Antimicrobial-resistance in Escherichia coli *isolated from wild pheasants (*Phasianus colchicus*)*

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Antibiotic,
Resistance,
Pheasant,
Wildlife.

Summary
The purpose of this study was to examine the prevalence of antimicrobial resistance among 180 *Escherichia coli* strains isolated from 200 wild pheasants caught in rural areas of the Czech Republic (Eastern Moravia) and Slovakia (Western Region). The isolates were also classified into phylogenetic groups by the multiplex PCR method. Our findings demonstrated that 130 strains were resistant to ampicillin (72%), 160 strains to cephalothin (89%), and 40 strains to tetracycline (22%). Ten strains were found to be resistant to chloramphenicol and sulfamethoxazole/trimethoprim (5.6%). In turn, all strains were sensitive to cefoperazone/sulbactam, ciprofloxacin, colistin, gentamicin, and piperacillin/tazobactam. Ten of the 180 isolates (5.6%) exhibited multi-resistant phenotypes, including resistances against beta-lactams, aminoglycosides, tetracyclines, sulphonamides, and chloramphenicol. As far as we know, this is the first report describing antimicrobial resistance in *E. coli* from pheasants.

Caratterizzazione di ceppi di Escherichia coli *isolati da fagiani (*Phasianus colchius*) nella Repubblica Ceca e Slovacca*

Parole chiave
Antibiotico,
Resistenza,
Fagiano,
Fauna selvatica.

Riassunto
Scopo di questo studio è stato esaminare la prevalenza della resistenza antibiotica nei confronti di 180 ceppi di *Escherichia coli* isolati da 200 fagiani selvatici catturati nelle aree rurali della Repubblica Ceca (Moravia orientale) e Slovacchia (regione occidentale). Con PCR multiplex gli isolati sono stati classificati anche in gruppi filogenetici. I risultati hanno dimostrato che 130 ceppi erano resistenti all'ampicillina (72%), 160 ceppi alla cefalotina (89%), 40 ceppi alla tetraciclina (22%), dieci ceppi al cloramfenicolo e al sulfametossazolo/trimetoprim (5,6%). Tutti i ceppi erano sensibili a cefoperazone/sulbactam, ciprofloxacina, colistina, gentamicina e piperacillina/tazobactam. Dieci dei 180 isolati (5,6%) mostravano fenotipi multi-resistenti, incluse resistenze contro i beta-lattamici, gli aminoglicosidi, le tetracicline, sulfamididolfonammidiche e il cloramfenicolo. Per quanto se ne sappia, questo è il primo rapporto sulla resistenza agli antibiotici nei fagiani.

Anthropogenic changes in an environment have the potential to affect different aspects of the world, globally. These types of changes may also enrich the population of resistant bacteria and facilitate the dissemination of antibiotic-resistant bacteria, and/or resistance genes to human pathogens (Martinez 2009). Several studies have indeed shown the presence of antimicrobial residues in human and animal waste and sewage (Kummerer 2009).

Wildlife is not directly exposed to clinical antimicrobial agents, but can get infected with antimicrobial-resistant bacteria through contact with humans, animals, and the environment. Water polluted with faeces appears to be the most vector of contamination (Kummerer and Henninger 2003).

By monitoring and characterizing the prevalence of antimicrobial-resistance in bacteria such as *Escherichia coli* in different populations, including animal, wildlife and humans, it is possible to have a better understanding upon the spread and ecology of antimicrobial-resistance in a One-health approach.

The aim of this research was to determine the antibiotic resistance features in 180 strains of *Escherichia coli* isolated from rectal swabs of wild pheasants in Eastern Moravia and Western Slovakia and compare results with those publicly available.

A total of 180 strains of *Escherichia coli* were collected between 2010 and 2013 from 200 wild pheasants (*Phasianus colchicus*, L. 1758) that were hunted in the regions of Eastern Moravia Czech Republic, (100 strains) and Western Slovakia (80 strains). The rectangular sample area for this study was defined by the following geographical points as shown in Figure 1: 1. Brno (49° 12´ N - 16° 37´ E), 2. Nitra (48° 19´ N - 18° 05´ E), 3. Prievidza (48° 46´ N - 18° 37´ E), and 4. Olomouc (49° 36´ N - 17° 15´ E).

The strains were cultured from rectal transport swabs on MacConkey agar. Suspected colonies were isolated and identified to the species level by using the ENTEROtest24 (ErbaLachema Brno, Czech Republic) commercial identification microsystem. The microbial species identification success rate was over 98%.

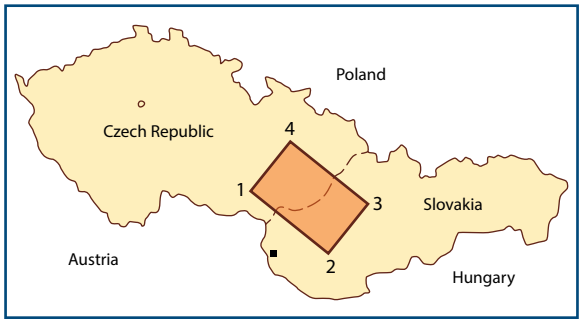

Figure 1. *The map showing the origin of hunted pheasants.*

Obtained data were evaluated using TNW Lite 6.5 software (ErbaLachema Brno, Czech Republic).

The *in vitro* susceptibility of the isolates against antimicrobial agents was determined using the standard disk diffusion procedure (CLSI 2012). The susceptibility, intermediate susceptibility, or resistance of individual isolates was determined according to the minimal inhibitory concentration breakpoints for *E. coli* established in the CLSI document M100-S22 (2012).

The following antibiotic discs (Oxoid Ltd., Besingstoke, Hampshire, GB) were used: aminoglycosides: gentamycin (10 µg), streptomycin (10 µg), neomycin (30 µg); penicillins: ampicilin (10 µg); β-lactamase inhibitor combinations: amoxicilin/clavulanic acid 2:1 (30 µg), piperacillin/tazobactam 10:1 (110 µg), sulbactam/cephoperazon (105 µg); cephems: cephalothin (30 µg); quinolones: ciprofloxacin (5µg); polymyxines: colistin sulphate (10 µg); phenicols: chloramphenicol (30 µg); folate pathways inhibitors: sulphamethoxazole/trimethoprim (25 µg); tetracyclines: tetracycline (10 µg).

Based on the presence/absence of these three fragments, an *E. coli* isolate could be assigned to one of the main phylo-groups, A, B1, B2 or D. The isolates were assigned to the phylogenetic groups according to presence of the genes chuA and yjaA and the DNA fragment TspE4.C2.: A(chuA-, TspE4.C2-), B1 (chuA-, TspE4.C2+), B2 (chuA+, yjaA+), or D (chuA+, yjaA-). (Clermont *et al.* 2000). A few colonies of each strain were solved in 100 µL of 1x PCR buffer (ThermoPol Buffer, NEB, USA), heat-treated to 95 °C for 20 minutes. Supernatant after centrifugation (10,000 g/L min) was taken as DNA template for all PCR tests. Amplicons were visualised by 1% agarose gel electrophoresis and strains were assigned to phylogenetic groups or subgroups (Escobar-Paramo *et al.* 2006, Carlos *et al.* 2010).

More than half of the strains belonged to A phylogroup (42% A0, 11% A1). Strains belonging to the B1 phylogroup were the 27% whereas the 20% of the strains fell within the combined phylogroups B2 and D (Table I). Out of 180 examined strains, 130 strains were ampicillin-resistant (72%) and the remaining 50 strains were ampicillin-intermediate; 160 strains (89%) showed resistance to cephalothin, the other 20 strains were ampicillin-intermediate. Ten *E. coli* strains were resistant to chloramphenicol and sulfamethoxazole/trimethoprim (5.6%) whereas the rest of strains were sensitive to these antibiotics. Intermediate sensitivity to amoxicillin/clavulanic acid was determined in 10 strains; all other strains were, in turn, showed to be sensitive. Forty strains were identified as intermediately sensitive to neomycin and streptomycin; all other strains were sensitive. All strains were sensitive to cefoperazone/sulbactam, ciprofloxacin, colistin, gentamicin, and piperacillin/

tazobactam. Forty strains (22%) were resistant to tetracycline, while 50 strains were intermediately sensitive and 90 strains were sensitive. Results are summarized in Table I.

The most common multidrug-resistance was observed for ampicillin + cephalothin (140 strains, 78%). Of these, 70 strains (39%) were determined to be resistant to another antibiotic, usually tetracycline. A total of 10 strains (5.6%) were multidrug-resistant to more than 3 classes of antibiotics, namely: ampicillin, cephalothin, chloramphenicol, neomycin (intermediate resistance), streptomycin (intermediate resistance), sulfamethoxazole/trimethoprim, and tetracycline. The multidrug-resistant strains belonged to groups A and B1. This finding is consistent with the study by Johnson and colleagues (Johnson *et al.* 2006), where isolates from phylogroups A and B1 are normally multi-resistant.

Our findings demonstrate that *Escherichia coli* isolated from pheasants are highly resistant to ampicillin and cephalothin. The study also demonstrates a considerable resistance to tetracycline.

This study shows that a significant antibiotic resistance also occurs in wild animals that have not previously been exposed to antibiotic treatment. Our work confirms the high prevalence of antimicrobial resistance to beta-lactam antibiotics in wildlife, which is consistent with other studies. A recent work has, indeed, reported up to 100% resistance to penicillin in *E. coli* isolated from gulls in Ireland (Smith *et al.* 2014). It can be assumed that the presence of resistant strains is a result of the increasing resistance of bacteria to antimicrobial drugs worldwide.

In the Czech Republic, antibiotic resistance is a serious concern. Between 2001 and 2005, the antibiotic resistance of *E. coli* to fluoroquinolones doubled; ciprofloxacin resistance increased from 8% to more than 20%, and the upward trend continues (Nyc *et al.* 2011). Our research showed no intermediate or resistant strains to ciprofloxacin antibiotics, nor cefoperazone, colistin, gentamicin, and piperacillin/tazobactam.

Between 1999 and 2000 the 51% of *Escherichia coli* strains isolated from poultry were found to be resistant to ampicillin, the 31% to piperacillin, and the 97% to tetracycline. In the 10% of the examined strains, an increasing resistance to ciprofloxacin and ofloxacin was observed (Kolář *et al.* 2002).

A study carried out in Slovakia examined the resistance to quinolone antibiotics (nalidixic acid, ciprofloxacin, and enrofloxacin) of commensal *Escherichia coli* isolated from healthy broiler chickens from several different farms. Isolates exhibited high resistance to nalidixic acid, ciprofloxacin, and enrofloxacin. The prevalence of resistance was approximately 80% for ciprofloxacin and 50% for enrofloxacin (Kmeť *et al.* 2007).

Wild birds are important in relation to antibiotic resistance in several different ways: 1) As sentinels, of human activities; 2) As reservoirs and melting pots for antibiotic-resistant bacteria and resistance genes; 3) As disseminators of antibiotic resistance through migration; 4) As sources of resistant bacteria colonising or infecting humans beings (Bonnedahl and Järhult 2014).

The results of our research demonstrate that

Table I. *Occurence of antibiotic resistence of* E. coli *in phylogenetic groups A, B1, B2, and D.*

	A n=95		B1 n=48		B2 n=22		D n=15		Total n=180	
	R/I*	**%**	**R/I***	**%**	**R/I***	**%**	**R/I***	**%**	**R/I***	**%**
[a]AMC	0/6	0/6	0/4	0/8	0/0	0/0	0/0	0/0	0/10	0/5.6
AMP	78/17	82/18	42/6	87/13	10/12	45/55	0/15	0/100	130/50	72/28
CEF	90/5	95/5	48/0	100/0	22/0	100/0	0/15	0/100	160/20	89/11
SCF	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
CIP	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
CST	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
GEN	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
CHL	7/0	7/0	3/0	6/0	0/0	0/0	0/0	0/0	10/0	5.6/0
NEO	0/21	0/22	0/6	0/12	0/4	0/18	0/0	0/0	0/30	0/17
TZP	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
STR	0/12	0/13	0/6	0/12	0/1	0/4	0/1	0/7	0/20	0/11
SXT	8/0	8/0	2/0	4/0	0/0	0/0	0/0	0/0	10/0	5.6/0
TET	21/24	22/25	13/17	27/35	6/9	27/41	0/0	0/0	40/50	22/28

*R/I = number of resistant/intermediate *E. coli* strains; [a]AMC = amoxicillin/clavulanic acid; AMP = ampicillin; CEF = cephalothin; SCF = cefoperazon/sulbactam; CIP =ciprofloxacin; CST = colistin; GEN = gentamycin; CHL = chloramphenicol; NEO = neomycin; TZP = piperacillin/tazobactam; STR = streptomycin; SXT = sulphametoxazole/trimethoprim; TET = tetracycline.

Escherichia coli in wild pheasants follows the trend of increasing antimicrobial resistance to antibiotics. Of the 180 examined *E. coli* strains, most were resistant to cephalothin and ampicillin (89% and 72% respectively), other strains were intermediately sensitive. No strains were sensitive to these antibiotics. Further studies are reasonably warranted in order to elucidate the spread of this phenomenon in other wild animals.

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