



Carriage of ESBL-producing Enterobacterales in urban and rural Hooded Crows in Hungary

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ABSTRACT

Background: Considering the increasing reports of antimicrobial resistance (AMR) in wildlife, highlighting its complexity, importance, and spread. We investigated the prevalence of extended-spectrum beta-lactamase (ESBL)-producing Enterobacterales in the hooded crow.

Methods: Faecal samples were collected from 52 rural and 212 urban wild crows in Hungary, caught using ladder traps, and tested for ESBL presence.

Bacterial species were identified using MALDI-TOF. Antibiotic susceptibility was tested using the disc diffusion method, and ESBL producers were detected based on double-disc synergy. ESBL-encoding genes were identified using PCR, and WGS was performed on isolated ESBL-producing *E. coli* (197/221 isolated ESBLs).

Results: Four of the sampled rural Hooded Crows and 130 urban ones (7.7% vs. 61%, chi-square $p < 0.0001$) yielded ESBL producers with the overwhelming dominance of *E. coli*. The *bla*_{CTX-M-1} group was predominant in both groups. In addition to CTX-M genes, genes encoding resistance to other antibiotic classes, such as *APHs*, *sul* genes, *tet* genes, etc., various virulence factors, and several incompatible plasmids were also detected. Most isolates belonged to the B1 and A phylogenetic groups. Overall, 22 sequence types (STs) and 33 distinct cgSTs were defined. The most prevalent ST was ST58, followed by ST10, S38, ST155, ST442, and more.

Conclusions: The much higher carrier frequency among urban crows points to the role of anthropogenic sources in the emergence of ESBL producers. Hooded Crows, due to their increasing presence in cities and proximity to humans, likely facilitate the dissemination of ESBL producers between the environment and humans.

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1. Introduction

The One Health concept recognises the interconnectedness of human, animal, and environmental health and promotes collaboration across these sectors to address shared health challenges. Among these, antimicrobial resistance (AMR) has emerged as one of the most pressing global threats, with the World Health Organization identifying it as a major One Health concern [1]. Lately, AMR has been increasingly reported in wildlife, with the latter being recognised as an important component of AMR ecology as wild

animals can act as reservoirs, sentinels, and potential vectors of resistant bacteria and resistance genes [2–6].

AMR bacteria were reported in the wild for the first time in 1975, when Sato (1978) isolated multiresistant *Escherichia coli* (*E. coli*) strains from wild pigeons. *E. coli* [7]. As a common commensal of the intestinal microbiota of humans and animals, *E. coli* is widely used as an indicator organism for monitoring AMR in both clinical and environmental settings [8–10]. Of particular concern is resistance to third-generation cephalosporins mediated by extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases. ESBL genes are frequently associated with mobile genetic elements, including plasmids and transposons, facilitating their dissemination among bacterial populations and across ecological niches [6,10,11].

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We aim to explore the occurrence of ESBL-producing Enterobacterales in the Hooded Crow (*Corvus cornix*, Linnaeus 1758), a urban-adapted, predominantly sedentary species that has established increasing populations in Hungarian cities [12]. Its close association with human settlements and frequent contact with anthropogenic food sources make it a suitable sentinel for investigating environmental dissemination of AMR. Despite reports of multidrug-resistant Gram-negative bacteria in other corvid species [13–15], information on ESBL-producing Enterobacterales in Hooded Crows remains lacking.

Therefore, this study investigated the occurrence and genomic characteristics of ESBL-producing Enterobacterales in free-ranging Hooded Crows from urban and rural areas of Hungary. We compared the prevalence of ESBL carriage between urban and rural birds and characterised the isolates using whole-genome sequencing to examine their resistance genes, sequence types, plasmid content, virulence-associated genes, and phylogenetic relationships. By doing so, we aimed to improve our understanding of the role of urban wildlife in the environmental dissemination of clinically relevant antimicrobial resistance within a One Health framework.

2. Material and methods

2.1. Sample collection

Free-ranging Hooded Crows (*Corvus cornix*) were captured between March and November 2020 using ladder entrance traps [16] in urban (Budapest and Debrecen) and rural areas (near Balmazújváros and Szakoly) in Hungary. Traps were baited with live decoys and food and monitored regularly. A total of 264 birds were sampled, comprising 212 urban and 52 rural individuals. Fresh faecal samples were collected using sterile cotton swabs in the Stuart transport medium and transported immediately to the Medical Microbiology Laboratory, University of Debrecen. No recaptures occurred during the study.

2.2. Bacterial isolation

Samples were cultured on eosin methylene blue agar supplemented with 2 mg/L cefotaxime to recover third-generation cephalosporin (3GC)-resistant isolates. Colonies with distinct morphologies from each sample were collected, processed, and identified using matrix-assisted laser desorption ionisation (MALDI)-time of flight (TOF) mass spectrometry (Bruker, Bremen, Germany). ESBL-producing isolates were identified by a double-disc synergy test using cefotaxime, ceftazidime, and cefepime. Susceptibility to carbapenem (i.e., ertapenem), fluoroquinolones (i.e., ciprofloxacin), aminoglycosides (i.e., amikacin, gentamicin, and tobramycin), and the trimethoprim-sulfamethoxazole combination was determined by the disc diffusion method following the EUCAST guidelines.

2.3. Resistance gene characterisation

Each isolate showing an ESBL phenotype was screened using PCR for *bla_{SHV}* and *bla_{TEM}* groups and for CTX-M-1, 2, 8, and 9 subgroups [17,18]. PCR products were resolved on 1.5% agarose gels and visualised using a Gel Doc XR imaging system with Image Lab v6.1 software (Bio-Rad).

2.3.1. Genomic DNA isolation and whole genome sequencing

All 197 isolated ESBL-producing *E. coli* were sequenced. Purified genomic DNA was extracted using the Quick-DNA Fungal/bacterial Miniprep Kit D6005 (ZYMO RESEARCH, USA), following the manufacturer's instructions.

Illumina-specific libraries were prepared using the Illumina Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA,

USA) and the Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA).

2.3.2. Genome assembly, AMR and virulence factors' identification, genetic diversity and relatedness of the strains

Raw Illumina reads were quality assessed, filtered, error-corrected, and assembled using FastQC, FastQP, Bloocoo, Unicycler, MEGAHIT, and GAM-NGS [19–21]. Assembly quality, completeness, and taxonomic purity were assessed using QUAST v5.2.0, CheckM, and Kraken2 with the k2_plusfp database [22,23]. AMR genes, sequence types, serotypes, plasmid replicons, virulence genes, cgMLST types, and phylogroups were identified using ABRicate (CARD; <https://github.com/tseemann/abricate>), mlst (<https://github.com/tseemann/mlst>), ECTyper (https://github.com/phac-nml/ecoli_serotyping), PlasmidFinder 2.1, VirulenceFinder 2.0, cgMLSTFinder 1.2, available from the cgE (Centre for Genomic Epidemiology), and ClermontTyping (<http://clermonttyping.iame-research.center/>) [24–31]. The genetic environments surrounding representative *bla_{CTX-M}* genes were manually inspected using RAST annotation and SnapGene v6.2 (<https://www.snapgene.com/>) to identify neighbouring genes and mobile genetic elements [32,33]. Finally, to predict the genomic origin (plasmid or chromosome) of *bla_{CTX-M}*-containing contigs, assemblies were analysed using the mlplasmids web-server application (<https://sarredondo.shinyapps.io/mlplasmids/>) [34].

3. Results

3.1. Occurrence and characteristics of ESBL-producing Enterobacterales in Hooded Crows

Overall, 51% (135/264) of the sampled Hooded Crows carried ESBL-producing Enterobacterales, including four rural birds (7.7%, 4/52) and 130 urban birds (61%, 130/212). ESBL-producers were detected in 108 birds from Debrecen (61.4%, 108/176) and 20 from Budapest (48.8%, 20/41). A total of 221 ESBL isolates were recovered, of which 197 (89.1%) were *E. coli*. The *bla_{CTX-M-1}* group were predominant (218/221), followed by *bla_{SHV}* and *bla_{CTX-M-9}*, found in two samples each.

While these baseline prevalence data define the scope of resistance within our dataset, a deeper investigation into their specific genomic architectures is required to elucidate how these elements are being maintained and disseminated.

3.2. Characteristics of ESBL-producing *E. coli*

Among the 197 phenotypically confirmed ESBL-producing *E. coli* isolates, whole-genome sequencing identified *bla_{CTX-M}* genes in 187 (94.9%) isolates. *bla_{CTX-M-1}* was the predominant allele (153/197), followed by *bla_{CTX-M-55}* (20/197), *bla_{CTX-M-15}* (10/197), and to a lesser extent, *bla_{CTX-M-32}* (3/197). While *bla_{CTX-M-14}* was found in a single isolate. CTX-M Groups 2, 8, and 9 were not detected. In the remaining ten isolates, WGS did not detect *bla_{CTX-M}* genes. Instead, five carried *bla_{CMY-59}*, three carried AmpC β -lactamase genes, one carried *bla_{TEM-1}*, and one lacked any detectable β -lactamase gene despite phenotypic ESBL confirmation and PCR detection of the *bla_{CTX-M-1}* group (Supplementary Table S1).

The 197 ESBL-producing *E. coli* isolates belong to 22 sequence types (ST) (Table 1). ST58 was the most prevalent (34/197), while the pandemic ST131 lineage was not detected. Coregenome (cg) MLST identified 33 distinct cgSTs, demonstrating considerable genomic diversity (Fig. 1). Nevertheless, closely related isolates belonging to the same ST were recovered from both Budapest and Debrecen.

Most ESBL isolates carried by Hooded Crows belonged to commensal phylogenetic groups (Table 2), primarily phylogenetic

Table 1
Distribution of the most frequent STs and cgSTs.

ST	cgST	ESBL genes	adult (%)	juvenile (%)	Budapest (%)	Debrecen (%)	Balmoazujvaros (%)
10	177,042	<i>bla</i> TX-M-55	–	3 (1.8%)	–	3 (1.7%)	–
10	196,882	<i>bla</i> CTX-M-1	–	8 (5.4%)	–	8 (4.5%)	–
10	85,754	<i>bla</i> CTX-M-1	–	1 (0.6%)	–	1 (0.6%)	–
38	52,633	<i>bla</i> CTX-M-15	–	2 (1.2%)	2 (12.5%)	–	–
57	4209	<i>bla</i> CTX-M-1	–	1 (0.6%)	–	1 (0.6%)	–
58	45,951	<i>bla</i> CTX-M-55	–	11 (6.5%)	–	11 (6.1%)	–
58	46,030	<i>bla</i> CTX-M-1	1 (3.4%)	22 (13.1%)	1 (6.3%)	22 (12.4%)	–
106	26,761	<i>bla</i> CTX-M-14	–	1 (0.6%)	–	1 (0.6%)	–
117	85,789	<i>bla</i> CTX-M-55	1 (3.4%)	–	1 (6.3%)	–	–
155	9117	<i>bla</i> CTX-M-1	1 (3.4%)	–	–	1 (0.6%)	–
155	27,078	<i>bla</i> CTX-M-1	2 (6.9%)	–	–	2 (1.1%)	–
155	81,555	<i>bla</i> CTX-M-15	3 (10.3%)	–	–	–	3 (75%)
155	136,648	<i>bla</i> CTX-M-1	1 (3.4%)	6 (3.6%)	–	7 (3.9%)	–
155	169,654	<i>bla</i> CTX-M-1	–	4 (2.4%)	–	4 (2.3%)	–
162	171,743	<i>bla</i> CTX-M-55	–	1 (0.6%)	–	–	1 (25%)
372	–	<i>bla</i> CTX-M-1	–	1 (0.6%)	–	1 (0.6%)	–
442	28,154	<i>bla</i> CTX-M-1	5 (17.2%)	28 (16.7%)	2 (12.5%)	31 (17.5%)	–
515	198,763	<i>bla</i> CTX-M-55	–	4 (2.4%)	–	4 (2.3%)	–
515	198,768	<i>bla</i> CTX-M-55	–	1 (0.6%)	–	1 (0.6%)	–
540	96,445	<i>bla</i> CTX-M-15	–	4 (2.4%)	–	4 (2.3%)	–
746	130,005	<i>bla</i> CTX-M-1	–	1 (0.6%)	–	1 (0.6%)	–
1288	184,707	<i>bla</i> CTX-M-15	1 (3.4%)	–	1 (6.3%)	–	–
1665	210,846	<i>bla</i> CTX-M-1	–	4 (1.8%)	3 (1.9%)	1 (0.6%)	–
1300	203,111	<i>bla</i> CTX-M-1	1 (3.4%)	–	–	1 (0.6%)	–
1718	143,413	<i>bla</i> CTX-M-1	6 (20.7%)	27 (16.1%)	–	33 (18.6%)	–
1723	56,789	<i>bla</i> CTX-M-1	4 (13.8%)	1 (0.6%)	–	5 (2.8%)	–
2485	161,907	<i>bla</i> CTX-M-1	–	10 (6.0%)	1 (6.3%)	9 (5.1%)	–
4421	44,522	<i>bla</i> CTX-M-1	–	6 (4.2%)	–	6 (3.4%)	–
5797	4894	<i>bla</i> CTX-M-1	–	9 (5.4%)	2 (12.5%)	7 (4.0%)	–
Unidentified	28,154	<i>bla</i> CTX-M-1	–	2 (1.2%)	–	2 (1.1%)	–
Unidentified	44,522	<i>bla</i> CTX-M-1	1 (3.4%)	–	–	1 (0.6%)	–
Unidentified	76,074	<i>bla</i> CTX-M-1	–	1 (0.6%)	–	1 (0.6%)	–
Unidentified	100,607	<i>bla</i> CTX-M-32	1 (3.4%)	–	1 (6.3%)	–	–
Unidentified	143,413	<i>bla</i> CTX-M-1	–	1 (0.6%)	–	1 (0.6%)	–
Unidentified	169,654	<i>bla</i> CTX-M-1	–	1 (0.6%)	–	1 (0.6%)	–
Unidentified	186,348	–	–	1 (0.6%)	–	1 (0.6%)	–
Unidentified	196,882	<i>bla</i> CTX-M-1	–	1 (0.6%)	–	1 (0.6%)	–
Unidentified	–	<i>bla</i> CTX-M-32	–	2 (0.6%)	2 (12.5%)	–	–
all STs			29 (100.0%)	168 (100.0%)	16 (100.0%)	177 (100.0%)	4 (100.0%)

An en-dash (–) indicates that no STs were detected (0.0%).

groups B1 (112/197) and A (55/197). A lesser number of isolates belonged to phylogenetic groups E (12/197), F (9/197), and D (3/197). One isolate each belonged to phylogroups B2 and G. Four strains were not affiliated with any phylogenetic group. Additionally, our finding showed that the four isolates from rural areas all belonged to the phylogenetic group B1, whereas the four *E. coli* isolates from Budapest belonged to groups B1 and A. Moreover, overall, 21 different serotypes were predicted for the 197 analysed ESBL producers. Eighty isolates had nondetectable O types and 14 different H types. For six isolates, the serotype was not determined.

Although our work focused mainly on ESBLs, additional resistance patterns to different antibiotic classes were also found in our isolates. For instance, genes conferring resistance to aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole, and additional β -lactams were frequently detected, particularly among isolates carrying *bla*CTX-M-1 and *bla*CTX-M-55 (Table 3).

Analysis of the genome assemblies identified ten plasmid incompatibility groups. IncI1-I (Alpha) (71.6%) and IncFII (49.0%) were the most common, whereas no plasmid replicons were detected in seven genomes (3.6%) (Table 3). Because short-read assemblies do not permit definitive plasmid reconstruction, physical linkage between *bla*CTX-M genes and specific plasmids could not be confirmed. Nevertheless, several *bla*CTX-M-containing contigs also contained IncI1-associated backbone genes, including conjugative transfer, partitioning, and shufflon-associated genes (Fig. 2). These findings suggest an association of *bla*CTX-M genes with

IncI1-like plasmids, although definitive plasmid assignment would require complete plasmid reconstruction.

Nonetheless, to systematically evaluate the genomic localisation of the ESBL-encoding genes across the entire cohort ($n = 187$), all assemblies were analysed using the machine learning classifier mlplasmids. mlplasmids predicted 139 (74.3%) *bla*CTX-M-containing contigs to be plasmid-derived and 48 (25.7%) to be chromosomal (Supplementary Material 2). Chromosomal localisations included *bla*CTX-M-1, *bla*CTX-M-14, *bla*CTX-M-15, and *bla*CTX-M-55 – and were detected across multiple sequence types. Interestingly, the presence of eight isolates containing *bla*CTX-M genes and plasmid replicon markers on the same contig, despite the contig being classified as chromosomal by mlplasmids, indicates a possible chromosomal integration of plasmid-derived DNA.

3.3. Virulence factors

Hooded Crow isolates harboured a diverse repertoire of virulence-associated genes. The prevalence of virulence genes ranged from 0% (PAI, vat, yfcV, stx1, stx2, and ipaH) to 97% (191/197) for *terC*. Genes commonly associated with plasmid-mediated virulence in avian pathogenic *E. coli* (APEC), including *hlyE* (93.4%), *iss* (78.2%), *ompT* (75.6%), *hlyF* (56.3%), together with *iroN*, *iucC*, *iutA*, *cvaC*, and *cib*, were frequently detected. In contrast, virulence genes commonly associated with extraintestinal pathogenic *E. coli* (ExPEC), particularly uropathogenic *E. coli* (UPEC), were de-

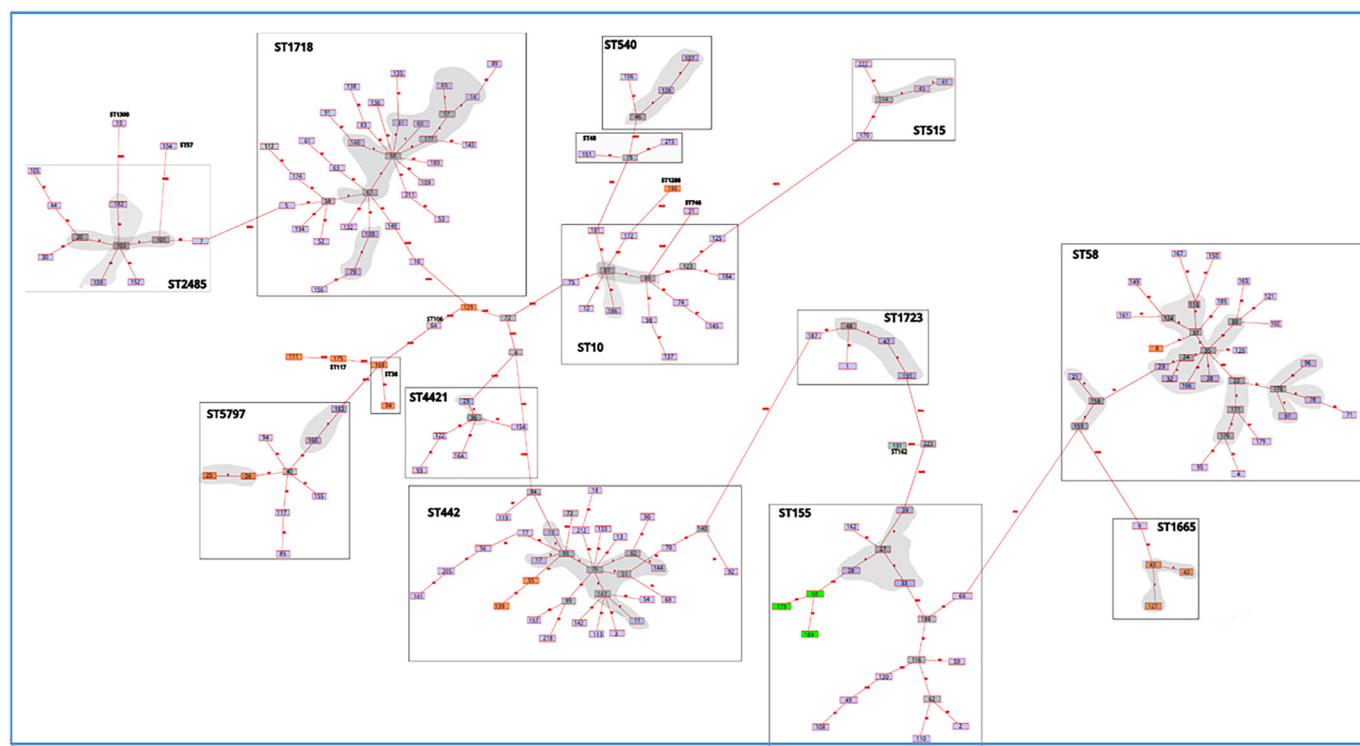


Fig. 1. Minimum spanning tree based on cgMLST allelic profiles of 197 sequenced ESBL-producing *E. coli* isolates. Each square represents an allelic profile based on sequence analyses of 2513 cgMLST target genes. The number on the connecting lines illustrate the number of target genes with different alleles in a pairwise comparison. The squares are named with isolate ID numbers and coloured according to their sampling area (grey = Debrecen, orange = Budapest, and green = Balmazújvaros). Closely related genotypes are shaded (<10 allele differences).

ected at much lower frequencies with *chuA*, *fyuA*, *irp2*, and *ibeA* detected in 13.2%, 19.8%, 19.8%, and 4.6% of isolates, respectively. Notably, neither *vat* nor *yfcV* was detected. Previous studies have shown that the cocarriage of *chuA*, *fyuA*, *vat*, and *yfcV* is strongly associated with efficient urinary tract colonisation and is considered a characteristic UPEC virulence signature [35]. Therefore, although a subset of our isolates carried individual ExPEC-associated virulence genes, the characteristic UPEC-associated virulence profile defined by the co-occurrence of *chuA*, *fyuA*, *vat*, and *yfcV* was absent from the dataset. This observation is consistent with the concept that the pathogenic potential of ExPEC strains depends on the accumulation of multiple complementary virulence determinants rather than the presence of individual genes alone [36].

4. Discussion

In this study, ESBL-producing *E. coli* occurred in 61% of Hooded Crows sampled, representing the first report in this species. This prevalence exceeds that reported in wintering rooks (33%) and Human faecal samples (1.7%) [15], as well as poultry (34.2%) [37], yet is slightly lower than porcine isolates [37] in Hungary. The marked difference between urban and rural birds (7.7% vs. 61%, respectively, $p < 0.0001$) strongly supports anthropogenic exposure as the primary driver of ESBL acquisition. Similar urban–rural contrasts have been widely reported in wildlife [37–41] and are generally attributed to environmental contamination with resistant bacteria originating from humans and livestock [15,42–45].

The *bla*_{CTX-M-1} gene was the predominant ESBL-encoding gene, followed by *bla*_{CTX-M-55} and *bla*_{CTX-M-15}, respectively. The prevalence of other ESBL types was rare. The genetic characteristics of ESBL-producing *E. coli* in Hooded Crows are, to some extent, similar to the characteristics of human isolates. In Hungary, human-isolated ESBL-producing *E. coli* carried predominantly *bla*_{CTX-M-15}

genes [17,18], while *bla*_{CTX-M-1} was reported to be the most common in farm animal isolates in the country [37,46], implying that Hooded Crow populations might potentially acquire resistance from human-related activities. Additionally, wild birds are the most studied wildlife hosts, where the most frequently found genes are *bla*_{CTX-M-1} and *bla*_{CTX-M-15} [6,47,48]. *bla*_{CTX-M-1} and *bla*_{CTX-M-15} were previously found dominant in rooks wintering in Europe [14,49] as well as gulls [50,51]. More recent European studies also reported the prevalence of *bla*_{CTX-M-55} in rooks [14,15,43] and gulls [51], a generally infrequent ESBL type in Europe, highlighting the role of bird migration in the introduction of novel genes to an area. Interestingly, the prevalence of *bla*_{CTX-M-55} in our Hooded Crows is noteworthy, considering their sedentary nature. We speculate that these birds acquire strains carrying ESBL genes prevalent in humans and animals from their surroundings, including visiting rooks.

The ESBL-producing isolates displayed high sequence-type diversity, with most STs previously associated with humans and animals [52], including globally disseminated strains like ST10, ST38, ST58, ST155, ST442, and ST746 [53–57]. Interestingly, only one isolate was identified as ST162, a major type sequence amongst wintering rooks in Europe [15] that was previously reported to cause infection in livestock and other farm animals [58]. The predominance of ST1718 (33/197) is particularly notable, as this lineage has rarely been reported and warrants further investigation [59]. Overall, the diversity of STs suggests repeated acquisition from multiple environmental sources rather than expansion of a single clone.

Yet, despite the overlapping STs of our crows and previously reported human and animal isolates, no such similarities were observed in the coregenome (cg) MLST revealed. Here, Hooded Crow isolates exhibited considerable coregenome diversity, comprising 33 cgMLST types. Multiple cgMLST lineages were detected within individual STs, indicating independent introductions rather than

Table 2
ESBL-producing *E. coli* present in the Hooded Crow (ONT= non-detectable O type).

STs	cgMLST	ESBL genes	Associated serotype	Number of isolates
10	196,882	<i>bla</i> CTX-M-1	ONT:H32	8
	177,042	<i>bla</i> CTX-M-55		3
	85,754	<i>bla</i> CTX-M-1		1
38	52,633	<i>bla</i> CTX-M-15	ONT:H12	2
57	4209	<i>bla</i> CTX-M-1	ONT:H26	1
58	46,030	<i>bla</i> CTX-M-1	O8:H25	23
	45,951	<i>bla</i> CTX-M-55	O8:H10	11
106	26,761	<i>bla</i> CTX-M-14	O15:H1	1
117	85,789	<i>bla</i> CTX-M-55	O114:H4	1
	136,648	<i>bla</i> CTX-M-1	O109:H51	8
	16,956	<i>bla</i> CTX-M-1	O116:H9	4
155	81,555	<i>bla</i> CTX-M-15	O9:H10	3
	27,078	<i>bla</i> CTX-M-1	ONT:H21	2
	9117	<i>bla</i> CTX-M-1	O9:H51	1
162	171,743	<i>bla</i> CTX-M-55	O8:H19	1
442	28,145	<i>bla</i> CTX-M-1	ONT:H21	27
		<i>bla</i> CTX-M-1	O91:H21	5
		<i>bla</i> CTX-M-1	O9:H21	1
515	198,763	<i>bla</i> CTX-M-55	O98:H2	5
540	96,445	<i>bla</i> CTX-M-15	O9:H30	4
746	130,005	<i>bla</i> CTX-M-1	ONT:H19	1
1288	184,707	<i>bla</i> CTX-M-15	O9:H9	1
1300	203,111	<i>bla</i> CTX-M-1	–	1
1665	210,846	<i>bla</i> CTX-M-1	O162/O101:H7	4
1718	143,413	<i>bla</i> CTX-M-1	O9:H31	33
1723	56,789	<i>bla</i> CTX-M-1	O141:H16	5
2485	161,907	<i>bla</i> CTX-M-1	O4:H45	9
		<i>bla</i> CTX-M-1	ONT:H47	1
		<i>bla</i> CTX-M-1	ONT:H7	5
4421	44,522	<i>bla</i> CTX-M-1	ONT:H42	9
5797	4894	<i>bla</i> CTX-M-1	ONT:H21	2
UnKnown	28,154	<i>bla</i> CTX-M-1	O91:H7	1
	44,522	<i>bla</i> CTX-M-1	ONT:H21	1
	76,074	<i>bla</i> CTX-M-1	O9:H21	1
	143,413	<i>bla</i> CTX-M-1	–:H9	1
	169,654	<i>bla</i> CTX-M-1	O69:H32	1
	196,882	<i>bla</i> CTX-M-1	O8:H6	1
	100,607	<i>bla</i> CTX-M-32	–	2
	–	<i>bla</i> CTX-M-32	–	2
	–	<i>bla</i> CTX-M-32	–	2
	–	<i>bla</i> CTX-M-32	–	2

clonal spread. Nevertheless, genetically related isolates were identified in both Budapest and Debrecen (ST58 cgMLST46030; ST442 cgMLST28154; ST1665 cgMLST 210,846; ST1718 csMLST143413; ST2485 cgMLST161907), suggesting occasional movement of strains between urban populations or widespread environmental dissemination. Although several STs appeared more frequently in juveniles, these differences were not statistically significant and likely reflect the limited number of adult samples (29 adult samples vs. 168 juvenile ones, and three of the adult samples are from the rural areas).

On the other hand, the absence of genetic similarities between Hooded Crow isolates and previously reported human and wintering rooks in Hungary [15], and the high diversity of STs could suggest the dissemination of genes encoding ESBLs rather than clonal transmission of resistant bacteria. ESBL-encoding genes transfer easily between bacteria through mobile genetic elements, mainly via horizontal transfer of plasmids [60]. This is highlighted by the association of *bla*CTX-M genes with a wide range of plasmid replicons previously reported [55,61]. For instance, the association of *bla*CTX-M-55 - IncN replicon type is noteworthy, since a similar observation was recently reported in wintering rooks in Hungary [15]. Such observations reinforce the idea that Hooded Crows might have picked up ESBL-encoding genes from a direct or indirect interaction with such rooks. Furthermore, our findings revealed that *bla*CTX-M-containing contigs share a high degree of similarity among the strains with the same STs. Plasmid prediction revealed that in the majority of the strains, plasmids like IncI1 were located near or in the same contigs as ESBL genes, indicating the potential plasmid-mediated transmission of these ESBL genes.

Interestingly, the demonstration of *bla*CTX-M variants—specifically *bla*CTX-M-1, *bla*CTX-M-14, *bla*CTX-M-15, and *bla*CTX-M-55—on both plasmid vectors and the host chromosome reveals a highly flexible evolutionary strategy within the studied *E. coli* population. While the majority of isolates maintain these ESBL genes on plasmids (such as the highly conserved IncI1 structures identified in our dataset), the clear presence of chromosomal integration across multiple distinct lineages indicates a significant shift in resistance dynamics.

While plasmid localisation promotes horizontal transfer, chromosomal integration ensures stable vertical inheritance and persistence even in the absence of antibiotic selection. The occurrence of chromosomal *bla*CTX-M genes in multiple unrelated lineages suggests that this event has arisen independently rather than through expansion of a single clone, highlighting the establishment of stable environmental reservoirs of ESBL resistance.

Hooded Crow isolates had a high prevalence of several Virulence Factors (VFs) encoded by genes including *hlyE*, *hlyF*, *iss*, *iroN*, *gad*, *cib*, *cvaC*, *ompT*, *mch* B,C,F, *lpfA*, *iucC*, *iutA*, and *afaD*, similar to previous reports in faecal *E. coli* [62,63]. Among the different VFs found, various factors were previously associated with avian pathogenic *E. coli* (APEC) as well as human ExPEC strains, which were already reported in poultry as well as wild birds [64,65]. The presence of isolates harbouring several key virulence genes (*iss*, *iroN*, *hlyF*, *ompT*, and *iutA*), such as ST10 and ST38, suggests that the Hooded Crow-derived isolates might be reservoirs of APEC and ExPEC virulence genes. APEC and ExPEC strains reportedly have similar virulence markers, such as different adhesins, invasins, or iron uptake systems [58,66], and neither pathotype has a spe-

Table 3
Resistance genes harboured by ESBL *E. coli* from Hooded Crows.

Area	ST	CgMLST	ESBL genes	Other genes						Plasmid	N° of isolates
				AMG	TMP	SA	Phe	Tet	FQ		
Debrecen	10	196,882	<i>bla</i> CTX-M-1							IncFIA; IncFIB(pB171); IncI1-I(Alpha)	9
Debrecen	10	85,754	<i>bla</i> CTX-M-1							IncFIA; IncI2 IncN	1
Debrecen	10	177,042	<i>bla</i> CTX-M-55	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>					<i>QnrS1</i>		1
Debrecen	10	177,042	<i>bla</i> CTX-M-55	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>					<i>QnrS1</i>	–	2
Budapest	38	52,633	<i>bla</i> CTX-M-15	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>	<i>sul2</i>	<i>dfrA14</i>		<i>tet(A)</i>	<i>QnrS1</i>	IncY	2
Debrecen	58	46,030	<i>bla</i> CTX-M-1	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>	<i>sul2</i>	<i>dfrA5</i>				IncFIB(AP001918); IncI1-I(Alpha); IncQ1	1
Budapest	58	46,030	<i>bla</i> CTX-M-1, <i>bla</i> TEM-1	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>	<i>sul2</i>					IncFIB(AP001918); IncFII(pSE11); IncI1-I(Alpha); IncQ1	1
Debrecen	58	46,030	<i>bla</i> CTX-M-1, <i>bla</i> TEM-1	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>	<i>sul2</i>	<i>dfrA5</i>				IncFIB(AP001918); IncI1-I(Alpha); IncQ1	21
Debrecen	58	45,951	<i>bla</i> CTX-M-55	<i>AAC(3)-IId</i> <i>ANT(3'')-Iia</i> <i>APH(3'')-Ib</i> <i>APH(6)-Id</i>	<i>sul2</i>		<i>floR</i>	<i>tet(A)</i>	<i>QnrS1</i>	IncFIA; IncFIB(AP001918)	1
Debrecen	58	45,951	<i>bla</i> CTX-M-55	<i>AAC(3)-IId</i>					<i>QnrS1</i>	IncFIA	1
Debrecen	58	45,951	<i>bla</i> CTX-M-55, <i>bla</i> TEM-150	<i>AAC(3)-IId</i> <i>ANT(3'')-Iia</i> <i>APH(3'')-Ib</i> <i>APH(6)-Id</i>	<i>sul2</i>		<i>floR</i>	<i>tet(A)</i>	<i>QnrS1</i>	IncFIA; IncFIB(AP001918); IncFII; IncFII; IncX1	9
Debrecen	155	27,078	<i>bla</i> CTX-M-1							IncFIB(AP001918); IncFII (pRSB107); IncI1-I(Alpha)	2
Debrecen	155	169,654	<i>bla</i> CTX-M-1							IncFIB(AP001918); IncI1-I(Alpha)	7
Debrecen	155	136,648	<i>bla</i> CTX-M-1							Col156; IncFII; IncI1-I(Alpha)	4
Debrecen	155	9117	<i>bla</i> CTX-M-1, <i>bla</i> CMY-59							Col(pHAD28); IncFIB(AP001918); IncFII; IncI1-I(Alpha); p0111	1
Bálmazújvaros	155	81,555	<i>bla</i> CTX-M-15, <i>bla</i> TEM-1	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>	<i>sul2</i>	<i>dfrA14</i>		<i>tet(A)</i>	<i>QnrS1</i>	Col156; IncY	3

(continued on next page)

Table 3 (continued)

Area	ST	CgMLST	ESBL genes	Other genes						Plasmid	N° of isolates
				AMG	TMP	SA	Phe	Tet	FQ		
Bálmazújvaros	162	171,743	<i>bla</i> CTX-M-55	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>					<i>QnrS1</i>	IncFIB(AP001918); IncFII(pCoo); IncI2; IncN	1
Debrecen	442	28,154	<i>bla</i> CTX-M-1							IncFII; IncI1-I(Alpha)	30
Budapest	442	28,154	<i>bla</i> CTX-M-1							IncFIB(AP001918); IncFII; IncI1-I(Alpha)	2
Debrecen	442	28,154	<i>bla</i> CTX-M-1	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>	<i>sul2</i>				<i>QnrS1</i>	IncFIB(AP001918); IncFII; IncI1-I(Alpha)	1
Debrecen	515	198,768	<i>bla</i> CTX-M-55							IncN	1
Debrecen	515	198,763	<i>bla</i> CTX-M-55	<i>APH(3'')-Ib</i> <i>APH(6)-Id</i>					<i>QnrS1</i>	IncN	4
Debrecen	540	96,445	<i>bla</i> CTX-M-55					<i>tet(A)</i>	<i>QnrS1</i>	–	4
Budapest	1665	210,846	<i>bla</i> CTX-M-1							IncFIB(AP001918); IncFIC(FII); IncI1-I(Alpha)	3
Debrecen	1665	210,846	<i>bla</i> CTX-M-1							IncFIB(AP001918); IncFIC(FII); IncI1-I(Alpha)	1
Debrecen	1718	143,413	<i>bla</i> CTX-M-1							IncFIA; IncFIC(FII); IncI1-I(Alpha)	33
Debrecen	1723	56,789	<i>bla</i> CTX-M-1							Col156; IncI1-I(Alpha); IncY	5
Debrecen	2485	161,907	<i>bla</i> CTX-M-1							IncFIB; IncI1-I(Alpha); IncX1	9
Budapest	2485	161,907	<i>bla</i> CTX-M-1							IncFIB; IncI1-I(Alpha); IncX1	1
Debrecen	4421	44,522	<i>bla</i> CTX-M-1							IncFIA; IncFIB(AP001918); IncFIC(FII); IncI1-I(Alpha)	6
Budapest	5797	4894	<i>bla</i> CTX-M-1	<i>aadA2</i>	<i>sul1</i>	<i>dfrA12</i>		<i>tet(A)</i>		IncFIB(AP001918); IncFII(pRSB107); IncI1-I(Alpha); p0111	2
Debrecen	5797	4894	<i>bla</i> CTX-M-1	<i>aadA2</i>	<i>sul1</i>	<i>dfrA12</i>		<i>tet(A)</i>		IncFIB(AP001918); IncFII(pRSB107); IncI1-I(Alpha); p0111	7

AGM, aminoglycoside; TMP, trimethoprim; SA, sulfonamide; Phe, phenicol; Tet, tetracycline; FQ, fluoroquinolone.

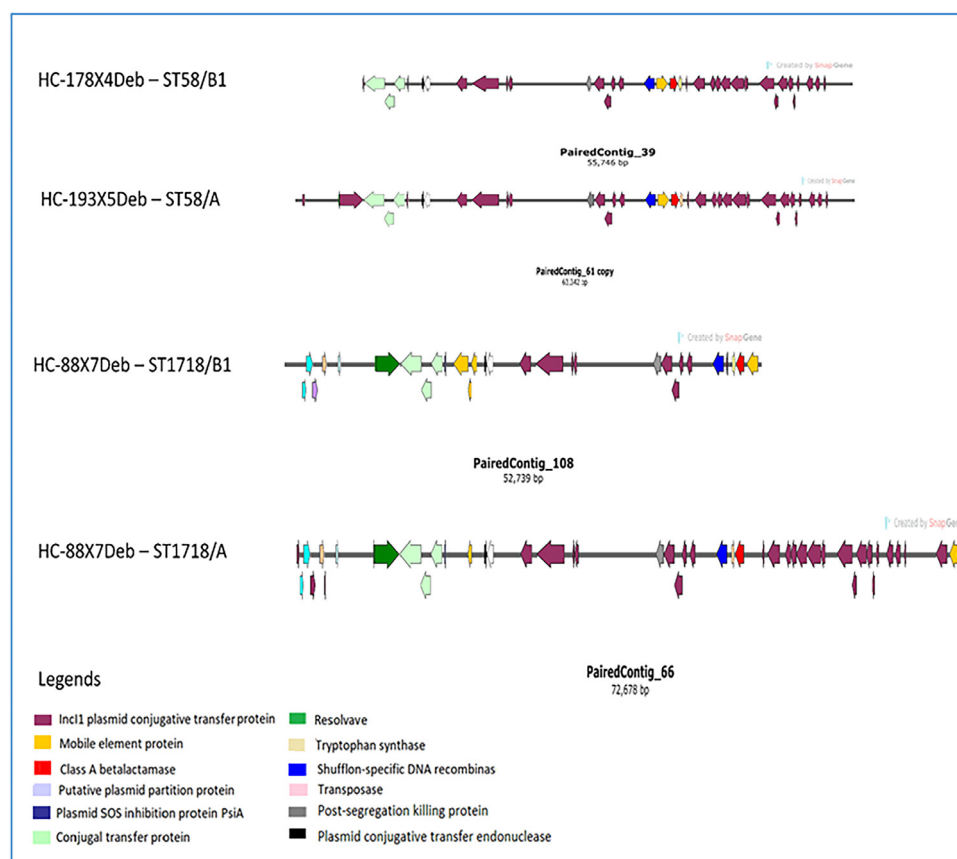


Fig. 2. Genetic environments surrounding *bla*_{CTX-M} genes in four representative *Escherichia coli* isolates. Each pair of isolates belongs to the same sequence type (ST). GenBank accession numbers and contig sizes are shown below each scheme. Open read frames (ORFs) are represented by arrows coloured by predicted function. The surrounding regions contain IncI1 plasmid-associated genes, including conjugative transfer, partitioning, and shufflon recombination genes, suggesting IncI1-like plasmid structures. Annotation was performed with RAST 2.0 and visualised using SnapGene v6.2 [32,33].

cific profile [66,67], emphasising the potential for a zoonotic transmission of such virulence between avifauna and humans. Thus, crows carrying these strains might pose a threat to both animal and human health. Additionally, in a phylogenetic typing of different ExPEC and APEC strains, several authors found that not all pathogenic avian strains are classified as B2 and are more likely classified as phylogenetic groups B1, C, and F [68,69]. Thus, our crow isolates are more likely APEC strains, since ESBL isolates belonged to phylogenetic group B1. Nonetheless, the prevalence of these ESBL producers could contribute to the spread of ESBLs and possible related gene transmission to other pathogens, making them a good indicator for the possible selective pressure behind such occurrences, thus providing insights on AMR emergence and dissemination [58].

Hooded Crow isolates also showed an interesting occurrence of non-beta-lactam resistance. For instance, similar to rook isolates [15], many *bla*_{CTX-M-15}⁺ and *bla*_{CTX-M-55}⁺ positive isolates also carried genes conferring resistance to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole. Such coresistance likely reflects the localisation of multiple resistance genes on transferable plasmids and further increases the clinical relevance of these isolates [70,71].

A small number of isolates (10/197, 5.1%) showed discordance between the phenotypic, PCR, and WGS results. Although all isolates were phenotypically confirmed as ESBL producers and were positive for the *bla*_{CTX-M-1} group by PCR, WGS failed to detect *bla*_{CTX-M} genes in these isolates. Instead, AmpC β -lactamase genes, *bla*_{CMY-59}, *bla*_{TEM-1}, or no β -lactamase gene were identified. As WGS was performed after the initial phenotypic and molecular

characterisation, plasmid loss during storage or subculturing cannot be excluded. In addition, limitations associated with short-read genome sequencing and draft genome assemblies may have contributed to the failure to detect resistance genes in a small number of isolates.

In conclusion, this study provides the first comprehensive genomic characterisation of ESBL-producing *E. coli* in free-ranging Hooded Crows. The high prevalence of ESBL-producing isolates, together with the diversity of *bla*_{CTX-M} variants, sequence types, plasmid replicons, and virulence-associated genes, highlights the role of urban Hooded Crows as reservoirs of clinically relevant antimicrobial resistance determinants. The predominance of plasmid-associated *bla*_{CTX-M} genes, alongside evidence of chromosomal integration in multiple lineages, underscores the dynamic mechanisms contributing to the persistence and dissemination of ESBL resistance in the environment. These findings reinforce the value of wild birds as One Health sentinels [72,73] and emphasize the need for continued genomic surveillance of wildlife to better understand the environmental transmission and spread of antimicrobial resistance.

Ethics approval and consent to participate: Ethical approval was not required for the study per local legislation and institutional mandates, as it constituted nonexperimental research on wild birds conducted in compliance with institutional rules. Furthermore, birds were collected for wildlife management in compliance with local and national legislation. These birds were handled in the least invasive manner, and did not suffer unnecessarily.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interests: The authors declare that they have no competing interests

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Author contributions: IB, LK, and GK contributed to conception and design of the study. IB and LK collected the fecal samples. IB analysed and interpreted the data regarding the AMR tests and the WGS and wrote the first draft of the manuscript. LL analysed and interpreted the data regarding the WGS. PG, LK and GK supervised the study. All authors contributed to the article and approved the submitted version.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.jgar.2026.08.004](https://doi.org/10.1016/j.jgar.2026.08.004).

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