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Clinically relevant antibiotic resistance in *Escherichia coli* from black kites in southwestern Siberia: a genetic and phenotypic investigation

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ABSTRACT Wild birds including raptors can act as vectors of clinically relevant bacteria with antibiotic resistance. The aim of this study was to investigate the occurrence of antibiotic-resistant *Escherichia coli* in black kites (*Milvus migrans*) inhabiting localities in proximity to human-influenced environments in southwestern Siberia and investigate their virulence and plasmid contents. A total of 51 *E. coli* isolates mostly with multidrug resistance (MDR) profiles were obtained from cloacal swabs of 35 (64%, *n* = 55) kites. Genomic analyses of 36 whole genome sequenced *E. coli* isolates showed: (i) high prevalence and diversity of their antibiotic resistance genes (ARGs) and common association with ESBL/AmpC production (27/36, 75%), (ii) carriage of *mcr-1* for colistin resistance on IncI2 plasmids in kites residing in proximity of two large cities, (iii) frequent association with class one integrase (IntI1, 22/36, 61%), and (iv) presence of sequence types (STs) linked to avian-pathogenic (APEC) and extra-intestinal pathogenic *E. coli* (ExPEC). Notably, numerous isolates had significant virulence content. One *E. coli* with APEC-associated ST354 carried *qnrE1* encoding fluoroquinolone resistance on IncHI2-ST3 plasmid, the first detection of such a gene in *E. coli* from wildlife. Our results implicate black kites in southwestern Siberia as reservoirs for antibiotic-resistant *E. coli*. It also highlights the existing link between proximity of wildlife to human activities and their carriage of MDR bacteria including pathogenic STs with significant and clinically relevant antibiotic resistance determinants.

IMPORTANCE Migratory birds have the potential to acquire and disperse clinically relevant antibiotic-resistant bacteria (ARB) and their associated antibiotic resistance genes (ARGs) through vast geographical regions. The opportunistic feeding behavior associated with some raptors including black kites and the growing anthropogenic influence on their natural habitats increase the transmission risk of multidrug resistance (MDR) and pathogenic bacteria from human and agricultural sources into the environment and wildlife. Thus, monitoring studies investigating antibiotic resistance in raptors may provide essential data that facilitate understanding the fate and evolution of ARB and ARGs in the environment and possible health risks for humans and animals associated with the acquisition of these resistance determinants by wildlife.

KEYWORDS *Milvus migrans*, *Escherichia coli*, ExPEC, APEC, wildlife, *qnrE1*, *mcr-1*, colistin resistance, IncI2, IncHI2

Wild animals of various species are increasingly reported to host bacteria with diverse antibiotic resistance and virulence phenotypes including isolates with resistance to last-line antibiotics (1). Migratory birds have the potential to acquire and transmit antibiotic-resistant bacteria (ARB) and the associated resistance determinants in their breeding and wintering habitats and through their long migratory pathways

Editor Patricia A. Bradford, Antimicrobial Development Specialists, LLC, Nyack, New York, USA

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The authors declare no conflict of interest.

See the funding table on p. 12.

Received 24 February 2023
Accepted 27 April 2023
Published 13 June 2023

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(2). Previous reports have indicated raptors including black kites as significant carriers of Enterobacterales isolates resistant to clinically important antibiotics including last-line drugs such as carbapenems and colistin (3–5).

The black kite (*Milvus migrans*) is a common raptor species with an estimated population of 6 million individuals (6). The species include several subspecies that are distributed across Eurasia, Africa, and Australia (6, 7). Black kites have a unique ecological flexibility, which support their prosperity in various natural and human-influenced landscapes (8, 9). The black-eared kite (*Milvus migrans lineatus*) is a subspecies of black kites that inhabits Asia (10, 11) and recently, they were also reported across Western Europe (12). Black-eared kites breed in Asian temperate regions and migrate in winter to the Indian peninsula (11, 13, 14). Black kites have an opportunistic feeding behavior that utilizes food from various natural (small vertebrates including carrions) and anthropogenic sources (human food refuse from landfills) to meet their diet requirement (8, 11, 15, 16). All these factors involving black kite's significant population, habitat, feeding habits, and long migration routes prime it to act as a model organism for investigating acquisition, prevalence, and transmission of ARB within avian wildlife.

E. coli with resistance to clinically important antibiotics were identified in human, environmental, domestic animals, and wildlife samples in Russia (5, 17, 18). A significant prevalence of *E. coli* resistant to quinolones and third-generation cephalosporins was reported in clinical and domestic animal samples from Russia (17, 19–21). In addition, several environment-focused studies have indicated Russian surface water and wastewater as a reservoir of ARB harboring resistance to a wide group of antibiotics including aminoglycosides, carbapenems, chloramphenicol, third-generation cephalosporins, and quinolones (18). However, studies investigating the carriage of ARB by Russian wildlife are scarce (5, 22). A recent study by our group indicated a black kite in Russian Siberia as a carrier of an *E. coli* isolate of sequence type (ST) 2280 harboring a mobile colistin resistance gene (*mcr-1*) that was located on conjugative IncI2 plasmid (5).

Here, we investigate the occurrence of antibiotic-resistant *E. coli* isolates from a large collection of Russian black kites (n = 55). The study utilized whole genome sequencing (WGS) data of 36 antibiotic-resistant *E. coli* from nestlings of black kites residing in three distinct habitats in the southwestern part of Russian Siberia. We investigated their phylogeny and carriage of antibiotic resistance genes (ARGs), virulence-associated genes (VAGs), and plasmid replicons and identified their sequence types (STs) and serotypes. Long read sequencing was utilized to investigate and characterize the plasmid content of one avian-pathogenic (APEC) *E. coli* isolate belonging to ST354.

MATERIALS AND METHODS

Sampling of black kites

Cloacal samples (n = 55) from free-living nestlings of black kites were collected in their natural habitat through two consecutive years in 2018 (n = 16) and 2019 (n = 39) using swabs with culture medium (Amies transport medium with activated charcoal, Czech Republic), then transported to the laboratory and stored at 4°C till the initiation of the enrichment protocol. Sampling was performed in three localities in Russia's southwestern Siberia (Fig. 1). Sampling localities included urban and agricultural areas around the cities of Biysk (n = 16, July 2018, Altai Krai) and Kyzyl (n = 33, June 2019, Republic of Tuva) and a small rural community in Kokorya (n = 6, June to August 2019, Altai Republic). (Refer to Table S1 for details on the sampling geolocation and collection year for each sample.)

Selection of *E. coli* isolates

E. coli isolates were selected by cultivation of primary cloacal samples enriched overnight in peptone buffer (37°C with shacking at 140 RPM) on MacConkey agar with cefotaxime (2 mg/L), ciprofloxacin (0.05 mg/L), or meropenem (0.125 mg/L). In addition,

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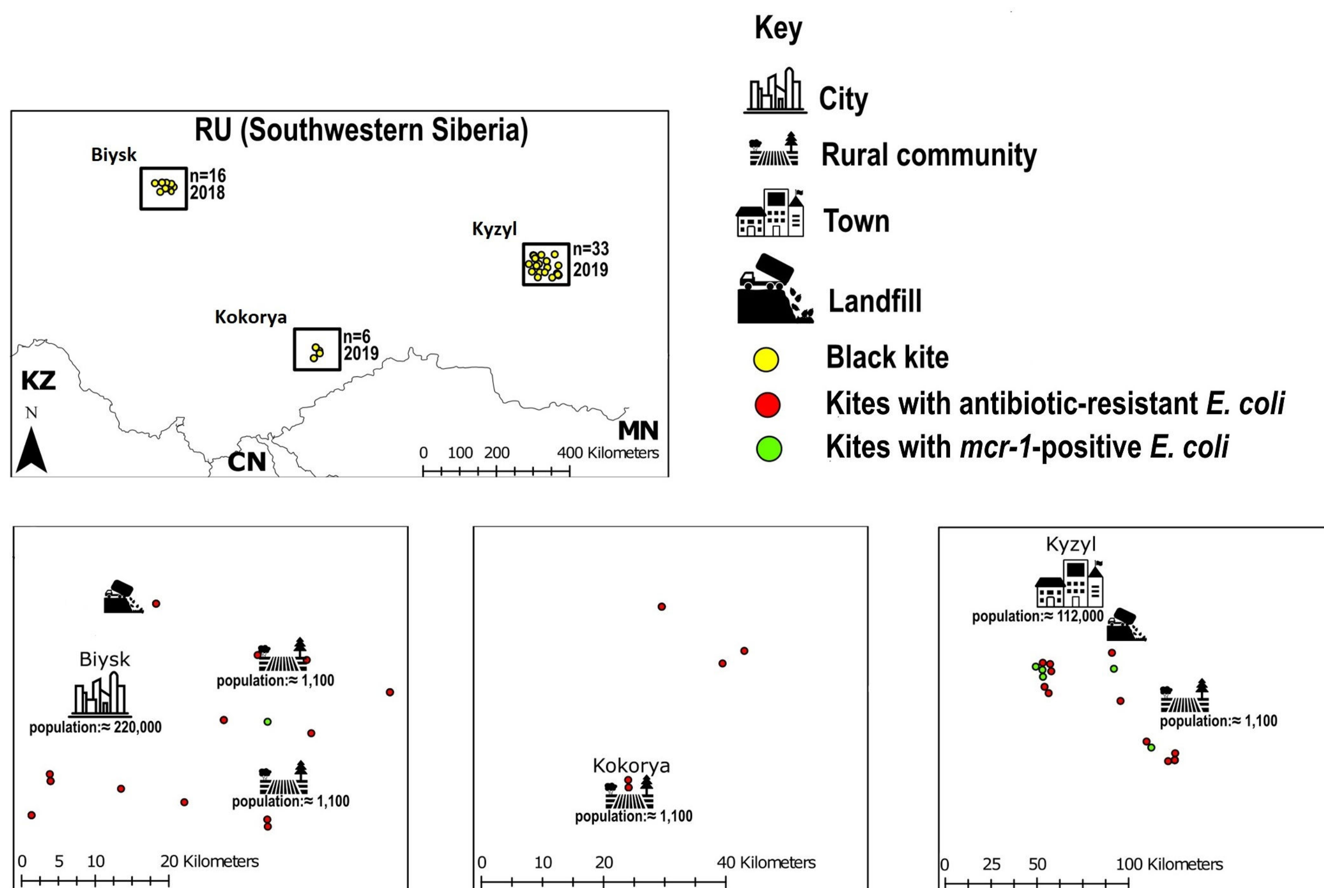

FIG 1 Schematic map showing the geographic distribution of black kites in southwestern Siberia including kites carrying antibiotic-resistant *E. coli* and *mcr-1*-positive isolates.

SuperPolymyxin medium (23) was utilized for the selection of isolates with resistance to colistin. One presumptive *E. coli* isolates from each plate was taken and identified to species level by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (24). Isolates identified as *E. coli* (n = 51) were subjected to further testing.

Identification of antibiotic resistance genes

PCR was used to identify ARGs encoding resistance to beta-lactams (bla_{ACC}, bla_{ACT}, bla_{BIL}, bla_{CMY}, $bla_{CTX\text{-}M}$, bla_{DHA}, bla_{FOX}, bla_{GES}, bla_{OXA}, bla_{LAT}, bla_{MOX}, bla_{SHV}, and bla_{TEM}) (25), quinolones (*aac(6′)-Ib-cr*, *qnrA*, *qnrB*, *qnrC*, *qnrS*, *qnrD*, *qnrE*, *qepA*, *oqxA*, and *oqxB*) (26, 27), and colistin (*mcr-1*-*mcr-9*) (5, 28). *E. coli* isolates with at least one confirmed ARG (n = 36) were selected for WGS.

Antibiotic susceptibility testing

Disc diffusion method was used to test the susceptibility of selected *E. coli* isolates from black kites to a set of 18 different antibiotics according to recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (29). The following antibiotic discs were used (Oxoid, Hants, UK): amoxicillin-clavulanic acid (20–10 µg), ampicillin (10 µg), azithromycin (15 µg), aztreonam (30 µg), cefazolin (30 µg), ceftazidime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), ertapenem (10 µg), fosfomycin (200 µg), gentamicin (10 µg), imipenem (10 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg) sulfonamide compounds (300 µg), streptomycin (10 µg), tetracycline (30 µg), and trimethoprim-sulfamethoxazole (1.25/23.75 µg). Measurement

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and interpretation of inhibition zone diameters of the tested isolates were based on EUCAST breakpoints (EUCAST 2019) or using breakpoints set by CLSI 2017 for antibiotics (azithromycin, cefazolin, tetracycline, nalidixic acid, sulfonamide compounds, and streptomycin) with no defined breakpoints in EUCAST 2019 (30, 31). EUCAST breakpoints are widely used in Europe, while CLSI breakpoints predominate in the USA. Both methodologies are recommended by World Health Organization; however, they lack harmonization with EUCAST breakpoints known to have higher resistance cut-offs and absence of intermediate resistance values or breakpoints for several antibiotics in *E. coli* and other bacterial genera compared to CLSI (32, 33). Susceptibility to colistin was assessed using colispot test (34) and interpreted based on EUCAST 2019 breakpoints (30). The production of AmpC beta-lactamase, extended-spectrum beta-lactamase (ESBL), and carbapenemase in *E. coli* isolates selected for WGS (n=36) was tested using AmpC, ESBL & Carbapenemase Set D72C (Mast Diagnostic, Merseyside, UK).

Whole genome sequencing

Whole genome DNA was extracted from 36 *E. coli* isolates using NucleosSpin Microbial DNA kit (Macherey-Nagel, Germany). Preparation of DNA libraries was performed using Nextera XT DNA library preparation kit followed by sequencing on the NovaSeq platform (Illumina, San Diego, California, USA). Trimming of short reads for quality (Q ≤ 20) and adaptor residues was carried out using Trimmomatic v0.36 (35). Short reads were assembled using SPAdes v3.12.0 (36). Complete and closed plasmids of one *E. coli* isolates (DR162-CEF) belonging to APEC-linked ST354 were obtained using long-read sequencing. Whole genome DNA was extracted using a QIAGEN midi kit (Qiagen, Hilden, Germany), and library preparation was performed using microbial multiplexing based on the manufacturers' recommendation. DNA was sheared using g-tubes (Covaris, Massachusetts, USA), but size selection was not performed for library preparation. Sequel 1 platform (Pacific Biosciences, California, USA) was used for sequencing followed by assembly using a Microbial Assembly pipeline in SMRT LNK v9.0 software (Pacific Biosciences, California, USA) with a minimum seed coverage of 30X. Quality control of obtained short- and long-read sequences was performed using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

In silico analysis of whole genome sequencing data

Publicly available tools were used for the analysis of sequenced *E. coli* isolates from black kites, which include FimTyper (v.1.0), SerotypeFinder (v.2.0), and MLST (v.2.0) (available at https://cge.cbs.dtu.dk/services/) to identify affiliation to serotype, *fimH* type, and ST, respectively. ARGs, VAGs, plasmid replicons, and their STs were determined using ResFinder (v.2.0) (available at https://cge.cbs.dtu.dk/services/), Virulence Factor Database (VFDB) (37), PlasmidFinder (v.2.0), and pMLST (v.2.0) (available at https://cge.cbs.dtu.dk/services/), respectively. Phylogroups of *E. coli* isolates were assigned using Clermont Typing (available at http://clermontyping.iame-research.center/). Automated annotation of complete and closed plasmids from DR162-CEF was performed using RASTtk (38) followed by manual curation by SnapGene (v.5.2.4, Biotech LLC, Chicago, USA) and BLASTn online tool (NCBI, Maryland, USA).

BRIG software (v.0.95) (39) and SnapGene (v.5.2.4, Biotech LLC, Chicago, USA) were used to perform a comparative analysis of plasmid sequences. A complete and closed *mcr-1*-positive-IncI2 plasmid (pDR164, GenBank accession no. MK542639) (5) originating from *E. coli* DR164-COL was used as a reference plasmid to investigate similar IncI2 plasmids within isolates from black kites' populations. In addition, a comparative analysis of pDR164 with similar IncI2 plasmids from GenBank database was performed using BLASTn online tool (NCBI, Maryland, USA) with coverage thresholds of ≥99%. Closed and complete plasmids within *E. coli* DR162-CEF (pDR162-CEF-A, pDR162-CEF-B, and pDR162-CEF-C) were investigated for similar plasmids in GenBank with BLASTn using a coverage threshold of ≥88% for pDR162-CEF-A and ≥98% for pDR162-CEF-B and pDR162-CEF-C. A similar identity threshold of ≥99% was applied to all compared

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plasmids. BLASTn alignments were performed to identify class one integrase gene (*intI1*) and insertion sequence (IS) *26* elements.

Phylogenetic analysis

Phylogenetic analysis based on single nucleotide polymorphisms (SNPs) was performed on all sequenced *E. coli* isolates from kites where *E. coli* K12-MG1655 was used as a reference genome. CSI Phylogeny 1.4 (available at https://cge.cbs.dtu.dk/services/) (40) was employed for SNPs analysis, and its results were visualized using iTOL (v.6.4) (41).

E. coli metadata

Short read sequences of *E. coli* isolates from black kites were deposited on GenBank (BioProject ID PRJNA702622) and on EnteroBase in the *Escherichia*/*Shigella* database (Table S1 for accession and barcode numbers, respectively).

Complete and closed sequences of four plasmids from DR162-CEF isolate were deposited in GenBank (accession nos. MW651977, MW651978, MW651979, and MW651980).

Transferability of quinolone and beta-lactam resistance

A conjugation assay based on filter mating method was performed on *qnrE1*-positive IncHI2-ST3 (pDR162-CEF-A) and bla_{CMY-2}-positive IncI1-Iα (pDR162-CEF-B) plasmids. The plasmid-free, rifampicin- and azide-resistant *E. coli* MT102 (42) was used as a recipient strain for the conjugative transfer of pDR162-CEF-A and pDR162-CEF-B. Incubation of mating strains was performed on Luri (LB) agar plates at 28°C for pDR162-CEF-A and 37°C for pDR162-CEF-B for 4 h. This was followed by selection of transconjugants on LB plates with ciprofloxacin (0.05 mg/L), rifampicin (25 mg/L), and sodium azide (100 mg/L) for pDR162-CEF-A and cefotaxime (2 mg/L), rifampicin (25 mg/mL), and sodium azide (100 mg/L) for pDR162-CEF-B with incubation overnight at 37°C. The presence of *qnrE1*, bla_{CMY-2}, IncHI2, and IncI plasmids in the transconjugants was confirmed by PCR (26, 43) and replicon typing (44). Transferability of *mcr-1*-positive IncI2 plasmid in one isolate (DR164-COL) was confirmed previously (5).

RESULTS

Carriage of multidrug-resistant *E. coli* is common among black kites

E. coli isolates (n = 51) with reduced susceptibility to clinically important antibiotics including cefotaxime (22/51, 43%), ciprofloxacin (22/51, 13%), and colistin (7/51, 5%) were recovered from cloacal samples of black kites (35/55, 63.6%). Notably, isolates with resistance to meropenem were absent from all kites. Most (48/51, 94.1%) isolates expressed MDR profiles with phenotypic resistance to three or more classes of antibiotics. ESBL/AmpC production was identified in the majority of sequenced isolates (27/36, 75%). Resistance to aminoglycosides, beta-lactams, fluoroquinolones, macrolides, phenicol, tetracycline, and trimethoprim was observed in numerous *E. coli* isolates, while isolates with colistin resistance phenotype (7/36, 19.4%) were identified in black kites residing in proximity to Biysk (1/16, 6%) and Kyzyl (6/33, 18%) cities. Fosfomycin resistance was only observed in isolates from samples collected around Biysk city (1/16, 6%) (Table S1).

Genomic profiles and phylogenetic analysis highlight high diversity of E. coli

WGS revealed high diversity of *E. coli* isolates in terms of their phylogroups, STs, serotypes, and *fimH* profiles. They were associated with all seven *E. coli* phylogroups (A, B1, B2, C, D, E, and F) with a predominance of phylogroups B1 (14/36, 38%) and A (11/36, 30%). A total of 30 different STs including four novel STs were observed, most of which were represented by single *E. coli* isolates while ST162 was the most common ST

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(4/36, 11.1%). Common *fimH* types included *fimH32* (10/36, 27%) and *fimH31* (4/36, 11%) (Table S1).

Phylogenetically, isolates from black kites had high heterogeneity (0–46714 SNP variants) with no observed clustering (Fig. 2). Two *E. coli* ST162 isolates with MDR and ESBL phenotypes from kites in Biysk region (DR164-CEF and DR167-CEF) were closely related (10 SNPs difference) and shared identical *fimH* type (32), serotype (O88:H10), and ARGs content. Similarly, two isolates originating from kites in proximity to Kyzyl city had two clonal *mcr-1*-positive isolates (DR356a-COL, and DR358b-COL with 0 SNPs difference) belonging to ST93 with *fimH*53 and serotype O21:H16. Both of the latter isolates were ESBL producers and shared identical ARGs and VAGs content and a highly similar plasmid profile. Two isolates from kites in proximity to Biysk and Kyzyl cities (DR161-CEF ST2197 and DR370-CEF ST12666, respectively) were closely aligned (103 SNPs difference) and had identical *fimH* type (23) and serotype (O128:H26) as well as virulence profile. However, their ARGs and plasmid content were distinct with the exception of sharing *tet*(*B*) and IncI1-I plasmid.

E. coli from black kites bear a notable pool of antibiotic resistance genes

Sequenced isolates carried between 0 and 16 ARGs with an average of 7 ARGs acting on 10 different classes of antibiotics. Most common ARGs included *tet*(*A*) (20/36, 55%), *sul2* (15/36, 41%), *aph*(*3''*)*-Ib* (14/36, 38%), *aph* (6)*-Id*, *sul1* (13/36, 36%, respectively), bla_{TEM-1B} (11/36, 30%), and *sul3* (10/36, 27%). All of the latter ARGs except for *tet*(*A*) and bla_{TEM-1B} were absent from isolates originating from black kites in Kokorya rural community.

Isolates with colistin resistance phenotype were identified in black kites residing around Biysk (1/16, 6.2%) and Kyzyl (6/33, 18.1%) cities, all harboring *mcr-1*-positive IncI2 plasmids (Fig. 3). Most sequenced isolates carried ESBL and AmpC genes (18/36, 50% and 9/36, 25%, respectively) but isolates from Biysk lacked acquired AmpC genes. Eight variants of bla_{CTX-M} gene were identified in the sequenced isolates with $bla_{CTX-M-1}$ (5/36, 13.8%) as the most common variant (Table S1). Among plasmid-mediated quinolone resistance (PMQR) genes, *qnrS1* was observed in three isolates of different STs originating from kites in Biysk, Kyzyl, and Kokorya (3/36, 8.3%). Other identified PMQR genes included *qnrB19* and *qnrE1* (2/36, 5.5%, respectively) that were exclusively present in individual samples from Biysk (1/15, 6%, respectively) and *qnrB4* (1/36, 2.7%) carried by

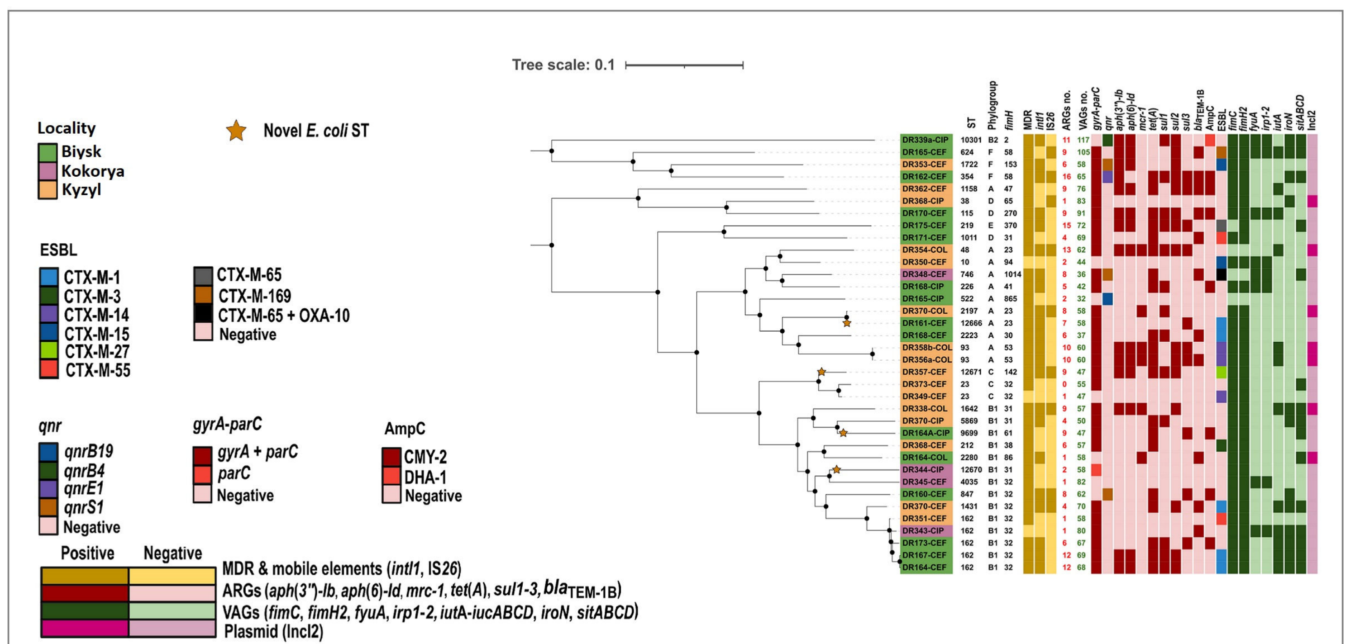

FIG 2 Clonal relationship of *E. coli* isolates (*n* = 36) from black kites (*n* = 55) in Biysk (*n* = 16), Kokorya (*n* = 6), and Kyzyl (*n* = 33).

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Plasmid-isolate legend

Plasmid ID	GenBank accession no.	Coverage %	Identity %	Isolate species	Isolate ST	Source	Year	Country	Region
pDR164	MK542639.1	100	100	*Escherichia coli*	2280	Black kite	2018	Russia	Altay Krai
DR338-COL	JALQAO000000000	ND	ND	*Escherichia coli*	1642	Black kite	2019	Russia	Altay Krai
DR354-COL	JALQAG000000000	ND	ND	*Escherichia coli*	48	Black kite	2019	Russia	Altay Krai
DR356a-COL	JALQAF000000000	ND	ND	*Escherichia coli*	93	Black kite	2019	Russia	Altay Krai
DR358b-COL	JALQAE000000000	ND	ND	*Escherichia coli*	93	Black kite	2019	Russia	Altay Krai
DR370-COL	JALPZY000000000	ND	ND	*Escherichia coli*	2197	Black kite	2019	Russia	Altay Krai
pSH16G4928	MH522426.1	100	99.9	*Salmonella enterica*	ND	Human	2016	China	Shanghai
pMCR-1_Msc_2	MT119279.1	99	99.9	*Escherichia coli*	ND	Human	ND	Russia	Moscow
pMCR-M19736	KY471314.1	99	99.9	*Escherichia coli*	ND	Human	ND	Argentina	ND
pMCR_WCHEC1604-IncI2	KY829117.1	99	99.3	*Escherichia coli*	ND	Sewage	2015	China	Chengdu

ND: Not defined

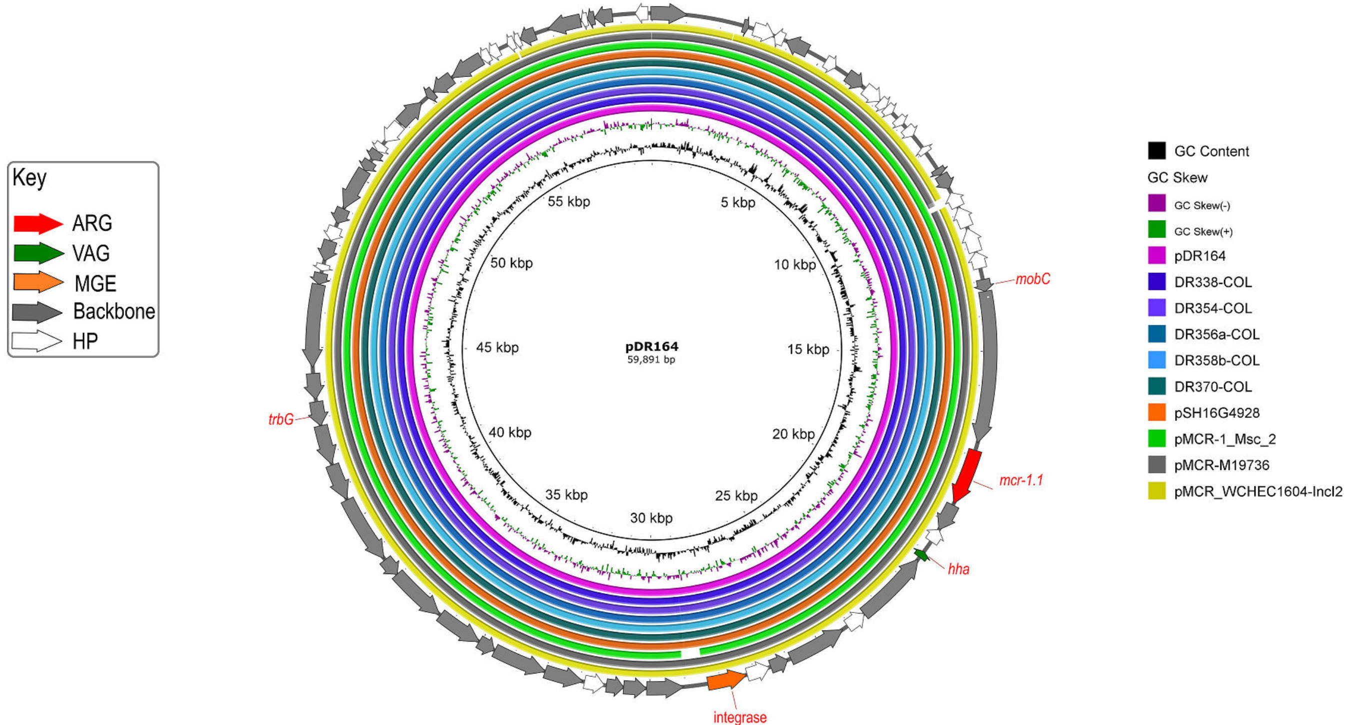

FIG 3 BRIG comparison of *mcr-1*-positive IncI2 plasmid pDR164 with similar plasmid sequences retrieved from isolates of black kites in Kyzyl ($n = 5$) and from GenBank. In the key, ARG: antibiotic resistance gene, VAG: virulence-associated gene, MGE: mobile genetic element, and HP: hypothetical protein.

an isolate originating from a kite in Kyzyl (1/17, 5%). Chromosomal mutations in *gyrA* (DNA gyrase subunit A) and *parC* (topoisomerase IV subunit) genes conferring resistance to fluoroquinolones were frequently found among sequenced isolates (29/36, 80.5% and 28/36, 77.7%, respectively) (Table S1).

Significant carriage of virulence-associated genes among black kite E. coli isolates

Several isolates (9/36, 25%) belonged to ExPEC and APEC-linked STs including ST10, ST23, ST38, ST93, ST354, ST624, and ST1011 and were distributed among kites from Biysk, Kyzyl, and Kokorya. Isolates carried 32 to 117 VAGs with an average of 63. Various isolates harbored genes associated with adhesion *fimH2* (35/36, 97%), *fimC* (32/36, 88%), and *papC* (3/36, 8%); siderophores *iroN*, *iutA, iucABCD* (12/36, 33%, respectively), *fyuA* (8/36, 22%) and *irp1-2* (8/36, 22%); and ferrous acquisition system *sitABCD* (15/36, 41%). Three isolates from kites in Biysk (DR344-CIP, ST12670) and Kyzyl (DR349-CEF and DR373-CEF, ST32, respectively) carried the *rhs*/PAAR toxin gene. One isolate (DR339a-CIP) with the highest number of virulence genes ($n = 117$) was sourced from a kite in Kyzyl and belonged to ST10301. Its virulence profile included several VAGs that were not identified in other isolates. This included genes encoding invasins (*afaD* and *afaE),* colibactin operon (*colABCDEFGHIJKLMNPQS*), toxins (*cnf1* for cytotoxic necrotizing factor type 1, *hylA* for α-hemolysin, *vat* encoding vacuolating autotransporter toxin, and *senB*

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for enterotoxin TieB), iron acquisition system (*chuA* for hemin receptor molecule), and a homologous Toll/interleukin-1 receptor (*tcpC*) (Table S1).

High diversity of plasmid replicons and spread of intI1 and IS26 in E. coli from black kites

In total, 18 different plasmid replicons were identified with *F* and various Col plasmids present in most isolates (28/36, 77% and 27/36, 75%, respectively). Sequenced isolates carried between zero and nine plasmids with an average of three plasmids (Table S1).

Corresponding replicon sequence types (RSTs) of all *F* plasmids were present in single isolates with several exceptions. F18:A-:B1 plasmid was identified in three isolates belonging to kites from Biysk, Kyzyl, and Kokorya. Two of these isolates (DR173-CEF from Biysk and DR343-CIP from Kokorya) shared a common ST162, while the isolate from Kyzyl (DR338-COL) was of ST1642. IncI-I plasmids were present in 11/36 (30.5%) isolates and belonged to clonal complex (CC) 2 and CC3. They belonged to six different STs with ST3 predominating (5/11, 45.4%). Two isolates carried HI2 plasmids, one belonged to ST3 carrying *qnrE1* gene (DR162-CEF) while another had a nontypeable ST with close relation to ST3. (Refer to Table S1 for characterization and distribution of replicon STs.)

Class one integrase gene *intI1* was common among the isolates (22/36, 61.1%) and present in *E. coli* from Biysk (*n* = 11), Kyzyl (*n* = 10), and Kokorya (*n* = 1). IS*26* (9/36, 25%) was only identified in isolates from kites in Kyzyl (*n* = 6) and Biysk (*n* = 3) (Fig. 2).

Resistance to colistin associated with mcr-1-IncI2 plasmids

The *mcr-1* gene in all six isolates from kites in Biysk and Kyzyl was carried on IncI2 plasmids with highly homologous structures (Fig. 3). Isolates harboring *mcr-1* were MDR, belonged to diverse STs (48, 93, 1,642, 2,197, and 2,280), phylogroups A (4/6) and B1 (2/6), and three of them were ESBL producers. Comparative analysis of IncI2 plasmids from black kites (Fig. 3) showed high identity and coverage with IncI2 plasmids originating from clinical samples in China (hosted by *Salmonella enterica*), Argentina, and Russia (hosted by *E. coli*) and a sewage sample in China (harbored in *E. coli*).

Plasmids of E. coli DR162-CEF carry genes for MDR, metal resistance, and colicin production

Isolate DR162-CEF originated from a black kite in Biysk was MDR and AmpC producer and belonged to ST354 and phylogroup *F* with *fimH*58 and serotype O1:HNT. Notably, in addition to plasmid-mediated resistance, DR162-CEF encoded chromosomal resistance to aminoglycosides (*aac* (3)-*Iva*, *aph*(*3"*)-*Ib*, *aph* (4)-*Ia*, and *aph* (6)-*Id*), phenicol (*cmlA*), sulfonamides (*sul1*, *sul2*, and *sul3*), tetracycline [*tet*(31), *tet*(A), and *tet*(M)] and trimethoprim (*dfrA7*). Its virulence content included genes associated with pathogenic *E. coli* including *fimH2*, *fimC, iroN,* and *sitABCD*.

Four complete and closed plasmids were recovered from isolate DR162-CEF of ST354 including pDR162-CEF-A (IncHI2-ST3), pDR162-CEF-B (IncI1-Iα/ST23 of CC2), pDR162-CEF-C (IncFII, replicon ST: F4*:A-:B37*), and pDR162-CEF-D (ColpVC). Plasmid pDR162-CEF-A (237,048 bp) was conjugative and had a typical HI2 backbone structure including regions for replication, stability, maintenance, and horizontal gene transfer (Fig. 4). It harbored five ARGs [$bla_{\text{TEM-1B}}$, *qnrE1*, *sul3*, *tet*(A), and *dfrA12*] and various metal resistance genes. Most of the ARGs except $bla_{\text{TEM-1B}}$ were part of a complex region containing *intI1* and an associated resistance region with copies of Tn*3,* IS*26*, IS*Ecp1,* and IS*256*. Similarly to its first report in a *Klebsiella pneumoniae* isolate (26), *qnrE1* was flanked by an IS*Ecp1* upstream and *araj* (transporter of major facilitator superfamily) and a truncated *ahp* (alkyl hydroperoxidase) downstream (Fig. 4). Comparative analysis of pDR162-CEF-A indicated six plasmids sharing similar IncHI2-ST3 backbone structure and tellurium resistance region. They all had antibiotic resistance regions with the absence of IS*Ecp1*-*qnrE1*-*araJ*-Δ*ahp* sequence, mostly originated from *E. coli* and were sourced from domestic animals and human sources in various Asian countries (China, Vietnam,

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Plasmid legend

Plasmid ID	GenBank accession no.	Coverage %	Identity %	Plasmid replicon	Isolate species	Source	Year	Country	Region
pDR162-CEF-A	MK651977.1	100	100	IncHI2-ST3	*Escherichia coli*	Black kite	2018	Russia	Altai Krai
pXH990_1	CP019356.1	92	99	IncHI2-ST3	*Escherichia coli*	Human	2016	China	Zhejiang
pP2-3T	MG014722.1	90	99	IncHI2-ST3/IncFIB	*Escherichia coli*	Pig	2008	China	ND
pCFSAN086837	CP039438.1	89	99	IncHI2-ST3	*Salmonella enterica*	Chicken	2017	Vietnam	Gia Lai
pCE1681-A	MT180430.1	89	99	IncHI2-ST3	*Escherichia coli*	Silver gull	2012	Australia	Sydney
pRS571-MCR-1.1	CP034390.1	88	99	IncHI2-ST3	*Escherichia coli*	Human	2018	Bangladesh	Dhaka
pGDP25-25	MK673547.1	88	99	IncHI2-ST3	*Escherichia coli*	Pig	2016	China	ND

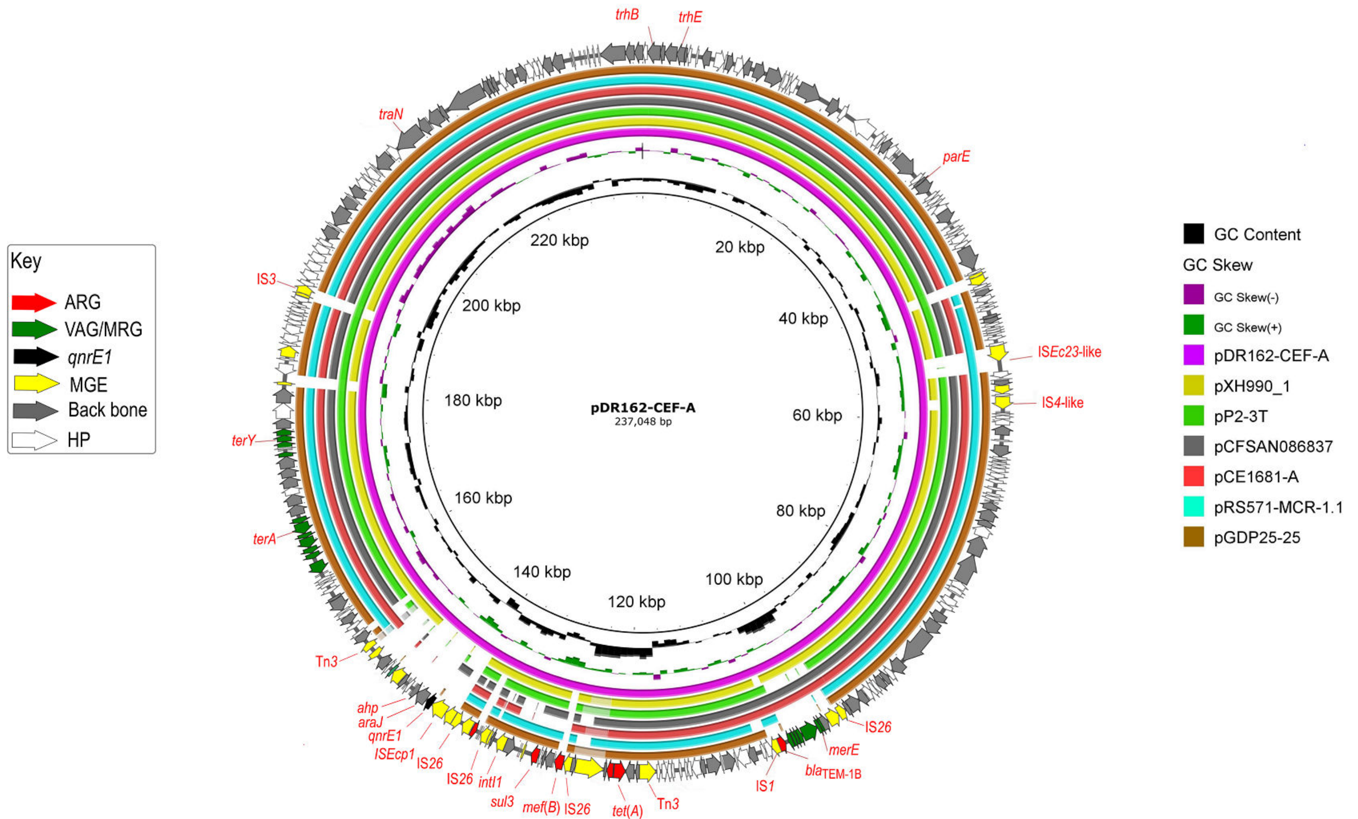

FIG 4 BRIG comparison of *qnrE1*-positive IncHI2-ST3 plasmid pDR162-CEF-A with similar sequences from GenBank. In the key, ARG: antibiotic resistance gene, VAG/MRG: virulence-associated gene/metal resistance gene, MGE: mobile genetic element, and HP: hypothetical protein.

and Bangladesh) and from a wildlife sample in Australia (Fig. 4). Notably, three of the compared plasmids (pXH990_1, pRS571-MCR-1.1, and pGDP25-25) carried acquired colistin resistance genes (*mcr-1*), while pCE1781-A hosted a carbapenem resistance gene *bla*$_{\text{IMP-4}}$.

Plasmids pDR162-CEF-B (96,414 bp) and pDR162-CEF-C (112,643 bp) carried various genes for colicin activity, colicin immunity, and metal resistance (Fig. S1 and S2). Plasmid pDR162-CEF-B had high coverage and identity with four similar *bla*$_{\text{CMY-2}}$-positive IncI1-Iα plasmids from domestic animals (pCVM22462 in *Salmonella enterica* from USA), wildlife (pCE1628_I1 in *E. coli* from Australia), food (pS10584 in *Salmonella enterica* from China), and an unknown source (plasmid_ 2). These plasmids were identical to pDR162-CEF-B except for an insertion of a reverse transcriptase in pDR162-CEF-B and all carried *bla*$_{\text{CMY-2}}$ (Fig. S1). Comparative analysis of pDR162-CEF-C identified highly related FII plasmids (pSUO2, p113.6k, and pIncF4-A-B1) with different RSTs, all originating from poultry in the USA and sharing plasmid backbone, metal resistance, and VAGs (Fig. S2).

DISCUSSION

Clinical and agricultural use and misuse of antibiotics are the main drivers for the emergence of antibiotic resistance that can contaminate the ecosystem including the

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environment and wildlife (45, 46). The existence of *E. coli* in wildlife with multidrug resistance (MDR) to clinically important antibiotics represents a potential hazard to human and animal health (47, 48), where wildlife can act as a reservoir and spreader of ARB in the human-environment-animal interphase (48, 49). Black kites in Russian western Siberia reside in localities with access to human food refuse (i.e., landfills) (50) and habitat intersection with agricultural elements (i.e., fertilized agricultural lands (51) and food animals), all of which are known to be common sources of ARB (52–54). These factors support the hypothesis of a potential anthropogenic spillover of ARB and antibiotic-resistant determinants to black kites in the Altai Krai region which warranted this study.

Through this investigation, we identified prominent occurrences of *E. coli* with antibiotic resistance including MDR and ESBL profiles in black kites residing in southwestern Siberia in proximity to human-influenced environments. The high occurrence of antibiotic-resistant *E. coli* in samples near major urban areas such as Biysk and Kyzyl supports the finding of previous studies (55–57) associating wildlife in environments influenced by anthropogenic activity with high carriage of ARB. The occurrence of antibiotic-resistant *E. coli* in Kokorya might implicate human populations in rural communities as carriers and possible transmitters of ARB that was observed in studies from rural regions in India (58) and Peru (59). Another possible source for the acquisition and carriage of antibiotic-resistant *E. coli* observed in black kites could be their wintering grounds that are located in the densely populated areas in India and Pakistan where they feed mainly on municipal waste from landfills (13). Considering that sampled kites were in their nestling stage, it is possible that their parents carried the antibiotic-resistant isolates from their wintering habitat in the Indian subcontinent and transmitted observed antibiotic- resistant *E. coli* via feeding to their offspring. Although, it is well known that bird nestlings can get inoculated by different bacteria from their environment particularly food provided by their parents which is mixed with the parents' saliva (60), reports investigating the carriage duration of bacteria in avian wildlife are still scarce (61, 62).

Our results indicate a high occurrence of antibiotic-resistant (35/55, 63%), MDR (48/51, 94%), and ESBL-producing (18/36, 50%) *E. coli* isolates in black kites in southwestern Siberia. In comparison, studies on wild birds from Europe (3, 63–65) show a relatively lower occurrence of antibiotic-resistant *E. coli* and ESBL producers while affirming the spread of MDR phenotype between these isolates. Plaza-Rodriguez et al. (63) identified ESBL-producing *E. coli* in 9.8% (10/102) of samples sourced from wild ducks (subfamily Anatinae) and geese (subfamily Anserinae) in Germany where most of these isolates (70%, 7/10) exhibited MDR profiles. Similarly, Guenther et al. (64) demonstrated the occurrence of ESBL-producing *E. coli* (5.2%, 9/171) in various raptor species in Germany, while a study on wild birds from Portugal (65) identified *E. coli* with ESBL profiles in 26.9% of samples (32/119), all of which were MDR. Skarzynska et al. (3) investigated a collection of samples sourced from free-living wild birds and birds from a rehabilitation center in Poland where they determined a high occurrence of antibiotic-resistant *E. coli* (50%, 35/70), most of which had MDR profiles (77%, 27/35). Several factors hinder proper comparative analysis and interpretation of such monitoring data on antibiotic resistance in wildlife. This includes a difference in study design (selective culturing and WGS versus whole sample metagenomics sequencing), the use of different sampling (cloacal and goiter swabs, fecal droppings, animal tissue) and culturing methods (selective versus nonselective), species and food preference of wild birds and geographic location of sampling.

Black kites in the Biysk region utilize available human food refuse from nearby landfills as their main food source (50). We predict a similar behavior for kites in Kyzyl where a landfill is located in the proximity to the city (Fig. 1). Based on these feeding behaviors, the observed ARB and their resistance profiles in these birds might be associated with anthropogenic spillover that was captured by black kites. This postulation is supported by results from a phylogenetic analysis that identified two clonal

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mcr-1-positive *E. coli* isolates (DR356a-COL and DR358b-COL) originating from two black kites in Kyzyl with zero SNPs difference indicating a recent common source of the two isolates. Notably, these isolates belonged to ST93 which is frequently reported in APEC and ExPEC (66, 67). Similarly, several isolates from kites in Biysk and Kyzyl belonged to STs associated with APEC (ST624 (68)), ExPEC (ST38 (69)), and overlapping APEC-ExPEC (ST10, ST23, ST354, and ST1011 (66, 69, 70)). Besides one isolate of ST162 that is commonly detected in livestock (71, 72), companion animals (73, 74), and wildlife (75, 76), black kites in Kokrya had no isolates with known STs linked to pathogenic *E. coli*. The association of avian wildlife foraging mainly on human food refuse with a significant prevalence of *E. coli* encoding resistance to clinically important antibiotics was well documented in studies involving wild bird species such as gulls (Laridae) (57, 77, 78), white storks (*Ciconia ciconia*) (79), and bald eagles (*Haliaeetus leucocephalus*) (78). These studies reported high diversity of ARGs encoding resistance to multiple classes of antibiotics including ESBL, which was also observed in black kites from Biysk and Kyzyl. The spread of ESBL/AmpC production, chromosomal quinolone resistance and considerable virulence content in sequenced isolates is concerning as it primes them as potentially pathogenic lineages.

Class one integrase gene *intI1* was suggested as a proxy for anthropogenic pollution and as a marker for antibiotic resistance (80). Based on that, the high occurrence of *intI1* in the sequenced isolates from kites in southwestern Siberia (22/36, 61.1%) may indicate a prominent anthropogenic influence on these populations. IS*26* has emerged as a critical element in the mobilization of ARGs in Gram-negative bacteria where it is usually found in complex resistance regions harboring resistance genes to multiple classes of antibiotics (81, 82). The presence of IS*26* in isolates originating from kites in Biysk and Kyzyl might be one of the factors contributing to the high diversity of their ARGs.

Mobile colistin resistance gene *mcr-1* is increasingly observed in avian wildlife including aquatic and migratory species (3, 83). Suggested ability of wild birds for prolonged carriage and transmission of ARB (61) is worrisome especially in the case of migratory birds as black kites harboring isolates with resistance to last-line antibiotics. The spread of identical *mcr-1*-positive IncI2 plasmids in isolates from black kites of different STs and locations might indicate mobilization of the plasmid through horizontal gene transfer or a gradual acquisition of *mcr-1*-positive isolates by black kites on multiple occasions from unknown sources. Identification of highly similar IncI2 plasmids originating from black kites and clinical samples in Russia, China, and Argentina and a sewage sample from China (Fig. 3) supports the reports of global spread of *mcr-1*-positive IncI2 plasmids (84).

The association of DR162-CEF with ExPEC-APEC and its carriage of MDR and virulent plasmids is concerning as *E. coli* ST354 was linked to serious clinical infections including urinary tract, prostate, and bloodstream infections (69, 84, 85).

To our best knowledge, we present the first report of *qnrE1* in *E. coli* from a wildlife source. Recently, quinolone resistance gene *qnrE1* was identified in a *Salmonella enterica* isolate from retail meat in China (86) and in *Enterobacter asburiae* from a clinical sample in Thailand (87). Most reports on *qnrE1* originate from South America where it was found in clinical (commonly on IncM plasmid) and domestic animal samples, hosted by *Klebsiella pneumoniae*, *Salmonella* Typhimurium, and *E. coli* (88–93). A recent study indicated a diseased parrot *Amazona aestiva* in Brazil as a carrier of *qnrE1*-positive IncM1 plasmid (94). However, the parrot was a companion animal [confirmed through written confirmation with the corresponding author (94)] which implies a possible human-companion animal transmission. The *qnrE1* gene was also identified in a MDR *K. pneumoniae* isolate from a native Amazonian fish in Brazil where it was harbored in a hybrid IncFIB/IncHI1B plasmid (95). *qnrE1* originated from the chromosome of *Enterobacter* spp. with a suggested role for IS*Ecp1* in its mobilization to *K. pneumoniae* (26). The association of *qnrE1* with IS*Ecp1* and its insertion in an MDR IncHI2-ST3 plasmid (pDR162-CEF-A) in *E. coli* ST354 may indicate an ongoing interspecies dissemination of *qnrE1*, facilitated by

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IS*Ecp1* and conjugative plasmids. In addition, the association of MDR IncHI2-ST3 plasmids with metal resistance and biocins might facilitate their persistence and spread in the absence of antibiotic selection pressure (96).

There are several limitations in our study including the uneven distribution of samples between black kites in the three sampled locations and the temporal difference in sampling of kites from Biysk (sampled in 2018) and those from Kyzyl and Kokorya (both sampled in 2019). The use of selective cultivation method results in targeting resistant bacterial isolates, thus hindering a better understanding of the avian microflora including common and persistent bacterial lineages. In addition, the unavailability of national clinical and environmental monitoring data on antibiotic resistance in southwestern Siberia prevented a comparative analysis that would potentially support determining possible sources and transmission routes of identified ARB and their associated ARGs.

Our results imply a human factor behind some of the observed diversity in resistance and plasmids profiles, it also demonstrates that even in environments with minimal human activity such as Altai rural communities, *E. coli* with considerable resistance and virulence determinants can be detected. The risk of carriage and transmission of these isolates on domestic animals and humans is not fully understood. We advise further investigation that adopts a One Health approach in investigating ARB in black kites and other avian species to determine the origin, spread, transmission routes, and persistence of these isolates in the human-domestic animal-environment-wildlife interphase. This in turn can help in determining their reservoirs and provide an informative concession of their health risk.

ACKNOWLEDGMENTS

We thank Jarmila Lausova, Dana Cervinkova, Katerina Kavanova, Katerina Rehakova, Zuzana Schovancova, and Martin Klvana for their help in the laboratory. We thank Jana Palkovicova for her support in bioinformatic preparation of sequenced raw data. We thank Sergey V. Vazhov, Roman F. Bachtin, Oleg Shiryaev, Stanislav Vyhnal, Valeriy Orgunov, and Sudur Orgunov for the cooperation in the field.

The project was supported by the Grant 2023ITA31 from the University of Veterinary Sciences Brno, Czech Republic.

The authors declare no conflict of interest.

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FUNDING

Funder	Grant(s)	Author(s)
ITA (University of Veterinary Sciences Brno)	2023ITA31	Hassan Tarabai

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Funder	Grant(s)	Author(s)
		Ivan Literak
		Monika Dolejska

AUTHOR CONTRIBUTIONS

Hassan Tarabai, Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review and editing | Simon Krejci, Investigation, Writing – original draft, Writing – review and editing | Igor Karyakin, Investigation, Methodology | Ibrahim Bitar, Investigation, Methodology, Writing – review and editing | Ivan Literak, Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review and editing | Monika Dolejska, Conceptualization, Data curation, Formal analysis, Funding acquisition, Resources, Supervision, Validation, Writing – original draft, Writing – review and editing

DATA AVAILABILITY STATEMENT

The sequencing data presented in this study are openly deposited in GenBank within Bioproject PRJNA702622.

ETHICS APPROVAL

All procedures and techniques used for sampling and handling of black kites in this study were conducted by trained personal in accordance to Russian federal law No. 52-FZ "On wildlife". Sampling of black kites was conducted in a manner to ensure the conservation of the bird species and their habitats and minimize the disturbance to black kite population. Precautions were implemented to minimize any potential harm or distress to the birds during handling. Black kites were released back into their natural habitat following sampling.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Material (mSphere00099-23-s0001.docx). Figure S1, Figure S2, and legend for Table S1.

Table S1 (mSphere00099-23-s0002.xlsx). Characteristics of *E. coli* isolates from black kites in southwestern Siberia.

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