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ORIGINAL ARTICLE

Characterization of antibiotic resistance in *Escherichia coli* isolates from Black-headed gulls (*Larus ridibundus*) present in the city of Novi Sad, Serbia

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Abstract

Despite common resistance to antimicrobials in *Escherichia coli* isolates from farm animals in Serbia, no data are currently accessible on its occurrence in *E. coli* isolated from gulls. Therefore, 67 cloacal swabs and 70 fecal samples from black-headed gulls were investigated for the presence of antibiotic-resistant *E. coli* isolates. Ninety-nine isolates were obtained during the study. Resistotyping and resistance gene typing has shown that 44 isolates harbor resistance to one or more antibiotics. Multidrug resistance was detected in 24 *E. coli* isolates. Ten isolates were resistant to extended-spectrum cephalosporin antibiotics and were studied in detail including virulence gene typing, phylogenetic and multilocus sequence typing, and mating. These ten isolates belonged to phylogenetic groups B2 (five isolates), D (four isolates) and B1 (one isolate). Five different sequence types (ST38, ST2307, ST224, ST162 and ST34) were detected in *E. coli* isolates with AmpC phenotype and genotype. One isolate carried the Inc I2/FIB replicon type plasmid with the $bla_{\text{CTX-M-1}}$ gene. Nine isolates had $bla_{\text{CMY-2}}$ genes, which were detected on conjugative plasmids in seven isolates. The virulence genes *hly*, *iroN*, *iss*, *ompT* and *cvaC* were detected in one transconjugant. Ten isolates were found to be resistant to ciprofloxacin, whose MIC ranged 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. So, Black-headed gulls from Serbia may be colonized by multidrug-resistant *E. coli,* some of which are resistant to critically important antibiotics in medicine.

Keywords *Escherichia coli* · Gulls · Resistance · Antibiotics · Virulence · Plasmid

Introduction

The escalating development of antimicrobial resistance in pathogenic and commensal bacteria is a well-known worldwide problem. There are several ways bacteria gain resistance to antibiotics, which include intrinsic, acquired and adoptive mechanisms (Adamassie et al. 2018). Acquired antimicrobial resistance genes are often associated with mobile genetic elements such as plasmids, transposons, integrons and insertion sequences. This complex genetic

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material can be easily transferred between bacterial species, which facilitates the spread of resistant bacteria in the environment (Levy and Marchall 2004; Wellington et al. 2013; Vandecraen et al. 2017). Hospital-acquired infections cause major concern in medicine, while in the food industry problems arise from persistent contamination of farms with pathogenic and multidrug-resistant bacteria. Therefore, the only way to reduce contamination and spread of bacteria is to use antibiotics prudently and continuously apply high hygiene standards. On the other hand, it is an extremely difficult to reduce dissemination of 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. It was established 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 isolated from 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, in some cases, mobile resistance genes have been detected in virulent *E. coli* from wildlife, but these may have originated from humans or farm animals (Hernandez et al. 2013; Atterby et al. 2016, 2017; Ahlstrom et al. 2018). Moreover, a de novo development of resistance in *E. coli* and other members of the *Enterobacteriaceae* family can also occur (e.g., to synthetic antibiotics such as fluoroquinolones), which may result from antibiotic residues and contamination of habitats due to anthropogenic activities. This is supported by the fact that fluoroquinolones can accumulate in natural environments, especially in soil and water (Grenni et al. 2018).

Extended-spectrum β-lactamase (ESBL)-producing *E. coli* is frequently detected in the livestock industry in both diarrheic and healthy animals (Faccone et al. 2019; Shabana and Al-Enazi 2020). Even well-established clones in humans, such as *E. coli* ST131, are occasionally detected in animals or the environment (Johnson et al. 2012; Nicolas-Chanonine et al. 2014). Moreover, ESBL-producing *E. coli* from gulls living in the vicinity of a dense human population were similar in genotypes to those found in human isolates in southern France (Bonnedahl et al. 2009) and Sweden (Bonnedahl et al. 2010; Atterby et al. 2017). Therefore, *E. coli* from gulls can serve as a biological indicator of environmental contamination owing to their tendency to live near humans and feed on landfills along or off the coast (Bonnedahl et al. 2009, 2014). The success of ESBL-producing *E. coli* in adaptation to different ecological environments depends on its virulence and antibiotic resistance mechanisms (Nicolas-Chanonine et al. 2014).

As early as in 2001, antimicrobial resistance (AMR), a global issue, prompted the World Health Organization to declare an urgent need for the implementation of the One Health approach to tackle all the aspects of resistance development in bacteria isolated from humans, animals, food and the environment. By establishing the One Health monitoring system in developing countries the gaps in knowledge about the dissemination of resistant bacteria are expected to be minimized (Queenan et al. 2016; Matheu et al. 2017). However, the evaluation of AMR in commensals is challenging. Thus, different approaches are incorporated into the global AMR monitoring systems all over the world. The monitoring of antimicrobial resistance in isolates from wildlife is also required. Wildlife may present sentinels of antimicrobial resistance and serve as vehicles for the broad dissemination of resistance genes in bacterial communities and the environment on our planet (White and Hughes 2019).

Given the necessity for comprehensive AMR monitoring, we analyzed the resistance mechanisms to different classes of antibiotics in *E. coli* isolates from black-headed gulls residing in the city of Novi Sad. In the isolates, the plasmids conferring resistance to cephalosporin antibiotics were studied in detail and it was determined whether they harbor virulence genes known to be significantly associated with extraintestinal and avian pathogenic *E. coli* (ExPEC and APEC).

Material and methods

Sampling

Sixty-seven cloacal swabs and seventy fecal samples (in total 137 samples) were obtained from black-headed gulls (*Larus ridibundus*) collected during the winter months in the city of Novi Sad in 2014. Cloacal swabs were taken on local landfills in the vicinity of the city during a bird-banding 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). Seventy fecal samples were collected at the local beach and at the Danube pier for isolation, identification of *E. coli* and resistotyping. Only antibiotic resistant isolates were included in the following research.

Isolation and identification of *E. coli*

E. coli was isolated by inoculating each single swab in10 mL of buffered peptone water (Oxoid, Basingstoke, Hampshire, United Kingdom). After 18 h of incubation at 37 °C, a full loop (10 µL) was used and the inoculum spread over McConkey agar (Oxoid, Basingstoke, Hampshire, United Kingdom). 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 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 (CLSI 2015). The following antibiotic disks with the recommended concentration of antibiotics were used: 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), cefoxitin 30 µg (FOX). The 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 (2015). The interpretation of phenotypic tests for the detection of plasmid-mediated pAmpC β-lactamase production included cefoxitin (30 µg) and cefepime (30 µg) disks as recommended by the EFSA Journal (2019). Testing of MICs of ciprofloxacin was done by the broth microdilution method in cation-adjusted Mueller Hinton broth (Oxoid, Basingstoke, Hampshire, United Kingdom). However, MICs for colistin were determined following the protocol by Gwozdzinski et al. (2018) in Mueller Hinton broth (Sigma-Aldrich, Schnelldorf, Germany) supplemented with calcium chloride dehydrate (Roth, Karlsruhe, Germany). *E. coli* ATCC 25,922 and *E. coli* NCTC 13,846 (for colistin) were included on each plate for quality control purposes.

Resistance and virulence gene screening and sequencing

E. coli isolates resistant to one or more antibiotics were included in the resistance gene screening with PCR. For all PCR assays, the DNA was obtained following the boiling procedure. *E. coli* was grown overnight in tryptic soy broth. The following day the bacteria were boiled 5 min at 100 °C, chilled on ice for 10 min and spin to obtain the DNA. The primer sequences, annealing temperatures and references used for the resistance and virulence gene screening including primers used for phylogenetic and replicon typing are presented in Supplementary Table 1. Briefly, the phylogeny typing was done according to Clermont et al. (2000) by using three pairs of primers in multiplex PCR to identify the four major phylogenetic groups (A, B1, D and B2) of *E. coli*. Resistance genes and integrons 1 and 2 were detected using the published primer sequences and applying PCR protocols for which the full references list is provided in the Supplementary Table 1. Seven pairs of primers at the identical annealing temperature of 63 °C were used for detection of virulence genes that are most commonly found in APEC and therefore considered predictors of the pathogenic phenotype of these bacteria for poultry (Johnson et al. 2008, 2010). Plasmid replicon typing was done according to the protocol by Carattoli et al (2005) which utilize 18 pairs of primers in five multiplex PCR reactions established at the same annealing temperature of 60 °C. The replicon typing is used to classify plasmids to different incompatibility groups (Inc) based on the systems that bacteria belonging to the *Enterobacteriaceae* family use to control their replication.

The master mix for PCR was prepared with a commercial kit One Taq Hot Start 2 × Master Mix M0484, (New England BioLabs, New England BioLabs, Frankfurt am Main, DE) or Qiagen Hot Star Taq Master Mix commercial kit (Qiagen, Hilden, Germany). The obtained amplicons of beta-lactam genes and topoisomerase genes (the *gyrA*, *gyrB*, *parC* and *parE* gene) were purified using the commercial kit Monarch, PCR and DNA Clean up Kit (New England, BioLabs, Frankfurt am Main, Germany) or a DNA purification step was done using the QiAquick PCR purification kit (Qiagen, Hilden, Germany). Clean DNA was then sent to Macrogen, Amsterdam, the Netherlands, for sequencing of both strands. The sequences of the $bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ genes and 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).

Multilocus sequence typing

Multilocus sequence typing (MLST) of seven *E. coli* isolates resistant to extended-spectrum cephalosporins was done by PCR amplification and sequencing of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) using primers and conditions defined on the EnteroBase *E. coli* MLST database (https://enterobase.readthedocs.io/en/latest/mlst/mlst-legacy-info-ecoli.html) (Wirth et al. 2006). Depending on the allele profile, the isolates were assigned to a specific sequence type (ST) in accordance with the EnteroBase database (Supplementary Table 2).

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. The HK225 strain was a generous gift from Professor Corinna Kehrenberg. It is a lactose-negative strain resistant to rifampicin and has been used as a recipient strain in mating experiments for several years. For these experiments, the Luria Bertani (LB) 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 LB medium for 30 min and the obtained transconjugants were processed 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 was processed as previously described (Jovcic et al. 2011). The DNA was either digested with the restriction enzyme *Xba*I or with S1 nuclease, or was not digested. PFGE was done with a 2015 Pulsafor unit (LKB Instruments, Bromma, Sweden) equipped with a hexagonal electrode array for 18 h at 300 V and 9 ℃. 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).

Results

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

E. coli was isolated from 46 out of 67 cloacal swabs. Twenty-four (No 1–24) *E. coli* isolates were found to be resistant to antibiotics and included in further studies. *E. coli* was isolated from 34 out of 43 fecal samples collected on the beach. Ten out of these 34 isolates (No 25–34) were included in the research due to their single or multidrug-resistant phenotype. On the local pier, 27 fecal samples were taken on two different locations and *E. coli* was isolated from 12 and 7 samples, respectively. Antimicrobial resistance was detected in 6 and 4 *E. coli* isolates (No 35–44), which were further processed. In total resistance to antibiotics was detected in 44 out of 99 *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). One isolate (No 7), which was resistant to six different antibiotics including one combination (AMP, CHL, STR, SA, TET, TMP/SXT), also carried class 1 integron. 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} (30 isolates), followed by $bla_{\text{CMY-2}}$ (9 isolates), while $bla_{\text{CTX-M-1}}$ was confirmed in only one isolate. The second most common resistance was found to tetracyclines, which was conferred by *tet*(A) or *tet*(B) genes alone (in 20 and 4 isolates, respectively) or by both (2 isolates). Some *E. coli* were found to be resistant to streptomycin (20 isolates). Resistance to trimethoprim was detected in 14 isolates, out of which five carried dihydrofolate reductase genes of type *dfrA7/17* and another five carried *dfrA5/14.* Two other genes coding for dihydrofolate reductase enzymes, *dfrA1* and *dfrA12,* were detected in three and one isolates, respectively. Resistance to chloramphenicol was mediated by the gene *cat1* (four isolates), another isolate carried the *cmlA* gene. The genes involved in resistance to sulfonamides were either *sul2* (in 14 isolates) or *sul3* (two isolates), but in four isolates both 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 (ESC) i.e., nine were resistant to cefotaxime, ceftazidime, cefpodoxime and cefoxitin, while one isolate (No 17) was resistant to cefotaxime and cefpodoxime (Table 2). Out of these ten isolates, three (No 11, 12 and 25) were co-resistant to quinolones and tetracyclines and therefore classified as multidrug-resistant. Virulence genes were found in six out of ten *E. coli* isolates exhibiting an ESC-resistant phenotype.

Nine isolates carried $bla_{\text{CMY-2}}$ gene single or in combination with the $bla_{\text{TEM-1}}$ gene.

The remaining isolate, 17, carried the $bla_{\text{CTX-M-1}}$ gene. However, this Inc I1/FIB plasmid was not transferred to the recipient strain in the conjugation experiments (Table 2). Furthermore, this isolate did not harbor any of the seven virulence genes commonly found in avian pathogenic *E. coli* (APEC). In contrast, seven out of the nine *E. coli* isolates were able to transfer the corresponding $bla_{\text{CMY-2}}$-bearing plasmid to the recipient strain. It was shown that isolate 11 carried a 95 kb conjugative plasmid, replicon type Inc I1/FIB, with the $bla_{\text{CMY-2}}$ gene and additional *hly*, *iroN*, *iss*, *ompT* and *cvaC* virulence genes (Table 2, Supplementary Fig. 1). The other conjugative plasmids were 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 in isolates 4, 14 and 35 (from 3.36×10^{-3} to 2.66×10^{-1} CFU), while in the rest of the isolates the conjugation transfer was lower (Table 2).

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
1	AMP, CIP, CHL, NAL, STR, SA, TET, TMP, SXT	0.5	bla_{TEM}, *cat1*,*strA*, *strB*, *sul2*, *tet*(B), *dfrA7/17, int1*
2	AMP, CHL, STR, SA, TET	0.5	bla_{TEM}, *cat1, strA*, *strB*, *sul2*, *tet*(B)
3	CIP, NAL, TET	0.25	*tet*(A)
4	AMC, AMP, CPD, CAZ, CTX, FOX	0.5	bla_{TEM}, bla_{CMY-2}
5	AMP, STR, SA, TET, TMP, SXT	0.5	bla_{TEM}, *strA*, *strB*, *aadA2*, *sul2*, *tet*(A), *dfrA12, int1*
6	AMP, CIP, CHL, NAL, STR, SA, TET, TMP, SXT	0.5	bla_{TEM}, *cat1*,*strA*, *strB*, *sul1*, *sul2*, *tet*(A), *dfrA7/17*
7	AMP, CHL, STR, SA, TET, TMP, SXT	0.5	bla_{TEM}, *cat1*,*strA*, *strB*, *sul2*, *tet*(A), *dfrA7/*17, *int1*, integron1
8	STR, TET	0.5	*strA*, *strB*, *tet*(B)
9	TET	0.5	*tet*(B)
10	AMC, AMP, CPD, CAZ, CTX, FOX	0.5	bla_{CMY-2}
11	AMC, AMP, CPD, CTX, CIP, NAL, TET, FOX	1	bla_{TEM} , bla_{CMY-2}, *tet*(A)
12	AMC, AMP, CPD, CAZ, CTX, CIP, NAL, TET, FOX	0.5	bla_{TEM}, bla_{CMY-2}, *tet*(A)
13	AMP, NAL, TET	0.5	*tet*(A)
14	AMC, AMP, CPD, CAZ, CTX, FOX	0.5	bla_{CMY-2}
15	AMP, CIP, NAL, TET	0.5	bla_{TEM}, *tet*(A), *tet*(B)
16	AMC, AMP, CPD, CAZ, CTX, FOX	0.5	bla_{TEM}, bla_{CMY-2}
17	AMP, CPD, CTX	0.25	bla_{TEM}, $bla_{CTX-M-1}$
18	CHL, STR, SA, TET	0.5	*cmlA*,*aadA1*, *aadA2*, *sul3*, *tet*(A), *int1*
19	AMP, NAL, STR, SA, TET, TMP, SXT	0.5	bla_{TEM}, *strA*, *strB*, *sul2*, *tet*(A),*dfrA5/14, int1*
20	AMP, CIP, NAL, STR, SA, TET, TMP, SXT	0.5	bla_{TEM}, *strA*, *strB*, *sul2*, *dfrA5/14*
21	STR, TET	0.5	*strA*, *strB*, *tet*(A), *tet*(B)
22	NAL, TET	0.5	*tet*(A)
23	AMP, CIP, NAL, STR, SA, TET, TMP, SXT	0.25	bla_{TEM}, *strA*, *strB*,*aadA1*, *sul1*, *sul2*,*tet*(A),*dfrA1, int1*
24	AMC, AMP, CPD, CAZ, CTX, FOX	1	bla_{CMY-2}
25	AMC, AMP, CPD, CAZ, CTX, NAL, TET, FOX	0.5	bla_{TEM}, bla_{CMY-2}, *tet*(A)
26	STR, SA	1	*strA*, *strB*, *sul2*
27	NAL	0.5	/
28	AMP, CIP, NAL, STR, SA, TET, TMP, SXT	0.5	bla_{TEM}, *strA*, *strB*, *aadA1*, *sul1*, *sul2*, *tet*(B),*dfrA1*
29	NAL	0.5	/
30	AMP, TET	1	bla_{TEM}
31	AMP, STR, SA, TET, TMP, SXT	2	bla_{TEM}, *strA*, *strB*, *sul2*, *tet*(A), *dfrA7/17*
32	AMP, CPD, CTX, SSS	1	bla_{TEM}, *sul2*
33	AMP, STR, SA, TET, TMP, SXT	1	bla_{TEM}, *strA*, *strB*, *sul2*, *tet*(A), *dfrA7/17*
34	AMP	1	bla_{TEM}
35	AMC, AMP, CPD, CAZ, CTX, FOX	0.5	bla_{TEM}, bla_{CMY-2}
36	AMP, STR, SA, TET	0.5	bla_{TEM}, *strA*, *strB*, *sul2*, *tet*(A)
37	AMP,STR, SA, TET	0.5	bla_{TEM}, *strA*, *strB*, *aadA2*, *sul2*, *tet*(A)
38	AMP,STR, SA, TET, TMP, SXT	1	bla_{TEM}, *strA*, *strB*, *sul2*, *tet*(A), *dfrA5/14*
39	AMP, STR, SA, TET, TMP, SXT	1	bla_{TEM}, *strA*, *strB*, *sul2*, *tet*(A), *dfrA5/14*
40	AMP,STR, SA, TET, TMP, SXT	0.5	bla_{TEM}, *strA*, *strB*, *sul2*, *tet*(A), *dfrA5/14*
41	NAL	1	/
42	AMP,CIP,NAL,STR,SA, TET, TMP,SXT	0.5	bla_{TEM}, *strA*, *strB*,*sul1*, *sul2, tet*(A), *dfrA1*
43	AMP,SA	0.5	bla_{TEM}, *sul3*
44	AMP	0.5	bla_{TEM}

MIC minimal inhibitory concentration, *CST* colistin, *AMC* amoxicillin/clavulanic acid, *AMP* ampicillin, *CPD* cefpodoxime, *CAZ* ceftazidime, *CTX* cefotaxime, *CIP* ciprofloxacin, *CHL* chloramphenicol, *GEN* gentamicin, *NAL* nalidixic acid, *STR* streptomycin, *SA* sulfonamides, *TET* tetracycline, *TMP* trimethoprim, *SXT* trimethoprim/sulfamethoxazole

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

Characterization of *E. coli*-donors							Characterization of *E. coli*-transconjugants					
Isolate No	Resistotype	Resist. genes	Virulence genes	Sequenc type	Phyl group	Inc group	Resistotype TR	Resist. gene transfer	Efficacy of conjugation	Virulence gene transfer	Plasmid size (kb)	Inc group
4	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	-	ST38	D	I1	AMC, AMP, CPD, CTX, CAZ, FOX,	bla_{CMY-2}	3.36×10^{-3}	-	95	I1
24	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	-	NP*	D	I1, FIB	-	-	-	-	-	-
10	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	*hlyF, ompT*	ND	D	I1	AMC, AMP, CPD,CAZ,CTX, FOX	bla_{CMY-2}	2.81×10^{-5}	-	110	I1
11	AMC, AMP, CPD, CAZ, CTX, CIP, NAL, TET, FOX	bla_{CMY-2}, $bla_{TEM-1,}$ *tetA*	*hly*, *iroN*, *iss*, *ompT*, *cvaC*	ST2307	D	I1, FIB	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	1.27×10^{-6}	*hly*, *iroN*, *iss*, *ompT*, *cvaC*	95	I1, FIB
12	AMC, AMP, CPD, CAZ, CTX, CIP, NAL, TET, FOX	bla_{CMY-2}, bla_{TEM-1}, *tetA*	*iroN*, *hlyF*, *iutA*, *iss,ompT*, *eitC*	ST224	B2	I1, FIB	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	3.33×10^{-4}	-	45, 96, 130	I1, FIB
14	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	*iroN*	ND	B1	I1, FIB	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	4.2×10^{-2}	-	95	I1, FIB
16	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2} bla_{TEM-1}	*iroN*, *hlyF*, *iutA*, *iss,ompT*, *eitC*, *cvaC*	ST162	B2	I1, FIB	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	3.3×10^{-5}	-	95, 170	I1, FIB
17	AMP, CPD, CTX	$bla_{CTX-M-1,}bla_{TEM-1}$	-	ND	B2	I1, FIB	-	-	-	-	-	-
25	AMC, AMP, CPD, CAZ, CTX, NAL, TET, FOX	bla_{CMY-2}, bla_{TEM-1} *tetA*	*iroN*, *hlyF*, *iutA*, *iss*, *ompT*, *cvaC*	NP*	B2	I1, FIB	-	-	-	-	-	-

Table 2 (continued)

Characterization of *E. coli*-donors						Characterization of *E. coli*-transconjugants				
35	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2} bla_{TEM-1}	-	ST34	B2	I1, FIB, Y	AMC, AMP, CPD, CAZ, CTX, FOX	bla_{CMY-2}	$2.66 \times ^{10-1}$	-	95	I1, FIB

AMC amoxicillin/clavulanic acid, *AMP* ampicillin, *CPD* cefpodoxime, *CAZ* ceftazidime, *CTX* cefotaxime, *FOX* cefoxitin, *CIP* ciprofloxacin, *NAL* nalidixic acid, *TET* tetracycline, *NP* not possible, *ST* designations could not be attributed to *E. coli* isolates No 24 and 25 (Supplementary Table 2), *ND* not done

Phylogenetic analysis and multilocus sequence typing were carried out on the isolates conferring resistance to ESC. It was determined that five isolates belonged to the phylogenetic-group B2, four 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 *E. coli* isolates resistant to cephalosporin antibiotics. Sequence type designation was not possible for two isolates, No. 10 and 14, which means that these may have belonged to some new sequence type(s).

Resistance to fluoroquinolone antibiotic-ciprofloxacin

In this study, high-level resistance to ciprofloxacin 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 ten *E. coli* 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 Ser83Leu or Asp87Asn. Plasmid-mediated quinolone resistance (PMQR) genes were not found.

Discussion

In the course of the present study, it was shown that 44 out of 99 *E. coli* isolates from black-headed gulls living in the wild carried resistant or multi-resistant *E. coli*. Several important mechanisms of resistance were determined in this collection of isolates. Resistance to beta lactam antibiotics was conferred by the genes bla_{TEM-1}, $bla_{CTX-M-1}$ and $bla_{CMY-2,}$ which encode beta lactamases. These enzymes hydrolyze the beta-lactam ring before antibiotics reach the penicillin-binding proteins. Resistance to streptomycin was mediated by the combination of the *strA* and *strB* genes, which encode aminoglycoside phosphotransferases APH(3″)/Ib and APH(6)/Id, respectively. Variants of dihydrofolate reductase gene (*dfr*) enabling resistance to trimethoprim and thus maintaining the reduction of dihydrofolate to tetrahydrofolate commonly found in livestock isolates were also found in *E. coli* isolates from gulls in this work. On the other hand the *tetA*, *tetB* and *cmlA* genes encode a specific transporter proteins conferring resistance to tetracyclines and chloramphenicol, respectively. The *cat1* gene encodes chloramphenicol acetyltransferase enzymes that inactivate the antibiotics by an acetylating mechanism. The antimicrobial resistance patterns, but also the resistance genes detected in the *E. coli* isolates were similar to those found in other studies, in which

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

Isolate No	*gyrA*	*parC*	*parE*	MIC-CIP mg/L
1	Ser83Leu, Asp87Asn	/	/	4
3	Ser83Leu, Asp87Asn	Ser80Ile	/	4
6	Ser83Leu, Asp87Asn	Ser80Ile	/	8
15	Ser83Leu, Asp87Asn	Ser80Ile	/	4
20	Ser83Leu, Asp87Asn	Ser80Ile	Leu416Phe	16
23	Ser83Leu, Asp87Asn	Ser80Ile	Ser458Ala	16
11	Ser83Leu Asp87Asn	/	/	32
12	Ser83Leu Asp87Asn	Ser80Ile	Ser458Ala	32
19	Ser83Leu	/	/	0.25
22	Asp87Tyr	/	/	0.125

PMQR genes (*qnrS*, *qnrA*, *qnrB*, *qnrC*,*qnrD*, *aac-Ib-cr*,*qepA, oqxA, oqxB*) were not identified in *E. coli* isolates. Sequencing of the QRDR was not done in isolates 28 and 42 resistant to CIP-ciprofloxacin and in isolates resistant to NAL-nalidixic acid (13, 25, 27, 28, 29, 41)

fecal samples were taken from waterfowl in the northeast part of the Czech Republic (Dolejska et al. 2007, 2009), on the Polish coast of the Baltic Sea (Literak et al. 2010) and in the Berlengas natural reserve in Portugal (Poeta et al. 2008). The CMY-2 plasmid carriers from gulls in Novi Sad were often resistant only to beta-lactam antibiotics. A similar result was obtained by Ahlstrom et al. (2018), who found only three out of eight *E. coli* isolates with a $bla_{\text{CMY-2}}$ gene from gulls and bald eagles from Alaska to be multi-drug resistant (Ahlstrom et al. 2018).

In this work, one *E. coli* isolate out of the 24 isolated from gulls at the local landfill carried the extended-spectrum β-lactamase-encoding gene $bla_{\text{CTX-M-1}}$. Nine out of 153 *E. coli* isolates from feces of yellow legged gulls in the south of France (Bonedahl et al. 2009) were identified as CTX-M-1 carriers as well, while one and two out of 83 *E. coli* isolates from black-headed gulls residing at the southeast coast of Sweden carried either $bla_{\text{CTX-M-15}}$ gene or $bla_{\text{CTX-M-14}}$ gene, respectively (Bonedahl et al. 2010). These genotypes are also found in human patients in Sweden, indicating the possibility of a limited clonal transmission (Atterby et al. 2017).

However, in *E. coli* isolates from food-producing animals, the $bla_{\text{CTX-M-1}}$ gene is prevalent in many European countries including the Mediterranean area, mainly due to the epidemic spread of the resistance plasmids (D'Andrea et al. 2013; Cantón et al. 2008; Dandachi et al. 2018). The $bla_{\text{CTX-M-1}}$ gene has also been detected in *E. coli* isolates from the Northern part of Serbia from cases of clinical bovine mastitis, from diseased pigs and wildlife (Todorović et al. 2018; Velhner et al. 2018) and now it was detected in one isolate from a gull. *E. coli* isolates from food-producing animals are often multi-resistant due to co-selection mechanisms (Michael et al. 2017), while *E. coli* isolates with the plasmid-borne AmpC gene ($bla_{\text{CMY-2}}$) will often appear resistant only to beta-lactam antibiotics (Atterby et al. 2016; Touzain et al. 2018).

In this work, it was observed that *E. coli* isolates with the CMY plasmid carried virulence genes which are often identified in APEC. These virulence factors 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*), aerobactin siderophore receptor gene (*iutA*), putative iron transport system gene (*eitC*) and the colicin V structural gene (*cvaC*), which is important for increasing the adhesion and invasiveness of *E. coli* and other bacteria of the *Enterobacteriaceae* family (Johnson et al. 2008, 2010). Our results are 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 $bla_{\text{CTX-M-1}}$ gene on IncI1/ST3 conjugative plasmids carried any of these virulence genes. However, *iutA*, *hlyF*, *ompT*, *iss* and *iroN* genes were found on CMY plasmid of the IncF replicon type.

In the mating experiment, only one transferable CMY plasmid (from a donor No. 11) carried *hly*, *iroN*, *iss*, *ompT* and *cvaC* virulence genes. This plasmid had the lowest efficacy of conjugation and was represented by the replicon type IncI1/FIB. It is possible that the other six *E. coli* isolates containing CMY plasmid (isolate No. 4, 10, 12, 14, 16 and 35) had also virulence plasmids but in vitro mating was successful only for the plasmid with $bla_{\text{CMY-2}}$ gene. On the other hand, the unsuccessful mating of two *E. coli* isolates (24 and 25) presumably indicates that the $bla_{\text{CMY-2}}$ gene was integrated into the bacterial chromosome or that this gene was located on non-mobilizable plasmids, since they do not possess the capacity required for self-transmissible conjugation (Smillie et al. 2010). The IncI group of plasmids exists in several variants. These are low copy number plasmids of a narrow host range, which vary in size (from 50 to 250 kb). In *E. coli* isolates from poultry, across Europe, these plasmids often carry ESBL or AmpC resistance genes (Rowandowicz et al. 2018). In this work IncI1-CMY type plasmids were cointegrated with the plasmid IncFIB. Therefore, the molecular mechanisms behind these genetic arrangements warrant future research.

In this strain collection phylogenetic and MLST analyses were used for molecular typing. It was shown that three isolates (No. 12, 16 and 35) from the phylogenetic group B2 belong to the sequence type ST224, ST162 and ST34 and possessed transferable IncI1/FIB-CMY plasmids, while isolate 4 belonging to the phylogenetic group D was identified as sequence type 38 and carried the IncI1/CMY plasmid. However, twelve *E. coli* isolates from gulls in Barrow, Alaska, were of sequence type ST38 but carried the $bla_{\text{CTX-M-14}}$ gene (Bonnedahl et al. 2014). In the European

Union, the ST38 lineage with the CMY plasmid is considered typical of poultry (Mo et al. 2016). The IncK, IncI and IncA/C plasmids were the most frequent bla_{CMY-2} gene carriers in *E. coli* isolates from humans, food-producing animals and food in Germany, while IncA/C plasmids were the most common CMY carriers in North America (Pietsch et al. 2018). It was determined that the successes in the proliferation of CMY-2 plasmids depend on the selective pressure posed by the use of antibiotics. In addition, larger plasmids, such as CMY carriers may be associated with a significant fitness cost which may lead to the loss of a plasmid in bacteria (Subbiah et al. 2011).

We detected high-level resistance to ciprofloxacin in several isolates. The resistance was induced by the mutations in the *gyrA*, *parC* and *parE* gene, which are commonly found in *E. coli* (Velhner and Stojanović 2012). Moreover, to the best of our knowledge, mutations in the *parE* gene (Leu416→Phe and Ser458→Ala) in *E. coli* isolates from gulls were for the first time reported in the current research. Resistance to ciprofloxacin was previously observed in *E. coli* isolates from mallards and hearing gulls residing at the Polish coast of the Baltic sea (Literak et al. 2010), from feces of gulls, pigeons and birds of prey in Portugal, Sweden and Spain (Vredenburg et al. 2013) and from silver gulls on beaches and foreshores in Australia (Mukerji et al. 2019). The plasmid-mediated quinolone resistance was not detected in *E. coli* isolates from gulls in the city of Novi Sad but was identified in the isolates from gulls in Poland, Netherlands and the United Kingdom (Literak et al. 2010; Veldman et al. 2013; Zendri et al. 2020).

Because of their feeding habits, water birds nesting in towns close to watercourses often carry multidrug-resistant bacteria and may contaminate the environment in the vicinity of their natural habitats. In addition, the global spread of epidemic *E. coli* clonal lineages may appear also in wild bird species and the spread of such bacteria needs to be closely monitored (Pitout and DeVinney 2017).

Conclusion

Antimicrobial resistance is detected in *E. coli* isolates from gulls living in the vicinity of the city of Novi Sad, Serbia. Some isolates are resistant to important classes of antibiotics. Most frequently, plasmid-mediated resistance to extended-spectrum cephalosporins and resistance to ciprofloxacin is identified. The gulls may have encountered resistant bacteria feeding at the local landfill, which is why they can be considered sentinels of antimicrobial resistance. Aimed at decreasing exposure to agents and substances hazardous to human and animal health, the scientific community and officials in Serbia should continuously support environmental health protection.

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Data availability The datasets generated in the current study are available from the corresponding author.

Declarations

Ethics approval Not applicable: fecal samples were taken from gulls by ornithologists during a routine ringing campaign and bird health check.

Consent to participate and consent for publication All authors agreed to participate in this work. They also approved the content of the research and the submission of the manuscript to the Veterinary Research Communication journal.

Conflict of interest The authors declare no conflict of interest.

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