Evolution of Resistance in Poultry Intestinal *Escherichia coli* During Three Commonly Used Antimicrobial Therapeutic Treatments in Poultry

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ABSTRACT The resistance rates of intestinal *Escherichia coli* populations from poultry were determined during treatment and withdrawal period with 3 antimicrobial agents commonly used as therapeutics in poultry medicine. A total of 108 chickens were considered: 18 were treated orally with enrofloxacin, 18 with doxycycline, and 18 with sulfonamides, whereas another 18 chickens were maintained as controls for each antimicrobial group. Fecal samples were taken during the treatment and after the withdrawal period, and *E. coli* were isolated through Fluorocult media plating. A total of 648 *E. coli* strains (216 per antimicrobial tested) were isolated and identified though biochemical methods. Minimal inhibitory concentrations to the antimicrobials used were also determined using a broth microdilution method. The resistance rates of intestinal *E. coli* to all of the antimicrobials tested significantly increased during the course of the therapeutic treatment. In addition, significant differences ($P = 0.0136$) in resistance rates persisted between the intestinal *E. coli* of the enrofloxacin-treated and control batches until the end of the withdrawal period, but this difference was not observed for the cases of doxycycline or sulfonamides treatments. Antimicrobial use in poultry medicine seems to select for antimicrobial-resistant strains of pathogenic bacterial species such as *E. coli.* In some cases, the higher frequencies of resistant strains may persist in the avian intestinal tract until the end of the withdrawal period, when it is legal to use these animals for human consumption.

Key words: *Escherichia coli*, poultry, resistance, antimicrobial, treatment

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INTRODUCTION

The risks associated with the use of antimicrobial agents for the therapeutic treatment of sick animals have been the subject of debate in recent years. Some scientists are very concerned about the potential consequences, such as the development and spread of bacterial resistance, whereas other researchers state that there is not enough evidence that might point toward such potential risk or even consider potential human health benefits derived from antibiotic use in food animals (Witte, 1998; Doyle, 2006).

In poultry farming, as well as with other intensively reared animals, antibiotics may be administered though feed or drinking water to whole flocks rather than to individual animals. In the European community (**EC**), the water- or feed-based administration of antimicrobials to animals (at lower doses than those employed for therapeutic purposes) to enhance animal growth has been completely banned since January 2006. After the ban of the use of avoparcin, bacitracin, tylosin, espiramicin, and virginiamicin as growth promoters in the 1990s, the level of bacterial resistance to such antimicrobials decreased considerably. Nevertheless, increasing amounts of antimicrobial agents have been used with therapeutic purposes in veterinary medicine since this ban (Monnet, 2000; Phillips et al., 2003). From among these agents, together with tetraciclines, quinolones, and sulfonamides, form some of the most widely used antimicrobial families in poultry therapy (Avrain et al., 2003; Phillips et al., 2003; Patel et al., 2004; Posyniak et al., 2005).

Escherichia coli is commonly found in the intestinal tract of humans and animals. Its use as an indicator bacterium is useful because this microorganism acquires antimicrobial resistance faster than other conventional bacteria. Thus, changes in the resistance of this species may serve as a good indicator of resistance in potentially pathogenic bacteria (Kijima-Tanaka et al., 2003; Von Baum and Marre, 2005).

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Poultry food products are an important source of *E. coli* because, at the time of slaughter, fecal contamination from the gut readily contaminates poultry carcasses. As a result, poultry meat may be contaminated with fecal material or ingesta and with bacteria associated with these contaminants (Sáenz et al., 2001; Van den Bogaard et al., 2001; Smith et al., 2007). Other authors have reported that vegetables may also be contaminated from animals via sewage and manure, which may act as a source of contamination by resistant bacteria (Phillips et al., 2003). These resistant bacteria may colonize the human intestinal tract and may also be involved in the transference of resistance genes to human endogenous microflora (Van den Bogaard et al., 2001). Hence, resistant *E. coli* from poultry selected during veterinary antimicrobial treatments can infect humans directly and via food.

The aims of the present work were to determine the degree of antimicrobial resistance of *E. coli* during the most commonly used antimicrobial treatments and after their withdrawal period. The withdrawal period is determined as a function of the persistence of antimicrobial residues in animal tissues. Thus, it is of crucial interest to investigate the resistance rates of the bacterial populations present in the animals' intestines because this may provide valuable information for establishing the safety of animal foods when the withdrawal period has been completed.

MATERIALS AND METHODS

Birds Employed

A total of 108 healthy male "label" chickens aged 4 to 6 wk obtained from the same commercial hatchery were used. The chickens were fed with the same antimicrobial-free feed before starting antimicrobial treatment. These chickens were split into 3 groups of 36 animals per group. In each of these 3 groups, 18 broilers were treated with a therapeutic antimicrobial dosage and another 18 were maintained as controls. None of the chickens had direct physical contact with any of the other chickens during the assays because they were housed in individual cages in the animal facility. Contamination between the treated and untreated batches was prevented by housing them in different rooms. The poultry were fed twice daily with antibimicrobial-free commercial poultry feed and had free access to the medicated water, in the case of the treated poultry, or to antimicrobial-free water, in the case of the control batches. Determination of water intake was performed at 12-h intervals.

All parts of this study were carried out according to EC council directives concerning the laws, regulations, and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes.

Enrofloxacin Treatment

Eighteen chickens were weighed and treated with a therapeutic dose of enrofloxacin in water (0.05 g/L) of Colmyc-E (S.P. Veterinaria, Tarragona, Spain), and another 18 were weighed and kept untreated as controls. The treatment was administered over 5 d, keeping a period of 12 d as the withdrawal time, in accordance with the instructions of the manufacturer. Each group of chickens was sampled immediately before starting treatment (d 0), on the first day of treatment (d 1), in the middle of treatment (d 3), on the last day of treatment (d 5), in the middle of the withdrawal period (d 11), and after the withdrawal time had ended (d 17).

Doxycycline Treatment

Eighteen chickens were weighed and treated with a therapeutic dose of doxycycline in water (1 g/L) of Doxidol (Fatro Uriach Veterinaria, Barcelona, Spain), and another 18 were weighed and kept as controls. The treatment was administered over 5 d, keeping a period of 7 d as the withdrawal time, in accordance with the manufacturer's instructions. Each group of chickens was sampled immediately before starting treatment (d 0), on the first day of treatment (d 1), in the middle of treatment (d 3), on the last day of treatment (d 5), in the middle of the withdrawal period (d 8), and after the withdrawal time had ended (d 12).

Sulfonamides Treatment

Eighteen chickens were weighed and treated with a therapeutic dose of a sulfonamides mixture (1.33 g of Sulfaquinoxaline + 1.66 g of Sulfamethazine + 1.66 g of Sulfameracine + 3.33 g of Sulfisoxazole/100 mL) in water (15 mL/L) from Cunisan Aviar (Arimany, Barcelona, Spain), and another 18 were weighed and kept as controls. The treatment was administered over 4 d, followed by 2 d of repose and another 3 d of treatment, in accordance with the instructions on the package insert. After treatment, a withdrawal period of 15 d was kept. Each group of chickens was sampled immediately before starting treatment (d 0), on the first day on treatment (d 1), on the fourth day of treatment (d 4), on the last day of treatment (d 9), in the middle of the withdrawal period (d 16), and after the withdrawal time had ended (d 24).

For the 3 antimicrobials tested, fecal samples were taken by swabbing the cloacae of each chicken with sterile swabs to obtain a minimum of 0.5 g of fecal matter, which was placed aseptically in a sterile tube. These samples were taken to the laboratory in an ice chest in less than half an hour for immediate processing.

Isolation and Identification of E. coli

The fecal samples of 3 animals belonging to the same group were placed together in a sterile masticator bag with an appropriate volume (1/9, wt/vol) of sterile buffered peptone water (Merck, Darmstadt, Germany) and subsequently homogenized with a masticator (Aes, Combourg, France) for 2 m. After homogenization, samples were tested for isolation and identification of *E. coli.* One

Table 1. Minimum inhibitory concentration (MIC) for 50% (MIC_{50}) and 90% (MIC_{90}), MIC ranges and resistance rates for *Escherichia coli* strains obtained from treated and control chickens during and after enrofloxacin application in the drinking water[1]

	Sampling day					
Item	0	1	3	5	11	17
Treated chickens						
n strains	18	18	18	18	18	18
MIC_{50}	0.5	4	4	8	4	4
MIC_{90}	8	16	16	8	8	16
Range	<0.125 to 8	<0.125 to 16	<0.125 to 16	<0.125 to 8	0.5 to 32	<0.125 to 8
n resistant (%)	6 (33.3)	13 (72.2)	15 (83.3)	16 (88.9)	16 (88.9)	16 (88.9)
Control chickens						
n strains	18	18	18	18	18	18
MIC_{50}	<0.125	<0.125	<0.125	<0.125	<0.125	<0.125
MIC_{90}	8	8	8	8	4	4
Range	<0.125 to 8	<0.125 to 8	<0.125 to 8	<0.125 to 8	<0.125 to 8	<0.125 to 8
n resistant (%)	4 (22.2)	6 (33.3)	9 (50)	6 (33.3)	3 (16.7)	4 (22.2)
P	0.4049	0.0450	0.2051	0.0368	0.0001	0.0136

[1]MIC_{50}, MIC_{90}, and ranges expressed in μg/mL.

milliliter of 10^{-3} to 10^{-7} dilutions of homogenates was tested in poured plates of Fluorocult agar prepared as specified by the manufacturer (Merck). After the agar had solidified, the plates were overlaid with 3 to 4 mL of melted Fluorocult and incubated at 44°C for 24 h. After incubation, pink to red colonies showing blue florescence after exposure to a 365-nm UV lamp were considered as *E. coli.*

After incubation, 3 typical colonies were harvested and transferred onto Columbia agar with 50 g/kg of sheep blood (BioMérieux, Marcy l'Etoile, France) and incubated at 44°C for 24 h to obtain pure cultures. This was done for each batch of chickens (3 chickens) on each sampling day (18 strains per group and day). Thus, a total of 648 strains were obtained (216 strains per antimicrobial tested).

These pure cultures were characterized by colony and cell morphologies, Gram stain, methyl red, citrate test, oxidase, and catalase activities, and indole production. Positive strains were confirmed by API 20E (BioMérieux).

All isolates were stored at –80°C until further analysis using maintenance freeze medium units (Oxoid, Basingstoke, UK).

Antimicrobial Susceptibility Testing of Bacteria

Antimicrobial susceptibility testing was performed using a broth microdilution susceptibility test in microtiter plates to determine minimum inhibitory concentrations (**MIC**). The MIC and levels of resistance were determined according to the recommendations of the Clinical and Laboratory Standards Institute (**CLSI**, formerly NCCLS, 2002). Standard antimicrobial dilutions were obtained with an enrofloxacin reference standard (Bayer AG, Leverkusen, Germany), doxycycline (Fluka, St Gallen, Switzerland), and sulfisoxazole (Sigma Chemical Co, St. Louis, MO). The final concentration ranges were 0.008 to 64 $\mu g \cdot mL^{-1}$ for enrofloxacin, 0.25 to 128 $\mu g \cdot mL^{-1}$ for doxycycline, and 4 to 2,048 $\mu g \cdot mL^{-1}$ for sulfisoxazole. Each tray also contained a positive and a negative growth control well.

The microtiter plates were incubated for 18 to 24 h at 37°C with a source of moisture to prevent dehydration. The MIC were determined by visual observation of the lowest concentration that yielded no visible growth for enrofloxacin and doxycycline in the wells. For sulfisoxazole, the MIC was defined as the concentration of the drug that elicited approximately 80% inhibition of growth in the well as compared with the growth in the control wells with no drug added. The breakpoints used were those recommended by the CLSI (formerly NCCLS, 2002) for veterinary pathogens: ≥2 $\mu g \cdot mL^{-1}$ for enrofloxacin, ≥16 $\mu g \cdot mL^{-1}$ for doxycycline, and ≥512 $\mu g \cdot mL^{-1}$ for sulfisoxazole. The MIC that inhibited 50% (**MIC_{50}**) and 90% (**MIC_{90}**) of total strains were calculated from the MIC values.

Target MIC ranges were verified with *E. coli* ATCC 25922 reference strain as quality control. Quality control was considered acceptable if the results obtained were within ranges recommended by the CLSI (NCCLS, 2002).

Statistical Analysis

The amounts of water ingested by the treated and control chickens were compared using an unpaired Student's *t*-test. The distributions of resistant strains were compared by means of the χ^2 test and Fisher's exact test. Differences were considered significant when probabilities were lower than 0.05. All statistical analyses were carried out using Statgraphics version 5.0.1. (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

The total consumption of the antimicrobials tested by the treated chickens during all of the treatments was 31.07 mg in the case of enrofloxacin (SD = 13.136) 42.97 mg in the case of doxycycline (SD = 13.28) and 870.02 mg in the case of sulfonamides (SD = 254.42). Taking into account

Table 2. Minimum inhibitory concentration (MIC) for 50% (MIC_{50}) and 90% (MIC_{90}), MIC ranges and resistance rates for *Escherichia coli* strains obtained from treated and control chickens during and after doxycycline application in the drinking water[1]

	Sampling day					
Item	0	1	3	5	8	12
Treated chickens						
n strains	17	18	16	18	18	18
MIC_{50}	2	4	8	4	8	8
MIC_{90}	16	8	16	16	16	16
Range	<0.25 to 16	<0.25 to 16	<0.125 to 16	0.5 to 16	<0.25 to 16	<0.25 to 32
n resistant (%)	3 (17.6)	2 (11.1)	6 (37.5)	4 (22.2)	6 (33.3)	8 (44.4)
Control chickens						
n strains	15	17	18	18	18	18
MIC_{50}	2	1	1	2	4	4
MIC_{90}	4	8	8	4	8	16
Range	<0.25–4	<0.25–16	<0.25–8	<0.25–8	0.5–32	<0.25–16
n resistant (%)	0 (0)	2 (11.8)	1 (5.6)	0 (0)	1 (5.6)	3 (16.7)
P	0.1773	0.4410	0.0214	0.0354	0.0272	0.1648

[1]MIC_{50}, MIC_{90}, and ranges expressed in μg/mL.

the average weights of the chickens employed for the treatments [552 g (SD = 114.32) for the case of enrofloxacin, 638 g (SD = 109.26) for the case of doxycycline, and 678 g (SD = 175.26) for the case of sulphonamides], the real dose ingested expressed as mg/kg of BW and day was 11.25 in the case of enrofloxacin, 13.47 in the case of doxycycline, and 183.32 in the case of sulphonamides.

The results obtained for the MIC_{50}, MIC_{90}, MIC ranges and resistance rates are shown in Tables 1 to 3 for enrofloxacin, doxycycline, and sulphonamides, respectively. There were no significant differences between the control and treated batches in regards to the MIC profile on d 0. Likewise, as expected there were no significant differences in the control batches between different days during the assay.

Immediately after starting the enrofloxacin treatment, pre-existing resistant *E. coli* populations presents in the chicken guts were rapidly selected and this resistance was maintained until the end of the withdrawal period (88.9 vs. 22.2% in the control batches). Thus, significant differences were obtained from d 1 to 17 with respect to the resistance rates of the treated and control chickens. The resistance rates reached their highest value for treated poultry on d 5 (88.9%), thereafter remaining at this level until d 17.

The *E. coli* resistance rates to enrofloxacin obtained in this work before starting treatment were higher than those reported by the European surveillance programmes. However, these rates are in agreement with previous data reported by other authors (Barrow et al., 1998; Moniri and Dastehgoli, 2005). In recent years, an increase in the resistance of *E. coli* to quinolones has been reported [i.e., the 10% ciprofloxacin resistance described in the Netherlands (Van den Bogaard et al., 2001) or the 38% ciprofloxacin resistance in *E. coli* isolated from Spanish supermarket poultry retail products in Spain (Sáenz et al., 2001)].

This increase in the resistance of *E. coli* to quinolones seems to be caused by the widespread use of these antimicrobials for veterinary purposes. It has been documented that ciprofloxacin resistance is higher in *E. coli* isolated from broilers than the microorganisms isolated from other sources, such as pigs and humans. This could be due to the higher use of quinolones in chickens than in

Table 3. Minimum inhibitory concentration (MIC) for 50% (MIC_{50}) and 90% (MIC_{90}), MIC ranges and resistance rates for *Escherichia coli* strains obtained from treated and control chickens during and after sulphonamides application in the drinking water[1]

	Sampling day					
Item	0	1	4	9	16	24
Treated chickens						
n strains	18	18	18	18	18	18
MIC_{50}	512	512	1,024	1,024	1,024	1,024
MIC_{90}	2,048	1,024	2,048	1,024	2,048	2,048
Range	<4 to 2,048	<4 to 2,048	512 to 2,048	512 to > 2,048	<4 to 2,048	<4 to > 2,048
n resistant (%)	11 (61.1)	13 (72.2)	18 (100)	18 (100)	16 (88.8)	17 (94.4)
Control chickens						
n strains	18	18	18	17	18	18
MIC_{50}	512	<4	<4	<4	<4	512
MIC_{90}	1,024	<4	1,024	1,024	1,024	1,024
Range	<4 to 2,048	<4 to 1,024	<4 to 1,024	<4 to 2,048	<4 to 2,048	<4 to 2,048
n resistant (%)	10 (55.6)	2 (11.1)	9 (50)	7 (41.2)	9 (50)	11 (61.1)
P	0.0663	0.0009	0.0018	0.0019	0.0153	0.0959

[1]MIC_{50}, MIC_{90}, and ranges expressed in μg/mL.

pigs or humans (Sáenz et al., 2001; Kijima-Tanaka et al., 2003). Also, a recent work has shown that quinolone resistance in *E. coli* isolated from broilers previously dosed with quinolones was significantly higher than the resistance of *E. coli* isolated from poultry without exposure to quinolones (49.5 vs. 33.7%, respectively; Moniri and Dastehgoli, 2005).

In the case of doxycycline treatment, although the increase in resistance rates in *E. coli* isolated from treated chickens was less evident than in the case of enrofloxacin, significant differences in the resistance rates of the treated and control chickens were observed on d 3, 5, and 8. These resistance rates reached their highest value for treated poultry at the end of the withdrawal period (44.4%).

In recent years, bacterial resistance to doxycycline has been widely documented in the case of certain respiratory pathogens (Chopra and Roberts, 2001; Cunha, 2003; Jones et al., 2004; Koeth et al., 2004). However, only a few authors have addressed such resistance in enteric pathogens of animal origin, such as *E. coli.* In this sense, it is crucial to gain a deeper knowledge about resistance of zoonotic bacteria to this antimicrobial agent because doxycycline is widely used in veterinary medicine, especially in the case of chickens and turkeys and specially in developing nations (Chopra and Roberts, 2001). The resistance rates determined in this work before the start of treatment were relatively low as compared with those found by other authors for tetracyclines in *E. coli* isolated from poultry feces [i.e., 75 or 43.8% of resistance of *E. coli* to tetracycline obtained by other authors for broilers in Spain (Sáenz et al., 2001; Bywater et al., 2004)]. Nevertheless, the resistance rates determined in our work may not contradict such reports because some tetracycline-resistant bacteria may be sensitive to doxycycline (NCCLS, 2002).

As in the cases described above, immediately after starting the sulphonamides treatment, pre-existing sulfphonamide-resistant *E. coli* populations were rapidly selected, and this resistance was maintained until the end of the withdrawal period (94.4 vs. 61.1% in the control batches). In addition, significant differences between the resistance rates of the treated and control chickens were observed from d 1 to 16. Resistance rates reached the highest values for the treated poultry on d 4 and 9 (100%). The resistance rates obtained in this study before starting treatment and in the control batches (55.6 and 61.1%, respectively) were in agreement with previously reported data, such as the 69.7% of sulfadimethoxine-resistant strains observed in *E. coli* isolated from poultry in Japan. Likewise, 52.1% resistance to trimethoprim-sulfamethoxazole was determined in *E. coli* isolated from broilers in Spain (Kijima-Tanaka et al., 2003; Bywater et al., 2004).

According to these results, during the 3 treatments evaluated, pre-existing resistant *E. coli* populations were rapidly selected and these resistances were maintained until the end of the withdrawal period. Taking into account that at the time of slaughter poultry meat may be easily contaminated with fecal *E. coli* (Sáenz et al., 2001; Van den Bogaard et al., 2001) and the fact that treated chickens are often sent to the slaughterhouse immediately after the withdrawal period, the resistant bacteria selected by the antimicrobial treatments could be a risk for public health after poultry slaughter and processing.

Moreover, the resistant *E. coli* strains selected by the antimicrobial treatments might reach humans via other animals, sewage, or other humans, such as farmers or slaughterers (Phillips et al., 2003). According to the results obtained in the present work, such indirect ways of transmission could be considered as potential ways of transmission of resistant bacteria to human beings.

Taking into account that after the EC banned the use of antimicrobials as growth promoters, an increment of quantities consumed of antimicrobials used as therapeutics was expected and an undesirable consequence of this prohibition would be the loss of efficacy of these antimicrobial agents. Thus, although more microbiological studies are necessary, it seems advisable to extend the withdrawal period after the implementation of antimicrobial poultry therapy, as well as an adequate control of sewage use.

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