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Prevalence and molecular characteristics of multi-resistant *Escherichia coli* in wild birds

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Abstract

Humans and animals share the same bacterial species including the resistant ones. For that reason, epidemiological studies in domestic and wild animals should be performed on a regular basis. Wild, particularly migratory birds, should be investigated as potential carriers of antimicrobial resistant bacteria that can be spread globally in a short time. The aim of this study was to investigate the prevalence and to characterize multi-resistant *Escherichia coli* in wild birds. Three hundred and ninety two samples were obtained from different bird species including gulls (*Larus* spp.), mallards (*Anas platyrhynchos)*, mute swans (*Cygnus olor)*, as well as other species of birds. Phenotypical and genotypical resistance of *E. coli* was investigated. In total 60 isolates from 179 tested were resistant to three or more antimicrobial classes and treated as multi-resistant (33.5%; 95% CI 21.56–45.44); the isolates were obtained from gulls, mallards, swans, and rooks. All of the strains demonstrated resistance to aztreonam and cefpodoxime. The most frequent resistance prevalence of the above-mentioned isolates *in vitro* was also demonstrated to ampicillin (82%), ampicillin/sulbactam (68%), cefazolin (66%), ceftriaxone (55%), and ciprofloxacin (47%). All *E. coli* isolates were susceptible to amikacin. The results of polymerase chain reaction confirmed the presence of the genes encoding resistance to beta-lactams, aminoglycosides, tetracycline, amphenicols, trimethoprim, and sulphonamides. Consequently, wild birds might constitute a potential hazard to human and animal health by transmitting multi-resistant *E. coli* strains to waterways and other environmental sources via bird faeces.

Antimicrobial resistance, genes, migratory birds

The resistance of bacteria to antimicrobials becomes a rising health problem in the world. The importance of wild birds as potential vectors of diseases has received renewed empirical interest recently, especially regarding human health. *Escherichia coli* is found in the environment, food and intestines of people and animals (Benskin et al. 2009; Wasinski et al. 2010). Due to their opportunistic and gregarious nature, gulls may be important reservoirs and vectors for anthropogenically derived faecal pathogens in coastal areas. Moreover, the migratory behaviour of birds has been proposed as a possible dissemination pathway of multi-resistant (MR) bacteria from human influenced habitats to remote places (Nelson et al. 2008; Guenther et al. 2012). Numerous wild bird species are attracted to untreated sewage, garbage dumps and manure, therefore, various pathogens such as *Escherichia coli, Salmonella enterica* and *Campylobacter* spp. might be prevalent in those birds (Quessy and Messier 1992; Hatch 1996; Moore et al. 2002; Fogarty et al. 2003; Waldenström et al. 2003). The aim of this study was to investigate the prevalence of and characterize the multi-resistant *Escherichia coli* in wild birds.

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Materials and Methods

Samples and place

Three hundred and ninety two samples of wild bird faeces were collected in 2015–2017 using sterile cotton swabs with a transport medium (Transwab® Amies, UK) in city parks and the Kaunas city dump site. Cloacal samples from passerines were taken on a ringing station of Ventes Ragas (55°20'28.1"N 21°11'25.3"E). Samples were delivered to the laboratory within 24 h. In total, 392 samples were collected from 29 different species of birds: herring gulls (*Larus argentatus*), black-headed gulls (*Chroicocephalus ridibundus*), rooks (*Corvus frugilegus*), hooded crows (*Corvus cornix*), common ravens (*Corvus corax*), mallards (*Anas platyrhynchos*), mute swans (*Cygnus olor*), European kingfishers (*Alcedo atthis*), Eurasian nuthatchs (*Sitta europaea*), Eurasian siskins (*Carduelis spinus*), long-tailed tits (*Aegithalos caudatus*), black redstarts (*Phoenicurus ochruros*), blackcaps (*Sylvia atricapilla*), yellow-browed warblers (*Phylloscopus inornatus*), great tits *(Parus major)*, dunnocks (*Prunella modularis*), European robins (*Erithacus rubecula*), common treecreepers (*Certhia familiaris*), wrens (*Troglodytes troglodytes*), barred warblers *(Sylvia nisoria),* bearded tits *(Panurus biarmicus),* chaffinches *(Fringilla coelebs),* chiffchaffs (*Phylloscopus collybita*), blackbirds (*Turdus merula*), song thrushes (*Turdus philomelos*), goldcrests (*Regulus regulus*), willow tits (*Poecile montanus*), common starlings (*Sturnus vulgaris*) and rock pigeons (*Columba livia*).

The ethical approval for this study was granted by the Lithuanian Environmental Protection Agency (permission number A4-8844).

Bacteriological and susceptibility testing

Material was inoculated onto McConkey Agar (Thermo Fisher, UK), for the screening of *E. coli*. Inoculated plates were incubated at +37 °C for 24 h. After incubation, the plates were screened for presumptive colonies. The randomly selected separate colonies (one colony per sample) were then identified using "Microgen Gram-Negative Plus" biochemical identification system (Microgen, UK).

The initial antimicrobial susceptibility testing was performed using the disc diffusion method according to Kirby-Bauer and the results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints except for cefazolin as no interpretative criteria were set for this antibiotic. Therefore, the clinical breakpoints for cefazolin were used as per recommendations of the Clinical Laboratory Standards Institute (CLSI 2015). The following discs with antimicrobials (Thermo Fisher, UK) were used: ampicillin (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), cefoxitin (30 µg), tetracycline (30 µg), imipinem (10 µg) and chloramphenicol (30 µg). Bacterial isolates resistant to three or more antimicrobial classes were then selected for the testing for minimal inhibitory concentrations (MIC's) using Sensititre® plates and ARIS 2X automated system (Thermo Scientific, UK) for amikacin, ampicillin, ampicillin/sulbactam, aztreonam, cefazolin, cefepime, cefotetan, cefoxitin, cefpodoxime, ceftazidime, ceftriaxone, ciprofloxacin, gatifloxacin, gentamicin, imipenem, nitrofuration, tobramycin and trimethoprim/sulphamethoxazole. Interpretation of the results was carried out using the manufacturer's software (SWIN®) adapted to the EUCAST clinical breakpoints.

Molecular testing

DNA (deoxyribonucleic acid) for molecular analysis was obtained after bacterial lysis. The cultures were grown on a Mueller Hinton Agar (Liofilchem, Roseto, Italy) for 24 h and afterwards a loopful of colonies was taken from the surface of the agar and transferred to phosphate buffered saline (pH 7.3). The content was centrifuged for 5 min. The supernatant was discarded and the pellet was re-suspended in TAE (tris-ethylenediaminetetraacetic acid) buffer. The suspension was heated using a thermomixer at 100 °C for 10 min. Boiled suspension was transferred directly on ice and diluted by 1:10 in TAE.

The resistant *E. coli* isolates were tested by PCR (polymerase chain reaction) for the detection of genes encoding antimicrobial resistance (Table 1). The PCR mix (25 µl) constisted of 12.5 µl of the Dream Taq Green PCR Master Mix (ThermoFisher Scientific, Lithuania), 1 µl of each primer, 2 µl of the DNA template and 8.5 µl of water. The PCR conditions were as follows: initial denaturation for 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 60 s at the temperature indicated for each primer pair in Table 1, 30 s at 72 °C; and a final extension step of 7 min at 72 °C.

The PCR products were determined by electrophoresis of 10 µl of the reaction products on 2% agarose gel with 1X TAE buffer at 105 V for 50 min.

Data analysis

Occurrences of multi-resistant (MR) isolates in faecal specimens were calculated by dividing the number of MR isolates by the total number of investigated specimens. For percentage estimates, Wilson (Score) 95% confidence intervals (CI 95%) and their ranges for true population proportions were calculated. The MR occurrence was determined by bird species as well. Antimicrobial resistance rates for each tested antimicrobial were given as numbers of resistant per total number of MR isolates.

Results

Resistant *E. coli* prevalence in bird faeces

During the study, *E. coli* resistant to one or more antimicrobials was recovered from 179 samples out of 392 tested (45.6%; 95% CI 40.67–50.53). Resistant isolates were recovered only exceptionally from gulls, swans, ducks and rooks (Fig. 1). No resistant isolates were detected in the faeces of small passerine birds.

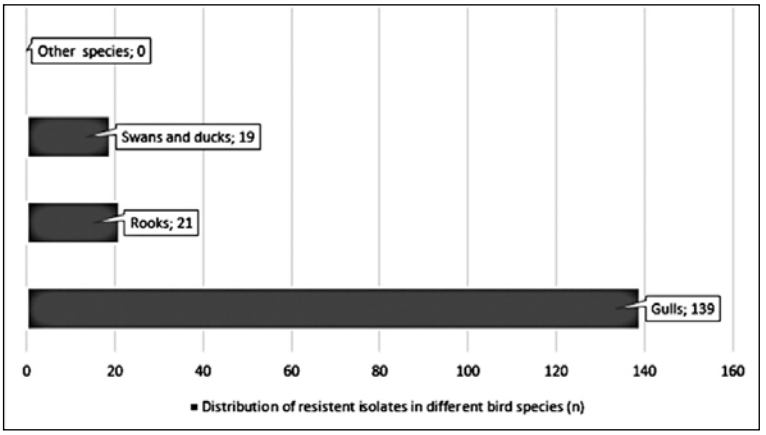

Fig. 1. Distribution of 179 *E.coli* isolates resistant to at least one antimicrobial in different wild bird species (n).

Antimicrobial susceptibility profiles

In total, 119 out of 179 isolates were resistant (66.4%; 95% CI 57.91–74.89) only to a single or two antimicrobial agents, and 60 isolates were resistant to three or more antimicrobial classes (33.5%; 95% CI 21.56–45.44). The multi-resistant (MR) isolates were obtained from gulls (n = 51), swans (n = 2), mallards (n = 2) and rooks (n = 5). (Plate II, Table 2) indicates the MIC distribution regarding multi-resistant *E. coli* isolates obtained from the wild birds.

All multi-resistant isolates were resistant to aztreonam and cefpodoxime. High resistance rates were also detected *in vitro* towards ampicillin (82%), ampicillin/subactam (68%), cefazolin (66%), ceftriaxone (55%) and ciprofloxacin (47%). All *E. coli* isolates were susceptible to amikacin.

Genes encoding resistance

The genes encoding antimicrobial resistance to antimicrobials are presented in Table 3.

The most prevalent genes were as follows: *aphA1 (*100%), *dfr*5 (84.6%), *tet*A, (85%) and bla_{TEM} (66.6%), encoding resistance to aminoglycosides, trimethoprim, tetracycline, and beta-lactams, respectively. The negative results were shown for all isolates following the genes *oxa*5, per_1 and per_2.

Discussion

The emergence and spread of multi-resistant bacteria in natural environments constitute a serious impact on animal and human health. We have detected 60 multi-resistant *E. coli* isolates in wild birds out of 176 tested (33.5%). The most prevalent resistance was detected towards aztreonam, cefpodoxime, other beta-lactams and fluoroquinolones.

Table 1. Antimicrobial resistance genes tested and oligonucleotide primers used in the study.

Primer name	Sequence (5‘-3‘)	Size, bp and t (°C)	Target gene	Source
*bla*TEM-F	GAGTATTCAACATTTTCGT	857 (50)	bla_{TEM}	Van et al. (2008)
*bla*TEM-R	ACCAATGCTTAATCAGTGA			
*bla*SHV-F	TCGCCTGTGTATTATCTCCC	768 (60)	bla_{SHV}	Ojdana et al. (2014)
*bla*SHV-R	CGCAGATAAATCACCACAATG			
*oxa*1-F	TCAACAAATCGCCAGAGAAG	276 (55)	bla_{OXA} group I	Bert et al. (2002)
*oxa*1-R	TCCCACACCAGAAAAACCAG			
*oxa*3-F	TTTTCTGTTGTTTGGGTTTT	427 (52)	bla_{OXA} group III	Bert et al. (2002)
*oxa*3-R	TTTCTTGGCTTTTATGCTTG			
OXA 5 group-F	AGCCGCATATTTAGTTCTAG	644(56)	bla_{OXA} group V	Bert et al. (2002)
OXA 5 group-R	ACCTCAGTTCCTTTCTCTAC			
CTX-M-F	ATGTGCAGYACCAGTAARGT	593 (50)	bla_{CTX-M}	Pagani et al. (2003)
CTX-M-R	TGGGTRAARTARGTSACCAGA			
*cmy*2-F	GCACTTAGCCACCTATACGGCAG	758 (58)	bla_{CMY}	Hasman et al. (2005)
*cmy*2-R	GCTTTTCAAGAATGCGCCAGG			
PER-1-F	ATGAATGTCATTATAAAAGCT	927 (48)	bla_{PER}	Celenza et al. (2006)
PER-1-R	TTAATTTGGGCTTAGGG			
PER-2-F	ATGAATGTCATCACAAAATG	927 (49)		Celenza et al. (2006)
PER-2-R	TCAATCCGGACTCACT			
*tet*A-F	GTGAAACCCAACATACCCC	887 (50)	*tet*(A)	Van et al. (2008)
*tet*A-R	GAAGGCAAGCAGGATGTAG			
*tet*B-F	CCTTATCATGCCAGTCTTGC	773 (50)	*tet*(B)	Van et al. (2008)
*tet*B-R	ACTGCCGTTTTTTCGCC			
*aad*B-F	ATGGACACAACGCAGGTCGC	534 (55)	*aad*B	Asadollahi et al. (2012)
*aad*B-R	TTAGGCCGCATATCGCGACC			
*aad*A-F	GTGGATGGCGGCCTGAAGCC	320 (55)	*aad*A	Asadollahi et al. (2012)
*aad*A-R	AATGCCCAGTCGGCAGCG			
*rmt*B-F	ATGAACATCAACGATGCCCT	769 (55)	*rmt*B	Yan et al. (2004)
*rmt*B-R	CCTTCTGATTGGCTTATCCA			
*arm*A-F	CAAATGGATAAGAATGATGTT	774 (55)	*arm*A	Galimand et al. (2003)
*arm*A-R	TTATTTCTGAAATCCACT			
*aph*A1-F	AAACGTCTTGCTCGAGGC	461 (55)	*aph*A1	Weill et al. (2004)
*aphA*1-R	CAAACCGTTATTCATTCGTGA			
*aacA*4-F	ATGACTGAGCATGACCTTGCG	487 (55)	*aacA4*	Odumosu et al. (2015)
*aacA*4-R	TTAGGCATCACTGCGTGTTCG			
aac(3)II-F	TGAAACGCTGACGGAGCCTC	369 (65)	*aac(3)II*	Sandvang and Aarestrup (2009)
aac(3)II-R	GTCGAACAG GTAGCACTGAG			
*str*A-F	CCTGGTGATAACGGCAATTC	546 (55)	*str*A	Lanz et al. (2003)
*str*A-R	CCAATCGCAGATAGAAGGC			
*str*B-F	ATCGTCAAGGGATTGAAACC	509 (55)	*str*B	Lanz et al. (2003)
*str*B-R	GGATCGTAGAACATATTGGC			
*cat*II-F	ACACTTTGCCCTTTATCGTC	495 (55)	*cat*II	Vassort-Bruneau et al. (1996)
*cat*II-R	TGAAAGCCATCACATACTGC			
*cml*A-F	TTGCAACAGTACGTGACAT	293 (55)	*cml*A	Keyes et al.(2000)
*cml*A-R	ACACAACGTGTACAACCAG			
*sul*1-F	TTCGGCATTCTGAATCTCAC	822 (55)	*sul*1	Cristabel et al. (2012)
*sul*1-R	ATGATCTAACCCTCGGTCTC			

Table 1 (continued). Antimicrobial resistance genes tested and oligonucleotide primers used in the study.

Primer name	Sequence (5‘-3‘)	Size, bp and t (°C)	Target gene	Source
*sul*2-F	CGGCATCGTCAACATAACC	722 (50)	*sul*2	Saenz et al. (2004)
*sul*2-R	GTGTGCGGATGAAGTCAG			
*sul*3-F	GAGCAAGATTTTTGGAATCG	792 (51)	*sul*3	Parreten et al. (2003)
*sul*3-R	CATCTGCAGCTAACCTAGGGCTTTGA			
dfr1-F	ACGGATCCTGGCTGTTGGTTGGACGC	254 (55)	*dfr*1	Gibreel and Skold (1998)
dfr1-R	CGGAATTCACCTTCCGGCTCGATGTC			
dfr5-F	GCBAAAGGDGARCAGCT	394 (44)	*dfr*5	Šeputienė et al. (2010)
dfr5-R	TTTMCCAYATTTGATAGC			
dfrA7-F	AAAATTTCATTGATTTCTGCA	471 (44)	*dfr*7	Navia et al. (2003)
dfrA7-R	TTAGCCTTTTTTCCAAATCT			

Other authors have reported data about resistant *E. coli* isolates obtained from different bird species including Canada geese, common buzzards, black kites, red kites, white-tailed eagles and goshawks (Middleton et al. 2005; Guenther et al. 2010; Radhouni et al. 2012). The main resistance patterns of the isolates in the above-mentioned studies were resistance combination to ampicillin, streptomycin, and sulphonamides. In Lithuania, the most important species carrying multi-resistant *E. coli* strains were herring gulls, black-headed gulls, rooks, hooded crows as well as waterfowl, in particular mallards and mute swans. Such data contradict the results of a previous study performed in Portugal where *E. coli* isolated from similar species were susceptible to most of the antimicrobials tested (Radhouani et al. 2010). In another study performed by Dolejska et al. (2007) in the Czech Republic, the rate of resistant isolates from black–headed gulls to at least one antimicrobial was 29.2% whereas 6.2% of the isolates were treated as multi-resistant ones.

There is evidence that international human travelling is one of the causes of the spread of multi-resistant *E. coli* worldwide (Peirano and Pitout 2010). Considering that migratory bird species, particularly gulls, are often carriers of multi-resistant bacteria, it might be assumed that they also pose a risk for the dissemination of multi-resistant isolates throughout different countries or regions. The recent discovery of colistin-resistant *E. coli* harbouring plasmid-mediated *mec*-1 gene in herring gulls in Europe (Ruzauskas and Vaskevičiute 2016) and in kelp gulls in South America (Liakopoulos et al. 2016) proves this opinion. The number of migrating birds worldwide has been estimated to be five billion a year (Berhold 2001) so the risk degree for the spread intensity of resistant bacteria is still undervalued. Beta-lactam antibiotics are the most frequently prescribed antibiotics worldwide to treat bacterial infections (Fernández-Aguadob et al. 2014). Therefore, it is not surprising that resistance to this class of antimicrobial agents poses increasingly complex problems for physicians. Extended-spectrum betalactamases (ESBLs) are a rapidly evolving group of beta-lactamases which share the ability to hydrolyze third-generation cephalosporins and aztreonam but are inhibited by clavulanic acid (Philippon et al. 1989). Our results demonstrated high rates of *E. coli* resistance to the 3rd generation of cephalosporins and aztreonam. The $bla_{CTX\text{-}M}$ gene was detected in 63.2% of the isolates resistant to the 3rd generation of cephalosporins.

The use of sulphonamides was restricted for food animals in the 1980s after a potential threat to human health from residues in foods of animal origin, and they are currently approved for use in treating calf scours. Resistance to sulphonamides is plasmid mediated but chromosomal mutations for sulphonamide resistance take place very slowly (Prescott et al. 2013). Different genes encoding resistance to this antimicrobial are already described.

Table 3. Presence of the tested genes encoding antimicrobial resistance in *E. coli* isolated from wild birds.

Antibiotic class	Groups of proteins	Genes	Primer	Genes		
				Investigated	Detected	%
		bla$_{TEM}$	*bla*TEM-F	57	38	66.6
			*bla*TEM-R			
		bla$_{SHV}$	*bla*SHV-F	59	29	49.1
			*bla*SHV-F			
		bla$_{OXA}$ 1 group	*oxa*1-F	56	6	10.7
			*oxa*1-R			
		bla$_{OXA}$ 3 group	*oxa*3-F	56	10	17.8
			*oxa*3-R			
		bla$_{OXA}$ 5 group	*oxa*5-F	56	0	0
Beta-lactams	Beta-lactamases		*oxa*5-R			
		bla$_{CTX-M}$	*ctx*M-F	49	31	63.2
			*ctx*M-R			
		bla$_{CMY}$	*cmy*2-F	49	1	2
			*cmy*2-R			
		bla$_{PER}$	*per*1-F	49	0	0
			*per*1-R			
			*per*2-F	49	0	0
			*per*2-R			
		tet(A)	*tet*A-F	33	28	85
Tetracycline	Tetracycline promoters		*tet*A-R			
		tet(B)	*tet*B-F	33	6	18
			*tet*B-R			
	Aminoglycoside adenylyltransferases	*aadB*	*aad*B-F	10	0	0
			*aad*B-R			
		aadA	*aad*A-F	10	1	10
			*aad*A-R			
	Aminoglycoside methyltransferases	*rmtB*	*rmt*B-F	10	0	0
			*rmt*B-R			
		armA				
			*arm*A-F	10	0	0
			*arm*A-R			
Aminoglycosides	Aminoglycoside phosphotransferases	*aphA1*	*aph*A1-F	10	10	100
			*aph*A1-R			
	Aminoglycoside acetyltransferases	*aacA4*	*aac*A4-*F*	10	1	10
			*aac*A4-*R*			
		aac(3)II	*aac(3)*II-F	10	5	50
			*aac(3)*II-R			
Streptomycin	Streptomycin phosphotransferases	*strA*	*str*A-F	55	20	36.3
			*str*A-R			
		strB	*str*B-F	55	21	38.1
			*str*B-R			
	Chloramphenicol acetyltransferases	*catII*	*cat*II-F	29	0	0
Amphenicols			*cat*II-R			
	Chloramphenicol promoters	*cmlA*	*cml*A-F	29	7	24.1
			*cml*A-R			

Table 3 (continued). Presence of the tested genes encoding antimicrobial resistance in *E. coli* isolated from wild birds.

Antibiotic classes	Groups of proteins	Genes	Primer	Genes		
				Investigated	Detected	%
		sul1	*sul*1-F *sul*1-R	49	11	22.4
Sulphonamides	Dihydropteroate synthases	*sul2*	*sul*2-F *sul*2-R	49	25	51
		sul3	*sul*3-F *sul*3-R	49	23	46.9
		dfr1	*dfr*1-F *dfr*1-R	13	2	15.3
Trimethoprim	Dihydrofolate reductase	*dfr5*	*dfr*5-F *dfr*5-R	13	11	84.6
		dfr7	*dfr*7-F *dfr*7-R	13	2	15.3

For instance, *sul*3 has been detected in *E. coli* isolates from different animals in Switzerland and Germany (Guerra et al. 2003; Perreten et al. 2003) while *sul*1 was the most prevalent among human isolates (Hammerum et al. 2006). Our study demonstrated that different *sul* genes were prevalent in *E. coli* carried by wild birds including *sul*1, *sul*2 and *sul*3, therefore, it suggests the possibility for birds to acquire antimicrobial resistant bacteria from different sources, including domestic animals and contaminated environment.

Tetracycline-resistance now occurs in anincreasing number of pathogenic, opportunistic, and commensal bacteria. Tetracycline-resistance is often due to the acquisition of new genes, which code for energy-dependent efflux of tetracyclines or for a protein that protects bacterial ribosomes from the action of tetracyclines. A limited number of bacteria acquire resistance by mutations, which alter the permeability of the outer membrane porins and/or lipopolysaccharides in the outer membrane, change the regulation of innate efflux systems, or alter the 16S rRNA (ribosomal ribonucleic acid) (Chopra and Roberts et al. 2001). The detection of *tet*A gene in almost 85% of tetracycline-resistant isolates in our study shows that the main mechanism of tetracycline resistance in *E. coli* isolates from wild birds is by active efflux. Similar results were published by Dolejska et al. (2007) where both *tet*A and *tet*B genes were detected in *E. coli* isolates.

This study demonstrated that wild birds, particularly those living in the urban enviroment are carriers of antibiotic resistant bacteria. These bacteria are often resistant to different classes of antimicrobials including those that are critically important for humans – aminoglycosides, aminopenicillins and fluoroquinolones. Moreover, microbiota of wild birds share the same antimicrobial resistance genes that are important in aquired resistance of bacteria prevalent in humans and domestic animals. It is still unclear whether *E. coli* prevalent in birds can colonize humans and domestic animals but there are some studies that show a possible zoonotic transfer of similar *E. coli* strains among different hosts (Van den Bogaard et al. 2013).

Control measures for disposal of antibiotics should be tightened as residues of antimicrobials and resistant bacteria from the environment can be passed onto wild birds.

Conflict of Interests

The authors declare that they have no competing interests.

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Table 2. Minimal inhibitory concentration distributions for multi-resistant *E. coli* isolates from wild birds (%), (n = 60).

Antimicrobial	MIC distributions (%) (mg/l)								
	0.5	1	2	4	8	16	32	64	128
Amikacin					100	0	0	0	
Ampicillin				18	0	2	80		
Ampicillin/Sulbactam				26	6	26	42		
Aztreonam					60	8	32		
Cefazolin				34	3	3	60		
Cefepime				88	2	5	5		
Cefoxitin				56	12	5	27		
Cefpodoxime			38	5	2	55			
Ceftazidime		54	12	8	18	3	5		
Ceftriaxone		45	0	8	7	3	12	25	
Cefuroxime				37	3	7	53		
Ciprofloxacin	53	5	42	0					
Gentamicin			83	0	0	17			
Imipenem			95	0	2	3			
Nitrofurantion						82	7	3	8
Ticarcillin/Clavulanic acid						74	8	18	
Tobramycin				88	12				
Trimethoprim/Sulfamethoxazole	51	5		22	22				

Green cells – susceptible; yellow– intermediate; red cells – resistant

MIC - minimal inhibitory concentration