Plasmid-Mediated Resistance to Extended-Spectrum Cephalosporins and Resistance to Fluoroquinolones in *Escherichia Coli* Isolates from Black-headed Gulls (*Larus Ridibundus*)

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Abstract

Although resistance to fluoroquinolones is common in *E. coli* isolates from farm and game animals in Serbia, currently no data are accessible on the occurrence of antibacterial resistances in *E. coli* isolates from gulls. Therefore, 45 cloacal swabs and 50 fecal samples from black-headed gulls were investigated for the presence of *Escherichia coli* isolates resistant to antibiotics. Multidrug resistance was detected in 22 *E. coli* isolates. High level resistance to fluoroquinolones was found in ten isolates with MIC values of ciprofloxacin ranging from 4 to 32 mg/L. Genotyping revealed single or double mutations in the quinolone resistance determining region (QRDR) of the *gyrA* or *gyrA*, *parC* and *parE* genes, respectively. Ten isolates showed resistance to extended-spectrum cephalosporin antibiotics. These ten isolates belonged to phylogenetic group B2 (five isolates), group D (four isolates) and group B1 (one isolate). An extended-spectrum β -lactamase resistance phenotype was detected in one isolate which carried the *bla*_{CTX-M-1} gene on a plasmid of the I2/FIB replicon type. Nine isolates carried *bla*_{CMY-2} genes, which were detected on conjugative plasmids in seven isolates. One transconjugant also carried *hly*, *iroN*, *iss*, *ompT* and *cvaC* virulence genes on the plasmid. Five different sequence types (ST38, ST2307, ST224, ST162 and ST34) were detected in *E. coli* isolates with ESBL or AmpC phenotype and genotype.

Introduction

The escalating development of antimicrobial resistance in pathogenic and commensal bacteria is a well-known worldwide problem. There are several ways in which bacteria can become resistant to antibiotics including point mutations within the bacterial chromosome, efflux mechanisms and reduced permeability or uptake of antimicrobials, enzymatic inactivation or alterations of the target site (Adamassie, 2018). Genes mediating antimicrobial resistance are often found inside mobile genetic elements such as plasmids, transposons, integrons and insertion sequences. This complex genetic material can be easily transferred between bacterial species which also facilitates the spread of resistant bacteria in the environment (Levy and Marchall, 2004; Wellington et al., 2013; Vandecraen et al., 2017). Pathogenic and multidrug-resistant bacteria can thus become widespread pathogens in hospitals or prevalent on production plants. In this case, the only way to reduce contamination and spread is to use antibiotics prudently and continuously apply the best standards of hygiene. On the other hand, it is an extremely challenging venture to reduce virulent and multidrug-resistant bacteria from the environment and wildlife.

Escherichia coli isolates from wild birds, mostly gulls, have been the focus of various studies and it was shown that wild birds carry bacteria that are often resistant to numerous antibiotics (Parker et al., 2016; Zurfluh et al., 2019). Multidrug-resistant *E. coli* have been identified in gulls residing in remote parts of the world (Hernandez et al., 2013; Ramey et al., 2018), on coastlines (Poirel et al., 2012 and beaches (Simões et al., 2010) worldwide. However, it is also important to note that in some cases mobile resistance genes have been detected in virulent *E. coli* from wildlife, but that these may have originated from humans or farm animals (Hernandez et al., 2013; Atterby et al., 2016; Atterby et al., 2017; Ahlstrom et al., 2018). In addition, a de novo development of resistance in environmental pathogens can also occur (e.g. to synthetic antibiotics such as fluoroquinolones), which can result from residues and contamination of habitats as a consequence of anthropogenic activities. This is supported by the fact that fluoroquinolones can accumulate in natural environments, especially in soil and water (Grenni et al., 2018).

It is important to note that resistance to fluoroquinolones has been detected in multidrug-resistant *E. coli* isolates from cattle, pigs and wild animals in Serbia (Todorović et al., 2018; Velhner et al., 2018). This type of resistance may be established also in *E. coli* isolates from wild birds which may facilitate the dissemination of fluoroquinolone-resistant *E. coli* in the environment.

Extended-spectrum β -lactamase-producing *E. coli* (ESBL) is frequently detected in the livestock industry in both diarrheic and healthy animals (Faccone et al., 2019; Shabana and Al-Enazi, 2020). Often ESBL-producing *E. coli* is also resistant to fluoroquinolones leaving fewer options for treatment of infections caused by the resistant bacteria in both human and veterinary medicine. Even well-established clones in humans, such as *E. coli* ST131-CTX-15, are occasionally detected in animals or the environment worldwide (Johnson et al., 2012; Nicolas-Chamoine et al., 2013). The adaptation of ESBL-producing *E. coli* to various niches could be explained by its mechanisms of virulence and the antibiotic selection mechanisms (Nicolas-Chamoine et al., 2013).

Therefore, we analyzed the resistance mechanisms to different classes of antibiotics including fluoroquinolones in *E. coli* isolates from black-headed gulls. Besides, the plasmid-mediated resistance to cephalosporin antibiotics was studied in detail and determined whether these *E. coli* isolates harbor virulence genes on their conjugative plasmids.

Material And Methods

Sampling

E. coli isolates were obtained from 45 cloacal swabs and 50 fecal samples from black-headed gulls (*Larus ridibundus*) collected during winter months in the city of Novi Sad in 2014. The cloacal swabs, which received the designation 1773, were taken on the local land field during a ringing campaign. For the trial, three permits were obtained, one from the Ministry of Agriculture and Environmental Protection of the Republic of Serbia (permit number 353-01-768/2014-08), one from the Ministry for Energy, Development and Environment Protection of the Republic of Serbia (permit number 353-01-845/2013-08) and one from the Institute for Nature Conservation of Vojvodina Province (permit number 1953 – 230). Twenty-four *E. coli* isolates from cloacal swabs that were resistant to antibiotics were included in further research. Thirty-four fecal samples were collected at a local beach ("Strand") and marked with 1774, while 16 were collected at the pier on the Danube and marked with 1775 and 1776. Twenty *E. coli* isolates obtained from feces and the 24 isolates from cloacal swabs that were resistant to antibiotics were included in the further research due to their single or multidrug-resistant phenotype.

Isolation and identification of *E. coli*

E. coli were isolated by inoculating a single swab in 10 mL of peptone water (Oxoid, Basingstoke, Hampshire, England CM1049). After 18 hours of incubation at 37°C, a full loop (10 µL) was used to spread the inoculum over McConkey agar. Single colonies were thus collected and subsequent passages were done to obtain a pure culture. *E. coli* isolates were stored at -80°C in tryptic soy broth with 20% glycerin until further use. Species confirmation was done by polymerase chain reaction using a protocol described by McDaniels et al. 1996 for the detection of the *gadA/B* gene encoding a glutamate decarboxylase in *E. coli*.

Resistotyping and MIC determination of ciprofloxacin and colistin

Resistotypes were determined as recommended by the Clinical and Laboratory Standards Institute (documents number M07 and M100, 2018). The following antibiotic disks with the recommended concentration of antibiotics were used in the study: ampicillin 10 µg (AMP), amoxicillin/clavulanic acid 20 µg + 10 µg (AMC), chloramphenicol 30 µg (CHL), ciprofloxacin 5 µg (CIP), gentamicin 10 µg (GEN), nalidixic acid 30 µg (NAL), streptomycin 10 µg (STR), sulfonamides 300 µg (SA), tetracycline 30 µg (TET), trimethoprim/sulfamethoxazole 1.25 / 23.75 µg (SXT), trimethoprim 5 µg (TMP), cefpodoxime 10 µg (CPD), cefotaxime 30 µg (CTX), and ceftazidime 30 µg (CAZ), ceftiofur 30 µg (FOX). Disks were purchased from BioRad (Marnes-la-Coquette, France). For quality control, *E. coli* ATCC 25922 was used. Multidrug resistance was assigned to isolates that were resistant to ≥ 3 antibiotics of different classes (Schwarz et al., 2010). Synergy tests for detection of extended-spectrum-β-lactamase producing *E. coli* were done as recommended in the CLSI document M100, 2018. The interpretation of phenotypic tests for the detection of plasmid-mediated pAmpC β-lactamase production included ceftiofur (30 µg) and ceftazidime (30 µg) disks as recommended by the EFSA Journal (2019). Testing of MICs of ciprofloxacin and colistin was done by the broth microdilution method in cation-adjusted Mueller Hinton broth (Oxoid, Basingstoke, Hampshire, England). However, MICs of colistin were determined following the protocol by Gwozdziński et al., 2018, in Mueller Hinton broth (Sigma-Aldrich, Schnelldorf, Germany) supplemented with calcium chloride dehydrate (Roth, Karlsruhe, Germany). *E. coli* ATCC 25922 and *E. coli* NCTC 13846 were included on each plate for quality control purposes.

Resistance and virulence gene screening and sequencing

The primer sequences, annealing temperatures and references used for the resistance and virulence gene screening including primers used for phylogenetic and replicon typing, by PCR are presented in Supplementary Table 1. The master mix for beta-lactam gene detection was prepared by using a commercial kit One Taq Hot Start 2x Master Mix M0484, (New England BioLabs, Ipswich, MA, USA) and amplicons were purified using the commercial kit Monarch, PCR and DNA Clean up Kit (New England, BioLabs, Ipswich, MA, USA) and then sent to Macrogen, Amsterdam, Holland for sequencing of both strands. For sequencing the quinolone determining regions (QRDRs) of the topoisomerase genes, the Qiagen Hot Star Taq Master Mix commercial kit (Qiagen, Hilden, Germany) was used for DNA extraction followed by a DNA purification step using the QiAquick PCR purification kit of the same manufacturer. The sequences of the *bla*_{CMY-2} and *bla*_{CTX-M-1} genes and of the QRDR genes were analyzed with the Basic Local Alignment Search Tool-nucleotide program-BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn>).

Mating experiment and S1 nuclease assay

The isolates that were resistant to extended-spectrum cephalosporins were included in mating experiments with the recipient strain *E. coli* HK225. For these experiments, Luria Bertani medium (Becton Dickinson, Sparks, MD, Le Pont de Claix, France) was supplemented with 2 mg/L cefotaxime and 100 mg/L rifampicin. The isolates were shaken in the liquid medium for 30 minutes and the obtained transconjugants were used for further analysis. DNA preparation and restriction enzyme digestion for Pulsed-field Gel Electrophoresis (PFGE) of seven *E. coli* isolates, the recipient strain and the resulting transconjugants were performed as previously described, (Jovicic et al., 2011). The DNA was either digested with the restriction enzyme *Xba*I or with S1 nuclease or was not digested. PFGE was performed with a 2015 Pulsafor unit (LKB Instruments, Bromma, Sweden) equipped with a hexagonal electrode array for 18 h at 300V at 9°C. The gels were stained with ethidium bromide and photographed under UV illumination. Transconjugants were confirmed based on the comparisons of *Xba*I macrorestriction profiles and the presence of plasmid bands in the S1 nuclease assay. The efficiency of conjugation was estimated according to Phornphisutthimas et al. (2007).

Multilocus sequence typing of transconjugants

Multilocus sequence typing (MLST) was performed by PCR amplification and sequencing of seven housekeeping genes (*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) using primers and conditions defined at the Enterobase *E. coli* MLST database (<https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html>) (Wirth et al., 2006). According to the allele profile, the isolates were assigned to a specific sequence type (ST) using the Enterobase database (Supplementary Table 2). Sequence type designation was not possible for two isolates (1773/47 and 1773/64), which means that these may belong to some new sequence type and thus must be analyzed by the whole genome sequencing approach for the final MLST confirmation.

Results

Multidrug resistance in *E. coli* isolates from black-headed gulls

Resistance to antibiotics was detected in 44 out of 95 *E. coli* isolates from black-headed gulls (Table 1). Resistance to three or more antibiotics of different classes was identified in 24 isolates. However, resistance to colistin was not found (Table 1). A class 1 integron was detected only in one isolate (1773/30) which was resistant to six different antibiotics including one combination of them: AMP, CHL, STR, SA, TET, TMP/SXT. Resistance to ampicillin was most frequently detected - in 34 isolates in total- and was associated with resistance to streptomycin, sulfonamides and trimethoprim in 15 isolates. The most frequently detected gene that was responsible for resistance to beta-lactams was *bla*_{TEM} (24 isolates), followed by *bla*_{CMY-2} (9 isolates), while *bla*_{CTX-M-1} was confirmed in only one isolate. The second most common resistance was found to tetracyclines, which was conferred by *tetA*(A) or *tetB*(B) genes alone (in 20 isolates and 4 isolates, respectively) or by both of them (2 isolates). Some *E. coli* were found to be resistant to streptomycin (20 isolates), which was mediated by the combination of *strA* and *strB* genes, which encode aminoglycoside phosphotransferases APH(3'')-Ib and APH(6)-Id, respectively.

Table 1
The resistotype and resistance gene detection in *E. coli* isolates from black-headed gulls

Isolate No	Resistotype	MIC(mg/L) CST	Resistance genes
1773/1	AMP, CIP, CHL, NAL, STR, SA, TET, TMP, SXT	0.5	<i>bla</i> _{TEM} , <i>cat1</i> , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet</i> (B), <i>dfrA7/17</i> , <i>int1</i>
1773/3	AMP, CHL, STR, SA, TET	0.5	<i>bla</i> _{TEM} , <i>cat1</i> , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet</i> (B)
1773/6	CIP, NAL, TET	0.25	<i>tet</i> (A)
1773/7	AMC, AMP, CPD, CAZ, CTX	0.5	<i>bla</i> _{TEM} , <i>bla</i> _{CMY-2}
1773/21	AMP, STR, SA, TET, TMP, SXT	0.5	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>aadA2</i> , <i>sul2</i> , <i>tet</i> (A), <i>dfrA12</i> , <i>int1</i>
1773/25	AMP, CIP, CHL, NAL, STR, SA, TET, TMP, SXT	0.5	<i>bla</i> _{TEM} , <i>cat1</i> , <i>strA</i> , <i>strB</i> , <i>sul1</i> , <i>sul2</i> , <i>tet</i> (A), <i>dfrA7/17</i>
1773/30	AMP, CHL, STR, SA, TET, TMP, SXT	0.5	<i>bla</i> _{TEM} , <i>cat1</i> , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet</i> (A), <i>dfrA7/17</i> , <i>int1</i> , integron1
1773/31	STR, TET	0.5	<i>strA</i> , <i>strB</i> , <i>tet</i> (B)
1773/40	TET	0.5	<i>tet</i> (B)
1773/47	AMC, AMP, CPD, CAZ, CTX	0.5	<i>bla</i> _{CMY-2}
1773/50	AMC, AMP, CPD, CTX, CIP, NAL, TET	1	<i>bla</i> _{CMY-2} , <i>tet</i> (A)
1773/52	AMC, AMP, CPD, CAZ, CTX, CIP, NAL, TET	0.5	<i>bla</i> _{CMY-2} , <i>tet</i> (A)
1773/59	AMP, NAL, TET	0.5	<i>tet</i> (A)
1773/67	AMC, AMP, CPD, CAZ	0.5	<i>bla</i> _{CMY-2}
1773/70	AMP, CIP, NAL, TET	0.5	<i>bla</i> _{TEM} , <i>tet</i> (A), <i>tet</i> (B)
1773/75	AMC, AMP, CPD, CAZ, CTX	0.5	<i>bla</i> _{CMY-2}
1773/80	AMP, CPD, CTX	0.25	<i>bla</i> _{CTX-M-1}
1773/85	CHL, STR, SA, TET	0.5	<i>cmlA</i> , <i>aadA1</i> , <i>aadA2</i> , <i>sul3</i> , <i>tet</i> (A), <i>int1</i>
1773/86	AMP, NAL, STR, SA, TET, TMP, SXT	0.5	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet</i> (A), <i>dfrA5/14</i> , <i>int1</i>
1773/87*	AMP, CIP, NAL, STR, SA, TET, TMP, SXT	0.5	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>dfrA5/14</i>
4	STR, TET	0.5	<i>strA</i> , <i>strB</i> , <i>tet</i> (A), <i>tet</i> (B)
16	NAL, TET	0.5	<i>tet</i> (A)
17	AMP, CIP, NAL, STR, SA, TET, TMP, SXT	0.25	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>aadA1</i> , <i>sul1</i> , <i>sul2</i> , <i>tet</i> (A), <i>dfrA1</i> , <i>int1</i>
22a	AMC, AMP, CPD, CAZ, CTX	1	<i>bla</i> _{CMY-2}
1774/1	AMC, AMP, CPD, CAZ, CTX, NAL, TET	0.5	<i>bla</i> _{CMY-2} , <i>tet</i> (A)
1774/2	STR, SA	1	<i>strA</i> , <i>strB</i> , <i>sul2</i>
1774/14	NAL	0.5	/
1774/18	AMP, CIP, NAL, STR, SA, TET, TMP, SXT	0.5	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>aadA1</i> , <i>sul1</i> , <i>sul2</i> , <i>tet</i> (B), <i>dfrA1</i>
1774/25	NAL	0.5	/
1774/29*	AMP, TET	1	<i>bla</i> _{TEM}
1774/37	AMP, STR, SA, TET, TMP, SXT	2	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet</i> (A), <i>dfrA7/17</i>
1774/42	AMP, CPD, CTX, SSS	1	<i>bla</i> _{TEM} , <i>sul2</i>
1774/43	AMP, STR, SA, TET, TMP, SXT	1	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet</i> (A), <i>dfrA7/17</i>
1774/45	AMP	1	<i>bla</i> _{TEM}
1775/17	AMC, AMP, CPD, CAZ, CTX	0.5	<i>bla</i> _{CMY-2}

* *tet*(A) and *tet*(B) genes were not detected in *E. coli* isolates number 1773/87 and 1774/29

Isolate No	Resistotype	MIC(mg/L) CST	Resistance genes
1775/20	AMP, STR, SA, TET	0.5	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet(A)</i>
1775/21	AMP,STR, SA, TET	0.5	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>aadA2</i> , <i>sul2</i> , <i>tet(A)</i>
1775/27	AMP,STR, SA, TET, TMP, SXT	1	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet(A)</i> , <i>dfrA5/14</i>
1775/30	AMP, STR, SA, TET, TMP, SXT	1	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet(A)</i> , <i>dfrA5/14</i>
1775/33	AMP,STR, SA, TET, TMP, SXT	0.5	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul2</i> , <i>tet(A)</i> , <i>dfrA5/14</i>
1776/4	NAL	1	/
1776/13	AMP,CIP,NAL,STR,SA, TET, TMP,SXT	0.5	<i>bla</i> _{TEM} , <i>strA</i> , <i>strB</i> , <i>sul1</i> , <i>sul2</i> , <i>tet(A)</i> , <i>dfrA1</i>
1776/19	AMP,SA	0.5	<i>bla</i> _{TEM} , <i>sul3</i>
1776/28	AMP	0.5	<i>bla</i> _{TEM}
* <i>tet(A)</i> and <i>tet(B)</i> genes were not detected in <i>E. coli</i> isolates number 1773/87 and 1774/29			

Resistance to trimethoprim was detected in 14 isolates among which five carried dihydrofolate reductase genes of type *dfrA7/17* or *dfrA5/14*, whose products catalyse the reduction of dihydrofolate to tetrahydrofolate. Two other genes, *dfrA1* and *dfrA12*, coding for dihydrofolate reductase enzymes, were detected in three and one isolates, respectively. Resistance to chloramphenicol was mediated by the gene *cat1* (four isolates), which encodes a chloramphenicol acetyltransferase protein that inactivates the antibiotic by an acetylation mechanism. Another isolate carried the *cmlA* gene encoding a specific transporter protein. The genes involved in resistance to sulphonamides were either *sul2* (in 14 isolates) or *sul3* (two isolates), while in four isolates both *sul1* and *sul2* genes were identified. In addition, the *int1* gene coding for class 1 integrase enzymes was found in six isolates (Table 1).

Characterization of *E. coli* isolates resistant to extended-spectrum beta-lactam antibiotics

Ten *E. coli* isolates were resistant to extended-spectrum cephalosporin antibiotics (nine isolates were resistant to cefotaxime, ceftazidime, cefpodoxime and ceftiofur, while one isolate (1773/80) was resistant to cefotaxime and cefpodoxime (Table 2). Out of these ten isolates, three (1773/50, 1773/52, 1774/1) were co-resistant to quinolones and tetracyclines and were therefore classified as multidrug-resistant. Six of the *E. coli* isolates carried the *bla*_{CMY-2} gene and exhibited a resistance phenotype with resistance only to beta-lactam antibiotics. The remaining isolate 1773/80 carried the *bla*_{CTX-M-1} gene. However, the plasmid Inc I1/FIB carrying this resistance gene was not transferred to the recipient strain in the conjugation experiments (Table 2). Furthermore, this isolate did not harbor any of the seven APEC virulence genes. In contrast, in the mating experiments, seven of the nine *E. coli* isolates carrying a *bla*_{CMY-2} gene were able to transfer the corresponding *bla*_{CMY-2}-bearing plasmid to the recipient strain. It was shown that isolate 1773/50 carried a 95 kb conjugative plasmid, replicon type Inc I1/ FIB, with the *bla*_{CMY-2} gene and additional *hly*, *iroN*, *iss*, *ompT* and *cvaC* virulence genes (Table 2). The other conjugative plasmids were either of replicon type I1 or I1/FIB but did not carry any of the virulence genes tested. The conjugation efficiency of these IncI or IncI/FIB type plasmids was moderate to high for isolates 1773/7, 1773/67 and 1775/17 (3.36×10^3 to 2.66×10^1 cfu), while for the rest of the isolates the conjugation transfer was lower (Table 2).

Table 2
Plasmid mediated resistance to extended-spectrum cephalosporin antibiotics in *E. coli* isolates from black-headed gulls

Characterization of <i>E. coli</i> -donors							Characterization of <i>E. coli</i> -transconjugants				
Isolate No	Resistotype	Resist. genes	Virulence genes	Sequen. type	Phyl. group	Inc group	Resistotype TR	Resist. gene transfer	Efficacy of conjugation	Virulence gene transfer	Plasmid size (kb)
1773/7	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	-	ST38	D	I1	AMC, AMP, CPD, CTX, CAZ, FOX,	<i>bla</i> _{CMY-2}	3.36 x 10 ⁻³	-	95
22a	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	-		D	I1, FIB	-	-	-	-	
1773/47	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	<i>hlyF, ompT</i>	ND*	D	I1	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	2.81 x 10 ⁻⁵	-	110
1773/50	AMC, AMP, CPD, CTX, FOX, CIP, NAL, TET	<i>bla</i> _{CMY-2} , <i>bla</i> _{TEM-1} , <i>tetA</i>	<i>hly, iroN, iss, ompT, cvaC</i>	ST2307	D	I1, FIB	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	1.27 x 10 ⁻⁶	<i>hly, iroN, iss, ompT, cvaC</i>	95
1773/52	AMC, AMP, CPD, CAZ, CTX, CIP, NAL, TET, FOX	<i>bla</i> _{CMY-2} , <i>bla</i> _{TEM-1} , <i>tetA</i>	<i>iroN, hlyF, iutA, iss, ompT, eitC</i>	ST224	B2	I1, FIB	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	3.33 x 10 ⁻⁴	-	40, 1140
1773/67	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	<i>iroN</i>	ND*	B1	I1, FIB	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	4.2 x 10 ⁻²	-	90
1773/75	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2} <i>bla</i> _{TEM-1}	<i>iroN, hlyF, iutA, iss, ompT, eitC, cvaC</i>	ST162	B2	I1, FIB	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	3.3 x 10 ⁻⁵	-	95, 118
1773/80	AMP, CPD, CTX	<i>bla</i> _{CTX-M-1} , <i>bla</i> _{TEM-1}	-		B2	I1, FIB	-	-	-	-	
1774/1	AMC, AMP, CPD, CAZ, CTX, FOX, NAL, TET	<i>bla</i> _{CMY-2} , <i>bla</i> _{TEM-1} , <i>tetA</i>	<i>iroN, hlyF, iutA, iss, ompT, cvaC</i>		B2	I1, FIB	-	-	-	-	
1775/17	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2} <i>bla</i> _{TEM-1}	-	ST34	B2	I1, FIB, Y	AMC, AMP, CPD, CAZ, CTX, FOX	<i>bla</i> _{CMY-2}	2.66 x 10 ⁻¹	-	95
*ND, ST designations could not be attributed to isolates <i>E. coli</i> 1773-47 and 1773-67 (Supplementary Table 2)											

Phylogenetic analysis was carried out on all the isolates conferring resistance to extended-spectrum cephalosporins. Five isolates belonged to the phylogenetic-group B2, four isolates belonged to group D and one isolate was assigned to the B1 group. Five different sequence types (ST38, ST2307, ST224, ST162 and ST34) were detected in these *E. coli* isolates.

Resistance to fluoroquinolones (FQ)

In this study, high-level resistance to fluoroquinolones was detected in ten isolates. The MIC values in FQ resistant strains ranged from 4 to 32 mg/L (Table 3). Mutations in the quinolone resistance determining region (QRDR) of the topoisomerase genes were investigated in a few selected isolates (Table 3). For these isolates it was shown that high MIC values of CIP were achieved due to multiple mutations in the *gyrA*, *parC* and/or *parE* genes (Table 3), while single point mutations in the *gyrA* gene were detected in isolates resistant to NAL, resulting in amino acid transitions Ser83→Leu or Asp87→Asn. Plasmid-mediated resistance (PMQR) determinants were not found.

Table 3: Mutations in topoisomerase genes in representative *E. coli* isolates from black-headed gulls

Isolate No	<i>gyrA</i>	<i>parC</i>	<i>parE</i>	MIC-CIP mg/L
1773/1	Ser83Leu, Asp87Asn	/	/	4
1773/6	Ser83Leu, Asp87Asn	Ser80Ile	/	4
1773/25	Ser83Leu, Asp87Asn	Ser80Ile	/	8
1773/70	Ser83Leu, Asp87Asn	Ser80Ile	/	4
1773/87	Ser83Leu, Asp87Asn	Ser80Ile	Leu416Phe	16
17	Ser83Leu, Asp87Asn	Ser80Ile	Ser458Ala	16
1773/50	Ser83Leu Asp87Asn	/	/	32
1773/52	Ser83Leu Asp87Asn	Ser80Ile	Ser458Ala	32
1773/86	Ser83Leu	/	/	0.25
16	Asp87Tyr	/	/	0.125

PMQR genes (*qnrS*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *aac-lb-cr*, *qepA*, *oqxA*, *oqxB*) were not identified in *E. coli* isolates. Sequencing of the QRDR was not done in isolates 1774/18 and 1776/13 resistant to CIP and in isolates resistant to NAL (1773/59, 1774/1, 1774/14, 1774/18, 1774/25, and 1776/4).

Discussion

In the course of the present study, it was shown that almost half of the black-headed gulls living in the wild carried resistant or multi-resistant *E. coli*. The resistance patterns, but also the resistance genes detected in the *E. coli* isolates were similar to those of other research studies, in which fecal samples from waterfowl were taken (Dolejska et al., 2007; Poeta et al., 2008; Dolejska et al., 2009; Literak et al., 2010; Tausova et al., 2012). However, the significant resistance to fluoroquinolone in *E. coli* isolates from gulls in Serbia implies the overuse of these antibiotics in human and veterinary medicine and the unsafe disposal of communal and medical waste in Serbia.

In the present collection of isolates, resistance to extended-spectrum cephalosporins was mediated by the plasmid-borne resistance gene *bla*_{CMY-2} except in one case where the *bla*_{CTX-M-1} gene was identified. Often the CMY-2 plasmid carriers were resistant only to beta-lactam antibiotics. A similar result was obtained in another study, in which only three out of eight cephalosporin-resistant *E. coli* isolates with a *bla*_{CMY-2} gene from gulls and bald eagles from Alaska were multidrug-resistant (Ahlstrom et al., 2018). However, in *E. coli* isolates from food-producing animals, the *bla*_{CTX-M-1} gene is widely distributed in the Mediterranean area (Dandachi et al., 2018). The CTX-M family is also prevalent in *Enterobacteriaceae* in many European countries, mainly due to the epidemic spread of resistance plasmids. It is important that CTX-M carriers were found not only in *E. coli* and *Klebsiella pneumoniae* isolates from hospital patients but also in patients with community-acquired infections (D'Andrea et al., 2013; Canton et al., 2014). The *bla*_{CTX-M-1} gene has also been detected in *E. coli* isolates from Serbia from cases of clinical bovine mastitis, from diseased pigs and wildlife (Todorović et al., 2018; Velhner et al., 2018), and in this work also in an isolate from a gull.

We detected several important virulence genes in the *E. coli* isolates conferring resistance to beta-lactam antibiotics. These virulence genes comprised genes encoding a siderophore receptor for the iron acquisition mechanism (*iroN*), an episomal outer membrane protease (*ompT*), a putative avian hemolysin (*hly*), a serum survival protein (*iss*) and the colicin V structural gene (*cvaC*), which is important for increasing the adhesion and invasiveness of *E. coli* and other species of the *Enterobacteriaceae* family and which are frequently identified in avian pathogenic *E. coli*-APEC isolates (Gilson et al., 1987; Johnson et al., 2008; Johnson et al., 2010).

In the analyzed collection of isolates, only those with the CMY plasmid carried APEC virulence genes. Our results are therefore similar to those presented in the research by Touzain et al. (2018). In their work none of the *E. coli* isolates from diseased broilers, which contained the *bla*_{CTX-M-1} gene on IncI1/ST3 conjugative plasmids, carried APEC virulence genes. However, virulence genes were found on *bla*_{CMY-2} plasmid of the IncF replicon type (Touzian et al., 2018).

The genotypes of the ESBL-producing *E. coli* from gulls living in the vicinity to a dense human population were similar to the genotypes found in human isolates in southern France (Bonnedaahl et al., 2009) and Sweden (Bonnedaahl et al., 2010). Therefore, *E. coli* from gulls can serve as a biological indicator of environmental contamination due to their habits of living near humans and feeding on landfills along or off the coast (Bonnedaahl et al., 2009; Bonnedahl et al., 2014).

Twelve *E. coli* isolates from gulls in Barrow, Alaska were of sequence type ST38 and carried the *bla*_{CTX-M-14} gene (Bonnedaahl et al., 2014). In this work only isolate 1773/7 was identified as sequence type 38 and this carried the IncI1/CMY plasmid. In the European Union, the ST38 lineage is considered typical for poultry but has also been found in human *E. coli* isolates. It was evident that in *E. coli* isolates from Germany IncK, IncI and IncA/C plasmids most frequently carry the *bla*_{CMY-2} gene, while IncA/C plasmids were the most common CMY carriers in North America (Pitech et al., 2018). Horizontal transfer via plasmids is perhaps the most likely mechanism of dissemination of the *bla*_{CMY-2} gene although the epidemiological spread of specific lineages such as CMY-2 producing ST131 is involved in the transmission of CMY plasmid as well. Nevertheless, a common ancestor of *E. coli* isolates carrying the *bla*_{CMY-2} gene has been identified in genetically distant strains suggesting that bacteria change over time due to their genome plasticity and diversity (Pitech et al., 2018). It was found that the successes in the proliferation of CMY-2 plasmids depend on the selective pressure posed by the use of antibiotics or on the size of the plasmid since

larger plasmids, such as CMY carriers, are associated with a significant fitness cost (Subbiah et al., 2011). Therefore, the long-term stability of the *bla*_{CMY-2} plasmids in *E. coli* isolates from gulls, which are discussed in this study, should be determined in the future using *in vitro* experimental approaches.

In this work, we detected high-level resistance to FQ in several isolates. However, resistance to FQ was also observed in *E. coli* isolates from mallards and hearing gulls residing at the Polish coast of the Baltic sea (Literak et al., 2010) and from feces of gulls, pigeons and birds of prey in Portugal, Sweden and Spain (Vredenburg et al., 2013). Resistance to FQ antibiotics and extended-spectrum cephalosporins was also recently found in extra-intestinal pathogenic *E. coli* (ExPEC) isolates from silver gulls residing at the coastline in Australia which included pandemic ExPECST131 strains belonging to clade C (C1-H30-R and C2-H30-Rx), (Mukerji et al., 2019).

Resistance to beta-lactam antibiotics and fluoroquinolones in bacteria isolated from wild animals, including wild birds, is of concern because the environment appears to be contaminated by anthropogenic activities, affecting the bacterial flora of wildlife (Mukerji et al., 2019). While ESBL-producing *E. coli* isolates from food-producing animals are often multi-resistant due to co-selection mechanisms (Michael et al., 2017), *E. coli* isolates from black-headed gulls carrying the plasmid-borne AmpC gene (*bla*_{CMY-2}) appear to be resistant only to beta-lactam antibiotics (Atterby et al., 2016; Touzian et al., 2018). It must also be pointed out that the global spread of epidemic *E. coli* clonal lineages is of great importance in medicine and the spread of such isolates needs to be closely monitored (Pitout and DeVinney, 2017).

Conclusion

In conclusion, multidrug resistance in *E. coli* isolates from black-headed gulls residing in the city of Novi Sad was detected in 22 out of 96 isolates. However, five isolates which were resistant only to beta-lactam antibiotics carried the *bla*_{CMY-2} gene on transferable plasmids. Virulence genes (*hly*, *iroN*, *iss*, *ompT* and *cvaC*) were detected on one conjugative CMY plasmid as well. In isolates with high resistance to fluoroquinolones, the resistance was due to multiple mutations in the topoisomerase genes. This result may have been caused by environmental contamination in Serbia.

Declarations

Author contribution

MV, BJ and MK designed the study and analyzed the results, DT, KN and GL performed the sampling and the experiments, MV and BJ wrote the manuscript, CK reviewed the manuscript.

Disclosure statement

No competing financial interest exists.

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References

- Admassie M (2018) Current review on molecular and phenotypic mechanisms of bacterial resistance to antibiotic. *Sci J Clin Med* 7:13-19.
- Ahlstrom CA, Bonnedahl J, Woksepp H, Hernandez J, Olsen B, Ramey AM (2018) Acquisition and dissemination of cephalosporin-resistant *E. coli* in migratory birds sampled at an Alaska landfill as inferred through genomic analysis. *Sci Rep* 8:7361, <http://doi:10.1038/s41598-018-25474-w>.
- D'Andrea MM, Arena F, Pallecchi L, Rossolini GM (2013) CTX-M type β -lactamases: A successful story of antibiotic resistance. *Int J Med Microbiol* 303:305-317.
- Atterby C, Ramey AM, Hall GG, Järhult J, Börjesson S, Bonnedahl J (2016) Increased prevalence of antibiotic-resistant *E. coli* in gulls sampled in Southcentral Alaska is associated with urban environments. *Infect Ecol Epidemiol* 6:32334-<http://dx.doi.org/10.3402/iee.v6.32334>.
- Atterby C, Börjesson S, Ny S, Järhult JD, Byfors S, Bonnedahl J (2017) ESBL-producing *Escherichia coli* in Swedish gulls-a case of environmental pollution from humans? *PLoS ONE*, 12(12): e0190380. <https://doi.org/10.1371/journal.pone.0190380>.
- Bonnedahl J, Drobni M, Gauthier-Clerc M, Hernandez J, Granholm S, Kayser Y, Melhus A, Kahlmeter G, Waldenström J, Johansson A, Olsen B (2009) Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. *PLoS ONE* 4(6): e5958. <https://doi:10.1371/journal.pone.0005958>.
- Bonnedahl J, Drobni P, Johansson A, Hernandez J, Melhus A, Stedt J, Olsen B, Drobni M (2010) Characterization, and comparison, of human clinical and black-headed gull (*Larus ridibundus*) extended-spectrum beta-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden. *J Antimicrob Chemother* 65:1939-1944.

- Bonnedahl J, Hernandez J, Stedt J, Waldenström J, Olsen B, Drobni M (2014) Extended-spectrum β -lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in gulls, Alaska, USA. *Emerg Infect Dis* 20:897-899.
- Canton R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, Coque TM (2008) Prevalence and spread of extended-spectrum β -lactamase producing *Enterobacteriaceae* in Europe *Clin Microbiol Infect* 14:144-153.
- Clinical and Laboratory Standards Institute, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard-tenth edition, CLSI document M07, 11th ed. Wayne, PA, USA, 2018.
- Clinical and Laboratory Standards Institute, Performance standards for antimicrobial susceptibility testing; Twenty-fifth Informational Supplement. CLSI document M100, 28th ed. Wayne, PA, USA, 2018.
- Dandachi I, Chabou S, Daoud Z, Rolain JM (2018) Prevalence and emergence of extended-spectrum cephalosporin-carbapenem-and colistin-resistant gram negative bacteria of animal origin in the Mediterranean basin. *Front Microbiol* 9:2299. <https://doi.org/10.3389/fmicb.2018.02299>.
- Dolejska M, Cizek A, Literak I (2007) High prevalence of antimicrobial-resistant genes and integrons in *Escherichia coli* isolates from black-headed gulls in the Czech Republic. *J Appl Microbiol* 103:11-19.
- Dolejska M, Bierošová B, Kohutová L, Literák I, Čížek A (2009) Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-spectrum beta-lactamases in surface water and sympatric black-headed gulls. *J Appl Microbiol* 106: 1941-1950.
- The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria for humans, animals and food in 2017. *EFSA Journal* 2019;17(2):5598, 2778 pp. <https://doi.org/10.2903/j.efsa.2019.5598> (page 41).
- Faccione D, Moredo FA, Giacoboni GI, Alborno E, Alarcón L, Nievas VF, Corso A (2019) Multidrug-resistant *Escherichia coli* harbouring mcr-1 and bla_{CTX-M} genes isolated from swine in Argentina. *J Glob Antimicrob Resist* 18:160-162.
- Gilson L, Mahanty HK, Kolter R, (1987) Four plasmid genes are required for colicin V synthesis export, and immunity. *J Bacteriol* 169:2466-2470.
- Grenni P, Ancona V, Caracciolo AB, (2018) Ecological effects of antibiotics on natural ecosystems: A review. *Microchem J* 136:25-39.
- Gwozdziński K, Azarderakhsh S, Imirzalioglu C, Falgenhauer L, Chakraborty T (2018) An improved medium for colistin susceptibility testing. *J Clin Microbiol* 56(5),56:e01950-17. <https://doi.org/10.1128/JCM.01950-17>.
- Hernandez J, Johansson A, Stedt J, Bengtsson S, Porczak A, Granholm S, González-Acuña Olsen B, Bonnedahl J, Drobni M (2013) Characterization and comparison of extended-spectrum β -lactamase (ESBL) resistance genotypes and population structure of *Escherichia coli* isolated from Franklin's gulls (*Leucophaeus pipixcan*) and humans in Chile. *PLoS ONE* 8(9):e76150. doi:10.1371/journal.pone.0076150.
- Johnson TJ, Wannemuehler Y, Doetkott C, Johnson SJ, Rosenberger SC, Nolan LK (2008) Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. *J Clin Microbiol* 46:3987-3996.
- Johnson TJ, Thorsness JL, Anderson CP, Lynne AM, Foley SL, Han J, Fricke WF, McDermott PF, White DG, Khatri M, Stell AL, Flores C, Singer RS (2010) Horizontal gene transfer of ColV plasmid has resulted in a dominant avian clonal type of *Salmonella enterica* serovar Kentucky. *PLoS ONE*, 5(12): e15524. <https://doi.org/10.1371/journal.pone.0015524>.
- Johnson JR, Nicolas-Chanoine M-H, DeRoy C, Castanheira M, Robicsek A, Hansen G, Weissman S, Urban C, Platell J, Trott D, Zhanel G, Clabots C, Johnston BD, Kuskowski MA and the MASTER investigators. Comparison of *Escherichia coli* ST131 pulsotypes, by epidemiologic traits, 1997-2009. 2012. *Emerg Infect Dis* 18:598-607.
- Jovcic B, Lepsanovic Z, Suljagic V, Rackov G, Begovic J, Topisirovic L, Kojic M (2011) Emergence of NDM-1 metallo- β -lactamase in *Pseudomonas aeruginosa* clinical isolates from Serbia. *Antimicrob Agents Chemother* 55:3929–3931.
- Levy SB, Marshall B (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* 10, (12 Suppl), S122-S129.
- Literak I, Dolejska M, Janoszowska D, Hrusakova J, Meissner W, Rzycka H, Bzoma S, Cizek A (2010) Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the Baltic Sea coast of Poland. *Appl Environ Microbiol* 76:8126-8134.
- McDaniels AE, Rice EW, Reyes AL, Johnson CH, Haugland RA, Stelma GN Jr. (1996) Conformational identification of *Escherichia coli*, a comparison of genotype and phenotype assays for glutamate decarboxylase and beta-D-glucuronidase. *Appl Environ Microbiol* 62:3350-3354.
- Michael GB, Kaspar H, Siqueira AK, Costa EF, Corbellini LG, Kadlec K, Schwarz S (2017) Extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* isolates collected from disease food-producing animals in the GERM-Vet monitoring program 2008-2014. *Vet Microbiol* 200:142-150.

Mukerji S, Stegger M, Truswell AV, Laird T, Jordan D, Abraham RJ, Harb A, Barton M, O'Dea M, Abraham S (2019) Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins. *J Antimicrob Chemother* 74:2566-2574, <https://doi.org/10.1093/jac/dkz242>.

Nicolas-Chanoine M-H, Bertrand X, Madec J-Y (2014) *Escherichia coli* ST131, an intriguing clonal group. *Clin Microbiol Rev* 27:543-574.

Parker D, Sniatynski MK, Mandrusiak D, Rubin JE (2016) Extended-spectrum β -lactamase producing *Escherichia coli* isolated from wild birds in Saskatoon, Canada. *Lett Appl Microbiol* 63:11-15.

Phornphisutthimas S, Thamchaipenet A, Panijpan B (2007) Conjugation in *Escherichia coli*. *Biochem Mol Biol Edu* 35:440-445.

Pietsch M, Irrgang A, Roschanski N, Michael GB, Hamprecht A, Rieber H, Käsbohrer A, Schwarz S, Rösler U, Kreienbrock L, Pfeifer Y, Fuchs S, Werner G, and RESET study group, 2018. Whole genome analysis of CMY-2 producing *Escherichia coli* isolates from humans, animals and food in Germany. *BMC Genomics* 19:601, <https://doi.org/10.1186/s12864-018-4976-3>.

Pitout JDD, DeVinney R (2017) *Escherichia coli* ST131: a multidrug-resistant clone primed or global domination. *F1000Research* 2017, **6**(F1000 Faculty Rev):195 (doi: 10.12688/f1000research.10609.1).

Poeta P, Radhouani H, Igrejas G, Gonçalves A, Carvalho C, Rodrigues J, Vinué L, Somalo S, Torres C (2008) Seagulls of the Berlengas natural reserve of Portugal as carriers of fecal *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases. *Appl Environ Microbiol* 74:7439-7441.

Poirel L, Potron A, De La Cuesta C, Cleary T, Nordmann P, Munoz-Price SL (2012) Wild coastline birds as reservoirs of broad-spectrum- β -lactamase producing *Enterobacteriaceae* in Miami Beach, Florida. *Antimicrob Agents Chemother* 56:2756-2758.

Ramey AM, Hernandez J, Tyrlov V, Uher-Koch BD, Schmutz JA, Atterby C, Järhult JD, Bonnedahl J (2018) Antibiotic-resistant *Escherichia coli* in migratory birds inhabiting remote Alaska. *EcoHealth* 15, 72–81, <https://doi.org/10.1007/s10393-017-1302-5>.

Schwarz S, Silley P, Simjee S, Woodford N, van Duijkeren E, Johnson AP, Gastra W (2010) Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *J Antimicrob Chemother* 65:601-604.

Shabana II, Al-Enazi AT (2020) Investigation of plasmid-mediated resistance in *E. coli* isolated from healthy and diarrheic sheep and goats. *Saudi J Biol Sci* 27:788-796.

Simões RR, Poirel L, Da Costa PM, Nordmann P (2010) Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. *Emerg Infect Dis* 16:110-112.

Subbiah M, Top EM, Shah DH, Call DR (2011) Selection pressure required for long term persistence of *bla*_{CMY-2} positive IncA/C plasmids. *Appl Environ Microbiol* 77:4486-4493.

Tausova D, Dolejska M, Cizek A, Hanusova L, Hrusakova J, Svoboda O, Camlik G, Literak I (2012) *Escherichia coli* with extended-spectrum β -lactamase and plasmid-mediated quinolone resistance genes in great cormorants and mallards in Central Europe. *J Antimicrob Chemother* 67:1103-1107.

Todorović D, Velhner M, Grego E, Vidanović D, Milanov D, Krnjaić D, Kehrenberg C (2018) Molecular characterization of multidrug-resistant *Escherichia coli* isolates from bovine mastitis and pigs in the Vojvodina Province, Serbia. *Microb Drug Resist* 24:95-103.

Touzain F, Le Devendec L, de Boissésou C, Baron S, Jouy E, Perrin-Guyomard A, Blanchard Y, Kempf I (2018) Characterization of plasmid harboring *bla*_{CTX-M} and *bla*_{CMY} genes in *E. coli* from French broilers. *PLoS ONE* 13(1):e0188768. <https://doi.org/10.1371/journal.pone.0188768>.

Vandecraen J, Chandler M, Aertsen A, Van Houdt R (2017) The impact of insertional sequence on bacterial genome plasticity and adaptability. *Crit Rev Microbiol* 43:709-730.

Velhner M, Todorović D, Grego E, Jovčić B, Prunić B, Stojanov I, Kehrenberg C (2018) Fluoroquinolone-resistant and extended-spectrum beta-lactamase producing *Escherichia coli* isolates from free-living wild animals. *Vet Microbiol* 223:168-172.

Vredenburg J, Varela AR, Hasan B, Bertilsson S, Olsen B, Narciso-da-Rocha C, Bonnedahl J, Stedt J, Da Costa PM, Manaia CM (2013) Quinolone-resistant *Escherichia coli* isolated from birds of prey in Portugal are genetically distinct from those isolates from water environments and gulls in Portugal, Spain and Sweden. *Environ Microbiol* 16:995-1004, doi:10.1111/1462-2920.12231.

Wellington EMH, Boxall ABA, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM, Williams AP (2013) The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect Dis* 13:155-165.

Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MCJ, Ochman H, Achtman M (2006) Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Molec Microbiol* 60:1136-1151.

Zurfluh K, Albini S, Mattmann P, Kindle P, Nüesch-Inderbilen M, Stephan R, Vogler BR (2019) Antimicrobial resistant and extended-spectrum β -lactamase producing *Escherichia coli* in common wild bird species in Switzerland. MicrobiologyOpen 2019;8:e845. <https://doi.org/10.1002/mbo3.845>.

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