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NOTE

Resistance to non-quinolone antimicrobials in commensal *Escherichia coli* isolates from chickens treated orally with enrofloxacin

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Abstract

The aim of the present study was evaluate how oral administration of enrofloxacin affected the frequency of resistance to different antimicrobials in commensal *Escherichia coli* isolates from healthy chickens. A further objective of this study was to characterize the mechanisms of resistance in these isolates. A trend towards increased resistance to enrofloxacin, doxycycline and amoxicillin of *E. coli* isolates from chickens after enrofloxacin administration was observed. The increase in the resistance to doxycycline and amoxicillin was probably due to a co-selection of tetracycline and β-lactam resistance genes by the administration of enrofloxacin. The detection of *tetM* was much higher than expected (50%), which indicates that this gene may play an important role in tetracycline resistance in *E. coli* from chickens.

Key Words: antimicrobial resistance, *E. coli*, poultry

Commensal and pathogenic *Escherichia coli* isolated from chickens are often resistant to quinolones, including enrofloxacin, tetracyclines, including doxycycline, and β-lactams, including amoxicillin[3,8–10,13,18)]. One possible origin of this problem might be the wide use of these antimicrobials in the oral treatment of poultry diseases, which may lead to resistance to the antimicrobials used[8,10,18)]. Another possible reason would be the co-selection of antimicrobial resistance, which could result in the selection of resistant *E. coli* not only to the drug being used but to other unrelated classes of antimicrobials as well[3,14,15)]. However, few studies published to date have examined systematically how administration of an antimicrobial affects the emergence and selection of resistant microorganisms to other antimicrobials. The aim of the present study was to evaluate the effect of the oral administration of enrofloxacin on the frequency of resistance to different antimicrobials among commensal *E. coli* isolates from healthy chickens. Another objective

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was to characterize the mechanisms of resistance in these isolates.

A total of 20 fifteen-day-old Single Comb White Leghorn male chickens, which were obtained from commercial hatcheries and without previous exposure to antimicrobials, were examined. Animals were randomly distributed into 4 groups of 5 chickens. Groups received different doses of enrofloxacin for different durations: a control group received no antimicrobial; a group was treated with 10 mg/l enrofloxacin in drinking water for 6 days (approved dose for enrofloxacin in poultry in Spain)[17]; a group of animals was treated with 5 mg/l enrofloxacin in drinking water for 12 days; and a group was treated with 20 mg/l enrofloxacin of drinking water for 3 days. Fecal samples were taken before initiating the treatment (BT), at the end of the treatment (AT), and at 4 weeks post-treatment (PT). After collection of PT samples, all chickens were humanely killed. The experiments followed the European Union principals for animal care and experimentation. Experimental procedures were in agreement and approved by the Ethical Committee for Animal Care and Experimentation of CReSA, UAB-IRTA, Barcelona, Spain.

To isolate *E. coli*, 1 g of each fecal sample was resuspended in 10 ml of buffered peptone water (Merck Millipore, Germany) and subsequently plated onto MacConkey agar (Merck Millipore). After overnight incubation, up to 10 colonies with the typical appearance of *E. coli* were randomly chosen from each sample; if 10 or fewer such colonies were present, all were selected. These isolates were identified as *E. coli* using a commercial identification system (Vitek Junior, bioMérieux, Spain).

Antimicrobial testing was performed using the disc diffusion method, according to the recommendations of the Clinical and Laboratory Standards Institute[2]. The discs contained 10 μg of enrofloxacin, 80 μg of doxycycline, and 30 μg of amoxicillin (Neo-Sensitabs, Rosco Diagnostica A/S, Denmark). Measurement of growth inhibition areas allowed the classification of each isolate as susceptible, intermediate or resistant, according to data provided by the manufacturer of the discs. As reference strain, *E. coli* ATCC 25922 was used.

E. coli isolates resistant to enrofloxacin were screened for mutations in the quinolone resistance-determining region of the *gyrA* and *parC* genes by PCR and sequencing, as previously described[6]. The presence of the major resistance genes for doxycycline (*tetA, tetB* and *tetM*) and amoxicillin (bla_{TEM}, $bla_{\text{CMY-2}}$, bla_{SHV} and $bla_{\text{OXA-1}}$) was determined in resistant isolates by PCR as previously described[11].

Significant differences in the frequencies of resistance and in the possession of the antimicrobial resistance genes were determined by the Fisher exact test. A P value less than 0.05 was considered significant. Analyses were performed using statistical software (IBM SPSS, Statistics, version 19, USA).

A total of 588 *E. coli* isolates from chickens were isolated in this study, corresponding to 200 isolates at BT, 190 at AT and 198 at PT (Table 1). The antimicrobial susceptibility of the 588 *E. coli* isolates is summarized in Table 1.

In the control group, a statistically significant increase in the frequency of resistance to enrofloxacin in the isolates from the PT stage compared with those from the AT and BT stages and in the frequency of resistance to doxycycline in the isolates from the BT stage compared with those from the AT and PT stages was observed (Table 1). Age-related differences in antimicrobial susceptibility of commensal *E. coli* strains from chickens have been previously reported[16]. Since no significant differences in the frequencies of antimicrobial resistance among the 3 groups treated were observed (data not shown), the isolates from the treated chickens were considered as a whole. In these isolates, a statistically significant increase in the frequencies of resistance to enrofloxacin, doxycycline and amoxicillin was observed at the AT and PT stages compared to the BT stage (Table 1). Taken together, our results show a trend towards

Table 1. Number (percentage) of commensal *Escherichia coli* isolates from control chickens and chickens treated orally with enrofloxacin that were resistant (R), intermediate resistant (I), or susceptible (S) to enrofloxacin, doxycycline and amoxicillin

Time of sample collection[a]		Enrofloxacin		Doxycycline		Amoxicillin	
		Control chickens	Treated chickens	Control chickens	Treated chickens	Control chickens	Treated chickens
BT	R	0 (0)[1]	12 (8)[1]	25 (50)[1]	35 (23.3)[1]	23 (46)	56 (37.3)[1]
	I	0 (0)	16 (10.7)	3 (6)	3 (2)	0 (0)	0 (0)
	S	50 (100)	122 (81.3)	22 (44)	112 (74.7)	27 (54)	94 (62.7)
AT	R	1 (2)[1]	116 (82.9)[2]	11 (22)[2]	67 (47.9)[2]	21 (42)	117 (83.6)[2]
	I	12 (24)	3 (2.1)	0 (0)	37 (26.4)	0 (0)	0 (0)
	S	37 (74)	21 (15)	39 (78)	36 (25.7)	29 (58)	23 (16.4)
PT	R	20 (40)[2]	129 (87.2)[2]	13 (26)[2]	88 (59.5)[2]	24 (48)	130 (87.8)[2]
	I	0 (0)	6 (4.1)	5 (10)	29 (19.6)	0 (0)	0 (0)
	S	30 (60)	13 (8.8)	32 (64)	31 (20.9)	26 (52)	18 (12.2)

Values with different numbers within a column are significantly different at $P < 0.05$.
[a]BT, before enrofloxacin administration; AT, at the end of enrofloxacin administration; PT, at 4 weeks after the end of enrofloxacin administration.

increased resistance to enrofloxacin, doxycycline and amoxicillin of *E. coli* isolates from chickens after enrofloxacin administration.

In agreement with our data, Miranda *et al*.[12] found a significant increase in the frequency of resistance to enrofloxacin in *E. coli* isolates from chickens after the oral administration of enrofloxacin. Thus, these studies indicate that enrofloxacin use in poultry increase the frequency of resistance to that antimicrobial in *E. coli* isolates. That increase is probably due to that the oral administration of enrofloxacin selects pre-existing quinolone-resistant *E. coli* populations present in the gut of chickens[12]. Resistance to fluoroquinolones in *E. coli* isolates is of special concern because they are critically important antimicrobials in human antimicrobial therapy[8]. For this reason, in the European Union there are currently recommendations for the prudent use of fluoroquinolones in food-producing animals[4].

It may be speculated that the increase in the resistance to doxycycline and amoxicillin found in this study was, at least partially, a consequence of the treatment with enrofloxacin. Mutations in *gyrA* and *parC* are located on the chromosomal while most of the tetracycline and β-lactam resistance genes are located on genetic mobile elements[6,9,10,18]. In addition to chromosomal mutations, several plasmids carrying quinolone resistance genes have been found to transmit low-level quinolone resistance[6,9]. Thus, it is possible that these plasmids content, in addition to quinolone resistance genes, some tetracycline and β-lactam resistance genes and that selective pressure due to the use of enrofloxacin may result in the co-selection of tetracycline and β-lactam resistance genes. Since transmissible genetic elements were not analyzed in this work, further studies are needed to confirm the role of such elements in the spread of resistance genes in poultry *E. coli*.

Because of the high number of isolates resistant to enrofloxacin, doxycycline and amoxicillin identified in this study (278, 239 and 371, respectively), 55 enrofloxacin-resistant isolates, 72 doxycycline-resistant isolates, and 191 amoxicillin-resistant isolates were randomly selected for characterizing the mechanisms of resistance.

47 of the 55 (85.5%) *E. coli* isolates resistant to enrofloxacin presented a double amino acid substitution in GyrA and a single amino acid substitution in ParC, while 2 *E. coli* isolates showed a single amino acid substitution in and a

Table 2. Number (percentage) of amino acid changes in the quinolone resistance-determining region (QRDR) of GyrA and ParC proteins and antimicrobial resistance genes in resistant commensal *Escherichia coli* isolates from control chickens and chickens treated with enrofloxacin

Antimicrobial	Mechanism of antimicrobial resistance	Control chickens	Treated chickens
	Amino acid changes in the QRDR of GyrA/ParC		
Enrofloxacin	Ser83→Leu/Ser80→Ile	0 (0)	2 (4)
	Ser83→Leu+Asp87→Asn/Ser80→Ile	2 (40)	45 (90)
	Ser83→Leu+Asp87→Asn/Ser80→Ile+Glu84→Gly	0 (0)	2 (4)
	Ser83→Leu+Asp87→Asn/Ser80→Ile+Val87→Ala	3 (60)	1 (2)
	Resistance genes		
Doxycycline	*tetA*	1 (7.7)	9 (15.3)
	tetB	1 (7.7)	2 (3.4)
	tetA+*tetB*	4 (30.8)	19 (32.2)
	tetA+*tetM*	6 (46.2)	10 (16.9)
	tetB+*tetM*	1 (7.7)	8 (13.6)
	tetA+*tetB*+*tetM*	0 (0)	11 (18.6)
Amoxicillin	bla_{CMY2}	14 (38.9)	5 (3.2)
	$bla_{\text{OXA-1}}$	0 (0)	2 (1.3)
	bla_{SHV}	7 (19.4)	0 (0)
	bla_{TEM}	2 (5.5)	32 (20.6)
	bla_{CMY2}+bla_{SHV}	0 (0)	3 (1.9)
	bla_{CMY2}+bla_{TEM}	10 (27.8)	86 (55.5)
	$bla_{\text{OXA-1}}$+bla_{TEM}	0 (0)	12 (7.7)
	Not identified	3 (8.3)	15 (9.7)

single amino acid substitution in ParC, and 6 isolates presented a double amino acid substitution in GyrA and a double amino acid substitution in ParC (Table 2). Our results confirm that mutations in *gyrA* at codons Ser83 and Asp87, and in *parC* at codon 80 are the most frequent *gyrA* and *parC* mutations found in human and animal isolates of *E. coli*[6]. To the authors' knowledge, this is the first report of Val87→Ala and Glu84→Gly substitutions in the ParC protein of *E. coli* isolates from chickens. Mutations in *parC* were always found together with mutations in *gyrA* in the present study, as reported for other *E. coli* isolates[6,9].

Some significant differences in the frequencies of antimicrobial resistance genes among *E. coli* isolates from control and treated chickens were found. Thus, bla_{TEM} was found significantly more frequently in the *E. coli* isolates from the treated chickens than in the *E. coli* isolates from the control group (83.9% and 33.3%, respectively (Table 2) ($P < 0.05$), and bla_{SHV} was found significantly more frequently in the *E. coli* isolates from the control group than in the *E. coli* isolates from the treated chickens (19.4% and 1.9%, respectively; $P < 0.05$). Differences in the detection of antimicrobial resistance genes among *E. coli* isolates from untreated and treated animals have been observed previously[1].

The predominant tetracycline resistance gene, *tetA*, was found alone or combined with other genes in 60 of the 72 (83.3%) isolates resistant to doxycycline. *tetB* and *tetM* were detected in 46 (63.9%) and 36 (50%) of the *E. coli* isolates, respectively (Table 2). In agreement with our results, previous studies have found that *tetA* is the tetracycline resistance gene detected most frequently in avian *E. coli*,

followed by *tetB*[3,5,13,14,18].

tetM is the most common tetracycline resistance gene in enterococci but has been infrequently found in *E.* coli[7]. However, in a recent study carried out on *E. coli* isolates from ducks[5] and in the present study, *tetM* was detected in a high percentage of *E. coli* isolates (48.2% and 50%, respectively). It has been suggested that the finding of *tetM* in *E. coli* isolates is due to a transfer of this gene from enterococci but it can not be ruled out that the transfer occur from other intestinal tract bacteria[7]. In agreement with the results found in ducks by Hu *et al*.[5] and in pigs by our group[7], in this study *tetM* was always found together with *tetA* and/or *tetB*. Hu *et al*.[5] observed that *tetM* was associated to a higher level of tetracycline resistance in *E. coli* isolates. Thus, the results found by Hu *et al*.[5] and in this study show that *tetM* may play an important role in tetracycline resistance in avian *E. coli*.

Predominant resistance genes in the isolates resistant to amoxicillin were bla_{TEM} and $bla_{\text{CMY-2}}$ (74.3% and 61.8%, respectively). These β-lactamase genes are among the most frequently detected in poultry *E. coli* isolates resistant to β-lactams[3,10,13–15]. In addition, 14 (7.3%) and 10 (5.2%) of the 191 isolates resistant to amoxicillin carried the $bla_{\text{OXA-1}}$ and bla_{SHV} genes, respectively. bla_{SHV} has been previously found in poultry *E. coli* isolates[10,13,14] but, to our knowledge, this is the first report of $bla_{\text{OXA-1}}$ detection in *E. coli* from chickens.

In conclusion, the oral administration of enrofloxacin seems to be associated with a significant increase in the frequencies of resistance to enrofloxacin, doxycycline and amoxicillin in commensal *E. coli* isolates from chickens. These data strengthen and endorse the recommendations to reduce the use of quinolones in poultry. In addition, the detection of *tetM* was much higher than expected, which indicates that this gene may make a significant contribution to tetracycline resistance in *E. coli* from chickens.

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