Investigations on Antimicrobial Resistance in Commensal *Escherichia Coli* Isolates from Waterfowl (Ducks) and Turkeys

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Abstract. *The aim of the present study was to investigate the prevalence of the phenotypic profiles and some genetic determinants of antimicrobial resistance to third generation cephalosporins, i.e., cefotaxime and ceftazidime, fluorinated quinolones and tetracyclines in commensal E. coli isolated from waterfowl (ducks) and turkeys.*

According to the requirements of the European Commission (Directive 2003/99), Bulgaria provides data on the phenotypic manifestations of antimicrobial resistance in Salmonella spp. and commensal Escherichia coli strains from the farm animals, but data on the genetic characteristics of resistant commensal Escherichia coli are limited. Due to limited data on resistance in commensal Escherichia coli isolates from ducks and turkeys in Bulgaria, we attempted to analyze data related to some genetic factors-determined resistance to chemotherapeutic drugs. In our study, we highlighted some of the most common genetic factors of resistance to cefotaxime and ceftazidime, bla $_{CTX-M-1}$ gene in poultry isolates in Europe. Also, plasmid-mediated resistance to fluorinated quinolones in Escherichia coli strains from poultry is often realized with the participation of genes determining resistance to cefotaxime, ceftazidime, gentamicin, tetracycline, etc.

From October 2020 to May 2021, 220 cloacal swab samples were collected in Stuart transport medium: 110 from waterfowl and 110 from turkeys. Ninety-three E. coli strains were isolated from the 110 waterfowl cloacal swabs, while 78 E. coli strains were isolated from the 110 turkey swabs. The E. coli isolates resistant to cefotaxime and ceftazidime were examined for the presence of bla$_{CTX-M-1}$ gene. Bacterial strains resistant to ciprofloxacin were examined for presence of plasmid-determined genes qnrS, qnrA and qnrB1, whereas those resistant to tetracycline were examined for tetA and tetB genes.

The highest percentage of waterfowl E. coli isolates exhibited resistance to tetracycline (81.7%), followed by resistance to ampicillin (75.3%). A high resistance was also observed with respect to ciprofloxacin (66.7%). The coli bacterial isolates from turkeys were most frequently resistant to tetracycline (71.8%) and ampicillin (70.5%), followed by ciprofloxacin (58.9%). As resistance genes were concerned, a significant prevalence was noted for tetA gene (81.7% and 71.8% in waterfowl and turkey strains) and qnrS gene (26.9% and 26% in waterfowl and turkey strains).

Introduction

The broad use of antimicrobial drugs creates appropriate conditions for the emergence and spread of resistance both among pathogenic bacterial microflora and commensal bacteria. The spread of antimicrobial resistance among zoonotic and commensal bacteria may pose risks related to a compromise of efficient therapy of human infectious diseases. In men, there are different mechanisms for transfer of zoonotic and commensal enterobacteria resistant to antimicrobial drugs, e.g. consumption of processing of contaminated foodstuffs, direct contact with animals and various environmental sources (soil, water, manure, etc.) (Argudìn et al., 2017). The monitoring of antimicrobial resistance in commensal enterobacteria, which are ubiquitously spread, is a good background for analysis of the selective pressure and early indicators for distribution of genetic determinants of resistance in different sectors of intensive livestock husbandry (EFSA, 2008).

The opinion of FAO/WHO/OIE (2008) is that the efficiency of third and fourth generation cephalosporins, as well as fluoroquinolones, which are all critically important for medicine, should not be compromised by their inadvertent use in the animal breeding and the agrarian sector. A negative example is the increasing spread of *E. coli* strains producing extended-spectrum beta-lactamases with a connection to the risk from horizontal transfer of genetic determinants of resistance to third and fourth generation cephalosporins (Saliu et al., 2017; Madec et al., 2017). Thus, for instance, the EFSA report (2020) discusses the very high prevalence of resistance to ciprofloxacin (73.5%) in commensal *E. coli* isolates from birds, i.e., the averagely high prevalence of *E. coli* from turkeys (34.8%). The report affirms that multi-drug resistant *E. coli* from broiler chickens were resistant to ciprofloxacin in 78.9% of cases, whereas isolates from turkeys were resistant in 71.7% of cases.

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On the other hand, tetracyclines are among the antimicrobial drugs that are most commonly used in veterinary medicine. What is more, the resistance to tetracyclines among commensal *E. coli* isolated from poultry and pigs is evaluated as high (Sengelov et al., 2003; ESVAC, 2019).

The aim of the present study was to investigate the prevalence of the phenotypic profiles and some genetic determinants of antimicrobial resistance to third generation cephalosporins, i.e., cefotaxime and ceftazidime, fluorinated quinolones and tetracyclines in commensal *E. coli* isolated from waterfowl (ducks) and turkeys.

Material and methods

From October 2020 to May 2021, 220 cloacal swab samples were collected in Stuart tranpsort medium: 110 from waterfowl and 110 from turkeys. Fifty cloacal swabs were obtained from 9-day-old turkey poults and 60 swabs from 12-month-old turkeys. The waterfowl samples originated from 2 farms located in South Bulgaria. In one of the farms, eggs were imported from France. The turkey samples originated from one farm from the central region of the country.

For the isolation of *E. coli*, we used MacConkey agar, and for the preliminary identification procedure, we used triple sugar iron agar. Respectively, biochemical identification of suspicious colonies was performed with the IMViC test (indole+/MR+/VP–/citrate–). *E. coli* isolates were identified by means of the semi-automated system Crystal (Becton, Dickinson, USA).

For phenotype analysis of *E. coli* resistance to antimicrobial drugs, the disc diffusion method and the MIC determination approach were used. The concentrations of chemotherapeutics for the disc diffusion method were as followed: ampicillin (10 μg), ampicillin/clavulanic acid (20/10 μg), ceftazidime (10 μg), cefotaxime (5 μg), gentamicin (10 μg), tetracycline (30 μg) and ciprofloxacin (5 μg) (manufactured by Himedia Biosciences, India). For MIC determination, the E-test was performed with test gradient strips manufactured by Liofilchem (Italy). For analysis of *E. coli* strains' resistance to cefotaxime and ceftazidime, a confirmatory test for MIC determination with the combinations cefotaxime+clavulanic acid and ceftazidime+clavulanic acid was done. The results were interpreted as per EUCAST criteria. Statistical data processing was performed with a Graph Pad program.

Resistant *E. coli* isolates were investigated for presence of the $bla_{CTX\text{-}M\text{-}1}$ gene. Bacterial strains resistant to ciprofloxacin were examined for presence of plasmid-determined genes *qnrS*, *qnrA* and *qnrB1*. The strains resistant to tetracyclines were examined for presence of *tetA* and *tetB* genes.

DNA was extracted with DNeasy Blood Tissue kit (Qiagen, Germany), and for detection of genes encoding resistance to tested antimicrobial drugs, real-time PCR was performed based on TaqMan hydrolysis probes (DNA Assay kits, Qiagen, Germany). The temperature regime of the amplification reaction comprised initial activation step, 1X at 95°C for 10 min, the second stage included 2 steps with 40 cycles of denaturation and annealing/elongation, 40X denaturation at 95°C for 15 s, annealing/elongation at 60°C for 2 min.

The positive DNA control had a cut-off of $C_T \leqslant 34$, and the positive control for amplification reaction: $C_T = 22 \pm 2$.

Results

Ninety-three *E. coli* strains were isolated from the 110 waterfowl cloacal swabs, while 78 *E. coli* strains were isolated from the 110 turkey swabs. From the latter, 34 strains were from 9-day-old turkey poults and 44 from 12-month-old turkeys.

Table 1 presents the results for the prevalence of resistance to tested antimicrobial drugs among waterfowl and turkey *E. coli* isolates. Table 2 presents separately the data on antimicrobial resistance in turkey isolates according to the age (9-day-old and 12-month-old birds).

The highest proportion of *E. coli* isolates from waterfowl were resistant to tetracycline (81.7%),

Table 1. Resistance distribution among indicator *Escherichia coli* strains isolated from waterfowl and turkeys (October 2020 – May 2021)

Antimicrobial	Resistant *E.coli* strains isolated from waterfowl (n = 93)	Confidence Limits (CL)	Resistant *E.coli* isolated from turkeys (n = 78)	Confidence Limits (CL)
Ampicillin	70 (75.3%)	66.0÷83.4	55 (70.5%)	60.0÷80.0
Amoxicillin/clavulanic acid	17 (18.3%)	11.1 ÷26.7	32 (41.0%)	30.4÷52.0
Cefotaxime	–	–	2 (2.6%)	0.4÷7.2
Ceftazidime	–	–	2 (2.6%)	0.4÷7.2
Gentamicin	16 (17.2 %)	10.2÷25.4	14 (17.9%)	10.3÷27.1
Tetracycline	76 (81.7%)	73.2÷88.9	56 (71.8%)	61.5÷81.1
Ciprofloxacin	62 (66.7%)	56.7÷75.7	46 (58.9%)	47.9÷69.5

followed by those resistant to ampicillin (75.3%). The waterfowl isolates were also outlined with a broad resistance to ciprofloxacin (66.7%). Among them, no resistance to cefotaxime and ceftazidime was found out; however, among turkey isolates, such a resistance was detected in 2 strains (2.6%), isolated form 9-day-old birds. *E. coli* isolates from turkeys also demonstrated a higher spread of resistance to tetracycline (71.8%) and ampicillin (70.5%), with higher percentages among the 9-day-old group (73.5%) compared with birds at 12 months of age (70.4%, 68.2%). Turkey isolates showed resistance more commonly to ciprofloxacin (58.9%), and again, the values were higher in 9-day-old turkey poults (70.5%). The broader spread of resistance to the combination amoxicillin/clavulanic acid among turkey isolates should be also noted (41.0%), as compared with waterfowl isolates (18.3%).

Table 3 and 4 present the MIC of tested antimicrobial drugs for waterfowl and turkey *E. coli* isolates. For waterfowl strains, MIC_{90} for ampicillin was 8 μg/mL, and higher values – 16 μg/mL – were determined for turkey strains. For both groups of *E. coli* strains, MIC_{90} of 4 μg/mL was observed with respect to amoxicillin/clavulanic acid. The detected MIC_{90} values for cefotaxime and ceftazidime among turkey isolates were 0.125 μg/mL and 0.25 μg/mL, respectively. The MIC_{90} for ciprofloxacin (0.5 μg/mL) and for gentamicin (2 μg/mL) were the same in both groups of strains. Higher MIC_{90} of 16 μg/mL for tetracycline was determined for turkey isolates; the respective MIC_{90} for tetracycline in waterfowl strains was 8 μg/mL.

Table 5 presents the spread of some specific genetic factors encoding resistance to cephalosporins, ciprofloxacin and tetracycline in *E. coli* isolated from waterfowl and turkeys. In 61.3% of waterfowl *E. coli* isolates (respectively 29.5% of turkey isolates), a multi-resistance profile including ampicillin, tetracycline and ciprofloxacin was determined. In 16.1% of waterfowl *E. coli* strains, the resistance profile including tetracycline and ciprofloxacin was observed. In 17.9% of turkey strains, the multi-resistance profile including ampicillin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, tetracycline and ciprofloxacin was found out.

In tested waterfowl and turkey *E. coli* isolates, the genes *tetA* and *qnrS* were the most prevalent: the *tetA* gene was found out in 81.7% of waterfowl strains while *tetB* was found in 48.4%. The *tetA* gene was detected in 71.8% of turkey isolates, but the prevalence of *tetB* gene was lower (17.9%). None of resistant *E. coli* isolates carried the *qnrA* gene. On the other hand, the prevalence of *qnrS* was observed in 26.9% of waterfowl and 26.0% of turkey isolates.

Table 2. Resistance distribution among indicator *Escherichia coli* strains isolated from turkeys at different ages (October 2020 – May 2021)

Antimicrobial	Resistance *E.coli* isolated from turkeys 9 days old (n = 34)	Confidence Limits (CL)	Resistance *E. coli* isolated from turkeys 12 months of age (n = 44)	Confidence Limits (CL)
Ampicillin	25 (73.5%)	57.6÷86.7	30 (68.2%)	53.8÷84.7
Amoxicillin/clavulanic acid	15 (44.1%)	28.1÷60.8	17 (38.6%)	24.9÷53.3
Cefotaxime	2 (5.9%)	0.6÷16.1	–	–
Ceftazidime	2 (5.9%)	0.6÷16.1	–	–
Gentamicin	11 (32.3 %)	17.8÷48.7	3 (6.8%)	1.3÷16.0
Tetracycline	25 (73.5%)	57.6÷86.7	31 (70.4%)	56.2÷82.8
Ciprofloxacin	24 (70.5%)	54.3÷84.3	22 (50.0%)	35.4÷64.5

Table 3. Minimum inhibitory concentration (MIC) distribution among indicator *Escherichia coli* strains isolated from waterfowl (n = 93)

	MIC μg/mL										
Antimicrobial	0.125	0.25	0.5	1	2	4	8	16	32	128	256
Ampicillin				4	9	10	17*	21	29	2	1
Amoxicillin/clavulanic acid			3	12	31	30	12*	4			
Gentamicin		1	3	34	39	16*					
Tetracycline				3	3	11	35*	30	8	1	
Ciprofloxacin	2	29	59*	1	2						

Legend: clinical breakpoints are marked with asterisks

Also, the *qnrB1* gene was more prevalent among turkey isolates, i.e., in 12.8% vs only 3 strains (3.2%) from waterfowl. The presence of $bla_{CTX\text{-}M\text{-}1}$ gene was detected in 2.6% of turkey *E. coli* isolates.

Figure 1 presents amplification plots of *qnrS* gene determined in *Escherichia coli* strains isolated from turkeys and Figure 2 presents amplification plots of *qnrS* gene determined in *Escherichia coli* strains isolated from waterfowl. Figure 3 presents amplification plots of *tetA* gene determined in *Escherichia coli* strains isolated from turkeys and respectively Figure 4 presents amplification plots of *tetA* gene determined

Table 4. Minimum inhibitory concentration (MIC) distribution among indicator *Escherichia coli* strains isolated from turkeys (n = 78)

	MIC μg/mL											
Antimicrobial	0.06	0.125	0.25	0.5	1	2	4	8	16	32	128	256
Ampicillin					5	3	15	23*	9	17	2	4
Amoxicillin/clavulanic acid				2	3	10	31	25*	7			
Cefotaxime	45	4	25	2		2*						
Ceftazidime	40	2	25	7	2		2*					
Gentamicin			4	10	5	45	12*	2				
Tetracycline					2	3	17	32*	21		3	
Ciprofloxacin		5	27	43*	3							

Legend: clinical breakpoints are marked with asterisks.

Table 5. Resistance phenotypes and genes determining resistance to antimicrobial agents in indicator *Escherichia coli* strains from waterfowl and turkeys (n = 171)

Genes determining resistance to beta-lactams, tetracycline and quinolone (n/%)							
Number of isolates	Resistance phenotypes	$bla_{CTX\text{-}M\text{-}1}$ gene	*tetA* gene	*tetB* gene	*QnrS* gene	*QnrA* gene	*QnrB1* gene
Waterfowl (n = 93)	Amp,T, CIP (57)	–	57 (61.3%)	40 (43.0%)	14 (15.1%)	–	3 (3.2%)
	T, CIP (15)	–	15 (16.1%)	3 (3.2%)	10 (10.7%)	–	–
	Amp, T (3)	–	3 (3.2%)	1(1.1%)	-	–	–
	Amp, G, T, CIP (1)	–	1 (1.1%)	1 (1.1%)	1 (1.1%)	–	–
Total:			76 (81.7%)	45 (48.4%)	25 (26.9%)	–	3 (3.2%)
Turkeys (n = 78)	Amp,T, CIP (23)	–	23 (29.5%)	13 (16.7%)	8 (10.2%)	–	5 (6.4%)
	Amp, AMC, T (14)	–	14 (17.9%)	1 (1.3%)	2 (2.6%)	–	
	Amp, AMC, T, CIP (14)	–	14 (17.9%)	–	3 (3.8%)	–	5 (6.4%)
	CIP (4)	–	–	–	2 (2.6%)	–	
	T, CIP (2)	–	2 (2.6%)	–	1 (1.3%)	–	
	Amp, G, T, CIP (2)	–	2 (2.6%)	–	2 (2.6%)	–	
	Amp, AMC, CAZ, CTX, T, CIP (1)	1 (1.3%)	1 (1.3%)	–	1 (1.3%)	–	
	Amp, AMC, CAZ, CTX (1)	1 (1.3%)	–	–	–	–	–
Total:		2 (2.6%)	56 (71.8%)	14 (17.9%)	19 (26.0%)		10 (12.8%)

Legend: Amp – ampicillin, AMC – amoxicillin/clavulanic acid, CTX – cefotaxime, CAZ – ceftazidime, G – gentamicin, T – tetracycline, CIP – ciprofloxacin.

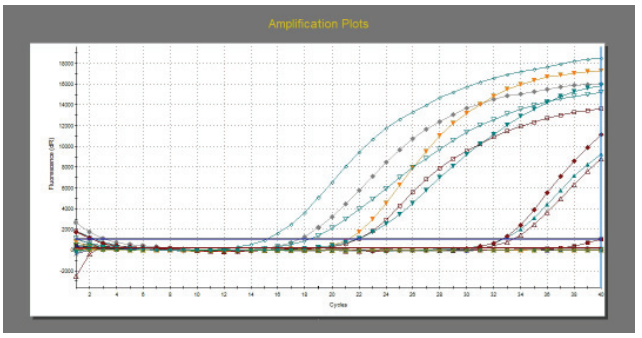

Fig. 1. Amplification plots of *qnrS* gene determined in *Escherichia coli* strains isolated from turkeys

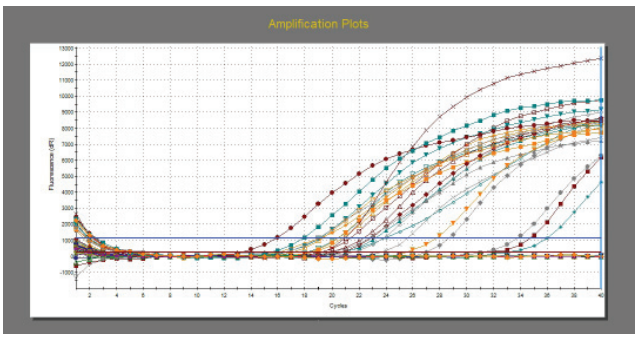

Fig. 2. Amplification plots of *qnrS* gene determined in *Escherichia coli* strains isolated from waterfowl

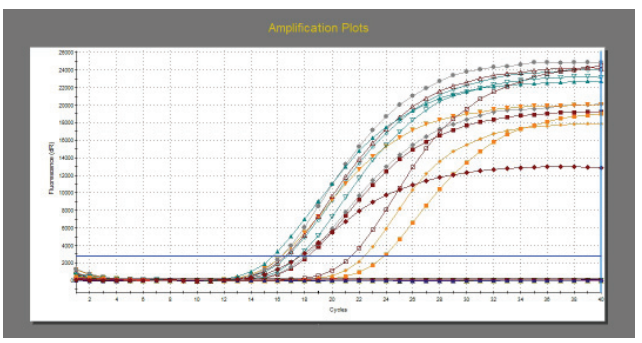

Fig. 3. Amplification plots of *tetA* gene determined in *Escherichia coli* strains isolated from turkeys

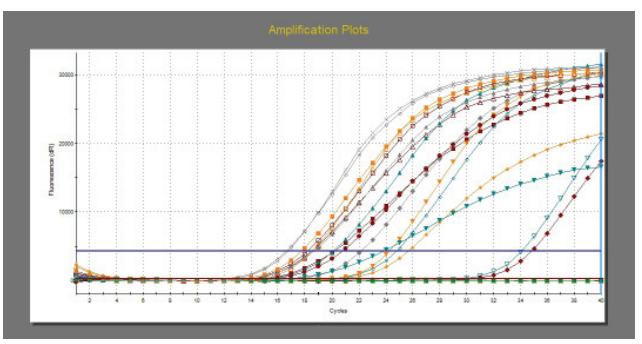

Fig. 4. Amplification plots of *tetA* gene determined in *Escherichia coli* strains isolated from waterfowl

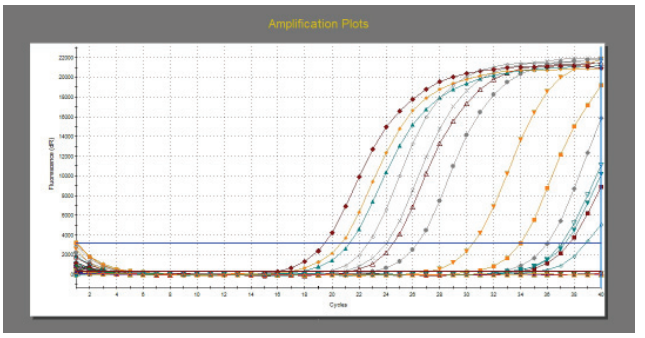

Fig. 5. Amplification plots of *tet B* gene determined in *Escherichia coli* strains isolated from waterfowl

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Discussion

The EFSA (2020) report declared a broad prevalence of resistance to ampicillin (66.8%), ciprofloxacin (56.5%) and tetracycline (61.2%) in commensal *E. coli* isolates from turkeys. According to the experts, the use of antimicrobial drugs at a population level in poultry farming was an argument in support of facts. On the other side, the report of ESVAC (2019) discusses data about the use of antimicrobial drugs in livestock husbandry, with a higher rate of increase for Bulgaria with respect to tetracycline (46.5 mg PCU) and lower levels for fluoroquinolones (5.7 mg PCU). The next report of EFSA and ECDC (approved in 2021) also mentioned that, in most EU member states, indicator *E. coli* isolates from broiler chickens and turkeys demonstrated rather high prevalence of resistance to ampicillin, sulfamethoxazole, trimethoprim and tetracycline. Data provided by Bulgaria for resistance to tetracycline in commensal *E. coli* isolates showed a tendency towards a decrease under 60.0% as compared with the data included in the preceding report from 2016. The multi-resistance profiles with the participation of tetracycline were found in 43.4% of tested *E. coli* isolates from broilers and 45.7% of isolates from turkeys (EFSA, 2021).

In confirmation of the EFSA data on distribution of resistance to tetracycline and ampicillin in commensal poultry E.coli isolates (annual report, 2020), in our study, we found a broader distribution of resistance to tetracycline, i.e., prevalence of genetic factors *tetA* gene (81.7%) and *tetB* gene (48.4%) in waterfowl commensal *E. coli* isolates. Similar data were reported for the presence of *tetA* (71.8%) in turkey strains, with lower prevalence of *tetB* gene (17.9%). Moreover, the phenotype analysis exhibited higher resistance to ampicillin in waterfowl strains (75.3%) as well as a high proportion of *E. coli* isolates from turkeys resistant to ampicillin (73.5%) and amoxicillin/clavulanic acid (44.1%). For example, in the Czech Republic, R derova et al. (2017) also

reported high percentages of resistance to ampicillin (96%) and tetracycline (90%) in commensal *E. coli* strains from turkeys. The authors also stated that ciprofloxacin-resistant isolates had MIC values from 0.25 μg/mL to 16 μg/mL and presence of *qnrS1* and *qnrB19* genes. In our study, MIC values higher than 2 μg/mL were not observed in resistant *E. coli* isolates with MIC_{90} values for strains from both species of 0.5 μg/mL. Also, the *qnrB19* gene was not detected. Another study from Norway (Slettemeås et al., 2019) also presented data about the high levels of MIC for ciprofloxacin (8 μg/mL) among 41% of *Escherichia coli* isolates from turkey meat. The authors take this fact into account as a result of imports of breeding animals in the country. In the present study we did not find statistically significant differences ($P \leqslant 0.001$) between ciprofloxacin resistant commensal *Escherichia coli* isolates from waterfowl in one of the 4 farms surveyed, which were imported Hungarian breeding animals.

In the present study, the resistance to ciprofloxacin was high: in 70.5% of isolates from turkeys and 66.7% from waterfowl. A study from the Czech Republic (Hricovà et al., 2017) presented data about the levels of resistance to fluorinated quinolones in commensal *E. coli* isolates from turkeys with considerably lower prevalence of resistance to ciprofloxacin: in 45% of strains. The authors observed the spread of *qnrB* and *qnrS* genes in 19% and 52% of turkey *E. coli* strain, respectively. In the UK, Gosling et al. (2012) reported a significantly lower spread of *qnrB* and *qnrS* genes among ciprofloxacin-resistant commensal *E. coli* isolates from turkeys: 3.7% and 1.4%, respectively. The authors also commented on the fact that in 88% of surveyed farms, isolates had multi-drug resistant profiles with the participation of ciprofloxacin. The authors also analyzed the broader spread of resistance to ampicillin and ceftazidime among commensal *E. coli* isolates from turkeys (84.9%; 4.7%) compared with the values obtained in the present study for both groups of *E. coli* bacteria (75.3%, 70.5%, 2.6%). With regard to the presence of the *qnrS* gene, the present study found out higher percentages among waterfowl and turkey strains (26.9%, 26.0%). A lower prevalence of the *qnrB* gene was observed among waterfowl isolates (3.2%). According to Gosling et al. (2012), the broad spread of multi-resistance profiles included up to 88.1% of turkey strains. We also found out a high percentage of multi-resistant strains in both waterfowl (62.4%) and turkeys (70.5%). As far as the beginning of this century, Van den Bogaard et al. (2001) reported multi-resistance profiles involving 5 and more chemotherapeutics among commensal *E. coli* isolates from turkeys.

Of interest is also the study of Skarzynska et al. (2021) in Poland, which established the distribution of a number of genes determining resistance to antimicrobial agents, including *qnrS* gene, *tetA* gene and *tetB* gene among wild birds, which the authors identify as a possible reservoir of resistance in environment. According to the authors, resistant profiles in *Eschericia coli* often refer to antimicrobials widely used in human and veterinary medicine.

Chuppava et al. (2018) observed high rates of resistance to ampicillin (42.0%) and enrofloxacin (48.0%) in day-old turkeys. For example, in the present study, the prevalence of resistance to aminopenicillins (73.5%) and ciprofloxacin (70.5%) was higher in *Escherichia* coli isolates from 9-days-old turkeys compared with the observed levels of resistance in isolates from turkeys 12 months of age. Multi-drug resistant profiles involving ceftazidime and cefotaxime were also observed in isolates from 9-days-old turkeys, respectively, related to $bla_{\text{CTX-M-1}}$ gene, *tetA* gene, and *qnrS* gene propagation. In accordance with the survey we conducted with farmers before our study, on the farm only ciprofloxacin was used in 9-days-old turkeys for prophylactic purposes. This fact may in some ways serve as an argument for the wider prevalence of ciprofloxacin resistance in commensal *E. coli* strains from 9-days-old turkeys. Of interest is also the fact that in commensal *E. coli* isolates from birds in this age category we found resistance to cefotaxime and ceftazidime, respectively, distribution of $bla_{\text{CTX-M-1}}$ gene. Probably the explanation for this fact can be found in the argument for the spread of plasmid-determinated genetic factors in multi-resistant *E. coli* strains.

Conclusion

The present study documented higher prevalence of resistance to tetracycline, ampicillin and ciprofloxacin among commensal *E. coli* isolates from turkeys and waterfowl (ducks) in comparison with average values for EC member states included in the EFSA report from 2020. The resistance to cefotaxime and ceftazidime was demonstrated in 2.6% of tested *E. coli* isolates from turkeys, as a part of multi-drug resistant profiles with the participation of aminopenicillins, third-generation cephalosporins, and one profile with tetracycline and ciprofloxacin at the same time. In our opinion, the wider prevalence of resistance to tetracycline, ciprofloxacin and ampicillin in commensal *E. coli* isolated from turkeys and ducks is probably based on the wider use of these chemotherapeutic drugs in our country than other countries in EU.

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