β-lactamase- and/or carbapenemase-producing *Escherichia coli* isolated from yellow-legged gulls from Barcelona, Spain. Antimicrob Agents Chemother 2017;61:e02071. doi:10.1128/AAC.02071-16.
[17] Sevilla E, Marín C, Delgado-Blas JF, González-Zorn B, Vega S, Kuijper E, et al. Wild griffon vultures (*Gyps fulvus*) fed at supplementary feeding stations: potential carriers of pig pathogens and pig-derived antimicrobial resistance? Transbound Emerg Dis 2020;67:1295–305. doi:10.1111/tbed.13470.
[18] Alcalá L, Alonso CA, Simón C, González-Esteban C, Orós J, Rezusta A, et al. wild birds, frequent carriers of extended-spectrum β-lactamase (esbl) producing *Escherichia coli* of CTX-M and SHV-12 types. Microb Ecol 2016;72:861–9. doi:10.1007/s00248-015-0718-0.
[19] Bécares J, Blas J, López-López P, Schulz H, Torres-Medina F, Flack A, et al. Migration and spatial ecology of the white stork in Spain. Migración y ecología espacial de la cigüeña blanca en España Monografía n° 5 del Programa Migra. SEO/BirdLife: Madrid:; 2019 http://hdl.handle.net/10261/217641.

[20] Clinical and Laboratory Standards Institute (CLSI) Performance Standards for Antimicrobial Susceptibility Testing. 32nd ed. Standards, USA: CLSI Supplement M100-Ed32. National Committee for Clinical Laboratory; 2022 https://clsi.org/standards/products/microbiology/documents/m100/.
[21] Martínez-Álvarez S, Sanz S, Olarte C, Hidalgo-Sanz R, Carvalho I, Fernández-Fernández R, et al. Antimicrobial resistance in *Escherichia coli* from the broiler farm environment, with detection of SHV-12-producing strains. Antibiotics (Basel) 2022;11:444. doi:10.3390/antibiotics11040444.
[22] Sáenz Y, Briñas L, Domínguez E, Ruiz J, Zarazaga M, Vila J, et al. Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. Antimicrob Agents Chemother 2004;48:3996–4001. doi:10.1128/AAC.48.10.3996-4001.2004.
[23] Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep 2013;5:58–65. doi:10.1111/1758-2229.12019.
[24] Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL. Threlfall EJ Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 2005;63:219–228. doi:10.1016/j.mimet.2005.03.018.
[25] Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 2017;13:e1005595. doi:10.1371/journal.pcbi.1005595.
[26] Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One 2014;9:e112963. doi:10.1371/journal.pone.0112963.
[27] Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: rapid annotations using subsystems technology. BMC Genomics 2008;9:75. doi:10.1186/1471-2164-9-75.
[28] Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, et al. Artemis: sequence visualization and annotation. Bioinformatics 2000;16:944–5. doi:10.1093/bioinformatics/16.10.944.
[29] Carver T, Thomson N, Bleasby A, Berriman M. Parkhill J DNAPlotter: circular and linear interactive genome visualization. Bioinformatics 2009;25:119–20. https://doi.org/10.1093/bioinformatics/btn578.
[30] Camacho M, Hernández JM, Lima-Barbero JF, Höfle U. Use of wildlife rehabilitation centres in pathogen surveillance: a case study in white storks (*Ciconia ciconia*). Prev Vet Med 2016;130:106–11. doi:10.1016/j.prevetmed.2016.06.012.
[31] Flament-Simon SC, Nicolas-Chanoine MH, García V, Duprilot M, Mayer N, Alonso MP, et al. Clonal structure, virulence factor-encoding genes and antibiotic resistance of *Escherichia coli*, causing urinary tract infections and other extraintestinal infections in humans in Spain and France during 2016. Antibiotics (Basel) 2020;9:161. doi:10.3390/antibiotics9040161.
[32] Fonseca EL, Morgado SM, Caldart RV, Vicente AC. Global genomic epidemiology of *Escherichia coli* (ExPEC) ST38 lineage revealed a virulome associated with human infections. Microorganisms 2022;10. doi:10.3390/microorganisms10122482.
[33] González-Santamarina B, Weber M, Menge C, Berens C. Comparative genomic analysis of antimicrobial-resistant *Escherichia coli* from South American camelids in Central Germany. Microorganisms 2022;10. doi:10.3390/microorganisms10091697.
[34] Brendecke J, Homeier-Bachmann T, Schmitz Ornés A, Guenther S, Heiden SE, Schwabe M, et al. Multidrug-resistant high-risk *Escherichia coli* and *Klebsiella pneumoniae* clonal lineages occur in black-headed gulls from two conservation islands in Germany. Antibiotics (Basel) 2022;11:1357. doi:10.3390/antibiotics11101357.
[35] Foster-Nyarko E, Alikhan NF, Ravi A, Thomson NM, Jarju S, Kwambana-Adams BA, et al. Genomic diversity of *Escherichia coli* isolates from backyard chickens and guinea fowl in the Gambia. Microb Genom 2021;7:mgen000484. doi:10.1099/mgen.0.000484.
[36] Skurnik D, Clermont O, Guillard T, Launay A, Danilchanka O, Pons S, et al. Emergence of antimicrobial-resistant *Escherichia coli* of animal origin spreading in humans. Mol Biol Evol 2016;33:898–914. doi:10.1093/molbev/msv280.
[37] Haenni M, Métayer V, Jarry R, Drapeau A, Puech MP, Madec JY, et al. Wide spread of $bla_{CTX-M-9}$/*mcr-9* IncHI2/ST1 plasmids and CTX-M-9-producing *Escherichia coli* and *Enterobacter cloacae* in rescued wild animals. Front Microbiol 2020;11:601317. doi:10.3389/fmicb.2020.601317.
[38] Madec JY, Haenni M. Antimicrobial resistance plasmid reservoir in food and food-producing animals. Plasmid 2018;99:72–81. doi:10.1016/j.plasmid.2018.09.001.
[39] Alonso CA, Michael GB, Li J, Somalo S, Simón C, Wang Y, et al. Analysis of bla_{SHV-12}-carrying *Escherichia coli* clones and plasmids from human, animal and food sources. J Antimicrob Chemother 2017;72:1589–96. doi:10.1093/jac/dkx024.
[40] Casella T, Nogueira MCL, Saras E, Haenni M, Madec JY. High prevalence of ESBLs in retail chicken meat despite reduced use of antimicrobials in chicken production, France. Int J Food Microbiol 2017;257:271–5. doi:10.1016/j.ijfoodmicro.2017.07.005.
[41] Lupo A, Haenni M, Saras E, Gradin J, Madec JY, Börjesson S. Is $bla_{CTX-M-1}$ riding the same plasmid among horses in Sweden and France? Microb Drug Resist 2018. doi:10.1089/mdr.2017.0412.
[42] Hayashi M, Matsui M, Sekizuka T, Shima A, Segawa T, Kuroda M, et al. Dissemination of IncF group F1:A2:B20 plasmid-harbouring multidrug-resistant *Escherichia coli* ST131 before the acquisition of bla_{CTX-M} in Japan. J Glob Antimicrob Resist 2020;23:456–65. doi:10.1016/j.jgar.2020.10.021.
[43] Liakopoulos A, Mevius D, Ceccarelli D. A review of SHV extended-spectrum β-lactamases: neglected yet ubiquitous. Front Microbiol 2016;7:1374. doi:10.3389/fmicb.2016.01374.
[44] Curiao T, Cantón R, Garcillán-Barcia MP, de la Cruz F, Baquero F, Coque TM. Association of composite IS*26*-*sul3* elements with highly transmissible IncI1 plasmids in extended-spectrum beta-lactamase-producing *Escherichia coli* clones from humans. Antimicrob Agents Chemother 2011;55:2451–7. doi:10.1128/AAC.01448-10.
[45] Castellanos LR, van der Graaf-van Bloois L, Donado-Godoy P, Mevius DJ, Wagenaar JA, Hordijk J, et al. Phylogenomic investigation of IncI1-Iγ plasmids harboring bla_{CMY-2} and bla_{SHV-12} in *Salmonella enterica* and *Escherichia coli* in multiple countries. Antimicrob Agents Chemother 2019;63:e02546. doi:10.1128/AAC.02546-18.
[46] Tian SF, Chu YZ, Chen BY, Nian H, Shang H. IS*Ecp1* element in association with $bla_{(CTX-M)}$ genes of *E. coli* that produce extended-spectrum β-lactamase among the elderly in community settings. Enferm Infecc Microbiol Clin 2011;29:731–4. doi:10.1016/j.eimc.2011.07.011.
[47] Moser AI, Kuenzli E, Campos-Madueno EI, Büdel T, Rattanavong S, Vongsouvath M, et al. Antimicrobial-resistant *Escherichia coli* strains and their plasmids in people, poultry, and chicken meat in Laos. Front Microbiol 2021;12:708182. doi:10.3389/fmicb.2021.708182.
[48] Findlay J, Mounsey O, Lee WWY, Newbold N, Morley K, Schubert H, et al. Molecular epidemiology of *Escherichia coli* producing CTX-M and pAmpC β-lactamases from dairy farms identifies a dominant plasmid encoding CTX-M-32 but no evidence for transmission to humans in the same geographical region. Appl Environ Microbiol 2020;87. doi:10.1128/AEM.01842-20.