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Screening of antimicrobial resistance and molecular detection of fluoroquinolone resistance mechanisms in chicken faeces-derived *Escherichia coli*

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ABSTRACT: This study was aimed at investigating the resistance to antimicrobial agents and to assess the predominant molecular mechanisms of fluoroquinolone resistance in faecal *E. coli* strains isolated from chickens farmed in central Poland. Bacterial strains were isolated from faecal samples of chickens reared on four conventional and one organic farm. The disk-diffusion method was applied to assess antimicrobial resistance and the prevalence of particular resistance mechanisms to fluoroquinolones was determined using specific polymerase chain reactions and sequencing of the *gyrA* and *parC* genes. Rep-PCR was used to determine the intra-specific variation of *E. coli* strains. The greatest resistance was observed for ß-lactams (e.g. from 25 to 100% of strains resistant to amoxicillin/clavulanate) and the smallest – for cephalotin (0 to 18.75% resistant strains). Three out of four conventional farms were characterised by very high resistance rates, particularly to enrofloxacin (from 87 to 93.3% of resistant isolates). The majority of multidrug-resistant strains were also isolated from these farms. The presence of plasmid-mediated quinolone resistance genes (*qnrB* and *qnrS*) was detected very frequently, even in strains that exhibited phenotypic susceptibility to fluoroquinolones. With respect to point mutations in quinolone resistance determining regions, Ser-83 substitution was observed in numerous strains. Some of the fluoroquinolone-resistant strains appeared to possess both *qnr* genes coupled with point mutations, which indicates that a high level of resistance can be affected by multiple factors. Nevertheless, excessive use of antimicrobial agents in food-producing animals decreases the susceptibility of commensal strains, even those that never had contact with antibiotics.

Keywords: antibiotic susceptibility; *E. coli*; rep-PCR; *qnr* genes; *parC*; *gyrA*

The use of antimicrobial agents in the treatment and prevention of poultry diseases in farming is considered to be one of the most important factors which is promoting the increase in the number of drug-resistant microorganisms in the environment (Witte 1998; Lebkowska 2009). The growth promoting effects of antimicrobial agents were first discovered in the 1940s, when chickens fed with tetracycline products were found to grow faster than those which were not fed with antimicrobial agents (Phillips et al. 2004). Since then, a large number of antimicrobials have been used to increase feed efficiency and average daily weight gain. Fluoroquinolones have been used in food-producing animals since the end of the 1980s (Veldman et al. 2011). In Poland, fluoroquinolones are the third most frequently used veterinary antimicrobials after tetracyclines and penicillins and they constituted 20% of the total antimicrobial agents used in poultry farming in 2011 (Dzierzawski and Cybulski 2012). The use of antibiotic growth promoters in animal farming has been restricted in the European Union since January 1st 2006. Despite this fact, the administered doses are often inflated or used contrary to the instructions of veterinarians. Additionally, the duration of treatment is often extended without any justification by farmers (Dzierzawski and Cybulski 2012). Therefore, the antimicrobial selection pressure for bacterial resistance in poultry is high and consequently, the faecal microflora of chickens contains relatively high proportions of resistant bacteria (Miles et al. 2006).

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There is a significant concern that resistant bacteria from food-producing animals may be transferred to humans either by direct contact or by consumption of contaminated food (Tollefson et al. 1997). What is more, animal waste, including manures which are dispersed into the soil or water, may contain high levels of resistant bacteria, thus causing further contamination of food, drinking water and the environment. Moreover, the use of fluoroquinolones in poultry farming may be inappropriate, as a result of cross-resistance, i.e. the resistance to one fluoroquinolone compound may compromise the effectiveness of other fluoroquinolones in the treatment of important human enteric infections (Blanco et al. 1997).

The two targets of fluoroquinolones are gyrase and topoisomerase IV, as they form ternary drug molecule-enzyme-DNA complexes that block replication fork movement (Hiasa et al. 1996), thus inhibiting bacterial DNA synthesis (Drlica and Zhao 1997). Single nucleotide polymorphisms (SNPs) in the quinolone resistance determining regions (QRDR) of *gyrA* and *parC* – the two genes encoding gyrase and topoisomerase IV, can lead to conformational changes in these enzymes that prevent the quinolone binding to the DNA-substrate complex (Hawkey 2003). In *Escherichia coli* and their related Gram-negative bacteria, DNA gyrase is the first target for fluoroquinolones and when *gyrA* has resistance-conferring mutations, the primary target switches to topoisomerase IV (Hopkins et al. 2005). Studies show that these mutations typically occur in *gyrA* first and then in *parC* (Namboodiri et al. 2011). QRDR mutations are the most commonly documented fluoroquinolone resistance mechanisms (Namboodiri et al. 2011). Another quinolone resistance mechanism includes the upregulation of efflux pumps, which export the antimicrobials out of the bacterial cell. Quinolone resistance can also be acquired horizontally through plasmid-mediated determinants, such as *qnr* genes. The *qnr* gene product inhibits quinolones binding to target proteins (Tran and Jacoby 2002). The remaining horizontally-acquired quinolone resistance genes include *aac(6')-Ib*, that encodes a fluoroquinolone acetylating enzyme, as well as *qepA* and *oqxAB*, encoding horizontally transmitted efflux pumps (Namboodiri et al. 2011).

The aim of this study was to investigate the prevalence of resistance to antimicrobial agents together with the assessment of the predominant molecular mechanisms of fluoroquinolone resistance in faecal *E. coli* strains isolated from chickens farmed in central Poland.

MATERIAL AND METHODS

Enumeration and isolation of *E. coli* strains. Fresh faecal samples were randomly collected from five poultry farms (A–E) in central Poland. The samples were taken twice during the six-week breeding period: the first sampling was conducted when the animals were one-week old and the second round of samples was collected two weeks later. Four of the investigated farms practiced a conventional, barn-production system of breeding (farms A–D) and one of the farms was organic (farm E). For farms A, B and C unofficial information about the use of enrofloxacin as an antimicrobial growth stimulator was acquired. Additionally, the farm owners provided information about the bacterial infections which occurred on their farms. The antimicrobial therapy was conducted and administered by a veterinarian: on farms A and D Amoxiclav 625 mg (amoxicillin + clavulanic acid) was used, on farm B – Floron 40 mg (florfenicol) and on farm C – Amoxymed 15 (amoxicillin). No antimicrobial therapy was performed on the organic farm.

The faecal samples were collected using sterile plastic applicators and pooled into tubes. Subsequently, the samples were diluted with saline solution and plated onto Endo agar medium (Sigma-Aldrich, Switzerland) and incubated for 18–20 h at 37 °C. After the incubation, presumptive *E. coli* (purple, with metallic sheen) colonies were enumerated. Between fifteen and twenty five *E. coli* isolates were selected for further examinations. All isolates were identified based on Gram staining, oxidase reaction and ß-glucuronidase activity on TBX medium (Oxoid, Great Britain).

Antimicrobial susceptibility testing. Antimicrobial susceptibility tests were performed using the standard disk-diffusion method in accordance with the recommendations of the Polish National Reference Centre for Antimicrobial Susceptibility (KORLD) (Gniadkowski et al. 2009). Cartridges of antimicrobial disks were obtained from Oxoid (Great Britain). Bacterial isolates were transferred to 5 ml sterile 0.9% saline solution to prepare the 0.5 MacFarland suspension standards, which were then streaked onto Mueller-Hinton II (Biocorp,

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Poland) agar plates within 15 min after adjustment of turbidity. Subsequently, the antimicrobial disks were applied and the plates were incubated for 18–20 h at 36 ± 1°C. The diameters of growth inhibition zones surrounding the antimicrobial disks were measured to mm and the inhibition zones were compared with the breakpoints recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2013). The enrofloxacin breakpoints were applied according to the Baytril® (enrofloxacin) technical bulletin (Bayer 2010). Antimicrobials used in this study and their breakpoints are given in Table 1. Quality control was performed using the *E. coli* strain ATCC 25922.

DNA extraction, PCR assay and DNA sequencing. DNA of *E. coli* isolates was extracted using the Genomic Mini kit (A&A Biotechnology, Poland) according to the manufacturer's instructions and was used as a template for all PCRs. Horizontally-acquired quinolone-resistance genes were identified using PCR. Primers from Cattoir et al. (2007) were used to screen for *qnrA*, *qnrB* and *qnrS* and the primers reported by Liu et al. (2008) were used for *qepA*. The quinolone-resistance determining regions of *gyrA* and *parC* genes were amplified using the primer pairs described in Wang et al. (2001). PCR reactions were carried out in a 25 µl volume and contained 50 ng of DNA template, 12.5pM of each primer, 2.5mM of dNTP, 1× PCR buffer and 1 IU DreamTaq DNA polymerase (ThermoScientific, US). The following temperature profile was used for the reactions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, annealing for 45 s at different temperatures, then extension at 72 °C for 1 min with final extension at 72 °C for 10 min and then cooling to 4 °C. PCR amplifications were performed in a T100™ Thermal Cycler (Bio-Rad, USA). The PCR products were electrophoresed for 60 min in 1% agarose gels run in 1 × TBE. Gels were stained with Simply Safe (0.5 mg/ml; EurX, Poland) and products were visualised under UV light and documented using the GelDoc system (BioRad, US).

For mutational analysis of the quinolone-resistance determining regions, the amplicons were purified using the CleanUp kit (A&A Biotechnology, Poland) and sequenced using gyrAF and parCF primers in an automatic 3500 sequencer (Applied Biosystems, US). The obtained sequences were analysed using MEGA 6 software (Tamura et al. 2013) and the translated protein sequences were compared to the corresponding genes from the U08817 (*gyrA*) and U08907 (*parC*) GenBank sequences to identify substitution mutations.

Rep-PCR was carried out using the BOXA1R primer (Versalovic et al. 1994) in two replicates. PCR reactions were performed in a total volume of 25 µl containing approximately 20 ng of DNA template, 12.5pM of the primer, 2.5mM of dNTP, 1 × PCR buffer and 1 IU DreamTaq DNA polymerase (ThermoScientific, US). PCR amplification was performed in a T100™ Thermal Cycler (Bio-Rad, USA) using the following temperature profile: initial denaturation at 94 °C for 5 min, followed by 25 touchdown cycles of denaturation at 94 °C for 30 s, annealing starting from 67.5 °C with the temperature decreasing by 0.5 °C in each cycle down to 55 °C for 30 s and elongation at 72 °C for 1 min and then 20 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min, and final elongation for 10 min. The PCR products were electrophoresed for 120 min in 1.5% agarose gels in 1 × TBE buffer, and stained with 0.5 mg/ml Simply Safe (EurX, Poland). After the electrophoresis the gel was analysed under UV light and using GelDoc (Applied Biosystems, US). Rep-PCR results were scored on agarose gels for two independent replicates. The bands present on both gels were scored and encoded in a presence-absence binary matrix.

Numerical analysis. A one-way ANOVA test ($P < 0.05$) was applied to determine the significance of differences between the numbers of *E. coli* colonies isolated from faecal samples. This was followed by the post-hoc Tukey test in order to verify the significance of differences in the bacterial numbers between each farm and collection dates. A chi-square test ($P < 0.05$) was used to estimate the differences between the percentages of resistance between groups of isolates from different farms. The tests were performed in Statistica v. 10 (StatSoft, US).

To verify the presence of clonal strains the Rep-PCR dataset was searched for the presence of individual haplotypes with FaBox (Villesen 2007). Strains carrying the same Rep-PCR haplotype and *gyrA* and *parC* sequences were considered clonal in our analysis. In order to assess the extent of intra- and inter-population variation AMOVA analysis was carried out using Arlequin 3.1.1. (Excoffier and Lischer 2010) with rep-PCR data coded as RFLP datatype and for both rep-PCR and *parC*/*gyrA* polymorphisms coded as standard datatype. Along with AMOVA analysis the basic differentiation in-

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Table 1. Percentage of *E. coli* isolates from different farms, susceptible (S), intermediate (I) and resistant (R) to antimicrobial agents assessed by the disk-diffusion method. Values given in bold type show the greatest resistance rates for individual antimicrobial agents

	Fluoroquinolones						β–lactams					
Antimicrobial (μg)	Ciprofloxacin (5)*			Enrofloxacin (5)*			Ampicillin (10)*			Amoxicillin/clavulanate (20/10)*		
Breakpoint (mm)	22/19			21/17			14			19		
	S	I	R	S	I	R	S	I	R	S	I	R
Farm A	12.5	0	87.5	0	12.5	87.5	0	–	**100**	0	–	**100**
Farm B	17.4	39.1	43.5	8.7	4.3	87.0	26.1	–	73.9	73.9	–	26.1
Farm C	6.7	0	**93.3**	6.7	0	**93.3**	13.3	–	86.7	46.7	–	53.3
Farm D	100	0	0	95.7	4.3	0	78.3	–	21.7	56.5	–	43.5
Farm E	75	15	10	55	25	20	40	–	60	75	–	25
	Aminoglycosides						Cephalosporins					
Antimicrobial (μg)	Gentamicin (10)*			Cephalotin (30)			Cefuroxime (30)			Cephazolin (30)		
Breakpoint (mm)	17/14			14/18			18			23/19		
	S	I	R	S	I	R	S	I	R	S	I	R
Farm A	18.75	68.75	12.5	81.25	0	**18.75**	81.25	–	18.75	0	31.25	68.75
Farm B	30.4	26.1	43.5	91.3	0	8.7	47.8	–	**52.2**	0	26.1	**73.9**
Farm C	33.3	6.7	**60**	86.7	0	13.3	53.3	–	46.7	0	26.7	73.3
Farm D	26.1	30.4	43.5	91.3	8.7	0	65.2	–	34.8	13.0	34.8	52.2
Farm E	35	35	30	95	0	5	65	–	35	5	50	45

*the differences between analysed groups are significant at $P < 0.05$

dexes and MST connections were obtained with Arlequin. The MST connections were further used for drawing a minimum spanning tree with Gephi 0.8.2. Strains isolated from the same farm were considered as a single population.

RESULTS

Enumeration and isolation of *E. coli* strains

The number of *E. coli* CFUs isolated from different farms is presented in Table 2. The greatest abundance was observed in the organic farm (farm E), while the smallest number was recorded in farm C, particularly on the second sampling date, after antimicrobial therapy, which included the application of amoxicillin. The differences in the numbers of bacteria between different farms and sampling dates are statistically significant (ANOVA $F = 19.36$, $P < 0.05$; Tukey test $P < 0.05$). The number of strains selected for further analysis is also given in Table 2.

Prevalence of drug resistance

The drug resistance test was conducted on a total number of 98 isolates, including the ATCC 25922 strain. It included all antimicrobial agents of the basic antibiogram of KORLD (Gniadkowski et al. 2009) for Enterobacteriaceae and two additional fluoroquinolone drugs, i.e. enrofloxacin and

Table 2. The occurrence of *E. coli* in faecal samples from different farms in various collection periods (CFU/g)

	Farm A	Farm B	Farm C	Farm D	Farm E
Term 1	20 667	3 318 889	880 555	297 667	1 016 667
Term 2	3 818 889	203 333	3 333	3 663 333	4 855 556
Mean	1 919 778	1 761 111	441 944	1 980 500	2 936 111
Number of isolates selected for further analysis	16	23	15	23	20

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Table 3. Prevalence of multidrug-resistant *E. coli* strains on the tested farms (%)

Number of antimicrobials	Farm A (n = 16)	Farm B (n = 23)	Farm C (n = 15)	Farm D (n = 23)	Farm E (n = 20)
0	0	4.35	0	21.74	20
1	0	4.35	0	30.43	30
2	6.25	13.03	0	8.70	15
3	0	17.39	20	13.04	10
4	25	21.74	13.33	17.39	5
5	50	17.39	33.33	4.35	10
6	6.25	8.70	6.68	4.35	0
7	6.25	4.35	13.33	0	10
8	6.25	8.70	13.33	0	0
Multidrug-resistant strains (to 3 or more antimicrobials)	93.75	78.27	100	39.13	35

ciprofloxacin. Table 1 shows the structure of antimicrobial susceptibility within the tested strains. Among the tested farms, farm A had the greatest prevalence of strains resistant to β-lactams (both ampicillin and amoxicillin with clavulanic acid – 100% resistance), on farm B there was the greatest percentage of strains resistant to cephalosporins (cefuroxime and cephazolin), while the greatest resistance to fluoroquinolones (both ciprofloxacin and enrofloxacin) and to genamicin (aminoglycosides) was observed on farm C. The two latter farms – conventional D and organic E were characterised

Table 4. Prevalence of different fluoroquinolone resistance determinants in *E. coli* isolates derived from different farms

Mechanism	Farm A (n = 16)		Farm B (n = 23)		Farm C (n = 15)		Farm D (n = 23)		Farm E (n = 20)	
	R	S (n = 2)	R (R^E, n = 10)	S (n = 3)	R	S (n = 1)	R	S (n = 23)	R (R^E, n = 2)	S (n = 16)
***GyrA* mutations**										
S83L	14	0	10 (10)	0 (2 I^E)	14	0	–	1	1 (1)	3 (3 I^E)
D87N	14	0	7	0	8	0	–	1	2	0
D87E	0	0	0	0	3	0	–	0	0	0
D87G	0	0	0	0	3	0	–	0	0	0
***parC* mutations**										
S80I	11	0	8	0	12	0	–	0	2	0
S80R	3	0	0	0	1	0	–	1	0	0
E84K	0	0	0	0	1	0	–	0	0	0
Mutations with unconfirmed effect										
gyrA V69D	0	0	0	1	0	0	–	0	0	0
*par*C H75Q	0	0	0	0	1	0	–	0	0	0
parC C82R	0	0	1 (1)	0	0	0	–	0	0	0
parC T121I	1	0	0	0	0	0	–	0	0	0
parC L131Q	0	0	0	0	0	0	–	4	0	0
PMQR										
qnrA	0	0	0	0	0	0	–	0	0	0
qnrB	13	2	7 (10)	0 (2 I^E)	3	0	–	15	2 (2)	12 (3 I^E)
qnrS	8	2	10 (10)	1 (2 I^E)	13	1	–	11	2 (2)	12 (3 I^E)
qepA	0	0	0	0	0	0	–	0	0	0

R^E = strains resistant only to enrofloxacin, I^E = strains with intermediate reaction to enrofloxacin

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Table 5. The results of AMOVA analysis for 98 isolates grouped into five farm-specific populations

Source of variation	Sum of squares	Variation components	Percentage of variation
Among populations	68.387	0.79442	30.59
Within populations	165.798	1.80216	69.41
Total	234.186	2.59658	100.00
Fixation index – F_{ST}	0.30595		

by the greatest number of strains susceptible to the tested antimicrobials. For instance, all strains isolated from farm D were susceptible to ciprofloxacin, and also no strains from this farm were resistant to enrofloxacin and cephalotin. The antimicrobial susceptibility testing also indicated the presence of multidrug-resistant *E. coli* strains isolated from farms A, B and C (Table 3). For example, on farm C there were no strains which were either susceptible to all antimicrobials, or resistant to one or two. The smallest percentage of multidrug-resistant strains (35%) was recorded on the organic farm E. On Farm C all strains were multidrug resistant. Additionally, on farms A, B and C there were no strains susceptible to cephazolin (Table 1).

Determination of SNP mutations in quinolone resistance determining regions (QRDR)

Table 4 summarises the prevalence of molecular determinants of fluoroquinolone resistance revealed in this study. All fourteen fluoroquinolone-resistant isolates from farm A harboured two mutations in *gyrA* (S83L and D87N), together with one mutation in *parC* (S80R or S80I). On farm B we recorded ten strains resistant to both tested fluoroquinolones and 10 resistant only to enrofloxacin, but all of them possessed the mutation in *gyrA* (S83L). An additional mutation in *gyrA* (D87N) was recorded in seven isolates resistant to both fluoroquinolones. The same strains also had yet another mutation in *parC* (i.e. S80I), which means that seven out of the twenty resistant isolates possessed three mutations in QRDR. We also identified the S83L mutation in *gyrA* in two isolates which were susceptible to ciprofloxacin and their inhibition zone classified them as intermediate to enrofloxacin. On farm C there were fourteen isolates resistant to both tested fluoroquinolones and all of them harboured two mutations in *gyrA* (S83L and either D87E, or D87N or D87G) and one of the mutations in *parC*. Interestingly, no isolates from farm D were resistant to either of the tested fluoroquinolones, but some mutations were still recorded, i.e. there was one strain, which harboured all three mutations (i.e. S83L, D87N and S80R). In the case of organic farm E, S83L in *gyrA* was recorded in three isolates susceptible to both of the tested fluoroquinolones and in three strains with intermediate reaction to enrofloxacin.

Detection of horizontally-transmitted quinolone resistance genes (PMQR)

Screening for *qnrA*, *qnrB*, *qnrS* and *gepA* genes was conducted for all ninety eight isolates and the reference strain. Out of the isolates evaluated, none carried either *qnrA* or *gepA*. On the other hand, *qnrB* and *qnrS* were very frequently detected, even in some of the strains which exhibited phenotypic susceptibility to the tested fluoroquinolones (Table 4).

Genetic variation of isolated strains

All ninety eight isolated *E. coli* strains along with the reference strain were subjected to Rep-PCR analysis in order to detect the possible clonality of the isolated strains. These data were used along with *gyrA* and *parC* sequences to detect the presence of unique haplotypes. This analysis revealed relatively high differentiation among our isolates as a total number of sixty five haplotypes were detected. Fifty two out of this number were found to be harboured by only one isolate. The 53rd single-isolate haplotype was carried by a reference strain. The most frequent haplotype was detected on farm A exclusively and was characteristic for eight isolates. Importantly, four haplotypes, observed for more than one isolate, were recorded on more than one farm at the same time. The most haplotype-rich *E. coli* population was that of organic farm E, where 0.95 haplotype per isolate was detected. An only slightly lower level was recorded on farm C,

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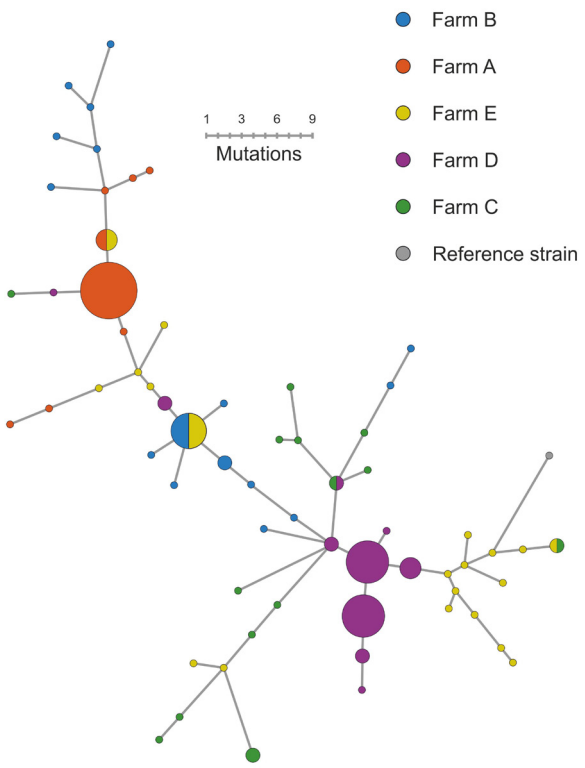

Figure 1. The minimum spanning tree acquired with Arlequin 3.1.1. and visualised with Gephi 0.8.2. The size of circles indicates the relative frequency of each haplotype

i.e. 0.93 haplotype per isolate. The opposite situation was observed for farms A and D, where only 0.5 and 0.39 haplotype per isolate were observed, respectively. The relative relationships between particular isolates are demonstrated on the minimum spanning tree (Figure 1). The position on the tree for farms A and D reflects their relative low genetic differentiation, as they are dominated by one (farm A) or two (farm D) most frequently detected haplotypes and the majority of other haplotypes is relatively closely situated. The haplotypes of organic farm E are distributed throughout the entire tree, reflecting their strong relative differentiation.

The overall genetic differentiation between farms was analysed according to AMOVA principles. The AMOVA results are presented in Table 5. Only rep-PCR data were used in AMOVA calculations, since *gyrA* and *parC* genes did not meet the requirement of independent traits, as they are subjected to strong selective pressure. The comparison of mean number of pairwise differences (MNPD) for each population revealed that two farms were characterised by considerably lower genetic diversity, i.e. farms A and D, with MNPD indices of only 2.591667 and 1.628458, respectively. For farms B, C and E the MNPD values were much higher (4.577075, 5.428571, 4.221053, respectively).

DISCUSSION

Bacterial resistance to antimicrobials and the reasons for the emergence of antimicrobial resistance are very well documented in the literature (Hopkins et al. 2005). Despite the fact that the European Union has now restricted the use of antimicrobial growth promoters in animal husbandry, some antimicrobials, including fluoroquinolones are still used for treatment under veterinary prescriptions and since enrofloxacin can be added to the drinking water of the whole flock for up to ten days, the use of antimicrobials in poultry farming remains on the increase (www.soilassociation.org 2012).

The number of *Escherichia coli* detected in chicken faeces was the greatest in farm E, which was an organic husbandry (Table 2). On other, conventional farms, these numbers were significantly lower. In the case of two conventional farms (B and C) the numbers of *E. coli* detected on the second sampling date were much lower than on the first date. It is important to note that on all conventional farms different infections were detected and antimicrobial therapy was administered.

Our data show that the resistance to ampicillin and both tested fluoroquinolones was the most frequently detected antimicrobial resistance (Table 1). Literature data confirm that resistance to ampicillin is one of the most frequently observed antimicrobial resistances (Namboodiri et al. 2011). Salehi and Bonab (2006) detected similarly high rates of both ampicillin and fluoroquinolone (enrofloxacin and ciprofloxacin) resistance in *E. coli* strains isolated from chickens in Iran. As in our study, the rate of resistance to both fluoroquinolones was similar, but was slightly higher for enrofloxacin than for ciprofloxacin (Table 1). Fluoroquinolones have been widely used in veterinary medicine since the early 1990s. In Europe, none of the fluoroquinolones licensed for use in humans is approved for use in veterinary medicine (Hopkins et al. 2005). However, our results demonstrate that ciprofloxacin resistance is highly correlated with the resistance to enrofloxacin (Table 1). Enrofloxacin is a methylester of ciprofloxacin and both agents are completely cross-resistant (van den Bogaard et al.

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2001). Enrofloxacin is commonly used in veterinary practice and in poultry farming for prolonged periods of time, causing the selection pressure which promotes the emergence and preservation of resistance to this antimicrobial agent and consequently, to the whole group of fluoroquinolones. In our study the greatest prevalence of drug-resistant strains was observed on three conventional farms – A, B and C (Table 1). On the other hand, the lowest resistance was observed on farms D and E, of which farm D was conventional and farm E was organic. These observations are most probably an effect of not using antimicrobial growth promoters on either of the farms. Moreover, one can assume that the antimicrobials administered on farm D were applied properly and with adherence to the veterinary recommendations. However, a low level of resistance to fluoroquinolones was detected on farm E and the resistance to amplicillin was quite high (i.e. 60%). This could be explained by the fact that the organic farm was located near the conventional farm C, where both ampicillin and fluoroquinolone resistance was very high, reaching 86.7% and 93.3% of strains, respectively (Table 1). Dead animals are often improperly utilised and the resistant bacteria could have been transferred with surface water. Similarly to our studies, Sapkota et al. (2011), when comparing antimicrobial resistance on organic and conventional poultry farms, observed that even though the conventional poultry farms contained a significantly greater share of drug-resistant bacteria (81% of *E. faecium* resistant to tetramycin), 12% of *E. faecium* population from the organic farm was also resistant to this antimicrobial. Despite preventive measures, antibiotic-resistant bacteria can reach the environment and persist there, although the selective pressure posed by antimicrobials is not present anymore. This situation may pose a risk of antimicrobial resistance spreading through horizontal gene transfer (Kaplan 2014).

Another disturbing observation made in this study was the widespread multidrug resistance (Table 3). On farm C there were no strains that were either susceptible to all eight antimicrobial agents, or resistant to only one or two of them. On farm A 50% of strains were resistant to five out of eight tested antimicrobials. Nonetheless, some of the strains isolated from the organic farm E were also multidrug-resistant (35% of strains resistant to three or more antimicrobial agents), despite the fact that no antibiotics are used on this farm. These observations may indicate that bacteria can acquire resistance to different antimicrobial agents from environmental exposure (Rysz and Alvarez 2004). Similar observations were made by Miles et al. (2006) in their study on *E. coli* isolates from broiler chickens raised on farms without recorded antimicrobial use. More importantly, van den Bogaard et al. (2001) proved that drug-resistant *E. coli* can easily spread from food-producing animals to farmers and consumers of poultry meat. Another important problem related to the high prevalence of multidrug-resistant bacteria in poultry faeces is related to the fact that animal waste is often dispersed into soil as natural fertiliser. Subsequently, resistant bacteria can eventually leach into groundwater, lakes and rivers and cause further contamination, not only of the environment but also of food (Moniri and Dastehgoli 2005). What is more, once bacteria seep into the ground there are countless opportunities for these populations to horizontally transfer their genetic material to a variety of other microbial species (Kaplan 2014).

SNP mutations in quinolone resistance determining regions together with Qnr plasmid determinants are the best documented mechanisms of fluoroquinolone resistance. In the present study the most frequent SNP mutation in *gyrA* was the Ser-83 substitution, as it occurred in fifty nine out of ninety eight tested isolates (Table 4). On farm A double substitutions in Ser-83 and Asp-87 were observed in as many as fourteen out of sixteen isolates, which resulted in very high resistance to both tested fluoroquinolones, i.e. 87.5% (Table 1). On farm C all fourteen resistant strains possessed two substitutions in *gyrA* (both Ser-83 and Asp-87) and one substitution in *parC* (Ser-80 in thirteen strains and Glu-84 in one strain). There was also one His-75 substitution in one resistant strain. On this farm the observed percentage of fluoroquinolone-resistant strains was the greatest of all farms (i.e. 93.3% of the tested strains resistant to both ciprofloxacin and enrofloxacin). Interestingly, some mutations in *gyrA* and *parC* were detected in susceptible strains, or in strains with intermediate reaction to enrofloxacin. However, according to Hopkins et al. (2005), a single mutation in *gyrA* may not be sufficient to ensure high-level resistance to the antimicrobial agents tested in this study and additional mutations in *gyrA* and/or *parC* are required for this type of resistance to occur. Our observations are concurrent with those of Abdi-Hachesoo et al. (2013), who concluded that the most frequent mutation in *gyrA*,

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i.e. Ser-83, is only the first step in the acquisition of fluoroquinolone resistance and results in high-level resistance to nalidixic acid. However, in order to obtain resistance to second-generation fluoroquinolones, such as ciprofloxacin and enrofloxacin, an additional mutation in *gyrA* or *parC* is needed.

In the present study the presence of *qnrB* and *qnrS* genes was detected in almost all isolates of *E. coli*, also in the ones that were susceptible to the tested fluoroquinolones according to the EUCAST breakpoints (Table 4). This is however consistent with the conclusions of Hopkins et al. (2005), who stated that the level of fluoroquinolone resistance provided by the plasmid-mediated Qnr genes is low and that the phenotypic susceptibility to fluoroquinolones can co-occur with the presence of these genes. However, the presence of the plasmid carrying the PMQR genes facilitates the selection of higher resistance by increasing the level of resistance mutation selection (Hopkins et al. 2005). Martinez-Martinez et al. (1998) observed that spontaneous mutations occurred over one hundred times more frequently in *E. coli* strains containing the plasmid than in the strains without it. The very frequent occurrence of PMQR-positive isolates in Polish *E. coli* strains is concurrent with the observations made by Veldman et al. (2011) in their study conducted in thirteen European countries. Similarly to our study, they observed that almost all *E. coli* isolates from Poland were qnrS-positive.

The haplotype analysis revealed a high overall genetic differentiation among the analysed strains. However, there are differences visible in the variation observed within each farm. As expected, the highest variation reflected by the haplotype abundance was observed on organic farm E. An opposite situation was observed on two conventional farms, i.e. farms A and D, as haplotypes specific to multiple strains dominated the sampled populations. Moreover, the haplotypes detected there were much more closely related to each other. This is supported more formally by the MNPD values that also indicate the considerably lower variation for these two farms. This picture indicates that the use of antimicrobial agents, apart from promoting resistance, may strongly decrease the biodiversity of digestive tract microflora. The extent of this effect is however hard to predict, as it was not recorded for the two other conventional farms B and C. More detailed investigation of this phenomenon was not possible in our study, as it requires controlled experimental conditions, or at least detailed knowledge about the antimicrobial treatment regime used.

In conclusion, the excessive use of antimicrobial agents in agriculture and animal husbandry has largely reduced the effectiveness of these drugs. The observed widespread resistance to fluoroquinolones in *E. coli* is an inevitable consequence of the use of enrofloxacin in food production animals. In addition to this, transformation and conjugation contribute to decreasing the antimicrobial susceptibility among commensal and environmental strains of *E. coli*, even those that did not have contact with antimicrobial agents.

Acquisition of high-level fluoroquinolone resistance appears to be a multifactorial process that includes acquisition of mutations in gyrase and topoisomerase genes, together with plasmid-mediated fluoroquinolone resistance determinants.

REFERENCES

Abdi-Hachesoo B, Asasi K, Sharifiyazdi H (2013): Rapid detection of Escherichia coli gyrA and parC mutants in one-day-old broiler chicks in Iran. Veterinaria Italiana 49, 291–297.

Bayer HealthCare LLC, Animal Health Division (2010): Baytril® (enrofloxacin) – Antibacterial injectable solution 2.27%. Technical Bulletin. Shawnee Mission, Kansas, USA.

Blanco JE, Blanco M, Mora A, Blanco J (1997): Prevalence of bacterial resistance to quinolones and other antimicrobials among avian Escherichia coli strains isolated from septicemic and healthy chickens in Spain. Journal of Clinical Microbiology 35, 2184–2185.

Cattoir V, Weill F, Poirel L, Fabre L, Soussy C, Nordmann P (2007): Prevalence of qnr genes in Salmonella in France. Journal of Antimicrobial Chemotherapy 59, 751–754.

Drlica K, Zhao XL (1997): DNA gyrase, topoisomerase IV and the 4-quinolones. Microbiology and Molecular Biology Reviews 61, 377–392.

Dzierzawski A, Cybulski W (2012): The need for prudent use of antibiotics in veterinary practice (in Polish). Veterinary Life 87, 316–321.

Excoffier L, Lischer HEL (2010): Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10, 564–567.

European Committee on Antimicrobial Susceptibility Testing (2013): Breakpoint tables for interpretation of MICs and zone diameters.

Gniadkowski M, Zabicka D, Hryniewicz W (2009): Recommended selection of the antimicrobial susceptibility tests

doi: 10.17221/8721-VETMED

for bacteria. Determination of Gram-negative rods susceptibility. National Reference Centre for Antimicrobial Susceptibility. Warsaw, Poland.

Hawkey PM (2003): Mechanisms of quinolone action and microbial response. Journal of Antimicrobial Chemotheraphy 51 (Suppl 1), 29–35.

Hiasa H, Yousef DO, Marians KJ (1996): DNA strand cleavage is required for replication fork arrest by a frozen topoisomerase-quinolone-DNA ternary complex. Journal of Biological Chemistry 271, 26424–26429.

Hopkins KL, Davies RH, Threlfall EJ (2005): Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. International Journal of Antimicrobial Agents 25, 358–373.

Kaplan T (2014): The role of horizontal gene transfer in antibiotic resistance. Eukaryon 10, 80–81.

Liu J, Deng Y, Zeng Z, Gao J, Chen L, Arakawa Y, Chen Z (2008): Coprevalence of plasmid-mediated quinolone resistance determinants QepA, Qnr and AAC(6')-Ib-cr among 16S rRNA methylase RmtB-producing Escherichia coli isolates from pigs. Antimicrobial Agents and Chemotherapy 52, 2991–2993.

Łebkowska M (2009): Antibiotic resistant bacteria in drinking water (in Polish). Environmental Protection 31, 11–15.

Martinez-Martinez L, Pascual A, Jacoby GA (1998): Quinolone resistance from a transferable plasmid. Lancet 351, 797–799.

Miles TD, McLaughlin W, Brown PD (2006): Antimicrobial resistance of Escherichia coli isolates from broiler chickens and humans. BMC Veterinary Research 2, 1–9.

Moniri R, Dastehgoli K (2005): Fluoroquinolone-resistant Escherichia coli isolated from healthy broilers with previous exposure to fluoroquinolones: is there a link? Microbial Ecology and Disease 17, 69–74.

Namboodiri SS, Opintan JA, Lijek R, Newman MJ, Okeke IN (2011): Quinolone resistance in Escherichia coli from Accra, Ghana. BMC Microbiology 11, 44.

Phillips I, Casewell M, Cox T, De Groot B, Friis C, Joness R, Nightingale C, Preston R, Waddell J (2004): Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. Journal of Antimicrobial Chemotherapy 53, 28–52.

Rysz M, Alvarez PJJ (2004): Amplification and attenuation of tetracycline resistance in soil bacteria: aquifer column experiments. Water Research 38, 3705–3712.

Salehi TZ, Bonab SF (2006): Antibiotics susceptibility pattern of Escherichia coli strains isolted from chickens with colisepticemia in Tabriz Province, Iran. International Journal of Poultry Science 5, 677–684.

Sapkota AR, Hulet RM, Zhang G, McDermott P, Kinney EL, Schwab KJ, Joseph SW (2011): Lower prevalence of antibiotic-resistant enterococci on U.S. conventional poultry farms that transitioned to organic practices. Environmental Health Perspectives 199, 1622–1628.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013): MEGA 6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30, 2725–2729.

Tollefson L, Altekruse SF, Potter ME (1997): Therapeutic antibiotics in animal feeds and antibiotic resistance. International Office of Epizootics 16, 709–715.

Tran JH, Jacoby GA (2002): Mechanism of plasmid-mediated quinolone resistance. Proceedings of the National Academy of Sciences of the United States of America 99, 5638–5642.

van den Bogaard AE, London N, Driessen C, Stobberingh EE (2001): Antibiotic resistance of faecal Escherichia coli in poultry, poultry farmers and poultry slaughterers. Journal of Antimicrobial Chemotherapy 47, 763–771.

Veldman K, Cavaco LM, Mevius D, Battisti A, Franco A, Botteldoorn N, Bruneau M, Perrin- Guyomard A, Cerny T, Escobar C de Frutos, Guerra B, Schroeter A, Gutierrez M, Hopkin K, Myllyniemi A-L, Sunde M, Wasyl D, Aarestrup FM (2011): International collaborative study on the occurrence of plasmid-mediated quinolone resistance in Salmonella enterica and Escherichia coli isolated from animals, humans, food and the environment in 13 European countries. Journal of Antimicrobial Chemotherapy 66, 1278–1286.

Versalovic J, Schneider M, De Bruin FJ, Lupski JR (1994): Genomic fingerprinting of bacteria uusing repetitive sequence-based polymerase chain reaction. Methods in Molecular and Cell Biology 5, 25–40.

Villesen P (2007): FaBox: an online toolbox for fasta sequences. Molecular Ecology Notes 7, 965–968.

Wang H, Dzinx-Fox JL, Chen M, Levy SB (2001): Genetic characterization of highly fluoroquinolone-resistant clinical Escherichia coli strains from China: role of acrR mutations. Antimicrobial Agents and Chemotherapy 45, 1515–1521.

Witte W (1998): Medical consequences of antibiotic use in agriculture. Science 279, 996–997.

https://www.soilassociation.org/LinkClick.aspx?fileticket=T_9ATk3bieI%3D&tabid=1715

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